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IDENTIFICATION OF E. COLI IN DRINKING WATER SAMPLES IN JAFFNA (VALIKAMAM, WEST) AREA AND DETECTION OF ANTIBIOTIC RESISTANCE

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ABSTRACT

Every year, 1.8 million people worldwide lose their lives due to the exposure to different pathogens in drinking water and foods. Many disease-causing bacteria, especially Escherichia coli (E.coli), have acquired resistance genes, which make antibiotic treatment ineffective. According to the retrospective study in Jaffna, 4% of cases are found with diarrheal illnesses. Therefore, ten samples of drinking water were collected from protected and unprotected wells in the Valikamam west area of Jaffna. E. coli was identified through membrane filtration in HiCrome E.coli agar, the isolated colonies were subjected to grams staining, and biochemical tests. The molecular identification was done targeting 16SrRNA, uidA and lacY genes using PCR. Further, the antibiotic resistance towards tetracycline(30µg), sulphonamide(300µg), and vancomycin (8µg) was analysed for the selected colonies of E. coli by using Kirby-Bauer disk diffusion test. Based on the disk diffusion test results, molecular detection was done for tetracycline targeting genes tetA and tet C by PCR. Out of 10 samples, five samples with bluish green colonies, and two samples with opaque colonies were selected from membrane filtered plates. The antibiotic susceptibility test was conducted on the following five colonies collected from a school in Chulipuram and from residential areas of Moolai and Pannagam, all five showed tetracycline and vancomycin resistance but sensitive to sulphonamide. Except a Moolai sample other four indicated only tetA-resistant gene. The presence of coliforms and other pathogenic organisms, as well as acquired antibiotic resistance genes, could jeopardize the health of community members. This also highlights the nature of water quality and multi-drug resistant bacteria in valikamam, west Jaffna area.

Keywords: Antibiotic resistance, Escherichia coli, Tetracycline, Polymerase Chain Reaction

INTRODUCTION

Globally, food and drinking water contamination is growing as significant issue in public health. Every year 1.8 million people and every day 5000 children are losing their life due to diarrheal diseases like cholera (National Academy of Sciences, 2007). Many disease-causing bacteria especially Escherichia coli (E.coli) has acquired resistance genes, which make antibiotic treatment ineffective. The drinking water must be free from pathogenic microorganisms. Presence of coliforms in aquatic environment indicates faecal contamination. Antibiotic Resistance Bacteria (ARB) is developed by overuse and misuse of human and veterinary antibiotics. It considered as a health issue, when they transfer their genes into non-pathogenic strains (Sanganyado and Gwenz, 2019). In England, E.coli

bacteraemia was increased by 33% within 4 years (Sabtu, Enoch, and Brown, 2015). In 2050, approximately 300 million people will be affected by antibiotics resistance with a loss of up to $100 trillion global economy. In USA there are at least 23,000 people die every year due to antibiotics resistance bacterial infections (Munita and Arias, 2016). Developing countries likes Sri Lanka is facing severe problems. According to the retrospective study which was done in Jaffna, 4% of cases found with diarrheal illness and out of it 35% were children (2.3 ±1 years) (Sathiadas, Mubarak and Arulmoli, 2016). According the mentioned data, detection of ARB and Antibiotic Resistant Genes (ARGs) study need to be carried out to safeguarding people with precautions.

LITERATURE REVIEW

Water is an essential source for all living beings. Ground water is one of the natural sources for water in Asia. It is used for drinking, agriculture, industry and other domestic purposes. In Sri Lanka, about 60% of the people use shallow dug wells as a drinking water source (Mahagamage, Manage and Manage, 2019). There are over 100,000 dug wells and no river, streams and springs in Jaffna peninsula. In Jaffna, the wells which are located near the coastal area have an increased salinity and it causes osmotic stress (Mikunthan et al., 2013). The metabolism pattern of the bacterium will be modified to facilitate changes in cytoplasmic water activity and it accumulates osmoprotectants under osmotic stress (Metris et al., 2014). Bacterial susceptibility to antibiotics also can be reduced in whole-population, because of high-salt condition (Zhu and Dai, 2018). Water borne diseases caused by various bacteria such as E.coli, Shigella spp, Vibrio cholera, and Salmonella are mainly responsible for gastrointestinal illnesses, which show the following symptoms diarrhoea, nausea, vomiting, fever and abdominal pain (Pandey et al., 2014).

E.coli is the most common indicator enteropathogenic bacteria in drinking water contamination. It usually found in environment, foods and the digestive tract of warm blooded animals. E.coli is a group of coliforms which is able to survive in aerobic and anaerobic environments (Wulffen et al., 2016). It appears pink in colour and rod in shape when viewed under a microscope. Most of E.coli strains are not harmful to humans and it helps in the food digestion process. However, 10% to 15% of E.coli acquired virulence factors (enterohemmorhagic verotoxin (Shiga-like toxin)) and has evolved into pathogenic strains that can cause disease in the gastrointestinal, urinary, and central nervous systems (Lim, Yoon and Hovde, 2013). Its survival rate is depend on temperature, pH, salinity, predation, streambed resuspension and sunlight intensity (Blaustein et al., 2012). During the rainfall, human and animals faecal waste with coliforms are washed-out and get into rivers, creeks, stream and ground water (Patoli, Patoli and Mehraj, 2010).

Traditional method of E.coli detection is culturing the sample on selective media with appropriate incubation conditions. Further it can be detected using its enzymatic activity such as tryptophanase (TnaA), β-D-glucuronidase (uidA), β-galactosidase (lac operon genes), acetate utilization, and β-lactamase and lysine decarboxylase. Biochemically, most of E.coli is positive for methyl red, nitrate reduction, indole and catalase reactions. It shows negative for urease, oxidase and citrate reactions (Aryal, 2018). The gene 16SrRNA has a universal distribution which enables the analysis of phylogenetic relationship among taxa. Its sequences are highly dissimilar in bacterial taxa (Vetrovsky and Baldrian, 2013). The 16SrRNA gene is present in all bacteria and it is a housekeeping genetic marker

(Janda and Abbott, 2009). Molecularly, E.coli is targeted through specific set of primers such as 16SrRNA, uidA and lacY, while the presence of these genes confirms the E.coli species. (Naphtali et al., 2019).

