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IDENTIFICATION OF ESCHERICHIA COLI FROM DRINKING WATER SAMPLES IN GOTHATUWA NEW TOWN AND DETECTION OF ANTIBIOTIC RESISTANT GENES

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ABSTRACT

Gothatuwa New town (GNT) is constantly affected with floods increasing the possibility for the presence of antibiotic resistant Escherichia coli (E coli). Hence, this study focuses on detecting tetracycline and sulphonamide resistant E coli from 10 drinking water sources of GNT with subsequent biochemical, morphological and molecular confirmation. Water collected from the tap, 3 unprotected wells and 1 protected well produced prominent colonies from the chromogenic media and 11 colonies (S1- S11) were subjected for testings. From this, 7/11 colonies showed high possibility to be E coli, providing expected results from Indole, Simmons' citrate tests as well as 16s rRNA, uidA and lac y genetic confirmations. From the rest (4/11) revealed a possibility to be E coli with tnA or citT gene mutations. However, the indole negative- citrate positive S4 colony from an unprotected well can be Salmonella species while indole positive-citrate positive S7, S8 and S9 colonies from a protected well can be Klebsiella species as well. Considering both biochemical and molecular identifications, S9 can be a uidA gene mutated K-12 strain E coli or Salmonella enterica with β-glucuronidase activity exerted by mutated β-galactosidase activity, while S7 and S8 can be lac y gene acquired Salmonella enterica. Furthermore, S4 can be a CitT gene mutant E coli K-12 BW25113 strain. Significantly, 7/11 colonies were tet A resistant while all were tet C negative with 5/11 colonies providing non-specific amplicons of 550 bp instead of 350 bp. Thereby, the water collected from the tap, 3 unprotected wells and 1 protected well revealed the highest possibility to have E coli with tetracycline efflux pumps coded by the tet A gene.

Key words: E coli, Drinking water, Tetracycline resistance.

INTRODUCTION

Antibiotic resistance; a global dilemma.

Antibiotics have immensely contributed to save innumerable lives ever since the discovery of penicillin in 1928. Antibiotics are efficient drugs for bacterial growth inhibition and death (Kraemer, Perron and Ramachandra, 2019). Since one hundred years past from inventing antibiotics, bacteria have evolved to gain resistance against antibiotics creating a global threat at present. Bacteria's capability to grow and proliferate within the nearness of normally dynamic antimicrobial medications is defined as 'antibiotic resistance'(AR) (Buza et al., 2016). In Europe, 250 000 individuals die annually from antibiotic resistance related infections (Barter et al., 2015). Moreover, antibiotic resistance is reported among 500 000 individuals with suspicious bacterial infections across 22 countries (WHO, 2018). If this crisis remains unsolved, even minor therapeutics will resist treatment. Consequently, an immediate solution is required before a

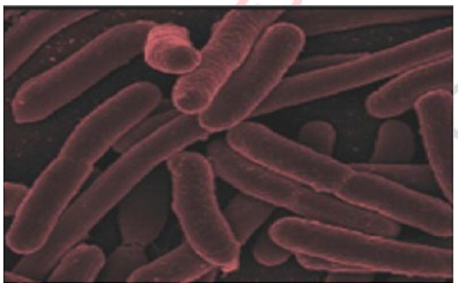
dawn of a post antibiotic era (Essack, Founou and Founou, 2017).

Role of drinking water in dissemination of antibiotic resistance

Drinking water sources; tanks, rivers lakes and tap water is an ideal setup for bacteria to gain AR due to accumulated growth factors and antibiotics (Esmaeilieh et al., 2018). Antibiotics and antibiotic resistant bacteria (ARB) released from industrial waste waters, agriculture, hospital effluents and from incomplete metabolism of humans and animals to drinking water sources, escalates AR in bacteria inhabited in water. Thus, consumption of ARB contaminated water propagates AR in commensal bacteria (Alvi et al., 2018; Larsson, 2014; Coleman, 2009).

E coli (figure 1) is a rod-shaped gram-negative coliform which inhabits the intestine of animals and humans (Buza et al., 2016). Though E coli is part of the gut microflora, some pathogenic E coli strains like Enteropathogenic E. coli and Enterohaemorrhagic E. coli can induce a spectrum of diseases including lethal hemorrhagic colitis (Blount, 2015). E coli contact the water via fecal matter and is a fecal indicator (Price and Wildeboer, 2016).

Figure 1.E coli viewed in scanning electron microscopy (Alves et al., 2017).



The main energy source of E coli is glucose while certain E coli K-12 strain mutants can anaerobically utilize citrate unlike other coliforms (Hofwegan, Hovde and Minnich, 2016). Furthermore, E coli possess distinctive genetic markers including the uidA gene (β-glucuronidase enzyme) which is present only in 94% of E coli and the lac y gene (lactose permease) which is absent in other enterobacteria (Balazsi et al., 2019; Gomez et al., 2015).

E coli gain AR by spontaneous mutations, mutating genes from selective pressure due to frequent exposure to sub lethal concentrations of antibiotics or from interactions with its native population or other microbial species by horizontal gene transfer (HGT). HGT occurs via one of the three mechanisms: conjugation, transduction and transformation. (Bountouni, Efstratiou and Kefalas, 2018). Conjugation is the main mechanism of E coli for AR dissemination. In conjugation, the intergrons harboring antibiotic resistant genes (ARG) are directly transferred to E coli in intestine or natural water sources via conjugative genetic elements including transposons and plasmids in which, the propagation is accelerated by vertical gene transfer (Berglund, 2015).

