GLOBAL ACADEMIC RESEARCH INSTITUTE

COLOMBO, SRI LANKA

GARI International Journal of Multidisciplinary Research

ISSN 2659-2193

Volume: 09 | Issue: 02

On 30th June 2023

http://www.research.lk

Author: Deshani Chethana Kumari Peiris, Uthpala Amarasekara

School of Science, BMS, Sri Lanka

GARI Publisher | Microbiology | Volume: 09 | Issue: 02

Article ID: IN/GARI/ICAS/2022/125 | Pages: 81-115 (35)

ISSN 2659-2193 | Edit: GARI Editorial Team

Received: 30.09.2022 | Publish: 30.06.2023

IDENTIFICATION OF E. COLI IN DRINKING WATER SOURCES AROUND BADULLA MUNICIPAL COUNCIL AREA AND DETECTION OF ANTIBIOTIC RESISTANCE GENES

M. Deshani Chethana Kumari Peiris, Uthpala Amarasekara

School of Science, BMS, Sri Lanka

ABSTRACT

At present, drinking water contamination with pathogenic bacteria through waste products such as human and animal feces has become a major issue in the world. Escherichia coli (E. coli) bacterial species plays a key role as a fecal contamination indicator, which has both harmful and harmless acts towards human. Harmless strains produce vitamin K2 inside the gut, while harmful strains cause diarrhea and even Uterine Track Infections (UTI). More importantly, some of the strains develop resistance for antibiotics due to prolong exposure. Specially, gut residing E. coli strains. Then treatment with common antibiotics become hopeless and that has threatened the health care globally. It is possible for other pathogenic bacteria including E. coli species to acquire these antibiotic resistance genes through horizontal gene transfer. Currently, non-clinical environments such as common aquatic resources have been recognized as a significant factor in dissemination of antibiotic resistance genes. In this study, water samples were collected from the water sources around Badulla Municipal Council, UVA province, Sri Lanka. At the first stage both biochemical and molecular analysis were performed to detect E. coli. Followed by, Antibiotic Susceptibility Testing (ABST) for the most probable E. coli samples to observe their antibiotic resistance against Tetracycline, Sulfonamide and Gentamicin. The tetracycline resistance strains were tested for the presence of Tet A and Tet C resistance genes by Polymerase Chain Reaction (PCR). Tet A gene was detected in five drinking water samples including common wells and a domestic tank.

Keywords: Drinking water, E. coli, Antibiotic Susceptibility Testing, Antibiotic resistance, Polymerase Chain Reaction (PCR), Tet A gene

INTRODUCTION AND LITERATURE REVIEW

Drinking water is a vital substance which is given by the nature, and it is recognized as a humanright by UN General Assembly. Improved safe water supply and sanitation, and better management of water resources, can increase countries' economic growth (WHO, 2019). However, in most of the countries, people don't have access to safe drinking water due to watercontamination with chemicals, heavy metals and due to animal and human feces which leads have pathogens in water (Ashbolt, 2004) such as bacteria, viruses and protozoa which linked totransmits diseases (figure 1). Diarrhoea is the most widely known disease linked to contaminated food and water and 829 000 people are estimated to die each year from diarrhea (WHO, 2019).

