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DETECTION OF TETRACYCLINE RESISTANCE IN COLIFORM BACTERIA FROM DRINKING WATER SAMPLES, OBTAINED FROM PANADURA, SRI LANKA

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ABSTRACT

The rise and spread of antibiotic resistance promoted by the overuse and misuse of antibiotics is a growing concern with regard to human health in the modern world we live in today. The consumption of water sources contaminated by bacteria, namely Escherichia coli (E. coli) pose a significant threat to health and to assess the safety for domestic human use by detection of antibiotic resistant E. coli in Panadura town, Sri Lanka, 10 drinking water samples were collected from distant locations within the said area. Microbiological techniques comprising membrane filtration, morphological analysis by gram-staining, biochemical tests – both Indole and Simmons's citrate test, followed by molecular assays; 16S rRNA, uidA and lacY gene PCRs were carried out for the confirmation of E. coli. Further to this, an antibiotic susceptibility test (ABST) was carried out using antibiotics tetracycline and sulfonamide following the Kirby-Baur protocol in accordance with CLSI standards for the determination of antibiotic resistance. Finally, the detection of antibiotic resistant genes for both tetA and tetC was carried out by PCR. Out of selected 7 samples of interest being subjected to subculturing after isolation by chromogenic media following biochemical, morphological and molecular assays, samples S1, S3, S6, S8, S9 and S11 are suspected to contain E. coli. With reference to ABST, all suspected samples had responded with sensitivity to sulfonamide. However, for tetracycline, samples S3 and S11 displayed intermediate resistance. Whereas, the samples S6, S8 and S9 indicated complete resistance. Samples S6, S8, S9 and S11 expressed tetA gene whilst the tetC gene assay produced non-specific amplicons. It was confirmed that the samples obtained from Keselwatta, expressing the tetA gene are tetA resistant E.coli.

Keywords: E. coli, Drinking water samples, Antibiotic resistance, Tetracycline, Sulfonamide

INTRODUCTION

In today's context of managing bacterial infections, there is a rapid emergence of antibiotic resistant bacteria, particularly imperiling the efficacy of antibiotics that have been transforming previous medical sciences over many decades. Statistically, nearly 700,000 people die per annum worldwide, due to drug resistant diseases (WHO, 2019). Narrowing it down to a town named Panadura in the country of Sri Lanka, is a residential area with a population of 33,375 as of the year 2020 (World Population Review, 2020). It has been estimated that there has been a high prevalence of acquiring antibiotic resistant genes from water resources, where naturally occurring antibiotic resistant genes and antibiotic resistant bacteria are

selected for and enriched by antibiotics found in sewage and agricultural run-off that result from the wide spread, and increased use of antibiotics (Xi et al., 2009). Some residents of the densely populated town, Panadura rely on consumption of water other than the water supplied by the municipal, such as well water, which is not subjected to prior treatment. In addition, to this day, some localities are situated in close proximity to the Bolgoda river - comprised of two interconnected lakes (North and South) presumed to be the largest national lake covering two-thirds of the Kalutara district (Figure 1), which is rich in bio-diversity (Pathiratne et al., 2007), where the water maybe conveniently made use of in day-to-day life, for domestic / direct purposes. Thereby, the town of Panadura was selected to be evaluated on the basis of the transference of antibiotic resistant genes to human beings.

This comparative study was carried out to assess the prevalence of antibiotic resistance in Panadura town in drinking water samples. The aims of this study comprised the identification of Escherichia coli (E. coli) incorporating microbiological techniques - membrane filtration, morphological analysis - grams staining and biochemical tests – Indole and Simmons' citrate tests as well as molecular assays - 16S rRNA, uidA and lacY gene PCR for the confirmation of E. coli , followed by antibiotic susceptibility testing (ABST) abiding CLSI standards using tetracycline and sulfonamide antibiotics, finally, the detection of antibiotic resistant genes (tetA and tetC) were carried out by Polymerase Chain Reaction (PCR).

Figure 1. The Bolgoda River (left), an unprotected well in Sarikamulla (center) and a protected well in Keselwatta (right)

E. coli and acquisition of antibiotic resistance

E. coli is classified phylogenetically as an Enterobacteriaceae, physiologically as a facultative anaerobe and in nature, E. coli resides ubiquitously as a constituent of the gut microbiome in mammals (Blount, 2015). However, E. coli is concerned with health issues such as urinary tract infections, enteric infections as well as systemic infections in humans (Poolman, 2017). The prominent pathogenic E. coli strain concerned with is the enterohaemorrhagic strain O157:H7 (Pervical and Williams, 2014). Apart from commensal strains, there exist other well-studied intestinal pathotypes classified by their virulence properties causing gastrointestinal diseases, where strains responsible are, for example. the enteropathogenic E. coli and the Enteroinvasive E. coli similar to Shigella strains (Jang et al., 2017).

A study by Lyimo et al. (2016) in Northern Tanzania, described that of 1819 of isolated E. coli tested from 71% of drinking water samples , 46.7% exhibited resistance to more than one antibiotic. Jiang et al. (2013) reported that the average concentrations of 11 antibiotic resistant genes in Shanghai drinking water sources that comprised of sulfonamide and tetracycline genes investigated in E. coli varied from 3.66 x 101 copy/mL to 1.62 x 101 copy/mL.

The 16SrRNA gene - a well-established, universal target gene and phylogenic molecular marker, is sequence specific in the genome of all bacteria. The possession of the identificatory key specific gene, uidA coding for β-glucuronidase enzyme, is a prominent feature of E. coli bacteria that accounts for much of the E. coli bacteria sequenced to

date. (Ragupath et al., 2017). On the other hand, the lacZ gene is unique to both E. coli and Shigella species and the two can be further distinguished upon the absence of the lacY gene in Shigella species (Løbersli et al., 2016). Enzymatic products of both lacY encoding lactose permease enzyme responsible for lactose transport across cytoplasmic membrane and lacZ encoding β-D- galactosidase for cleavage of the disaccharide lactose into both glucose and galactose are both necessary for lactose fermentation (Horakova et al., 2008).