Most antibiotic compounds are naturally-produced molecules that can inhibit or kill the bacterial growth and cells (Anderson, 2019). Tetracycline, Sulfonamides and β-lactams are widely used in old medicine for treating human and veterinary bacterial infections (Chen et al., 2017). Antibiotic resistant bacteria cannot be fully inhibited or killed by an antibiotic, even though the drug may have worked effectively before the resistance occurred. Antibiotic resistance can be developed by overuse of antibiotics, using antibiotic for viral infections, not consuming the entire prescribed dosage, poor hygiene, lack of sanitation and genetic mutation. ARB not only develops in the hospital environment, but also can develop in residential area, homes, school and in other public environments (Sabtu, Enoch, and Brown, 2015). ARB can spread by humans or other animals by the mechanisms shown in Figure 1 (Rather et al., 2017). According to Kibret and Abera research work, E. coli isolates were sensitive to gentamicin, nitrofurantoin, ciprofloxacin and chloramphenicol. Further it showed high resistance rates of above 80% to erythromycin, amoxicillin and above 60% to tetracycline (Kibret and Abera, 2011). Resistance to sulfonamides and β-lactams are also detected in E.coli isolates (Chen et al., 2017).

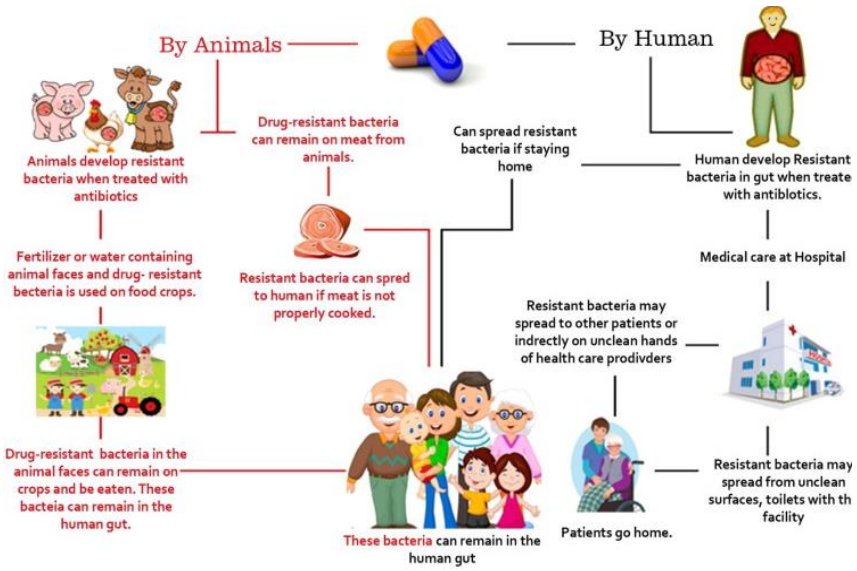

Figure 1: The pathways that lead to the development and spreading of antibiotic resistant bacteria (Rather et al., 2017).

Bacteria have a remarkable genetic response to environmental threats as the presence of antibiotic molecules that may jeopardize their existence. Bacteria can apply two major genetic strategies to adapt to the antibiotic attack. One is the, mutations in genes often associated with the mechanism of action of the compound and the second one is acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (Munita and Arias, 2016).

The bacterial cell which is susceptible for antibiotic can develop mutations in its genes and cause resistance for antimicrobial molecules. The antibiotics eliminate all susceptible bacteria and predominate mutated bacterial population. Predominant bacteria cause antibiotic resistance through following mechanisms, modifications of the antimicrobial target, a decrease in the drug uptake, activation of efflux mechanisms to extrude the harmful molecule and global changes in important metabolic pathways via modulation of regulatory networks (Grossman, 2016). Horizontal Gene Transfer helps bacterial evolution through acquisition of foreign DNA material and also responsible for the development of antimicrobial resistance. Basically horizontal transfer can occur through transformation, transduction and conjugation (Munita and Arias, 2016).

Tetracycline is the most important broad spectrum antibiotic. It is used for treating a variety of human, veterinary and agriculture infections caused by Gram-negative and Gram-positive bacterial pathogens, along with atypical pathogens through inhibiting bacterial protein synthesis by binding to the 16SrRNA of the 30S bacterial ribosome subunit (Markley and Wencewicz, 2018). Bacteria can develop many resistance genes against tetracycline. Its resistance is mediated by four mechanisms such as efflux, ribosomal protection, enzymatic inactivation, and the target modifications which are showed in figure 2 with its respective genes (Tuckman et al., 2007). In this study, tetracycline resistance tetA and tetC genes are considered for detection. Tetracycline-specific efflux pumps, which are transmembrane proteins pumps out toxic materials like intracellular drug in an energy-dependent fashion. It is preventing accommodation of incoming aminoacyl tRNAs at the acceptor site (A-site) (Markley and Wencewicz, 2018). Tet efflux pump belongs to the major facilitator superfamily (MFS). These efflux proteins have six groups based on amino acid sequence. Group 1 includes Tet(A), Tet(B), Tet(C), Tet(D), Tet(E), Tet(G), Tet(H), Tet(Z), and probably Tet(I), Tet(J), and Tet(30)(Chopra1 and Roberts,2001).

Sulfonamides are widely used in clinical and veterinary fields to cure bacterial and protozoal infections. They act as a structural analogue of ρ-amino-benzoic acid and bind to DiHydroPteroate Synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway and cause in the inhibition of dihydrofolic acid formation. E.coli develops sulfonamides resistance through the mutations in the DHPS gene (folP) or the acquisition of an alternative DHPS gene (sul1, sul2 and sul3) (Bailey et al., 2007).

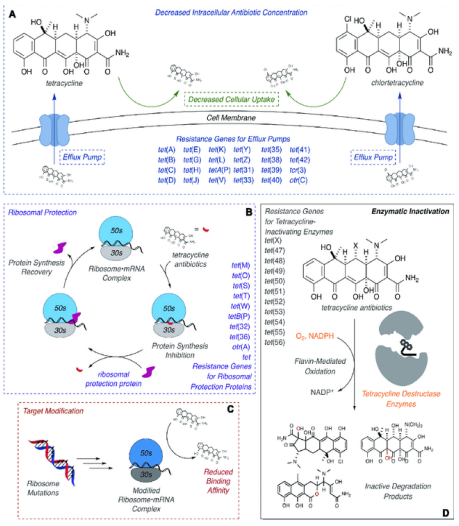

Figure 2: Molecular mechanisms of tetracycline resistance. (A) Efflux, exclusion, (B) ribosome protection, (C) ribosome modification, (D) enzymatic inactivation. Documented resistance genes associated with each type of tetracycline resistance are provided (Markley and Wencewicz, 2018).