Name of micro-organisms	Major diseases	Major reservoirs and primary sources
Bacteria		
Salmonella typhi	Typhoid fever	Human faeces
Salmonella paratyphi	Paratyphoid fever	Human faeces
Other *Salmonella*	Salmonellosis	Human and animal faeces
Shigella spp.	Bacillary dysentery	Human faeces
Vibrio cholera	Cholera	Human faeces and freshwater zooplankton
Enteropathogenic *E. coli*	Gastroenteritis	Human faeces
Yersinia enterocolitica	Gastroenteritis	Human and animal faeces
Campylobacter jejuni	Gastroenteritis	Human and animal faeces
Legionella pneumophila and related bacteria	Acute respiratory illness (legionellosis)	Thermally enriched water
Leptospira spp.	Leptospirosis	Animal and human urine
Various mycobacteria	Pulmonary illness	Soil and water
Opportunistic bacteria	Variable	Natural waters
Enteric viruses		
Enteroviruses		
Polio viruses	Poliomyelities	Human faeces
Coxsackie viruses A	Aseptic meningitis	Human faeces
Coxsackie viruses B	Aseptic meningitis	Human faeces
Echo viruses	Aseptic meningitis	Human faeces
Other enteroviruses	Encephalities	Human faeces
Rotaviruses	Gastroenteritis	Human faeces
Adenoviruses	Upper respiratory and gastrointestinal illness	Human faeces
Hepatitis A virus	Infectious hepatitis	Human faeces
Hepatitis E virus	Infectious hepatitis; miscarriage and death	Human faeces
Norovirus	Gastroenteritis	Fomites and water
Protozoa		
Acanthamoeba castellani	Amoebic meningoencephalitis	Human faeces
Balantidium coli	Balantidosis (dysentery)	Human and animal faeces
Cryptosporidium homonis, C. parvum	Cryptosporidiosis (gastroenteritis)	Water, human and other mammal faeces
Entamoeba histolytica	Amoebic dysentery	Human and animal faeces
Giardia lamblia	Giardiasis (gastroenteritis)	Water and animal faeces
Naegleria fowleri	Primary amoebic meningoencephalitis	Warm water
Helminths		
Ascaris lumbricoides	ascariosis	Animal and human faeces

Figure 1. The water borne pathogens and major disease caused by them (Ashbolt, 2004)

Escherichia coli (E. coli) is a very common bacteria which can be found in human and animal feces and discovered by German microbiologist Theodor in 1884 (Blount, 2015). E. coli comes under phylum proteobacteria, family enterobacteria and a type of coliform bacteria. E. coli is agram-negative bacteria and rod shaped (about 2.0µm long, 0.25-1.0µm in diameter and cell volume is about 0.6-0.7µm3), where the cell wall is composed of a thin layer of peptidoglycan which causes the loss of crystal violet staining and retention of safranin during gram staining (pink) (Blount, 2015). It can produce ATP by aerobic respiration if oxygen is present but also capable in swapping to produce ATP by fermentation when oxygen is not present (facultative anaerobic) (Wassenaar, 2016). In the structure of E-coli it possesses a flagellum and has a peritrichous. Optimum growth occurs at 370C (98.60F) and 7.2-7.8 pH. Most of the e-coli strainsare harmless, reside in animal and human gut system and beneficial in producing Vitamin K2 (helps in blood clotting) (Kaper, Nataro and Mobley, 2004). They can live outside the body for a limited time which makes it as a potential organism as a fecal contamination of foodand water (Edberg et al., 2000).

The existence of E. coli in the environment is a major concern because its relationship with humans is not completely gentle. E. coli infections through the fecal oral contamination route isa main cause of diarrhea, peritonitis, colitis, bacteremia, infant mortality, and urinary tract infections. Treatments cost more than several billions world widely

and nearly 2 million peopleper each year die (Kaper, Nataro and Mobley, 2004).

The most notorious strain of E-coli is O157: H7, an entero hemorrhagic strain that produces a Shiga-like toxin (Liu et al., 2019) and attacks small blood vessels in intestine and causes bloodydiarrhea and as well as hemolytic uremic syndrome (HUS). It is a potentially deadly condition that may involve blood clots in capillaries, thrombocytopenia, and renal failure (Griffin et al., 1988). Various types of antibiotics are the main treatment for E. coli infections (figure 2).

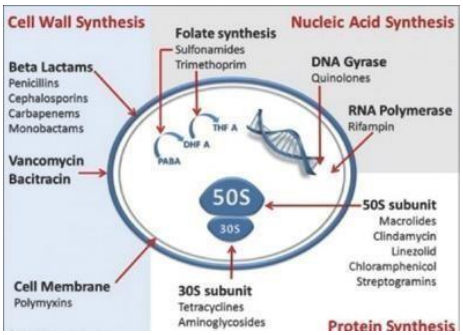


Figure 2. Mechanism of action of antibiotics (Kapoor, Saigal and Elongavan, 2017)