As bacteria have a very short life cycle compared to that of eukaryotes, it allows for rapid emergence of new adaptations in short periods of time. Originally susceptible bacteria may become resistant bacteria through mutations or by acquiring resistant genes. When multicellular organisms, humans or animals (livestock) are subjected to antibiotic therapy, drastic shifts occur in the symbiotic communities. The richness and diversity of human-associated microbiota decreases, exerting selection pressure by antibiotics allowing evolution of antibiotic resistant bacteria harboring antibiotic resistant genes (Zhang et al., 2011). Such entities of bacteria reach the aquatic environments through improper waste water discharges / sewage treatment or disposal (Hernandes et al., 2013). As water bodies are sites of genetic exchange, potentially by horizonal gene transfer (HGT) comprising conjugation, transduction and transformation between phylogenetically distant gram-negative and gram positive bacteria, where surrounding environmental bacteria interact with the resistant bacteria in the vicinity originating from humans or animals through water contaminated by fecal matter (Saima et al., 2020) When antibiotic resistant genes that are not encoded in the bacterial genome, rather in mobile genetic elements such as integrons, transposons or even plasmids, the ability of such entities to be transferred are enhanced (Sommer and Dantas, 2011). Thus, such bacteria harboring resistance genes opportunistically colonize the body of human beings upon consumption of such concerning water sources, via several domestic, direct and indirect routes (Wellington et al., 2013). The propagation of antibiotic resistance is promoted by pollution; by improper wastewater discharge, hospital effluents, farms that make use of large doses antibiotics as growth promoters in animals.

Molecular mechanism of tetracycline resistance include ribosomal modification, enzyme inactivation, efflux pumps and ribosomal protection (Markley and Wencewicz, 2018). Of 40 characterized tet genes to date, tetA, tetB, tetD, tetE and tetG are concerned with the said mechanisms in gram-negative bacteria (Hedayatianfard et al., 2014). Principle tetracycline resistant mechanisms include the efflux pump coded by tetA and tetC genes (Al-Bahry et al., 2016). The well characterized tetracycline efflux pump – TetA is the most frequently occurring tetracycline resistance determinant in gram negative bacteria has an inward-outward mechanism of action within the bacterial cell membrane that exchanges a proton (H+) for the tetracycline molecule against a concentration gradient (Nguyen et al., 2014).

Resistance to sulfonamides by a chromosomal mutation in the folP gene of E. coli, leads to the substitution of phenylalanine residue in the 28th position by an isoleucine residue, resulting in a mutated form of the original DHPS enzyme with a low affinity towards sulfonamide (Tačić et al., 2017). Currently, there are discovered sulfonamide resistant genes sul1, sul2 and sul3 known to be located in different plasmids of the bacteria, coding for alternative forms of DHPS enzyme conferring resistance to sulfonamide (Shin et al., 2015). The sul1 gene being the most

prevalent, is usually located on the 3' conserved region of a class 1 integron, sul2 on small non-conjugative plasmids and where, sul3 is a rare plasmid-borne gene (Byrne-Bailey et al., 2008).

METHODOLOGY

Measurement of physicochemical parameters

The 10 water samples (Table 1) were collected within 48 hours, from distant locations within the same town and were measured for their pH, temperate and chlorine concentrations.

Table 1. Water samples and their sources

Water Sample	Location	Source
1	Sarikamulla	well water (protected)
2	Sarikamulla	tap water
3	Sarikamulla	well water (protected)
4	Sarikamulla	well water (unprotected)
5	Keselwatta	Tube well water (protected)
6	Keselwatta	well water (protected)
7	-	Bolgoda river
8	Keselwatta	well water (unprotected)
9	Keselwatta	well water (unprotected)
10	Keselwatta	well water (protected)

Membrane filtration

Duplicate petri plates were prepared by transferring (100 mL each) of the 10 water samples onto 0.45 µm nitrocellulose pore membrane filters placed on top of Buchner funnels. The membrane filters were placed at center of the plates, containing 25mL of the solidified E. coli chromogenic media and were incubated at 37℃ for 24 hours.

Isolation of E. coli

10 colony colours of interest were obtained separately, and were inoculated into Luria Broth (LB). The tubes were incubated at 37℃ for 24 hours following quadrant streak plating on nutrient agar that were subjected to incubation at the same conditions.

Biochemical tests (Simmons's citrate test and Indole test)

Indole test

The LB cultures were inoculated into test tubes containing tryptone broth, followed by incubation at 37℃ for 24 hours. 5ml of Kovac′s reagent was pipetted into each inoculant and the colour changes were observed.

Simmons's citrate test

The LB cultures were inoculated Simmons's citrate agar test tubes followed by

incubation at 37℃ for 24 hours

Gram-staining / Morphological analysis

The LB cultures were inoculated onto water drops placed on microscopic glass slides, heat fixed and allowed to cool. Crystal violet (1 minute). Grams Iodine (1 minute and 2 seconds), Grams decolouriser and Safranin (1 minute) were added to the slides ensuring the slides were washed prior to each step and were observed under the microscope at 40x and 100x after air drying.

Genomic DNA extraction – (Promega kit)

1ml of each LB culture was subjected to centrifugation at 1300rpm, following the addition of nuclei lysis, RNAse and protein precipitation solutions until threads of DNA strands were obtained. Following centrifugation, the DNA pellet was aspirated with 70% ethanol.