E coli survive well in aquatic environments and is highly adapted for HGT, which makes E coli a prominent vector for ARG dissemination via water (Chen et al., 2017). The prevalence of antibiotic resistant E coli is gradually increasing due to the consumption of water contaminated with AR E coli, which is released again to the environment as a cycle by feces. This is because the resistant bacteria entered to the gut via water can disseminate the ARG to gut E coli as well (Buza et al., 2016).

Tetracycline Resistance

Tetracycline is a broad-spectrum bacteriostatic antibiotic. It interacts with a conserved sequence in 16s rRNA of the

30s ribosomal subunit resulting in interference of aminoacyl-transfer RNA required for protein synthesis (Markley and Wencewicz 2018; Grossman, 2016). Even though tetracycline is not practiced against E coli infections, tetracycline resistance is well reported in commensal E coli which implies that it is due to a bystander effect caused by resistant pathogens treated with tetracycline in humans and animals (Adlerberth et al., 2006). Moreover, this is accelerated as tetracycline is capable to remain undegraded in the aquatic environments for a prolonged period due to the hydrophilicity nature (Daghir and Drogui, 2013).

Ribosome protection, tetracycline efflux, enzymatic inactivation and tetracycline modification are mechanisms of tetracycline resistance. The generalized mechanism for E coli is efflux pumps, which regulates the internal environment by pumping antibacterial drugs out of the cell (Soto, 2013). These pumps are encoded by mobile tet A, tet B, tet C, tet D and tet G genes (Idris, Olowe and Taiwo, 2013).

Sulphonamide Resistance

Sulfonamide is a synthetic antibiotic which arrests growth of bacteria by inhibiting di-hydropteroate synthase (DHPS) enzyme activity in the folic acid pathway of E coli. The most common mechanism of sulfonamide resistance in E coli governs by mutations in the folP gene which codes for the DHPS enzyme (Barbe et al., 2019). Mutated DHPS enzyme provides a lower affinity to sulfonamide while, increasing or maintaining the affinity for the p-aminobenzoic acid (Hassanein, 2019). Clinical resistance to sulfonamide is predominated by plasmid-borne mechanisms mediated by mobile sul 1, sul 2, sul 3 and sul 4. Each sul gene encode for an altered DHPS enzyme providing reduced affinity for sulfonamide (Gundogdu et al., 2011).

The significance of the project.

'Antibiotic resistance' is a current emergence requiring proper acknowledgement. Among various modes of ARG dissemination including food, agriculture and livestock, the water sources hold a prominent place due to the high microbial diversity (Loh et al., 2018, Moreira, Nunes and Manaia, 2014). Thus, scrutinizing the profile of AR in the water holds a timely significance to discover a solution (Pruden et al., 2018). This study focuses on isolating and detecting tetracycline and sulfonamide resistant E coli in drinking water from the highly populated GNT (figure 2), which is constantly affected with floods making it a possible hotspot for antibiotic resistant E coli (National Disaster Management Centre, 2012). This can facilitate future research to comprehend the dissemination of the ARG in the environment. Thereby, strengthens the prospects in discovering solutions to overcome the global crisis of antibiotic resistance related infections.

Figure 2. Location of the GNT (Lankaland n.d.)

METHODOLOGY

Sample Collection and measurement of the physical parameters.

Drinking water was collected from 10 locations in GNT (table 1) within 48 hours prior commencement. The physical parameters including, temperature, pH and the chlorine concentration of each water sample was measured.

Table 1. The 10 locations of GNT

Sample label	Sub town	Well status
1	Kajughawatta	Tap water
2	Kajughawatta	Protected well
3	Kajughawatta	Protected well
4	Kajughawatta	Protected well
5	Kotikawatta	Unprotected well
6	Kotikawatta	Unprotected well
7	Kotikawatta	Protected well
8	IDH town	Protected well
9	Kotikawatta	Protected well
10	Kotikawatta	Unprotected well

Membrane filtration

From the 10 water samples, 100 ml were transferred onto Buchner funnels containing 0.45 µm pore membrane which was then placed on the HiCrome E. coli media creating duplicates for each sample including the positive and the negative control. The petri plates were incubated at 37 ˚C for 24 hours.

Isolating E coli by culturing techniques.

From the chromogenic media, 10 significant colonies were inoculated in LB and were incubated at 37˚C for 24 hours. The incubated broths were quadrant streaked on NA and were incubated at 37˚C for 24 hours. The most isolated 10 colonies from each plate and an extra 11th colony were inoculated in LB and were incubated at 37˚C for 24 hours.

Biochemical analysis.

Indole test

The LB cultures were inoculated on tryptone medium containing test tubes. They were incubated at 37˚C for 24 hours. Afterwards, 5 ml of the Kovac's reagent was poured to each inoculant and the colour change was observed.

Simmons' citrate test

The LB were inoculated on Simmons'-citrate medium using a sterilized inoculation loop and were incubated at 37˚C for 24 hours.

Morphological analysis; gram staining.