Antibiotic resistance (AR) has become a great risk and a challenge in treating bacterial infections. Overuse and misuse of antibiotics are the common reasons for developing AR (Fernando et al, 2016). There are two main ways of obtaining resistance either by decreasing theaffinity of the target for the antibiotic or diminishing the concentration of the antibiotic inside the cell (Blanco et al, 2016). Over the last few years have shown the presence of E. coli with ARgenes in the broader environment, including water supplies and soil samples but not in gut systems (Fernando et al, 2016). But normal strains also can acquire this ability by 'Horizontal Gene Transfer (HGT) when the gut is exposes to water which contains those strains with AR genes (Hasegawa, Suzuki, and Maeda, 2018). E. coli and other some bacteria show several AR mechanisms,

1. Efflux pump mediated decreased the drug uptake (Tetracycline resistance),

E. coli try to decrease absorption of antibiotics such as Tetracycline which is capable of inhibiting protein synthesis and try to increase discharge of them by pumping out using efflux pumps. Efflux-mediated resistance of E. coli against Tetracycline was first detected in 1970s. The substrate specificity of efflux pumps differs widely (figure 3) (Blanco et al., 2016). Doxycycline, Augmentin, and ampicillin are common antibiotics in the Tetracycline class and Tet A, B, C, D, E, Y, and I genes can be detect in genome of these E. coli strains which show resistance against them (Karami et al., 2006).

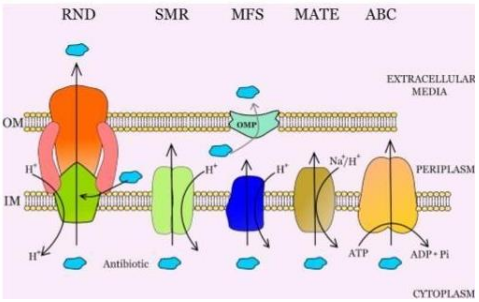


Figure 3. Schematic representation of the main types of bacterial efflux systems (Blanco etal., 2016)

1.Target modification (sulfonamide resistance),

By inhibiting the production of H2pteroate from 6-hydroxymethylpterin pyrophosphate and p- aminobenzoate (PABA), Sulfonamide prevent the biosynthesis of folate compounds bacteria.

E. coli show a common way of approach to show Sulfonamide resistance via mutation of the structural gene encoding H2pteroate synthase, so that the altered enzyme product is a sulfonamide resistant form that discriminates more effectively between PABA and sulfonamidesthan the wild type of enzyme. Sul1, sul2, and sul3 are the genes which are responsible for this resistance and sulfamethazine and sulfacetamide are common antibiotics from this class (Byrne-Bailey et al., 2009).

METHODOLOGY

Sample collection

All the water samples were collected (figure 4) into label sterilize glass bottles (400ml). They were secured in zip lock bags separately.