Visualization of extracted Genomic DNA

5 µL of each extracted genomic DNA sample was mixed with 2 µL of 5x Taq buffer and were loaded into wells of 0.8% agarose gel immersed in 1X TAE buffer. The resulting bands were visualised by the UV-trans illuminator.

PCR amplification and visualization of 16S rRNA, uidA and lacY gene assays Three separate master mixes (total volume 25 µL) for each of the gene PCR assays were prepared by pipetting 5 µL of 5x PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 0.625 µL of 10 mM dNTP, 2.5 µL of both forward and reverse primers. This was followed by the separate addition of 0.25 µL Taq polymerase, 1.5 µL genomic DNA and 11.125 µL distilled water. Primer information for the three PCR assays are provided in Table 2.

Table 2. Primer information for 16S rRNA, uidA and lacY genes (Gao et al., 2012, Pavlovic et al., 2011)

Primers	Primer sequence	Amplicon size (bp)
16SrRNA forward	AGAGTTTGATCCTGGCTCAG	424
16SrRNA reverse	GGTTACCTTGTTACGACTT	
*uid*A forward	TGGTAATTACCGACGAAAACGGC	147
*uid*A reverse	ACGCGTGGTTACAGTCTTGCG	
*lac*Y forward	ACCAGACCCAGCACCAGATAAG	104
*lac*Y *reverse*	TTCTGCTTCTTTAAGCAACTGGC	

Reaction mixtures in micro-centrifuge tubes were amplified for 35 cycles using a thermocycler for each of the three gene amplifications (16S rRNA, uidA and lacY). Conditions for 16S rRNA gene PCR - initial denaturation at 94℃ for 30 seconds, denaturation at 94℃ for 30 seconds, annealing at 55℃ for 45 seconds, extension at 72℃ for 1 minute and 30 seconds with final extension at 72℃ for 10 minutes. uidA gene PCR - annealing at 58℃ for 30 seconds, extension at 72℃ for 1 minute and final extension at 72℃ for 10 minutes. LacY gene PCR – initial denaturation at 94℃ for 3 minutes, denaturation at 94℃ for 1 minute, annealing at 58℃ for 30 seconds, extension at 72℃ for 1 minute and final extension at 72℃ for 5 minutes. The amplicons (2 µL each) were run in 2% agarose gel with a 3 µL 100 bp ladder loaded alongside. Agarose gel electrophoresis was carried out for 15 minutes at 50V, then 20 minutes for 45V following the visualisation of the bands under a UV-transilluminator.

Antibiotic sensitivity test (ABST)

1 mL of LB cultures from samples positive for the 16S rRNA, uidA and lacY

gene PCR assays, were inoculated into 5 mL distilled water test tubes. Turbidities were compared with a 0.5 Mc Farland standard,

following their equalization by appropriate amounts of distilled water. All inoculants were spread plate streaked on Muller Hinton agar plates, following the placements of 30 µg tetracycline and 300 µg sulfonamide antibiotic discs alongside distilled water discs used as a negative control, subjected to incubation at 37℃ for 24 hours. The diameters of the relevant zones were measured and classified according to CLSI standards provided in Table 3.

Table 3. Interpretation of zone of inhibition diameters from antibiotics tetracycline and sulfonamide (Hudzicki, 2009)

Antibiotic	**Tetracycline(mm)**	**Sulfonamide(mm)**
Sensitive	Equal or greater than 15	Greater than 16
Intermediate	12 -14	11-15
Resistant	Equal or less than 11	Equal or less than 10

PCR amplification and visualization of tetA and tetC genes

For the samples that resulted in both intermediate resistance and resistance for tetracycline antibiotic in the ABST assay – two separate master mixes were prepared for both tetA and tetC (total volume 25 µL) as previous. Primer information for the two PCR assays are provided in Table 4.

Table 4. Primer information for tetA and tetC genes (Chen et al., 2013)

Primers	**Primer sequence**	**Amplicon size (bp)**
***tet*A forward**	GCTACATCCTGCTTGCCTTC	210
***tet*A reverse**	CATAGATCGCCGTGAAGAGG	
***tet*C forward**	TGCGTTGATGCAATTTCTATGC	335
***tet*C reverse**	GGAATGGTGCATGCAAGGAG	

Reaction mixtures in microcentrifuge tubes were amplified for 35 cycles using a thermocycler for each of the 2 gene PCR assays (tetA and tetC). Initial denaturation and denaturation conditions for tetA and tetC gene PCR - 95℃ for 5 minutes, followed by 20 seconds at the same temperature. Annealing at 60℃ for 30 seconds (tetA) and 64℃ for 40 seconds (tetC). Extension and final extensions at 72℃ for 30 seconds and 10 minutes at the same temperature, respectively. The

amplicons (2 µL each) were run in 2% agarose gel with a 3 µL 100 bp ladder loaded alongside. Agarose gel electrophoresis was carried out for 15 minutes at 50V, then 20 minutes for 45V following the visualisation of the bands under a UV-transilluminator.