Disc diffusion test is an antibiotic susceptibility test in which, antibiotic discs are placed in the agar containing the bacteria. Inhibition of growth around the disc signifies the susceptibility of the bacteria to the antibiotic. Zone of inhibition can be measured to determine susceptibility or resistance according to the Clinical and Laboratory Standards Institute (CLSI) (Giuliano et al., 2019). resistance could be easily prevented by educating people about potential risks associated with improper administration of antibiotics and also providing proper instruction on dosage, frequency of administration and treatment course to the prescribed patient (Rather et al., 2017).

MATERIALS AND METHODOLOGY

Before beginning any work, the working area, equipments were disinfected with 70% ethanol and all the glass wares autoclaved in 1340C and 0.22MPa to maintain aseptic environment. All the microbiological work was done within a sterile field created by a Bunsen burner.

Sample collection and analysis of physiochemical parameters

Ten samples of Drinking water were collected in 375mL bottles from protected and unprotected wells in Valikamam area of Jaffna district (Table 1). The samples were stored in a dark at room temperature until analysis was carried out

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Table 1: Sample collection details for this study

sample	collection area	well appearance	Date of collection	Time (pm)
S1	Pannagam Home	Protected	13.10.2019	12.00
S2	Chulipuram Temple	Unprotected		12.15
S3	Chulipuram School	Protected		12.27
S4	Chulipuram Home	Unprotected		12.39
S5	Moolai Home	Protected		01.09
S6	Moolai Home	Unprotected		01.35
S7	Pannagam Cultivation	Protected		01.48
S8	Sithankerny Temple	Protected		02.00
S9	Pannagam Home	Unprotected		02.15
S10	Pannagam Home	Unprotected		03.00

The temperature, pH and chlorine concentration were analysed in the laboratory using thermometer, pH meter and cloritest papers respectively.

Isolation of E-coli

Samples were analysed using standard membrane filtration. Approximately 100ml of each sample were filtered through filter paper which was kept on the Porcelain funnels. Then filter paper was placed on HiCrome E. coli agar plate. Triplicates were prepared from each sample, E-coli ATCC 25922 strains (Positive control ‘P’) and autoclaved

distilled water (Negative control 'N'). Finally all the plates were incubated at 370C for 18-24 hours.

Subculture

Luria Broth (LB) was dispensed into 15ml falcon tubes. The selected bluish green and colourless colonies from the HiCrome agar plate were inoculated into the broth. Then tubes were then incubated overnight at 37°C for 24 hours. After incubation, all the falcons were stored in the refrigerator. Each subculture was renewed once in two weeks.

Morphological indentification of E.coli by gram staining

Drop of water and subculture were placed on the middle of the slide. Then it was smeared with a loop and heat fixed. Next, few drops of crystal violet were added on to the smear and washed after one minute. Following that, gram's iodine was added and washed after a minute. Then gram's decolouriser was added and immediately washed. Finally safranin was added for minute and rinsed with water. Then it was allowed to air dry and observed under light a microscope at 100x oil immersion.

Biochemical analysis

Indole test

Approximately 5mL Tryptone broth was added into each test tubes. Then isolated bacterium was inoculated into broth. The test tubes were covered with foil paper and placed in the incubator at 370C for 24 hours. After incubation, 500µL of Kovac's reagent was added and colour change was observed.

Citrate Utilization test

Simmons citrate agar 15 mL of was dispensed into each tube and allowed to cool in a slanted position. The tubes were inoculated by streaking the organisms once across the surface. Then all the tubes were incubated 370C for 24 hours, after incubation colour changes was observed.

DNA Extraction by the Promega Wizard® Genomic DNA Purification Kit and boiling cell method and visualization

The genomic DNA was extracted from all selected samples according to promega manufacturer's instructions and boil cell procedure.

The 0.8% agarose gel was used for Genomic DNA visualization. The electrophoresis chamber was filled with 1×TAE buffer and the gel was placed in the tray. Then 5µL of extracted DNA samples were mixed with 2µL of loading dye and loaded into the wells. Then the chamber was connected to a power of 60V for 30 minutes.

Selection of primers and PCR amplification for detection of E-coli

The following sets of primers (16SrRNA and uidA, LacY) were used for the detection of E.coli. The Primer details are given in the following Table 2

Table 2: Primer sets used in this study for detection of E-coli

Gene	Primers (5' – 3')	PCR product Size	Reference
16s rRNA	F: GTTGTAAAGCACTTTGAGTGGTGAGGAAGG	424bp	Nakano *et al*., 2003

	R: GCCTCAAGGGCACAACCTCCAAG		
uidA	F: TGGTAATTACCGACGAAAACGG R: ACGCGTGGTTACAGTCTTGCG	147bp	Fatemeh *et al.*, 2014
lacy	F: ACCAGACCCAGCACCAGATAAG R: TTCTGCTTCTTTAAGCAACTGGC	104bp	Lobersli *et al.*,2016

Table 3: Components of PCR mix for 16SrRNA and uidA, LacY PCR (Adapted Molina et al.,2015).

Reagents	volumes ×1	volumes ×15
sterile distilled water	11.125µL	166.875 µL
5×PCR buffer	5µL	75 µL
10mM dNTPs	0.625µL	9.375 µL
25mM $MgCl_2$	1.5µL	22.5 µL
2.5 mM forward primer	2.5µL	37.5 µL
2.5 mM reverse primer	2.5µL	37.5µL
5U/µL Taq polymerase	0.25 µL	3.75 µL
DNA	1.5µL	-
	25 µL	-

To carry out PCR, the master mix was prepared by adding the respective components except for DNA in given order as shown in the Table 3. Then 23.5µL master mix was transferred into each PCR tube respectively and 1.5 µl of respective DNA sample was added. Then they were kept in the PCR machine and PCR cyclic conditions were set up according to primers as given in Table 4.