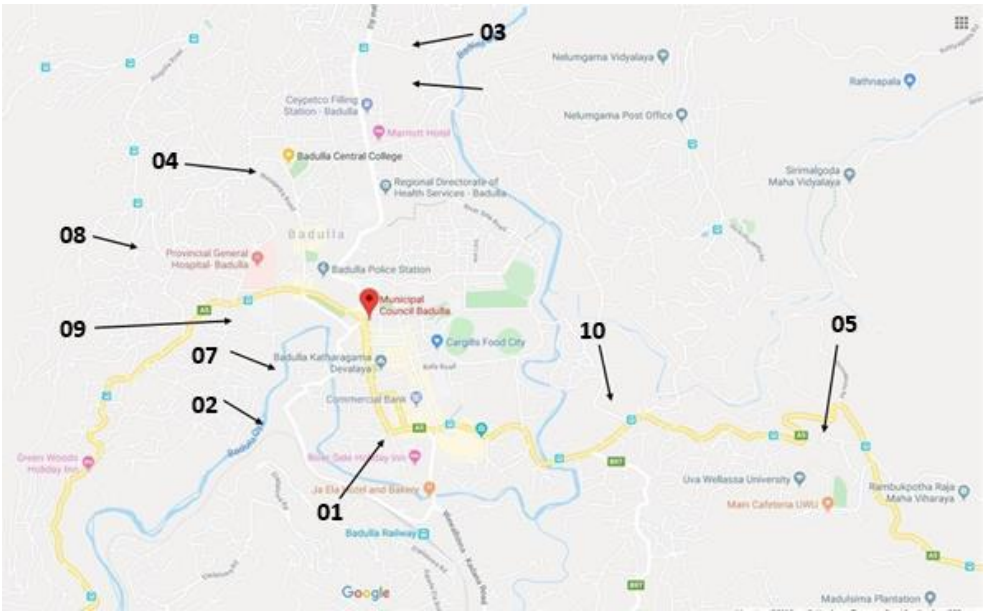


Figure 4. Map of Badulla Municipal Council Area with the water samples collected places(table 4.)

Methodology for measuring pH.

From the 1st sample, 75ml was taken into a beaker. The pH meter pen was dipped inside distilledwater to calibrate. Then the pen was dipped in the sample and waited until fluctuation stopped. The pH value was recorded. The same procedure was repeated for all ten samples.

Methodology for measuring Temperature.

The thermometer was dipped inside the beaker containing water sample and waited for 2 minutes. The temperature reading was recorded. Same procedure was repeated for all ten samples.

Methodology for measuring Chlorine Concentration.

The Chlorine strip was dipped inside the beaker containing water sample and waited for 2 – 3 seconds. Then waited for the color change and recorded. Same procedure was repeated for all ten samples.

Membrane filtration and culturing in Hi- crome agar.

Hi crome agar was prepared in to 30 petri dishes according to triplicate technique on a sterilized work bench. The Buchner funnel was placed on the conical flask and the filter paper was placed on the funnel. Then 100ml from 1st sample was filtered. Then the filter paper was placed on the hi-chrome agar petri dish and para filmed. Then it was labeled according to the sample details and placed inside the incubator at inverted position for 24 hours at 37°C. Sameprocedure was repeated for all samples.

Preparation of sub culturing in Luria Broth.

Luria Broth (LB) was prepared and 8ml was added into each falcon which were labeled. Then colony was obtained using a sterilized loop and dipped into the respective falcon.Then the falcon was para filmed and incubated for 24 hours at 37°C. Same procedure was repeated for all other samples. After the incubation they were stored in a refrigerator below 20oC.

Gram staining.

At a sterilized area, a thin bacterial smear was made on a microscopic slide and heat fixed. Thesmear flooded with crystal violet for 1 minutes and gram Iodine for 1 minute. Then few drops of decolorize (95% ethanol) were added and washed off immediately. Finally, the smear was flooded with safranin for 45 seconds. After each step it was washed with distilled water. Then the slide was air dried and observed under 40X and 100X (oil immersion) using the microscope.Repeated the same procedure for all other 13 samples.

Indole test.

Tryptophan broth was prepared and 5ml of broth was poured in to 14 labeled testtubes. Then the colonies were inoculated into the test tubes using the loop separately and test tubes were closed properly using foil and para films and incubated for 24 hours at 37°C. The same procedure was repeated.

Citrate test.

Simon's citrate agar was prepared and a cotton swab was used to inoculate the bacterial cultures from the falcons into the agar. Then they were secured using foil and para filmand incubated at 37°C for 24 hours.

Promega Kit based DNA extraction.

The DNA was extracted from 14 samples along with the positive control and negative controlusing Promega Wizard DNA extraction kit, according to the kit's instructions.

Detection of genomic DNA using 0.8% Agarose Gel Electrophoresis (AGE).

Agarose gel (0.8%) was prepared and placed inside the chamber with 1X TAE buffer. The DNA samples were thawed and followed by a spin for 2 seconds and 5µl of each sample mixed with 2µl of loading dye and loaded in to each well separately. The lid was closed and allowed to run for 25 minutes at 60V, and the gel image was obtained using gel documentation system. The DNA which was extracted using both kit and boiled

cell methods were visualized using AGE in separate gels.

Amplification of 16srRNA (424 bp), uidA and Lac Y genes using PolymeraseChain Reaction (PCR) and detection of genes using 2% AGE.