RESULTS

All 10 water samples had temperatures of both 29℃ and 30℃, pH ranging from 6.53-7.10 and chlorine concentrations of less than 5 ppm for all sources. 10 individual colonies of interest were selected out of specific 20 membrane filtration petri plates – a single blue colony from sample petri plates 3, 3A, 7, 7A and 9A, a single pink colony from sample petri plates 5, 9 and 9A, a single opaque colony from sample petri plates 6 and 10A that were subjected to quadrant streak plating (Table 5 and Table 6). Further to this, an extra opaque colony was selected from sample quadrant petri plate 4. Of all samples – sample S5 was only negative for indole test (Figure 3) whereas samples S4 and S11 only resulted in blue colour for Simmons' Citrate test (Figure 4) All 11 colonies resulted in bands for the lacY gene (104 bp) (Figure 7). In contrast, out of 11 colonies subjected to the remaining 2 gene PCR assays, 8 colonies (S1, S2, S3, S5, S8, S9, S10 and S11) resulted in bands for 16S rRNA gene (424 bp) (Figure 5) and colonies except for colony S7 resulted in bands for uidA gene (147 bp) (Figure 6). All colonies were sensitive to sulfonamide in ABST, contrasting them from tetracycline where 5 colonies (S1, S6, S8, S9 and S10) were resistant, 2 colonies were intermediately resistant (S3 and S11) and 1 colony (S2) was susceptible for tetracycline antibiotic (Table 7). 3 colonies (S1, S3 and S6) resulted in bands for tetC (335 bp) (Figure 10) whereas 4 colonies (S6, S8, S9 and S11) resulted in bands for tetA (210 bp) (Figure 9). Table 8 summarizes the results obtained in this study.

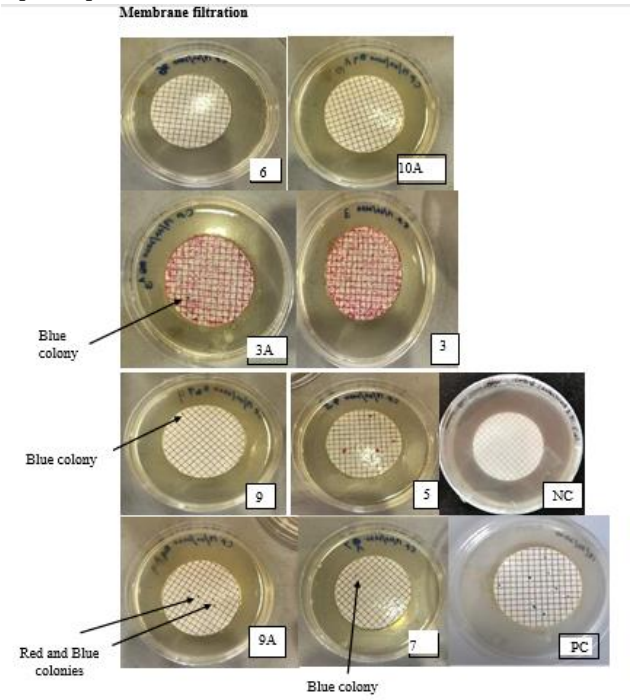

Figure 2. Selection of desired colonies from membrane filtration on E. coli chromogenic agar including positive E. coli control ATCC 25922 (PC) and negative control - distilled water (NC)

Table 5. Results summary table for membrane filtration highlighting the selected colonies

Petri plates	Opaque	Blue	Pink
1	Many	-	-
1A	Many	-	-
2	Many	-	-
2A	Many	-	-
3	Many	One (1 selected)	Many
3A	Many	One (1 selected)	Many
4	Many	-	Many
4A	Many	-	Many
5	Many	-	4 (1 selected)
5A	Many	-	-
6	Many (1 selected)	-	-
6A	Many	-	-
7	Many	One (1 selected)	-
7A	Many	One (1 selected)	-
8	Many	-	-
8A	Many	-	-
9	Many	-	2 (1 selected)
9A	Many	2 (1 selected)	4 (1 selected)
10	Many	-	-
10A	Many (1 selected)	-	-

Table 6. Selected colonies and their identities from the quadrant streak plate

Colony label	Sample identity
S1 (Plate 1)	3 Blue
S2 (Plate 2)	3A Blue
S3 (Plate 3)	5 Opaque in Pink
S4 (Plate 4)	5 Pink
S5 (Plate 5)	6 Opaque
S6 (Plate 6)	7 Blue
S7 (Plate 7)	7A Blue
S8 (Plate 8)	9A blue
S9 (Plate 9)	9A Pink
S10 (Plate 10)	9 Pink
S11 (Plate 11)	10 A Opaque

Biochemical tests

Indole test

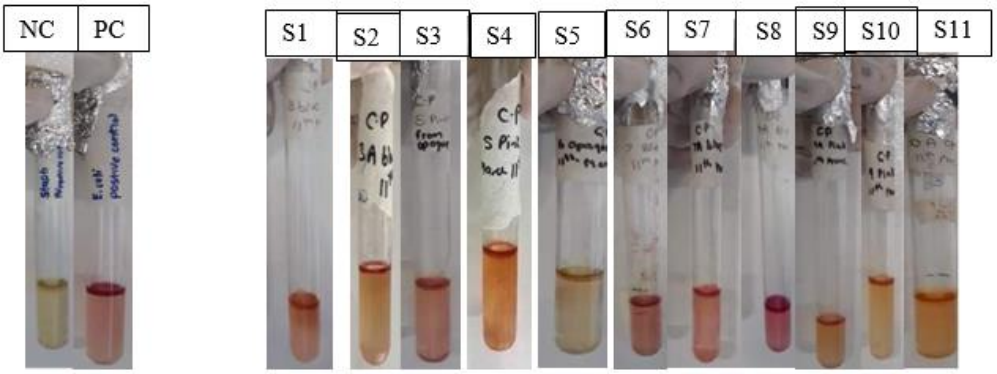

Figure 3. Indole results for - NC (Staphylococcus aureus, ATCC 25923), PC (E. coli, ATCC 25922) and samples S1-S11