The LB cultures were mixed with the water drop placed on each glass slide, was heat fixed and was left to cool down. Crystal Violet, Grams Iodine, Gram's decolorizer, and Safranin were poured to the slide and was kept for 1 minute, 1 minute, 2 seconds and 1 minute respectively prior washing at each step. After air drying, they were observed at 40x and 100x.

DNA extraction (Promega-Kit).

LB inoculants of 1 ml were subjected to a series of centrifugation at 1300 rpm followed by adding nuclei lysis, RNAse, and protein precipitation solutions respectively till thread like DNA were visible. After centrifugation, 70% ethanol was used to aspirate the DNA pellet.

Visualization of the extracted genomic DNA.

Extracted genomic DNA of 5 µl was combined with 2 µl of 5x Taq buffer. They were loaded into the wells of the 0.8% agarose gel immersed in 1X TAE. The gel

electrophoresis was proceeded for 20 minutes at 45 V. The bands were visualized using the UV-trans-illuminator.

Amplification and visualization of the 16srRNA, uidA and lac y genes.

Three separate master mix was prepared for a total volume of 25 µl each by adding a reaction mixture containing 5 µl of 5 x PCR buffer, 1.5 µl of 25 mM MgCl2, 0.625 µl of 10 mM dNTP, 2.5 µl of 2.5 µM forward and reverse primers respectively to each master mix according to table 2 followed by the addition of 0.25 µl of 5µl-1 Taq DNA polymerase, 1.5 µl of genomic DNA and 11.125 µl of distilled water.

Table 2. The primer sequences of 16s rRNA, uidA and lac y genes (Molina et al., 2015, Lobersli et al., 2016).

Gene	Primer sequence	Amplicon length (bp)
16s rRNA	F: GTTGTAAAGCACTTTGAGTGGTGAGGAAGG R: GCCTCAAGGGCACAACCTCCAAG	424
uidA	UAL (F): TGGTAATTACCGACGAAAACGG UAR: ACGCGTGGTTACAGTCTTGCG	147
lac y	F: ACCAGACCCAGCACCAGATAAG R: TTCTGCTTCTTTAAGCAACTGGC	104

The reaction mixture in micro-centrifuge tube was amplified for 35 cycles at each amplification in a thermocycler PCR system by following separate PCR parameters for the 16s rRNA. uidA and lac y gene amplifications. For the 16s rRNA PCR the initial denaturation of 94 ˚C for 2 minutes, denaturation at 94 ˚C for 30 seconds, annealing at 55 ˚C for 45 seconds, extension at 72 ˚C for 1 minute and 30 seconds and final extension at 72 ˚C for 10 minutes were carried out. For the uidA gene PCR initial denaturation at 95 ˚C for 3 minutes, denaturation at 95 ˚C for 30 seconds, annealing at 58 ˚C for 30 seconds, extension at 72 ˚C for 1 minute and final extension at 72 ˚C for 10 minutes were carried out. For lac y gene amplification, initial denaturation at 94 ˚C for 3 minutes, denaturation for 1 minute, annealing at 58 ˚C for 30 seconds, extension at 72 ˚C for 1 minute and the final extension at 72 ˚C for 5 minutes were carried out.

Each amplicon of 2 µl was loaded into the wells of the 2% agarose gel followed by 3 µl of 100bp ladder. The gel electrophoresis was proceeded for 15 minutes and then for 20 minutes at 50 V and 45 V respectively and the bands were visualized using the UV-transilluminator.

Antibiotic sensitivity test.

Overnight LB cultures of the S1, S2, S3, S4, S5, S6, S7, S8, S10, S11 colonies which produced amplicons for all the 3 genes, were inoculated in 1 ml quantity on test tubes containing 5 ml distilled water. The turbidity was compared with the 0.5 Mc Farland solution and was equalized by adding the relevant LB.

The inoculants were streaked using cotton swabs on Muller Hinton agar plates. The antibiotic discs of tetracycline, sulfonamide, and paper dipped in distilled water (negative control) were placed on the relevant section of the plate labeled and was incubated at 37˚C for 24 hours. The diameters of the zones were measured

and was categorized according to the CLSI standard according to table 3 (Hudzicki, 2009).

Table 3. The CLSI standard of classification for tetracycline and sulfonamide resistance, intermediate and sensitivity.

Antibiotic	Tetracycline (mm)	Sulfonamide (mm)
Sensitive	Equal or 15<	>16
Intermediate	12 -14	11-15
Resistant	Equal or 11>	Equal or 10>

Amplification and visualization of tet A and tet C genes.

Two separate master mix were prepared with a total volume of 25 µl by adding a reaction mixture containing 5 µl of 5 x PCR buffer, 1.5 µl of 25 mM MgCl2, 0.625 µl of 10 mM dNTP, 2.5 µl of 2.5 µM forward and reverse primers for tet A and tet C respectively to each master mix according to table 4 followed by the addition of 0.25 µl of 5µl-1 Taq DNA polymerase, 1.5 µl of genomic DNA and 11.125 µl of sterile distilled water.

Table 4. The primer sequences of tet A and tet C genes (Chen et al., 2013).