The PCR tubes were label and 1.5µl from each sample were allocated into respective tube along with positive and negative controls. Then 23.5 µl (table 1) of prepared master mixturewith respective primers sets were allocated into all PCR tubes and they were tapped carefully. The tubes were placed inside the PCR machine. The protocols (cycling conditions) were already saved as in the table 2 and they were run according to the respective protocol.

Table 2. PCR Cycling conditions (Aditi, Rahman, and Hossain, 2017)

Cycling Reaction	**16srRNA (424 bp)**		
	Temperature (ºC)	**Time (min)**	**No. of Cycles**
Initial denaturation	94	2.00	35
Denaturation	94	0.30	
Annealing	55	0.45	
Extension	72	1.30	
Final Extension	72	10.00	
Hold	4	∞	
Cycling Reaction	**uidA**		
	Temperature (ºC)	**Time(min)**	**No. of Cycles**
Initial denaturation	95	3.00	35
Denaturation	95	0.30	
Annealing	55	0.30	
Extension	72	1.00	
Final Extension	72	10.00	
Hold	4	∞	
Cycling Reaction	**Lac Y**		
	Temperature (ºC)	**Time(min)**	**No. of Cycles**

Initial denaturation	94	3.00	30
Denaturation	94	1.00	
Annealing	55	0.30	
Extension	72	1.00	
Final Extension	72	5.00	
Hold	4	∞	

Table 1. Volumes of reagents for PCR master mixture preparation

Reagents	Volumes of the reagents according to the numberof samples	
	1× (µl)	17× (µl)
Distilled water	11.125	189.125
5X PCR buffer	5	85
dNTPs	0.625	10.625
$MgCl_2$	1.5	25.5
F primer	2.5	42.5
R primer	2.5	42.5
Taq	0.25	2.25
Total without DNA	23.5	397.5

Then, 2% agarose gels were prepared and the 3 µl of PCR products were loadedinto wells separately after mixing with 2µl of loading dye. The ladders were loaded, and the gelwas placed inside the gel chamber and the lid was closed properly. The PCR products were run for 45 minutes under 45V and 15 minutes under 50V. The gel images were visualized using the gel documentation system.

Antibiotic Sensitivity Test (ABST)

First, Muller Hinton Agar was prepared and poured into petri plates and left to dry. Then the plate was divided into 4 areas. To an empty test tube 2ml of autoclaved distilled water was added with 200µl of 24 hours cultures from samples until the turbidity of the samplesbecomes like the pre-prepared 0.5M McFarland Standard. The turbidity was observed against a lined paper. Tetracycline, Sulfonamide and

Gentamicin were used as antibiotics with autoclaved filter paper was used as the control. 4 drops of the sample were placed on the surface of the agar and a cotton swab was used to spread it as a thin layer. The antibiotic discs and controls were placed on the middle of each respective areas as in the belowself-illustrated plot. Then the plates were incubated at 37oC for 24 hours to obtain the zones of inhibitions.

After 24 hours, with the aid of a ruler the diameters were measured in mm as illustrated in the plot (figure 5) and samples were categorized either Resistance or Intermediate resistance or Susceptible according to the measurements under the guidance of CLSI standards for E. coli (ATCC 25922)and respective antibiotic.

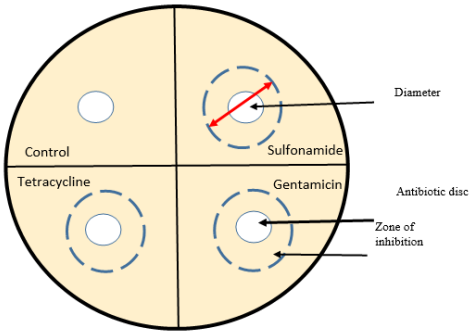


Figure 5. Self-illustration of an ABST disc structure

Amplification of Tet A, Tet C and Sul 1 genes using PCR and detection of genesusing 2% AGE.

Conditions in table 3 were used to run PCR.