Simmons' citrate test

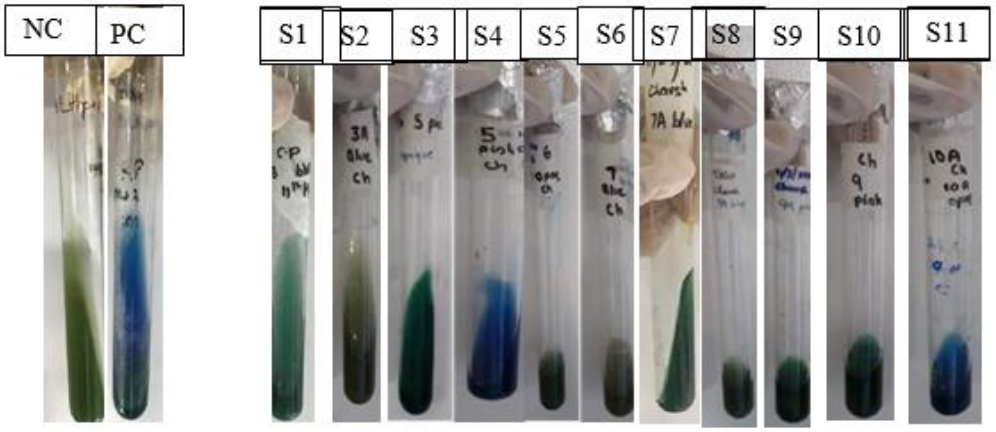

Figure 4. Simmons's citrate results for - NC (E. coli, ATCC 25922), PC (Staphylococcus aureus, ATCC 25923) and samples S1-S11

Gram-staining / Morphological analysis

Pink cocci were observed in samples S1, S4, S5, S8, S9, S10 and S11 whereas pink rods were observed in samples S2, S3, S6 and S7.

PCR (16S rRNA, uidA and LacY gene PCR)

Key - Well 1 : S1, Well 2 :S2, Well 3: S3, Well 4 : S4 , Well 5: S5, Well 6: S6,
Well 7: S7, Well 8: S8 Blue, Well 9: S9, Well 10: S10, Well 11: S11, Blank : Distilled water, PC : E. coli ATCC25922

16S rRNA (424 bp) PCR gel image

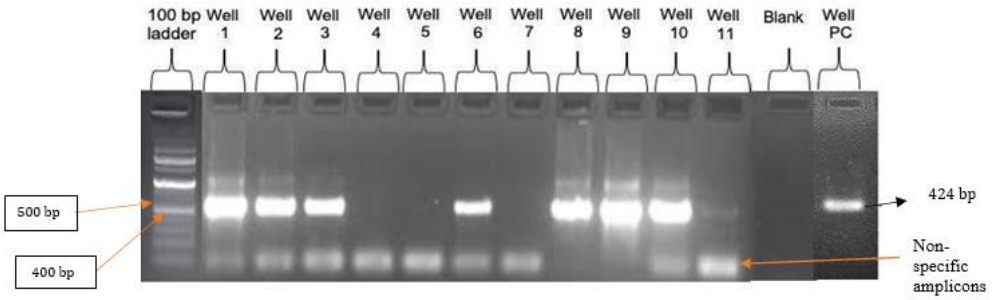

Figure 5. 2.0% agarose gel containing 100 bp DNA ladder, 424 16S rRNA PCR products for S1-S11, blank (distilled water) and positive control (E. coli ATCC 25922)

uidA gene PCR gel image

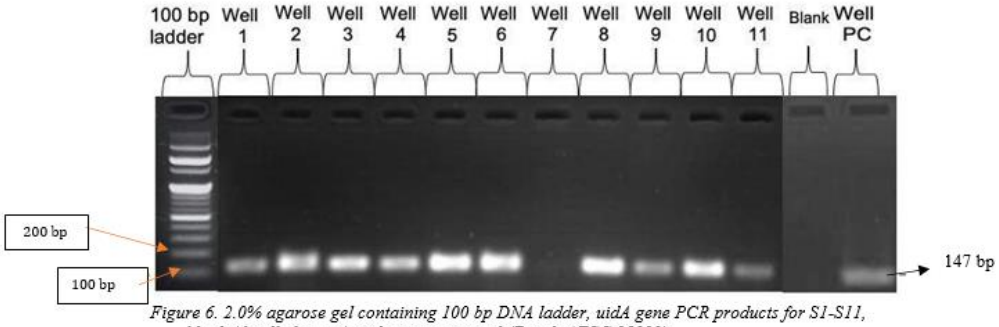

Figure 6. 2.0% agarose gel containing 100 bp DNA ladder, uidA gene PCR products for S1-S11, blank (distilled water) and positive control (E. coli ATCC 25922)

lacY gene PCR gel image

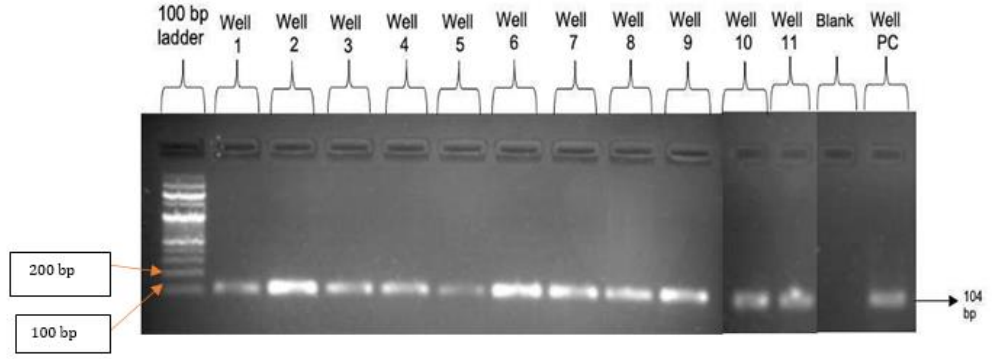

Figure 7. 2.0% agarose gel containing 100 bp DNA ladder, lacY gene PCR products for S1-S11, blank (distilled water) and positive control (E. coli ATCC 25922)