Gene	Primer sequence	Amplicon length (bp)
tet A	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	210
tet C	F: TGCGTTGATGCAATTTCTATGC R: GGAATGGTGCATGCAAGGAG	335

The reaction mixtures in micro-centrifuge tube were amplified for 40 cycles at each amplification in a thermocycler PCR system by following separate PCR parameters for the tet A and tet C gene amplifications. For tet A PCR, initial denaturation at 95 ˚C for 5 minutes, denaturation at 95 ˚C for 20 minutes, annealing at 60 ˚C for 30 seconds, extension at 72 ˚C for 30 seconds and final extension at 72 ˚C for 10 minutes were followed. For tet C PCR, initial denaturation at 95 ˚C for 5 minutes, denaturation at 95 ˚C for 20 seconds, annealing at 64 ˚C for 40 seconds, extension at 72 ˚C for 30 seconds and final extension at 72 ˚C for 10 minutes were followed.

Each amplicon of 2 µl was loaded into the wells of the 2% agarose gel followed by 3 µl of 100bp ladder. The gel electrophoresis was proceeded for 15 minutes and then for 20 minutes at 50 V and 45 V respectively and the bands were visualized using the UV-transilluminator.

RESULTS

Membrane filteration on HiCrome media

Figure 3. Membrane filtration duplicates of the HiCrome petri plates of the selected 10 colonies and positive control (PC) (E coli ATCC 25922) on HiCrome

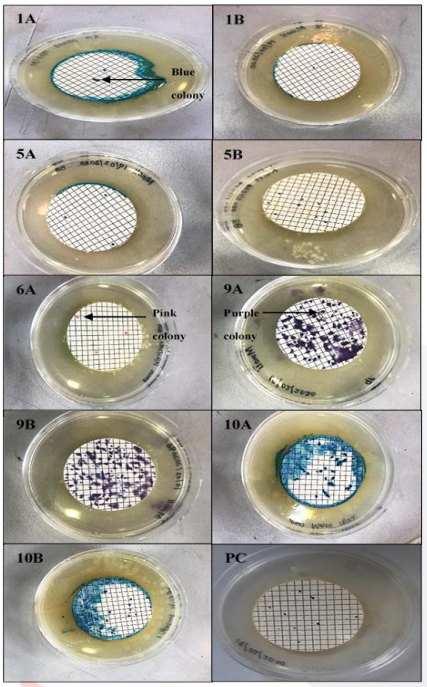


Table 5. The HiCrome media plates with the chosen colonies

Sample Identity	Blue	Purple	Pink	Opaque
1A	✓	-	-	-
1B	✓	-	-	-
2A	-	-	-	-
2B	-	-	-	--
3A	-	-	-	-
3B	-	-	-	-
4A	-	-	-	-
4B	-	-	-	-
5A	✓	-	-	✓
5B	✓	-	-	-
6A	-	-	✓	-
6B	-	-	-	-
7A	-	-	-	-
7B	-	-	-	-
8A	-	-	-	-
8B	-	-	-	-
9A	-	✓	-	-
9B	-	✓	-	-
10A	✓	-	-	-
10B	✓	-	-	-

Sample name	Colony colour indication	✓ :A chosen colony
The chosen blue colony		- :Not choosen
The chosen purple colony		
The chosen pink colony		
The chosen opaque colony		

Table 6. Chosen colonies from the S1 – S11 the streak plates.

Plate Number	Sample identity	Colony identity
1	1A Blue	S1
2	1B Blue	S2
3	5A Blue	S3
4	5A Opaque	S4
5	5B Blue	S5
6	6A Pink	S6
7	9A Purple colony 1	S7
8	9A Purple colony 2	S8
9	9B purple	S9
10	10A Blue	S10
11	10B Blue	S11

Biochemical analysis.
Indole test

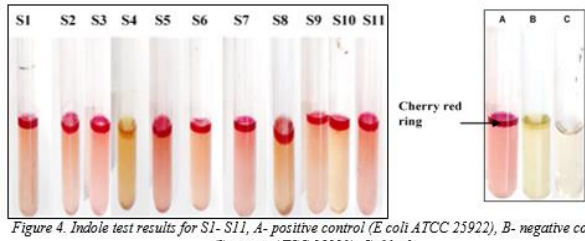


Figure 4. Indole test results for S1- S11, A- positive control (E coli ATCC 25922), B- negative control (S aureus ATCC 25923), C- blank.

Simmons' Citrate test.

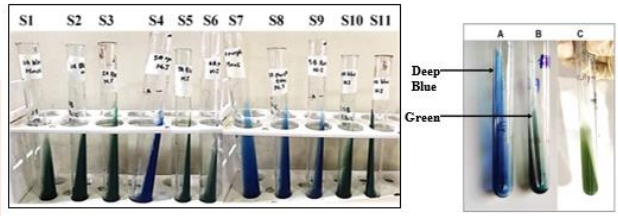
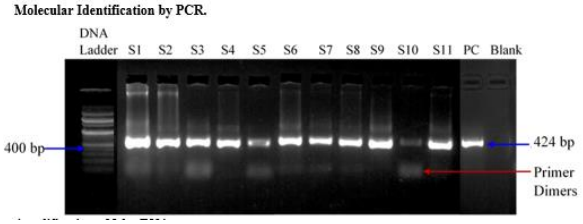


Figure 5. Simmons' citrate test; A– Positive control (E coli ATCC 25922), B-negative control (S aureus ATCC 25923) C-Blank.

Table 7.The summative of the biochemical and the morphological analysis.