Table 4: PCR cyclic conditions for detection of E. coli

Cyclic reactions	*16SrRNA* (Nakano *et al.*, 2003)		*uidA* (Molina *et al.*,2015)		*lacy* (Lobersli *et al.*,2016)	
	Temperature	Time (min)	Temperature	Time (min)	Temperature	Time (min)
Initial denaturation	94^0C	2.00	95^0C	3.00	94^0C	3.00
Denaturation	94^0C	0.30	95^0C	0.30	94^0C	1.00
Annealing	55^0C	0.45	58^0C	0.30	58^0C	0.30
Extension	72^0C	1.30	72^0C	1.00	72^0C	1.00
Final extension	72^0C	10.00	72^0C	10.00	72^0C	5.00
Hold	4^0C	∞	4^0C	∞	4^0C	∞
	35 cycles		35 cycles		30 cycles	

Detection of antibiotic resistance

Disc diffusion technique

The inoculum E.coli was prepared according to 0.5 McFarland turbidity standard its turbidity with standard 0.5 McFarland.. Then inoculum was spread using cotton swabs onto the Muller-Hinton agar plates. The antibiotic discs of tetracycline (30µg), sulfonamide (300µg) and vancomycin (10µg) were selected. The petri plates were divided into four and placed the antibiotic discs and control in the respective side. The plates were incubated at 37°C for 24 hours. At last, zone of inhibition was measured.

PCR amplification

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Tetracycline resistant genes such as tetA and tetC were selected for amplification. The following primers as mentioned in table 5 were used. tetA primer master mix and tetC master mix (Table 6) were prepared and carried out PCR according to the Table 7 PCR cyclic parameters.

Agarose gel electrophoresis

The 2µl PCR products and 3µl of 100bp ladder were loaded in to 2% agarose gel and run at 45V for 35 minutes, 50V for 25 minutes. Once the procedure was done gel image was visualized by using UV transilluminator

Table 5: Antibiotic resistance primers used in this study

Gene	Primers (5' – 3')	PCR product Size	Reference
TetA	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	210bp	Cesare *et al*., 2015
tetC	F: TGCGTTGATGCAATTTCTATGC R: GGAATGGTGCATGCAAGGAG	335bp	Tamminen *et al*., 2011

Table 6: Components of PCR mix (Adapted Molina et al., 2015)

Reagents	volumes ×1	volumes ×15
sterile distilled water	11.125µL	166.875 µL
5×PCR buffer	5µL	75 µL
10mM dNTPs	0.625µL	9.375 µL
25mM $MgCl_2$	1.5µL	22.5 µL
2.5 mM forward primer	2.5µL	37.5 µL
2.5 mM reverse primer	2.5µL	37.5µL
5U/µL Taq polymerase	0.25 µL	3.75 µL
DNA	1.5µL	-
	25 µL	-

Table 7: PCR cyclic conditions for detection of tetracycline resistance

Cyclic reactions	*tet A* (adapted Cesare *et al.*, 2015)		*tet C* (adapted Tamminen *et al.*, 2011)	
	Temperature	Time (min)	Temperature	Time (min)
Initial denaturation	95^0C	5.00	95^0C	5.00
Denaturation	95^0C	0.20	95^0C	0.20
Annealing	60^0C (40 cycles)	0.30	64^0C (40 cycles)	0.40
Extension	72^0C	0.30	72^0C	0.30
Final extension	72^0C	10.00	72^0C	10.00
Hold	4^0C	∞	4^0C	∞

The evaluation of DNA concentrations obtained by the two DNA extraction methods using statistical analysis

Both extracted DNA samples absorbance (260nm, 280nm, 320nm) were obtained using spectrophotometry (10µL sample, 2990µL TE buffer). Then in independent t- test statistical analysis, DNA concentration, both methods (1-kit extraction, 2-boiling cell) were considered as dependent (testing), independent (grouping) variable respectively. Significance difference between two extracted DNA concentrations mean was tested at 5% level of significance.

RESULTS

In this study E.coli was identified by biochemical and molecular techniques on the basis of some enzyme activities.16SrRNA, uidA, lacY primer sets were used for PCR for molecular confirmation of E.coli. The antibiotic resistance of E.coli totetracycline sulfonamide and vancomycin antibiotics was checked through disc diffusion and molecular techniques.

Physiochemical analysis of collected sample

Samples	PH	Temp ^{0}C	[Cl] ppm
S1	7.25	29	‹5
S2	7.35	29	‹5
S3	7.23	29	‹5
S4	7.22	29	‹5
S5	7.52	29	‹5
S6	7.40	29	‹5
S7	7.62	28	‹5
S8	7.55	28	‹5
S9	7.50	28	‹5
S10	7.09	28	‹5
Negative Control (N) autoclaved distilled water	7.05	30	‹5
Positive control (P) *E-coli ATCC25922*	7.06	30	‹5

Table 8: physiochemical parameters obtained results

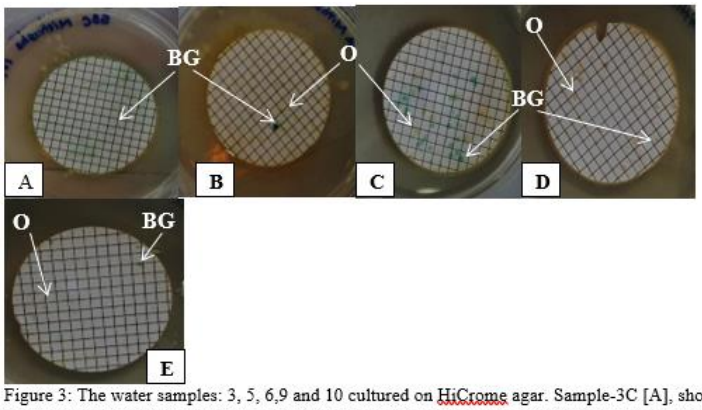

Figure 3: The water samples: 3, 5, 6,9 and 10 cultured on HiCrome agar. Sample-3C [A], showed only bluish green while Sample-5A [B], Sample-6B [C], Sample-9A [D] and Sample-10A [E] showed bluish green and opaque colonies.

Table 9: Summary table of membrane filtrations

Sample	Triplicate						colony selected for sub culture with its label	
	A		B		C			
	BG	O	BG	O	BG	O	BG	O
S1	+	+	+	+	+	+	S1C 'BG'	*
S2	+	+	-	+	-	+	S2A 'BG'	S2B'O'
S3	+	-	+	-	+	-	S3C 'BG'	*
S4	+	+	+	+	+	+	S4A 'BG'	*
S5	+	+	-	+	-	+	S5A 'BG'	*
S6	-	-	+	+	-	-	S6B 'BG'	S6B 'O'
S7	-	-	+	-	-	-	*	*
S8	-	+	-	+	-	+	*	S8C 'O'
S9	+	+	-	+	-	+	S9A 'BG'	*
S10	+	+	-	+	-	+	S10A 'BG'	S10 A'O'
P	-	+	-	+	-	+	*	*
N	-	-	-	-	-	-	*	*

+	colony obseved	BG-Bluish Green colony
-	colony not obseved	O -Opaque colony
*	subculture was not performed	

(Isolation of E-coli ATCC 25922 strains (Positive control 'P') result was not given expected output in membrane filtration. Further analysed was carried out with E-coli ATCC 25922 strains which was given by laboratory supervisor directly.)