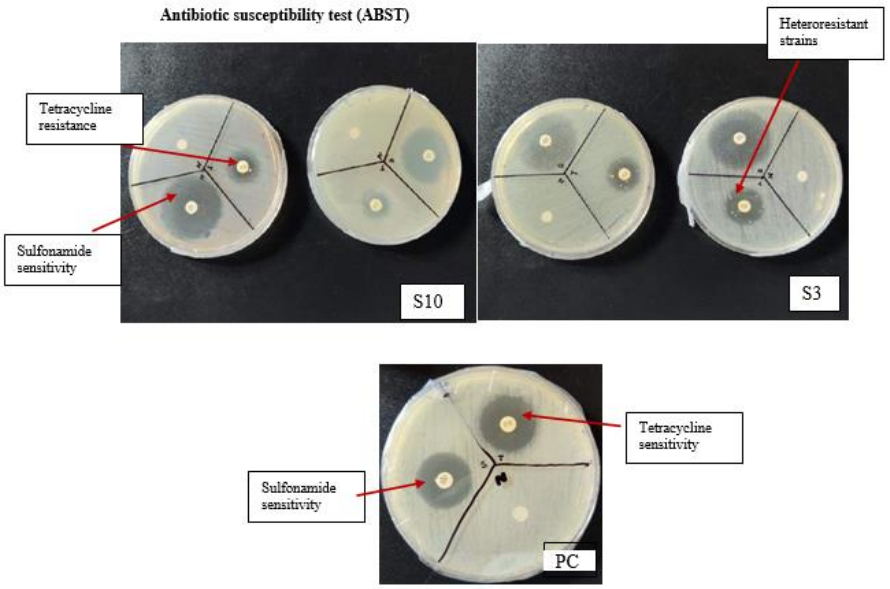

Figure 8. ABST results for S10, S3 and Positive control (PC)
(T – Tetracycline 30 µg, S – Sulfonamide 300 µg, N – Distilled water)

Table 7. Summary for tetracycline ABST results

	Plates 1 and 2	
Results	**30 mg Tetracycline (mean diameter and standard deviation)**	**Inference (According to CLSI standards)**
S1	12 mm ± 1.4	Resistant to tetracycline
S2	28 mm ± 2.1	Sensitive to tetracycline
S3	17 mm ± 2.8,	Intermediate resistance to tetracycline
S6	8.0 mm ± 0.0	Resistant to tetracycline
S8	9.5 mm ± 0.7	Resistant to tetracycline
S9	11.0 mm ± 0.0	Resistant to tetracycline
S10	8.5 mm ± 0.7	Resistant to tetracycline
S11	17 mm ± 1.4	Intermediate resistance to tetracycline
PC	22.8 mm	Sensitive to tetracycline

Tet gene PCR

Key - Well 1 : S1, Well 2 : S3, Well 3 : S6, Well 4 : S8, Well 5 : S9, Well 6: S10, Well 7: S11, Blank: Distilled water.

tetA PCR

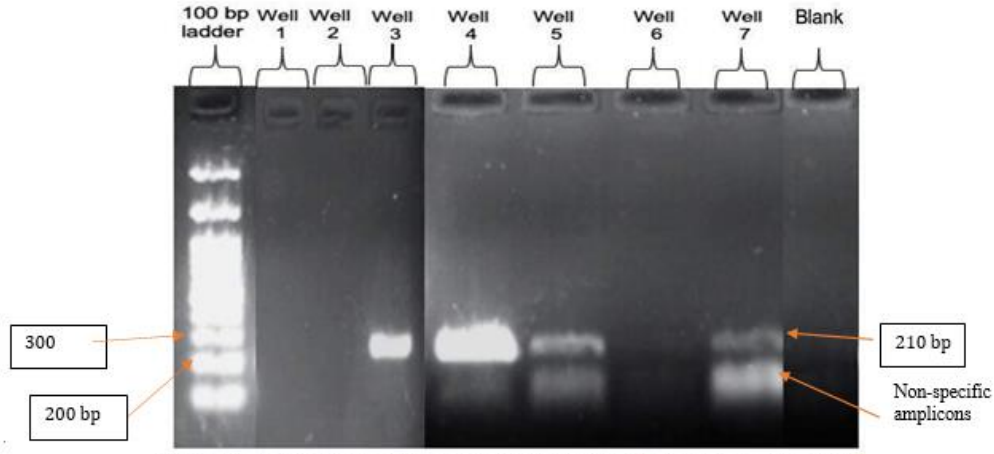

Figure 9. 2.0% agarose gel containing 100 bp DNA ladder, tetA PCR products, including the blank well (distilled water)

tetC PCR

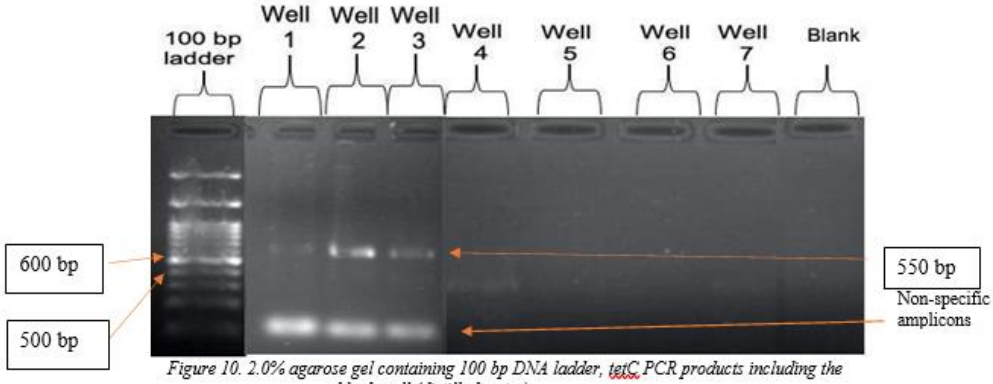

Figure 10. 2.0% agarose gel containing 100 bp DNA ladder, tetC PCR products including the blank well (distilled water).