Colony identity	Indole test	Simmons' citrate test	Gram staining test
S1	✓	X	Pink, rods & cocci
S2	✓	X	Pink, rods
S3	✓	X	Pink, rods
S4	X	✓	Pink, rods & cocci
S5	✓	X	Pink, rods
S6	✓	X	Pink, rods
S7	✓	✓	Pink, rods
S8	✓	✓	Pink, rods
S9	✓	✓	Pink, rods
S10	✓	X	Pink, rods & cocci
S11	✓	X	Pink, rods

Interpretation	Symbol
Positive indication	✓
Negative indication	X
Not considered	-



Amplification of 16s rRNA.

Figure 6. 2% agarose gel image for 16s rRNA gene amplification. Well 1-band present(100 bp DNA ladder), Well 2 -band present (S1), Well 3 -band present (S2), Well 4 -band present (S3), Well 5 -band present (S4), Well 6 -band present (S5), Well 7 -band present (S6), Well 8 -band present (S7), Well 9 -band present (S8), Well 10 -band present (S9), Well 11 -band present (S10), Well 12 -band present (S11), Well 13- band present (positive control; PC), Well 14- band absent (blank).

Amplification of uidA gene

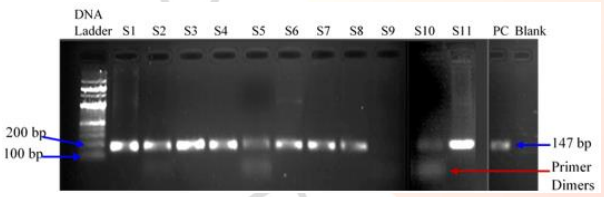


Figure 7. 2% agarose gel image for uidA gene amplification. Well 1-band present (100 bp DNA ladder), Well 2-band present (S1), Well 3 -band present (S2), Well 4 -band present (S3), Well 5 -band present (S4), Well 6 -band present (S5), Well 7 -band present (S6), Well 8 -band present (S7), Well 9 -band present (S8), Well 10 -band absent (S9), Well 11 -band present (S10), Well 12 -band present (S11), Well 13- band present (positive control; PC), Well 14- band absent (blank).

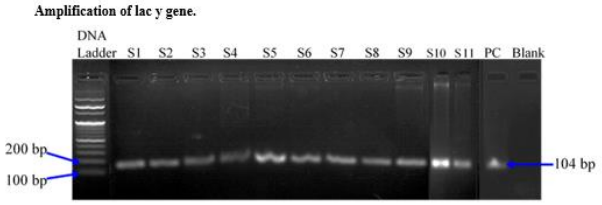


Figure 8. 2% agarose gel image for 16s rRNA gene amplification. Well 1-band present (100 bp DNA ladder), Well 2 -band present (S1), Well 3 -band present (S2), Well 4 -band present (S3), Well 5 -band present (S4), Well 6 -band present (S5), Well 7 -band present (S6), Well 8 -band present (S7), Well 9 -band present (S8), Well 10 -band present (S9), Well 11 -band present (S10), Well 12 -band present (S11), Well 13- band present (positive control; PC), Well 14- band absent (blank).

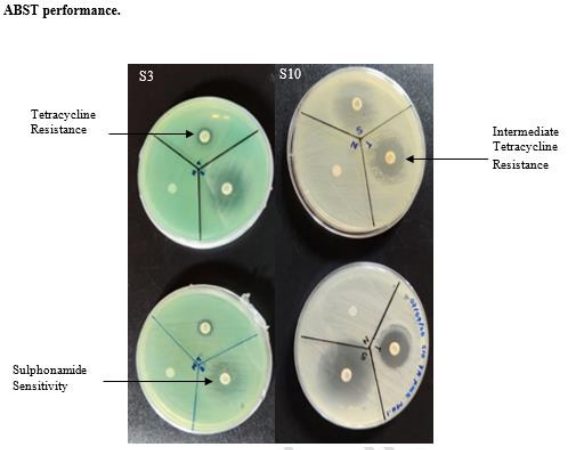


Table 8.The zone diameters measured for tetracycline resistance.

Colony Identity	Mean (mm) ± Standard deviation	CLSI category
S1	10.75 ± 2.44	Resistant
S2	08.75 ± 0.35	Resistant
S3	08.25 ± 0.35	Resistant
S4	10.00 ± 0	Resistant
S5	11.00 ± 4.24	Resistant
S6	07.50 ± 0	Resistant
S7	10.50 ± 0.71	Resistant
S8	07.75 ± 0.35	Resistant
S9	-	-
S10	13.50 ± 0.71	Intermediate Resistant
S11	13.00 ± 4.24	Intermediate Resistant

Visualisation of the tet genes amplification.
tet a.

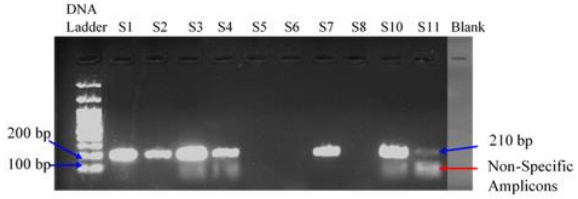


Figure 10. 2% agarose gel image for tet A gene amplification. Well 1-band present (DNA ladder 100 bp), Well 2 -band present (S1), Well 3 -band present (S2), Well 4 -band present (S3), Well 5 -band present (S4), Well 6 -band absent (S5), Well 7 -band absent (S6), Well 8 -band present (S7), Well 9 -band absent (S8), Well 10 -band present (S10), Well 11 -band present (S11), Well 12- band absent (blank).

tet C.