Table 3. PCR Cycling conditions (Chen et al, 2013)

Cycling Reaction	Tet A		
	Temperature (^{o}C)	Time (min)	No. of Cycles
Initial denaturation	95	5.00	40
Denaturation	95	0.20	
Annealing	60	0.30	
Extension	72	0.30	
Final Extension	72	10.00	
Hold	4	∞	
Cycling Reaction	Tet C		
	Temperature (^{o}C)	Time (min)	No. of Cycles
Initial denaturation	95	5.00	

Denaturation	95	0.20	40
Annealing	64	0.40	
Extension	72	0.30	
Final Extension	72	10.00	
Hold	4	∞	

DATA ANALYSIS (RESULTS)

Sample collection and physiochemical parameters.

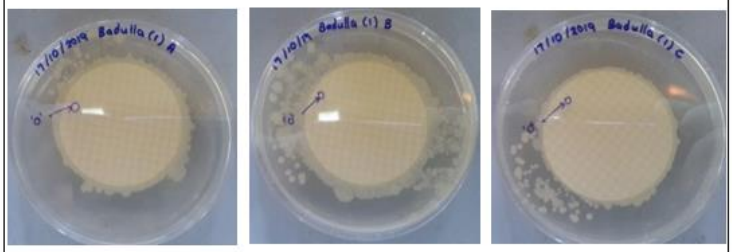
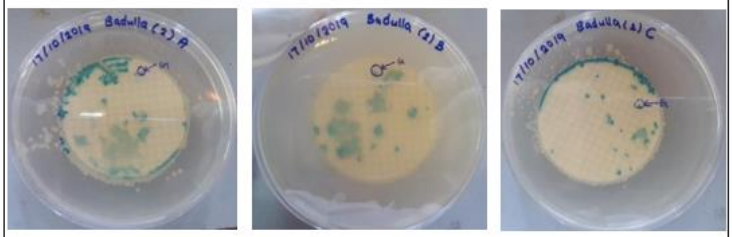
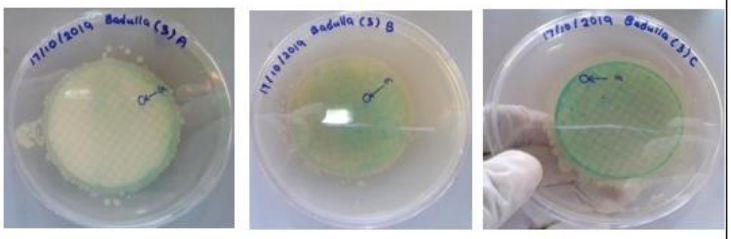
Table 4. Locations, sources, and physio chemical parameters of the collected samples

Sample / Label	Location	Sources of water	pH value	Temperature (° C)	Chlorine concentration
Badulla 1	Lower king's street	Tap water (Well - not protected)	7.24	31	< 5 ppm
Badulla 2	Badulu oya	River	7.21	30.5	< 5 ppm
Badulla 3	Rideepana	Common well (Not protected)	7.10	31	< 5 ppm
Badulla 4	Wellagedara	Common well (Not Protected)	7.25	31	< 5 ppm
Badulla 5	Wewassa	Waterfall	7.44	31	< 5 ppm
Badulla 6	Puwakgodamulla	Common well (Not protected)	7.13	30	< 5 ppm
Badulla 7	Deiyannewela	Well (Protected)	7.55	31	< 5 ppm

Badulla 8	Silver land	Well (Protected)	6.29	31	< 5 ppm
Badulla 9	Peelipothagama road	Well (Not Protected)	6.96	30	< 5 ppm
Badulla 10	Hindagoda	Spring water (Not protected)	7.13	31	< 5 ppm

Membrane filtration and LB sub culturing

Table 5. Results of membrane filtration

Sample	Results of membrane filtration
Badulla (1)	 ***Figure 6.*** Membrane filtration results of Badulla (1) sample, only opaquecolonies were observed an opaque colony was selected from plate A.
Badulla (2)	 ***Figure 7.*** Membrane filtration results of Badulla (2) sample, green and opaque colonies were observed in plate all 3 plates. A green colony from plate A was selected.
Badulla (3)	 ***Figure 8.*** Membrane filtration results of Badulla (3) sample, green and opaque colonies were observed in plate all 3 plates. A green colony from plate B was selected.