Table 8. Results summary
(key – positive: +, negative: - and not carried out: N/A)

Sample	Indole test	Simmons citrate test	Grams' staining	16S rRNA	uidA	lacY	Tetracycline ABST	tet A	tet C
S1	+	-	Pink cocci	+	+	+	+	-	-
S2	+	-	Pink rods	+	+	+	-	N/A	N/A
S3	+	-	Pink rods	+	+	+	+	-	-
S4	+	+	Pink cocci	-	+	+	N/A	N/A	N/A
S5	-	-	Pink cocci	-	+	+	N/A	N/A	N/A
S6	+	-	Pink rods	+	+	+	+	+	-
S7	+	-	Pink rods	-	-	+	N/A	N/A	N/A
S8	+	-	Pink cocci	+	+	+	+	+	-
S9	+	-	Pink cocci	+	+	+	+	+	-
S10	+	-	Pink cocci	+	+	+	+	-	-
S11	+	+	Pink cocci	+	+	+	+	+	-

DISCUSSION

In this pilot study, water samples were obtained from distinct areas with high possibilities of contamination considering water sources which are unprotected, protected and in close proximity to waste disposal sites, including the Bolgoda River.

E. coli are able to survive and thrive in secondary habitats with conditions ranging from temperatures of 7℃- 47℃, minimum pH of 4 (Petersen and Hubbart, 2020) and chlorine concentrations less than 5 ppm (Owoseni et al., 2017). In this study, a positive control containing E. coli ATCC 25922 and a negative control containing Staphylococcus aureus ATCC 25923 were taken into consideration. Isolation of E. coli was carried out using membrane filtration technique using E. coli chromogenic agar in order to trap and concentrate the E.coli colonies exhibiting characteristic colony colours to aid in obtaining them (Forster and Pinedo, 2015). Membrane filters of 0.45 µm were used for the retention of E. coli with widths of 1 µm and lengths 2 µm. The chromogenic media used, selective for E. coli growth, inhibits growth of gram-positive bacteria by the presence of bile salts inducing widespread protein aggregation and pro-oxidixing shifts, resulting in disulphide stresses E. coli have the survival advantage of possessing the cystolic chaperone Hsp33 against the highly potent gram-positive microbial

activity of bile salts (Cremers et al., 2014). Duplicate membrane filtration petri plates were

prepared for each sample to improve the validity of the results, which resulted in blue, pink and opaque colonies, respectively. The blue colonies obtained in membrane filtration petri plates 3, 3A, 7A, 9A resulted due to the cleavage of the chromogenic agent X-glucuronide (5-bromo-4-chloro-3-indoxyl-β-D-glucuronide) by the activity of the intracellular enzyme β-D-glucuronidase encoded by the key specific gene uidA native to E. coli (Lange et al., 2013). However, resulting pink-red colonies further may have arisen due to an impurity in the chromogenic media, by the cleavage of halogenated isoform derivative 5-bromo-6-chloro indoxyl instead (Perry and Freydière, 2007). Opaque colonies on the other hand, were a product resulted by the absence of cleaving enzymes (Brown et al., 2011) leading to the absence of colour – likely Salmonella Enteritidis (Manal et al., 2015). Desired colonies from membrane filtration plates were sub-cultured in Luria broth to obtain high yields of viable cell cultures (Low et al., 2013) A non-selective media could be prepared in parallel with the selective media, to help analyze the recovery of the bacteria concerned with, to compare the medias if unexpected results are observed (Alikhani et al., 2007).

Quadrant streak plating was carried on specific colonies following membrane filtration to increase the reliability of the isolated colonies to obtain pure colonies and separate out any mixed colonies obtained during inoculation (Sanders, 2012). Biochemical tests were carried out as primary identification tests in order to aid in the indication of E. coli on the basis of their utilization abilities (Al Human, 2016). The bacteria were tested for their ability to utilize citrate as a source of energy by the enzyme citritase, resulting in acetate and oxaloacetate, where oxaloacetate breaks down further into pyruvate and carbon dioxide. The enzyme oxaloacetate dehydrogenase, converts carbon dioxide into sodium carbonate by shifting towards an alkaline pH resulting in the colour change from green to blue of the pH indicator bromothymol blue (Lupindu, 2017). E. coli generally lacks the citrate transporters so therefore they do not answer the citrate utilization test (Jiang et al., 2020). Samples S4 and S11 resulted in a colour change to blue that might indicate the presence of E. coli K-12 strains (Hofwegen et al., 2016). A mutation involving a tandem duplication of the rnk-citG region of E. coli chromosome, which includes the citrate: succinator antiporter gene, citT may also be responsible, for citrate utilization under anaerobic conditions, resulting in a colour change (Leon et al., 2018) as observed in Sample S11. The indole test was carried out as a classic test to characterize E. coli from coliforms (MacWilliams, 2009) and other enteric bacteria (Chu et al., 2012). The indole test recruited the tryptophanase enzyme to utilize the amino acid tryptophan, releasing indole along with ammonia and pyruvate, leading to the formation of a cherry-red ring at the top of the tube, upon addition of Kovac's reagent (Darkoh et al., 2015). However, the Sample 5 was negative for the indole test. The tnaA gene situated in the tnaCAB operon is responsible for the production of tryptophanase enzyme (Li and Young, 2013). A tetramer assembly disruption by a point mutation may either reduce the efficacy or inactivates the tryptophanase enzyme activity, resulting negative for indole test (Li and Young, 2015). Gram-staining was performed to distinguish gram negative bacteria from gram positive bacteria on the basis of higher lipid content. As E. coli are gram-negative bacteria, during the gram stain procedure, upon application of the decolourizer, the lipid layer dissolves completely, aiding in the retention of the counter-stain, safranin

resulting in red-pink rods morphologies (Tripathi and Sapra, 2020). However, cocci morphologies were resulted. The transformation of rod to cocci may have taken place due to stresses such as heat fixation (Furchtgott et al., 2011). The rod shape of E. coli is maintained by an elongasome (Liu et al., 2019). As a protective mechanism against lethal treatments, the transition in morphologies from rod to cocci may have taken place (Markova et al., 2010).