Morphological indentification of E. coli by gram staining

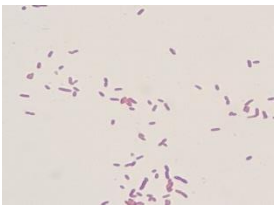

Figure 4: Gram staining of S5A Bluish Green Microscopic view under ×100 showed presence of rod shape pink colour bacteria.

Biochemical analysis

Indole test

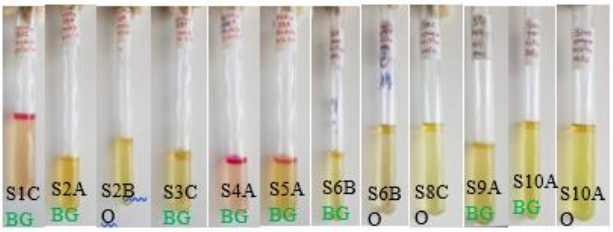

Figure 5: Samples S1A, S4A, S5A bluish green colonies (BG) E-coli ATCC25922 showed pink colour ring. While samples S2A, S3C, S6B, S9A, S10A bluish green colonies and samples S2B, S8A, S10A opaque (O) showed colorless ring formation.

Citrate utilization test

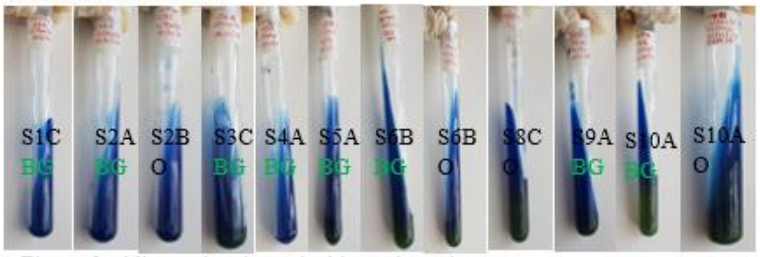

Figure 6: All samples showed a blue colour change.

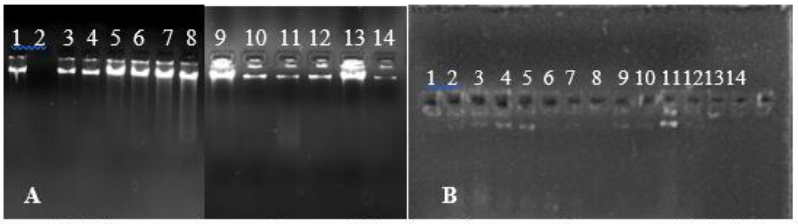

Figure 7: 0.8% agarose gel image of genomic DNA obtained by promega kit extraction (A), boiled cell method extraction (B). All samples' including positive control genomic DNA band was obtained. Negative control showed no band. Sample details showed in Table 3.5

Table 10: Results obtained from genomic DNA electrophoresis both extractions

Well	Sample	Results of DNA extraction	
		kit method	boiled cell
1	Positive control	+	+
2	negative control	-	-
3	S1C Bluish green	+	+
4	S2A Bluish green	+	+
5	S2B colourless	+	+
6	S3C Bluish green	+	+
7	S4A Bluish green	+	+
8	S5A Bluish green	+	+
9	S6B Bluish green	+	+
10	S6B colourless	+	+
11	S8C colourless	+	+
12	S9A Bluish green	+	+
13	S10ABluishgreen	+	+
14	S10A colourless	+	+
+	Genomic DNA was observed		
-	Genomic DNA was not observed		

Results obtained for molecular identification of E. coli

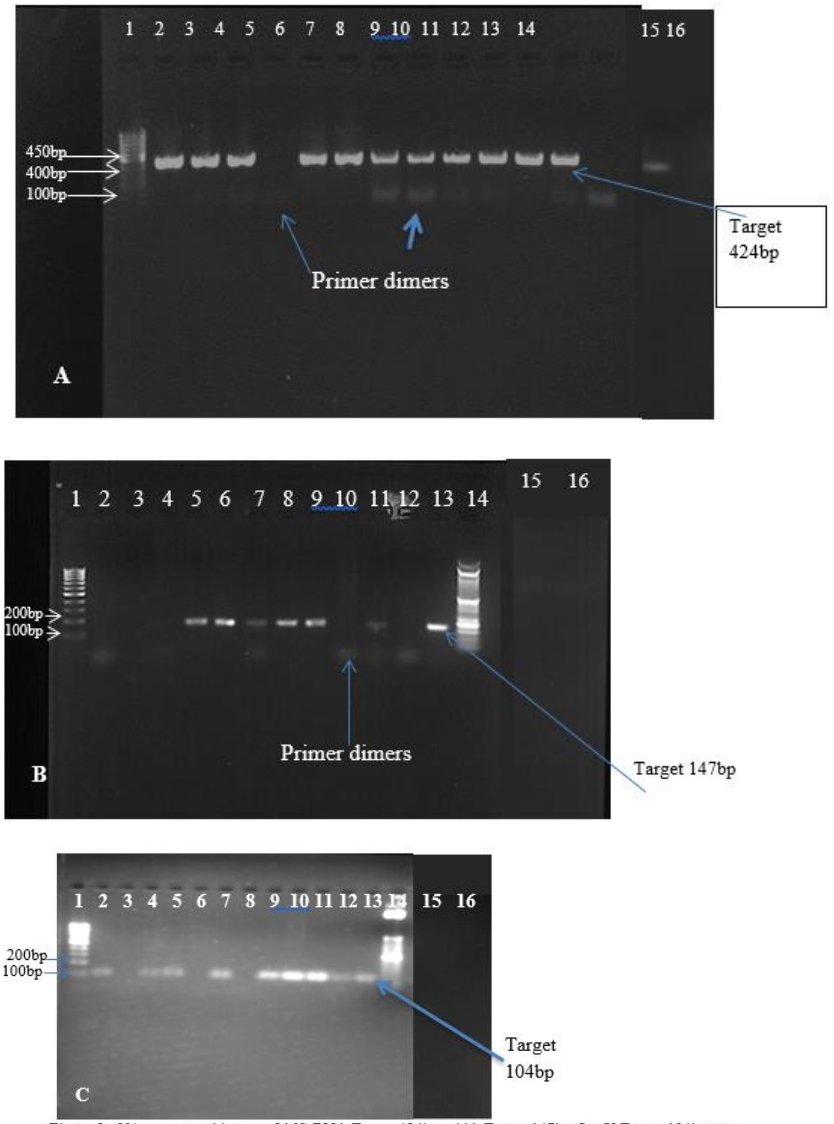

Figure 8: 2% agarose gel image of 16SrRNA Target 424bp, uidA Target 147bp, LacY Target 104bp are shown in [A], [B], and [C] respectively. Sample details showed in Table 3.6