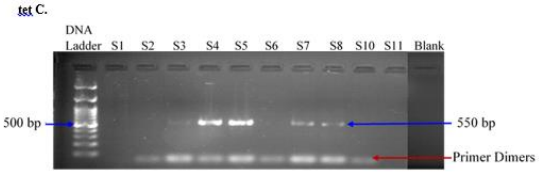


Figure 11. 2% agarose gel image for tet C gene amplification. Well 1-band present (100 bp), Well 2 -band absent (S1), Well 3 -band absent (S2), Well 4 -band present (S3), Well 5 -band present (S4), Well 6 -band present (S5), Well 7 -band absent (S6), Well 8 -band present (S7), Well 9 -band present (S8), Well 10 -band absent (S10), Well 11 -band absent (S11), Well 12- band absent (blank).

Table 9.The summative of the net analysis

Colony identity	Indole test	Simmons citrate test	Grams staining	16s rRNA	uid A	lac y	ABST Tetracycline	tet A	tet C
S1	✓	X	Pink, rods & cocci	✓	✓	✓	✓	✓	X
S2	✓	X	Pink, rods	✓	✓	✓	✓	✓	X
S3	✓	X	Pink, rods	✓	✓	✓	✓	✓	X
S4	X	✓	Pink, rods & cocci	✓	✓	✓	✓	✓	X
S5	✓	X	Pink, rods	✓	✓	✓	✓	X	X
S6	✓	X	Pink, rods	✓	✓	✓	✓	X	X
S7	✓	✓	Pink, rods	✓	✓	✓	✓	✓	X
S8	✓	✓	Pink, rods	✓	✓	✓	✓	X	X
S9	✓	✓	Pink, rods	✓	X	✓	-	-	-
S10	✓	X	Pink, rods & cocci	✓	✓	✓	✓	✓	X
S11	✓	X	Pink, rods	✓	✓	✓	✓	✓	X

The 8/10 water samples had tempreature as 29 °C while 2 had 30 °C. The pH was in the range between 6.5-7.9 while the chlorine concentration of all the samples were below 5 ppm. From the 10 colonies choosen from the 20 HiCrome duplicates, 1 blue colony from the plates 1A, 1B, 5A, 5B, 10A and 10B, 1 purple colony from 9A and 9B , 1 opaque colony from the 5A and 1 pink colony from the 6A (table 5, figure 3) were selected for streak plating, in which 10 colonies and 1 extra colony from the 9A plate was selected (table 6).The colonies S1-S11 revealed cherry red colour for the indole test except S5 (figure 4).The S1,S2, S3, S5, S10 and S11 revealed green colour, while S4, S7, S8 and S9 revealed blue colour in the Simmons' citrate test (figure 5). All the 11 colonies were positive for 16s rRNA, uidA and lac y gene amplifications (figure 6, 7, 8) except the S9 colony, which was negative for the uidA gene and was excluded for further testings. From the ABST, S1-S8 demonstrated zone diameters below 11 mm and S10 and S11 between 12-14 mm for tetracycline and above 16 cm for sulphonamide for all the colonies (figure 9, Table 8). S5, S6 and S8 produced amplicons for tet A (figure 10) while all were tet C absent producing 335 bp non

specific bands for S4, S5, S7 and S8 (figure 11).

DISCUSSION

The optimum temperature, pH and the chlorine concentration for the E coli growth were 37℃, 6.50 – 7.08 and 0-5 ppm respectively (Philip et al., 2018; WHO, 2018). The physical parameters observed revealed that all the samples were suitable for E coli growth.

The 0.45 µm cellulose membrane retains coliforms in water including E coli (2-2.5µm) (Zhu, Mustafi and Weisshaar, 2020; Ottenhall et al., 2018).The bile salts in the HiCrome media allows only gram negative E coli growth due to less cell membrane permeability in gram positive bacteria (Cremers et al., 2014).The samples 1, 5 and 10 provided blue colonies (figure 3, table 5) which can be due to the hydrolysis of X-glucuronide in the medium by the beta-glucuronidase enzyme (Lange, Strathmann and Oßmer, 2013). Sample 9 produced purple colonies, which can be due to indoxyl interactions with the cleaved X-glucuronide. This can be further halogenated to produce 5-bromo-6-chloro-indoxyl which can be the reason for the pink colonies observed (Perry, 2017; Perry and Freydie, 2006). However, opaque colonies were also expressed, which have a chance to be Salmonella or Shigella species as certain species does not express the beta-glucuronidase enzyme (Molina et al., 2015). To have a more confirmatory E coli isolation, MacConkey agar can be parallelly utilized which can isolate E. coli from other non-lactose fermenting coliforms based on the lactose fermentation ability (Lupindu, 2017).

Prior performing identification tests, pure colonies were extracted by sub culturing chosen colonies in NA and subsequently obtaining 11 isolated colonies (table 6). Thereby, the specificity and the reliability for testing was increased (Lagier et al., 2015). Furthermore, in future, parallel utilization of MacConkey agar can differentiate E coli from other enterobacteria which would provide a more precise analysis for E coli colony isolation (Wanger et al., 2017).