Molecular assays were performed in order to achieve high specificities and sensitivities in the identification of E. coli on the basis of the expression of 3 known genes sequenced to date. The 16S rRNA gene PCR assay as observed in the agarose gel image of Figure 6 was carried out as it is a highly conserved sequence specific gene in bacteria (Jenkins et al., 2011). Unlike expected, some bacterial colonies did not result in amplicons possibly due to primer target mismatches, resulted by mutations in the 16S rRNA gene sequence, affecting primer annealing (Sambo et al., 2018). Primer dimers were also observed, possibly due to excess primer quantity used (Miyazaki et al., 2017). Bright bands in few samples were obtained due to high DNA content during pipetting. As the uidA gene is a prominent feature of E. coli encoding the enzyme β-D-glucuronidase responsible for blue colonies in membrane filtration, that distinguishes it from other coliforms, this was carried out as observed in Figure 9. Of all amplicons observable, Sample 7 which resulted in a blue coloured colony in E. coli chromogenic media did not produce a band in the uidA gene PCR assay possibly due to a primer-target mismatch or a pipetting error (Lorenz, 2012). The uidA gene is found in 97.7% of E. coli and 2% of Shigella species (Molina et al., 2015). In addition, it is found in low percentages in Salmonella species (Ud-Din and Wahid, 2014). In order to distinguish E. coli species from that of Shigella on the basis that the former harbors the LacY gene and therefore are lactose fermenters (Martínez-Gómez et al., 2012), the LacY gene PCR assay was carried out for all the samples (Ud-Din, and Wahid, 2015).

Antibiotic sensitivity testing by Disc diffusion method was carried out in order to categorize E. coli bacteria on the basis of their sensitivities, in relation to their zones of inhibition for 30 μg tetracycline and 300 μg sulfonamide antibiotic discs used. According to CLSI standards, equal concentration of microbes using the 0.5 McFarland standard, were added to each petri plate containing Mueller Hinton agar in order to maintain the homogeneity of the samples. Mueller Hinton agar is considered to be an overall reliable medium for the susceptibility testing of non- fastidious bacteria, as it is low in sulfonamide, tetracycline and trimethoprim inhibitors (Hudzicki, 2009). In samples S3, S1 and S10 heteroresistance strains had grown within the inhibition zones of 30 μg tetracycline induced by selective pressure of tetracycline antibiotics or by the evolution of new drifts (Gefen et al., 2017). Since all samples were sensitive to sulfonamides, they were disregarded for the sulfonamide gene assay. The samples that gave rise to intermediate and resistant zones of inhibition for tetracycline were appropriate for further analysis, thereby selected for both tetA and tetC PCR assays. Most samples expressed the tetA gene in this study and according to similar studies carried out, proved E. coli conferring the tetA gene majorly, therefore it can be proposed that the said gene predominantly occurs in resistant E. coli bacteria encoding the efflux pump (Huang et al., 2019). As observed in Figure 10, non-specific amplifications of 550 bp were obtained instead of 335 bp in the tetC gene assay. The primers used for tetC, may have incorrectly annealed to a non-target sequence, resulting in primer target mismatches (Green et al., 2015).

Therefore, it can be suggested that, a new set of primers can be designed to eliminate the issue of such non-specific amplicons (Riley et al., 2013). The non-specific amplicons observed in Figure 10 may have resulted due to excessive cycles, long extension / annealing times or low annealing temperatures (Villalba et al., 2017).

CONCLUSION

Samples S6 (Bolgoda river) , S8 (unprotected well) , S9 (unprotected well) and S11, (protected well) obtained from Keselwatta, harboring the tetA gene, confirms the presence of tetA resistant E. coli.

Research limitations, improvements and suggestions

There are a very few biochemical properties that can distinguish enteroinvasive E. coli from Shigella . Therefore, the use of specific markers can be combined with a multiplex PCR for the improved detection of E. coli using 16S rRNA, uidA and lacY genes, taking into consideration that the primers in each assay, do not overlap one another (Kheiri, 2017). In addition, a quantitative PCR assay, can be performed for the quantification of antibiotic resistant genes, in place of a conventional PCR – which is limited to indicating just their presence. The water samples can be utilised directly for the quantitative PCR assay, in order to assess an overall rough estimate of the abundance of tetA and tetC genes loads, from the microbes responsible for harbouring the antibiotic resistant genes, without subjection to tedious post-microbiological techniques. Furthermore, more reliable gene sequences can be assessed. A study conducted by Deng et al 2014., experimentally confirmed that the ycjM gene is enteric specific and the ycjM assay was superior to the commonly used uidA- based PCR method in differentiating enteric E. coli from β-D-glucuronidase positive environmental bacteria. In this study, the number of water samples taken into consideration was low, in order to reinforce the reliability of the outcomes of the study, more samples, covering wider areas can be taken into consideration in the future. Antibiotics other than sulfonamides and tetracyclines can be used, for example amoxicillin and ampicillin antibiotics likewise in relation to different concentrations of the said antibiotics, to see how the corresponding resistant genes respond, towards those antibiotics.

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