well no	loaded Samples	Molecular detection		
		16SrRNA	*uidA*	*Lac Y*
1	100bp ladder			
2	S1C 'BG'	+	-	+
3	S2A 'BG'	+	-	-
4	S2B 'O'	+	-	+
5	S3C 'BG'	-	+	+
6	S4A 'BG'	+	+	-
7	S5A 'BG'	+	+	+
8	S6B 'BG'	+	+	-
9	S6B 'O'	+	+	+
10	S8C 'O'	+	-	+
11	S9A 'BG'	+	+	+
12	S10A 'BG'	+	-	+
13	S10A 'O'	+	+	+
14	50bp ladder	*	50bp ladder	
15	positive control	+	-	-
16	negative control	-	-	-
+	Target base pair was observed			*not used
-	Target base pair was not observed			
	selected as *E. coli*			

Detection of antibiotic resistance

Disc diffusion (Antibiotics susceptibility test)

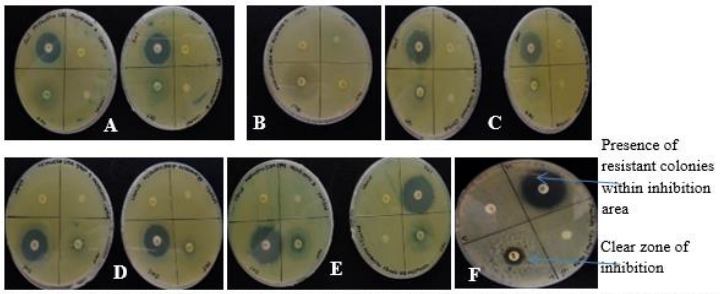

Figure 9: Sample 3C bluish green (duplicate) [A], S6B Opaque (duplicate)[C], S9A bluish green (duplicate) [D], S10A Opaque (duplicate) [E], E-coli ATCC25922 [F] indicated a clear zone of inhibition for sulfonamide (300µg) and Tetracycline (30µg) and vancomycin (8µg) showed presence of resistant colonies. Whereas sample S5A bluish green [B] indicated presence of resistant colonies for tetracycline (30µg), sulfonamide (300µg) and vancomycin (8 µg).

Table 12: Zone of inhibition and interpretation of each antibiotic; Resistant (R), intermediate (I), Sensitive (S)						
Sample	Tetracycline mm	Interpretation	sulfonamide mm	Interpretation	vancomycin mm	Interpretation
S3C 'BG'	9.5±0.71	R	21±0	S	0	R
S5A 'BG'	8.5±0.71	R	28.5±1.5	S	0	R
S6B 'O'	8.5±0.71	R	24.5±0.5	S	0	R
S9A 'BG'	8±0	R	21±0	S	0	R
S10A 'O'	11±0	R	26.5±0.5	S	0	R
Positive control	11±0	R	21±0	S	0	R

Figure 3.6.3: Sample S6B Opaque (duplicate) indicated a clear zone of inhibition for sulfonamide (300µg). Tetracycline (30µg) and vancomycin showed presence of resistant colonies

Detection of antibiotic resistance gene using PCR

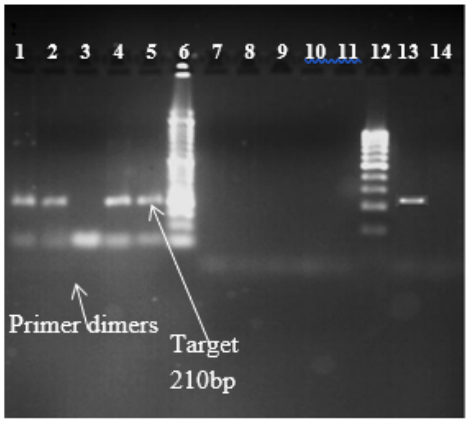

Figure 10 : 2% agarose gel image of (210bp) and *tetC* (335bp) *TetC* gene was not detected. Positive control showed *tetA* band. Sample detains showed in Table 3.7

Table 13.: Gel loading diagram for Figure 10

well	sample	results
tecA 210bp		
1	S3C Bluish green	+
2	S5A Bluish green	+
3	S6B Opaque	-
4	S9A Bluish green	+
5	S10A Opaque	+
6	50bp ladder	
tecC 335bp		
7	S3C Bluish green	-
8	S5A Bluish green	-
9	S6B Opaque	-
10	S9A Bluish green	-
11	S10A Opaque	-
12	100bp ladder	
13	*E.coli ATCC25922* (P) *tetA*	+
14	Negative control (N)	-
+	Target was observed	
-	Target was not observed	

T-Test

Table 14: Group Statistics

	method	N	Mean	Std. Deviation	Std. Error Mean
DNA concentration	1	12	170.0000	208.47	60.17966
	2	12	23.7500	75.80252	21.88230

Levene's test for equality of variances 0.008 which is less than 0.05 so, both DNA concentration equal variances not assumed t statistic value 2.28
The degree of freedom 13.86 Significance value (p) .039

t (13.86) = 2.28, p=0.039 The study found that the kit extracted DNA concentration (170+/- 208.47µg/ml) was numerically significantly higher than the boiling cell extracted DNA concentration (24+/-75.80µg/ml), t (13.86) = 2.28, p=0.039

Table 15: Independent Samples Test

	Levene's Test for Equality of Variances		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
								Lower	Upper
DNA concentration Equal variances assumed	8.461	.008	2.284	22	.032	146.25000	64.03457	13.45043	279.04957
Equal variances not assumed			2.284	13.859	.039	146.25000	64.03457	8.77811	283.72189