E coli expresses tryptophanase enzyme coded by the tnaA gene which cleaves L-tryptophan into pyruvic acid, indole and NH3 (Percival and Williams, 2014).Upon addition of the Kovac's reagent, indole positivity can be determined by cherry red condensation, while no colour change (yellow) indicate indole negativity. S1-S11 were indole positive except S4 (figure 4, table 7) (Kriega and Padgett, 2011).However, apart from E coli, Salmonella is indole positive, while Klebsiella species and Shigella species are indole negative (Abdallah et al., 2016; Al-mohanna, 2011). The indole negativity of S4 colony can be due to the mutations in the active sites; D1, D2 and D3 domains in the tryptophanase enzyme or pyridoxal phosphate coenzyme mutations resulting loss of indole production (Li and Young, 2015).

E coli cannot utilize citrate as a carbon source under aerobic conditions as it lacks the citrate transporter though it possesses required enzymes in the tricarboxylic acid cycle for citrate utilization (Reynolds and Silver, 1983). Hence, E coli is Simmons's citrate test negative expressing no colour change (green) which was observed in S1, S2, S3, S5, S6, S10, and S11 (figure 5, table 7). However, E coli reveals possibilities for citrate utilization expressing blue colour indicated by the bromothymol blue detecting alkaline byproducts produced, which was observed from S4, S7, S8 and S9 (Pal, Khatun and Banjari, 2017). This can be due to an insertion in the upstream of citT gene allowing aerobic expression of the citrate transporter (Hofwegen, Hovde and Minnich, 2016). Moreover, they can be mutant K-12 strain E coli expressing a

semi constitutive citrate transport system enabled by citB and citA gene mutations (Hall, 1982). Simmons' citrate test distinguishes E coli from citrate positive Salmonella species and Klebsiella species, while Shigella species show Simmons' citrate negativity (Mikolet, 2014; Al-mohanna, 2011).

As discussed, it can be predicted that the S1, S2, S3, S5, S6, S10, S11 colonies have a high possibility to be E coli while S7, S8 and S9 colonies have a chance to be Salmonella species other than being E coli with a citT gene mutation (Mikolet, 2014). Moreover, the S4 colony can be Klebsiella species other than being E coli with both the tnA and citT gene mutations (Al-mohanna, 2011; Haque and Sao, 2015).

The microorganisms are classified as gram positive and gram negative based on the cell membrane permeability and the cell wall thickness (Thairu, Nasir and Usman, 2014). The polysaccharide layer in the cell wall of the gram negative; E coli dissolves by the decolorizer allowing Safranin to stain prominently, expressing 'pink' colour (Budin et al., 2012).

E coli is 'rod' shape due to the circumferential arrangement of the peptidoglycan cell wall (Chang and Huang, 2014). Morphologically, all the 11 colonies have a high possibility to be E coli as pink rods were present in all (table 7). Nevertheless, cocci shapes were also visualized, which can be due to the shifting of the elongosome (rod) into divisasome (cocci) by the dominated expression of tubulin-like filaments over actin-like filaments governed by the heat stress exposed from the 'heat fixing' during grams' staining (Weiss, 2013). The biochemical and the morphological analysis only provides a general estimation. Hence, a genetic profile analysis is essential for a precise identification of E coli presence.

DNA extracted from the Promega-kit was present in all the colonies except S3, S4, S5 and S10 providing faint bands which can be due to less DNA quantity or by pipetting errors (Lee et al., 2012).

All S1-S11 colonies can be bacteria due to 16S rRNA positivity (figure 6). 16S rRNA region is highly conserved in bacteria facilitating bacterial genus identification (Johnson et al., 2019). Additionally, 16s-23s rRNA sequence internal transcribed spacer region can provide E coli 'species' identification (Magray et al., 2011). High molecular weight smears can be due to excessive amplification, which can be reduced by reducing the cycle number (Hashim, 2016).

All the colonies were uidA positive except S9 (figure 7). uidA gene coding for the β- glucuronidase enzyme is present in 97% of E coli aiding differentiation from other Enterobacteriaceae species (Al-zuwainy and Abid, 2012; Lasalde et al., 2005). Furthermore, β-glucuronidase enzyme plays an important role in forming gall stones in humans. It deconjugates bilirubin diglucuronide producing glucuronic acid and bilirubin that precipitates with calcium ions creating calcium bilirubinate, which is the key component of brown pigmented gallstone and biliary sludge (Al-zuwainy and Abid, 2012). Approximately 94% of E coli species express uidA gene except the pathogenic hemorrhagic E. coli of serotype 0157:H7 (Al-zuwainy and Abid, 2012; Lasalde et al., 2005). Nevertheless, uidA gene is also present in Shigella species (44%) and Salmonella species (29%) including certain species of Yersinia, Citrobacter, Edwardsiella, Hafnia, Staphylococcus, Streptococcus, Corynebacteria, and Clostridium bacteria (Al-zuwainy and Abid, 2012; Barbau-Peidnoir et al., 2018). Hence, a more specific gene is required for amplification to detect E coli more specifically.