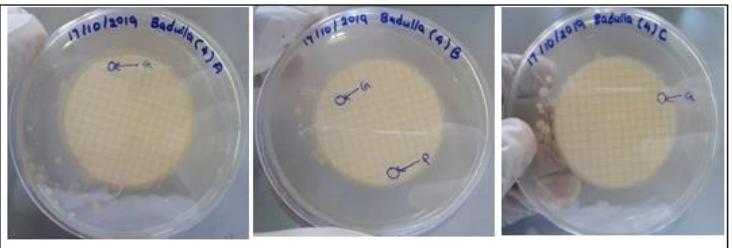
Badulla (4)	

Figure 9. Membrane filtration results of Badulla (4) sample, green and opaque colonies were observed in plate A and C. Green, opaque and pink colonies were observed in plate B. A green colony from plate A was selected. Apink and a green colony were selected from plate B.

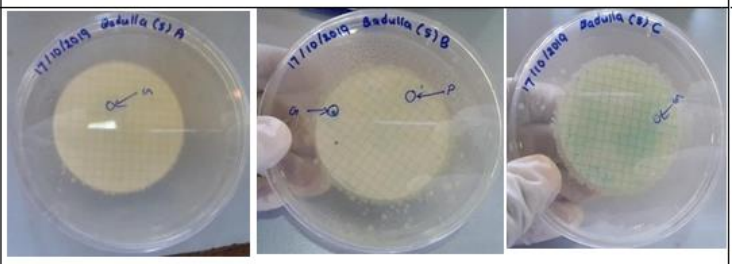
Badulla (5)	

Figure 10. Membrane filtration results of Badulla (5) sample, green and opaque colonies were observed in plate A and C. Green, opaque and pink colonies were observed in plate B. A green colony from plate A was selected. Apink and a green colony were selected from plate B.

Badulla (6)	

Figure 11. Membrane filtration results of Badulla (6) sample, only opaquecolonies were observed in all 3 plates and an opaque colony from plate A was selected.

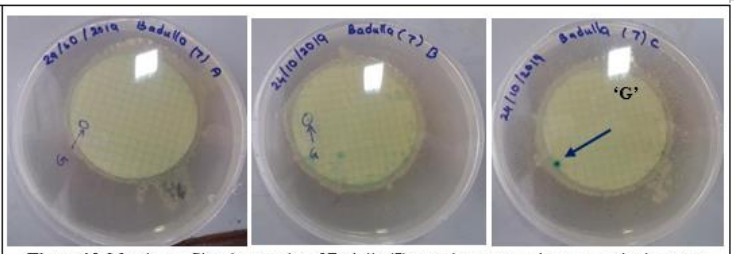
Badulla (7)	

Figure 12. Membrane filtration results of Badulla (7) sample, green and opaque colonies were observed in all 3 plates. Green colonies were selected from all 3 plates.

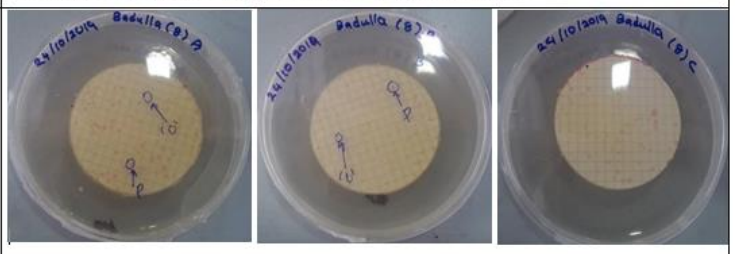
Badulla (8)	

Figure 13. Membrane filtration results of Badulla (8) sample, pink and opaque colonies were observed in all 3 plates. Pink and opaque colonies were selected from plate A and B.