Table 16: Both extracted DNA concentration and purity

Sample	Promega kit extraction		Boil cell extraction	
	Concentration (µg/ml)	Purity (A_{260}/A_{280})	Concentration (µg/ml)	Purity (A_{260}/A_{280})
S1C BG	-75	-1.25	15	-
S2A BG	135	1.50	15	-
S2B O	465	2.58	90	1.50
S3C BG	435	2.64	180	0.44
S4A BG	165	1.83	-15	1.00
S5A BG	-135	-2.25	60	0.80
S6B BG	165	2.20	15	0.25
S6B O	180	1.33	90	1.00
S8C O	315	2.10	15	0.11
S9A BG	435	2.23	0	0.00
S10A BG	0	0.00	-75	1.67
S10A O	-45	0.75	-105	-0.43
positive control	165	0.14	75	0.63

DISCUSSION

The faecal contamination of drinking water sources and the presence of antibiotic resistant bacteria increase the risk to human health (Chen et al. 2017). In this study, we were interested in investigating the presence of E.coli and prevalence of antibiotic resistance gene in water samples from valikamam west community. According to the findings, five samples were considered as E.coli positive, and those five samples were found to be tetracycline, vancomycin resistance and sulfonamide sensitive. Further only four samples have tetracycline resistance gene tec A. Detection of E-coli was done using membrane filtration, biochemical and molecular techniques.

According to WHO guidelines for Drinking and Water quality, E.coli should not be in detectable in 100ml of drinking water sample (Bain et al., 2014). Persistence of E.coli population in water would require suitable environmental conditions. E.coli is able to grow in water within the range of pH 6 to 8 and the temperature within 4 to 450C (Zhao et al., 2006). It cannot be colonized at 5ppm chlorinated water (Bozaslan et al., 2016). According to the physiochemical parameter analysis of this study, concentration of chlorine was significantly lower than recommended amount 5ppm, pH and temperature also were within the ranges, which were facilitating the growth of E.coli. This indicates physiochemically E. coli has high survival rates in all collected samples.

Triplicate technique is important for decreasing uncertainty of the data, clear identification of expected results and increases the precision of the results (Spierling, 2017). Cellulose nitrate membrane filters 0.45µm pore size was used to easily trap E.coli which is small in size about ~1 × 3 µm in enough (Reshes, et al., 2007).

Hicrome agar distinguishes E.coli from other bacteria on the basis of β-glucuronidase enzyme activity (Abid and zuwiny,2012). It gives bluish green colouration when E-coli cells absorb X-glucuronide and breakdown the bond between chromophore and glucuronide using its enzyme glucuronidase. Presence of bile salts mixture in the medium inhibits the growth of gram positive bacteria (Cremers et al., 2014). The uidA gene which is present more than 97% of E. coli isolates, encodes β-glucuronidase enzyme (Maheux et al., 2015). PCR analysis of using uidA set of primers; more validate the results obtained by isolation of E.coli in chromogenic media (Abid and zuwiny, 2012).

According to the hicrome agar and uidA PCR results, samples S3C, S4A, S5A, S6B, S9A bluish green colonies, S6B,S10A opaque colonies were positive for the uidA PCR. This shows that some bacteria (S6B,S10A opaque colonies) do not exhibit β-glucuronidase (GUD) activity in chromogenic medium although the uidA gene sequence is present in Escherichia coli serotype 0157:H7 which is able to grow in sterile fresh water (NandaKafle et al., 2018) (Monday,Whittam and Feng, 2001). On the other hand samples S1C, S2A, S10A bluish green colonies showed false negative for uidA. PCR inhibitors such as bile salts, ethanol, and proteins can interfere with different steps of PCR cycle or affect taq polymerase enzyme and cause false negative results in PCR (Schrader et al., 2012). In sample S2B, S8C opaque colonies could be Salmonella enteritidis ATCC13076 or some Klebsiella spp (Himedia laboratory) (Molina et al., 2015). Furthermore β-glucuronidase activity not only observed in E. coli isolates but also other gram negative bacteria such as Shigella species 44%, Yersinia species and Salmonella species 29%(Abid and zuwiny, 2012) (Public Health England, 2014). So further specific

detection need to be carried out. In the case of the entire sample colony forming units could not be measured because due to the high the microbial count (David and Davidson, 2014). Morphological analysis using gram staining of all samples confirms presence of gram negative rod shape-bacteria. Presence of gram negative bacilli also could be caused due to E.coli, Salmonella spp, Shigella spp, Klebsiella and Pseudomonas aeruginosa group of bacteria which were mostly prevalent in drinking water samples (Cleven et al., 2006).

Indole production is an important phenotypic characteristic to identify bacteria using its tryptophanase catalytic deamination reaction which is encoded by TnaA gene (Darkoh et al., 2015). E.coli can be differentiated from other bacteria seeing that 96% of its indole positive whereas many entero-bacteria are negative (Rezwan,Lan and Reeves,2009). Samples S2A, S3C, S6B, S9A, S10A bluish green colonies gave negative results for indole. Some E.coli tnaA mutant strains could result in lack of tryptophan biosynthesis (Yoshida et al., 2009). On the other hand presence of glucose also repress TnaA gene expression. Less amount of E..coli colonies, interference of other bacteria, presence of anaerobic conditions, insufficient tryptophan and less incubation period can lead to lack of indole production by E.coli (Han et al.,2010).

Most of the E.coli strains are citrate negative even though this study showed all the samples were citrate positive and only E.coli ATCC25922 positive control showed citrate negative. Staphylococcus aureus showed positive. This indicates no contamination occurred in the tryptone broth and presence of gram negative bacterial mix colonies in subculture resulted in citrase enzyme activity. Klebsiella pneumonia, Proteus mirabilis and E. coli K-12 strain are some citrate positive gram negative bacilli that might have resulted in citrate positive results, which are mostly present in aquatic environments. Also some E.coli can use citrate under anaerobic conditions via expression of the CitT citrate/succinate antiporter (Hofwegen, Hovde and Minnich, 2016). To detect the E.coli by only using culture, microscopic and biochemical techniques have more limitations. Due to hindrance of other Enterobacteriaceae and lack of precision, molecular technique was used for further confirmation of E.coli because of its accurate, rapid and sensitivity (Chen et al. 2017,)

16SrRNA gene is found in all bacteria (universality) which encodes the RNA smaller subunit of the bacterial ribosome. The gene sequences are very helpful to study bacterial phylogeny and taxonomy (Janda and Abbott, 2007). There is a minute changes (hyper variable regions) in 16SrRNA sequences during evolutionary process within the species and were highly conserved. Even though certain bacteria are difficult to identify because they share more than 99% identity in their 16SrRNA gene sequence. For example, it was not possible to distinguish between the 16SrRNA sequences of E. coli and Shigella sp (Jenkins et al., 2012). The results showed that except sample S3C bluish green, other samples were positive for 16SrRNA (424bp) primer which confirmed the presence of bacteria. 16SrRNA gel image had primer dimers which decrease the efficiency of amplification of 16SrRNA target gene band and also insufficient concentration of template could be caused absence of band. Broad range of 16srRNA PCR primers are designed to detect Enterobacteriaceae and vibrioaceae families because of that 16srRNA alone would not be ideal to confirm the presence of E.coli (Nakano et al., 2003).