Subsequent amplification of the lac y gene coding for the lactose permease enzyme, differentiate E coli subjecting the lactose fermentation ability, which is

generally absent in Salmonella and Shigella species. lac y gene is one of the 3 functional genes in the lac operon, which codes for the lactose permease enzyme (Pavlovic et al., 2011; Eswarappa et al., 2009; Lobersli, 2016). S1-S11 colonies were lac y positive (figure 8). However, Shigella sonnei and Shigella dysenteriae possess lac y gene without the enzymatic activity (Devanga et al., 2018). Hence, Cyd gene which codes for the cytochrome bd complex, which is a vital component in aerobic respiration in E coli can be amplified to differentiate E coli from Shigella species (Awadha, Mawla and Jebor, 2014).

lac y PCR (figure 8) expressed crooked bands, which can be due to gel preparation errors (Abadi, 2015). Faint bands were expressed in uidA and lac y PCR (figure 7, 8), which can be due to insufficient template quantity, poor primer quality and insufficient cycle number (Lorenz, 2012). Optimizing reagent components and the PCR parameters can be implemented as solutions (Hashim, 2016).

As discussed above, from the 16S rRNA, uidA and lac y gene positive amplifications, 10 colonies revealed highest possibility to be E coli. However, the uidA negative S9 expressed purple colour in the chromogenic media. This can be due to a mutation in the primer annealing site which led to non-amplification while expressing the β-glucuronidase enzyme which is identified as a possibility in mutant K-12 E coli strains (Monday, Whittam and Feng, 2001). This model is also supported by the Simmons' citrate test for S9 as S9 was citrate positive implying the presence of mutant K-12 strain E coli (Novel and Novel, 1976). Moreover, S9 have another possibility to be a mutant Salmonella enterica which has lac z gene expressing β-galactosidase enzyme (Leonard, Lacher and Lampel, 2015). Hence, the purple colour expression from the Hi Crome media can also be due to the β-galactosidase enzyme exerting as β-glucuronidase enzyme by chance which is resulted as lac Z and uidA genes are paralogs (Molina et al., 2015; Xiong et al., 2007). In addition when considering both biochemical and molecular analysis in combination, S7 and S8 can be Salmonella enterica with uidA gene, which had also acquired the lac y gene (Miller et al., 2020; Leonard, Lacher and Lampel, 2015). Moreover, S7 and S8 can also be E coli with citT gene mutations (Hall, 1982). Furthermore, S4 can be E. coli K-12 BW25113 strain which lacks indole biosynthetic pathway due to a tnA gene mutation and as a K-12 E coli strain, there is a chance for citT gene mutations (Fernandez et al., 2011; Hall, 1982).

ABST is a useful technique for the identification of the antibiotic resistance in microorganisms by standardization of the antibiotic concentration (Balouiri, Sadiki and Ibnsouda, 2016). There, Mueller Hinton agar is used as it is a loose agar which facilitate better antibiotic diffusion and thereby leads to truer zones (Nassar, Hazzah and Bakr, 2019). The colonies which were positive for all the three PCR amplifications (16s rRNA, uidA and lac y gene), S1- S11 except S9 were proceeded with ABST as these colonies possess the highest possibility to be E coli. There, for tetracycline, S1-S7, S8 colonies revealed resistance while S10 and S11 revealed intermediate resistance (figure 9, table 8). All the colonies were sensitive for Sulfonamide. However, the CLSI classification for the zone diameters is only a categorical interpretation about resistance undermining the necessity for a further certification (Flanagan and Steck, 2017).

The tet A (26%) is predominant in E coli compared to other tet genes; tet C, tet D, tet E, and tet M which is 4% collectively (Tuckman et al., 2007). tet A gene is commonly identified in E coli strains affecting humans and ducks (Hu et al., 2012). The abundance of tet A in E coli

perhaps can be associated a higher dissemination in the environment (Al-Bahry et al., 2016). S1-S4, S7, S10 and S11 were tet A positive (figure 10, table 9). tet C was negative for all the colonies while S3, S4, S5, S7 and S8 expressed 550 bp nonspecific amplicons instead of 350 bp bands (figure 11) (Chen et al., 2013). Non-specific amplicons (figure 11, table 9) can be reduced by choosing a different primer (Hashim, 2016). Primer dimers (figure 6, 7, 11) can be due to complementary primer annealing by chance. To reduce primer dimer formation, the primer concentration can be reduced as the high concentration of the primers in the PCR drives the formation of an initial ternary complex of Taq DNA polymerase with the two oligonucleotides producing primer dimers(Poritz and Ririe, 2014).In addition, different primers can be selected to prevent chance annealing of complementary bases as primer dimers can also be produced due to primer manufacture defects such as sequences containing more than three repeats of sequences of G or C (Biocompare, 2013).

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CONCLUSION

The sample 1, 5, 6, 9 and 10 (10/11 colonies) collected from the tap, 3 unprotected wells and 1 protected well respectively, revealed a higher possibility to possess E coli from molecular identifications and out of this, 7/11 colonies provided expected results as E coli for both the biochemical and the molecular identifications. Furthermore, S9 can be a mutant K-12 E coli with a mutation in the uidA gene or a mutant Salmonella enterica expressing beta glucuronidase activity facilitated by β-galactosidase. The S7 and S8 colonies can be Salmonella enterica with uidA gene and acquired lac y gene or E coli with citT gene mutations. The S4 can be a citT gene mutant E coli K-12 BW25113 strain. Importantly, 7/11 colonies revealed high possibility to possess efflux pumps coded by tet A gene.

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