E. coli have β-galactosidase enzyme activity. In the absence of glucose, E. coli utilize lactose as a metabolite and split it into glucose and galactose by beta-

galactosidase. The lac operon have genes lacZ, lacY and lacA, encode for β-galactosidase, lactose permease and thiogalactoside transacetylase, respectively (Ullmann,2009). All the E. coli species harbour lacY and other Enterobacteriaceae mostly lack the lactose permease lac Y gene. E. coli is closely related to shigella spp and it can be differentiated from others species with the presence of beta-glucuronidase (uidA) and beta-galactosidase (lacY) enzyme activity (Rezwan, Lan and Reeves, 2004). According to the uidA and lacY gel image (Figure 3.2.4 and 3.2.5) samples S1C bluish green, S10A bluish green and S2B, S8C opaque colonies showed β-glucuronidase (uidA) negative and beta-galactosidase (lacY) positive. Opaque colonies which have lacY could be caused by presence of Klebsiella spp which are beta-glucuronidase negative and have lactose fermentation. Bluish green colonies might cause uidA negative due to lack of DNA template and PCR inhibitors. In the case of uidA positive and lacY negative (S4A, S6B Bluish green), confirmed absence of E.coli and presence of other □-glucuronidase positive species such as Vibrio, Salmonella, Pseudomonas aeruginosa which are non-lactose fermenting genera (Shanson and Path,2014). Bluish green colonies negative for both genes negative (S2A bluish green) could be cause by false negative for uidA. Finally five samples S3C, S5A, S9A bluish green colonies and S6B, S10A Opaque colonies were selected as E-coli based on presence of both uidA and lac Y PCR detection (Ragupathi et al.,2017).

The prevalence of resistance to tetracycline, sulfonamide and vancomycin was significantly higher than other tested antibiotics in environmental samples and tetracycline resistant strains of E.coli have increased in Sri Lanka (Jayasekera et al., 2019). Antibiotics disc diffusion technique for sulfonamide (zone of inhibition) resulted in clear inhibitory zones for sample S3C, S9A bluish green and S6B, S10A opaque colonies and this revealed no resistance by E.coli to 300µg sulfonamide. Antibiotics are highly variable in natural environment. Bacteria response to antibiotics according to its surrounding and they develop resistance strains for antibiotics (Munita and Arias, 2016). Sample S5A BG did not have a clear zone of inhibition. It has antibiotics resistance and sensitive mixed colonies. In sample S5A BG one plate was rejected from duplicate due to an experimental error. So the result was not reliable and this sample was not considered for PCR analysis.

All the samples (S3C, S5A, S9A bluish green colonies and S6B, S10A Opaque colonies) were tetracycline (30µg) resistance in disc diffusion technique. Except sample S6B opaque other four showed tetracycline resistance due to the tetA gene expression. Other genes such as tetB, tetD, tetE, tetO and tetM might have been responsible for conferring resistance for tetracycline in S6B opaque samples. At the same time the gel image showed that there was so much of primer dimers formed especially S6B opaque. This can be indicating the concentration of DNA template is very low and could cause tetA negative due to insufficient primers (Tukman et al., 2009). Here we also observed the multi-drug resistance which is generally caused by combination of different mechanism due to resistance for vancomycin (Reller et al., 2009).

DNA concentration and purity is important to validate our research work and those can be assessed through spectrophotometric absorbance and agarose gel analysis. Impurity can cause inaccurate results and low concentration of DNA? can lead to primer dimer formation in PCR (Nzilibili et al., 2018). Comparison of the two extraction methods of genomic DNA gel images (figure 3.2.1 and3.2.2) showed that the band observed from kit

method is more bright and clear than the boiling cell extraction. According this visual observation, boiling cell extracted samples have low concentration and purity. Further these two methods were statistically analysed through independent t-test and those results revealed that there is a significant difference present in standard deviation between both extractions in group statistics and also the independent sample test 'Levene's test for equality of variances' significance value is 0.008 which is less than 0.05 and one of the assumption of homogeneity of variance is not assumed. The P value (DNA concentration equal variances not assumed) is 0.039 which is less than 0.05. This suggests that the differences between two means are statistically different at 5% level of significance. Therefore, kit DNA extraction was considered as a better method than boiled cell method (Hoekstra, Kiers and Johnson,2012). In this study investigated prevalence of tetracycline(tetA) and vancomycin resistance in E.coli presence in source of water commonly used by people especially children (presence in school drinking water S3C) in Jaffna valikamam west. This also highlights the critical nature of the poor water quality and drug resistance in particular area.

Further work in future

Detection of waterborne bacteria is essential as it helps to develop strategies to quantify their numbers and obtain pathogen-free safe water for human consumption. Conventional methods for detection of coliforms in water samples are selective but may yield false-positive results and are laborious and time-consuming. In future this study will be carried out with more sensitive and specific techniques. In the PCR there is problem with free and extraneous DNA detection. NGS approaches, for example, metagenomics, metatranscriptomics, single-cell genomics, and comparative genomics must be integrated in future to study the freshwater microbiomes to set the benchmark for real-time water quality monitoring. Immunological methods such as ELISA also will be used for indentification (Deshmukh et al.,2016). The major limitation of molecular and immunological methods is the need of trained personnel and costly specialized instruments. Biosensor-based methods are rapid, easy to run, and do not require trained professionals, and these methods can be used to detect and quantify waterborne bacteria. However, the specificity and resolution of detection of biosensor technology need to be amplified for on-site monitoring of environmental water samples (Nurliyana et al., 2017).

Researchers have expressed concern over the increase in the number of patients who are developing untreatable invasive infections due to antibiotics resistant bacteria each year, and the increase in the mortality rate resulting from such conditions. It is therefore important to highlight that there is an imminent need to develop new antibiotics since there has been an increase in pan-drug-resistant bacteria worldwide which is a dangerous crisis leading to millions of death worldwide (Rather et al.,2017).

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