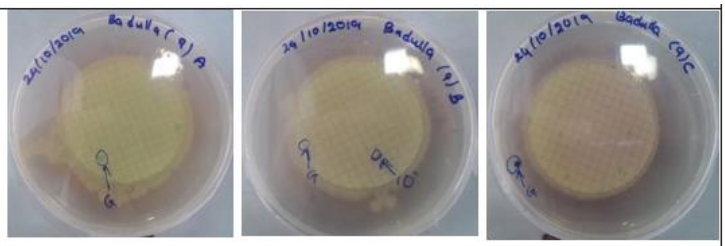
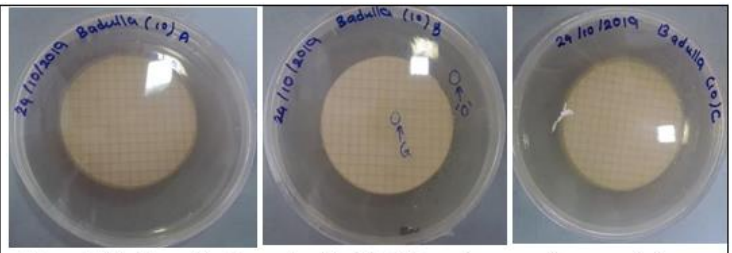
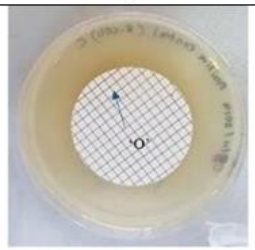
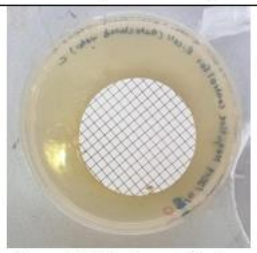
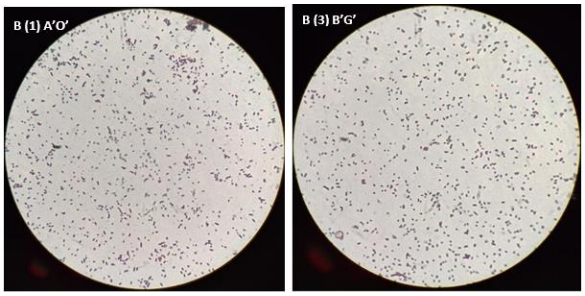
Badulla (9)	 ***Figure 14.*** Membrane filtration results of Badulla (9) sample, green and opaque colonies were observed in all 3 plates. Green colonies were selected from plate A and C. Green and opaque colonies were selected from plate B.
Badulla (10)	 ***Figure 15.*** Membrane filtration results of Badulla (10) sample, green and opaque colonies were observed in all 3 plates. Green and opaque colonies were selected from plate B.
Positive Control (*E. coli*- ATCC 25922)	 ***Figure 16.*** Membrane filtration results of positive control (*E-coli* ATCC 25922), only opaque colonies were present in all 3 plates. An opaque colony was selected.
Negative control (Auto-claved Distilled water	 ***Figure 17.*** Membrane filtration results of negative control (Autoclaved distilled water), colonies were not observed in all 3 plates.

Table 6. LB sub culturing, culture selection for biochemical analysis and gram staining

Sample	LB falcon label according to the colony	Selected / Not selected
Badulla 1	Badulla (1) A ‘O’	Selected
Badulla 2	Badulla (2) A ‘G’	Selected
	Badulla (2) B ‘G’	Not selected
Badulla 3	Badulla (3) A ‘G’	Not selected
	Badulla (3) B ‘G’	Selected
Badulla 4	Badulla (4) A ‘G’	Not selected
	Badulla (4) B ‘G’	Selected
	Badulla (4) B ‘P’	Selected
Badulla 5	Badulla (5) A ‘G’	Selected
	Badulla (5) B ‘G’	Not selected
	Badulla (5) B ‘P’	Not selected
Badulla 6	Badulla (6) A ‘O’	Selected
Badulla 7	Badulla (7) A ‘G’	Selected
	Badulla (7) B ‘G’	Not selected
	Badulla (7) C ‘G’	Not selected
Badulla 8	Badulla (8) A ‘O’	Not selected
	Badulla (8) A ‘P’	Not selected
	Badulla (8) B ‘O’	Selected
	Badulla (8) B ‘P’	Selected
Badulla 9	Badulla (9) A ‘G’	Not selected
	Badulla (9) B ‘G’	Selected
	Badulla (9) B ‘O’	Selected
	Badulla (9) C ‘G’	Not selected
Badulla 10	Badulla (10)B ‘G’	Selected
	Badulla (10)B ‘O’	Selected

Selected colonies -

Gram staining



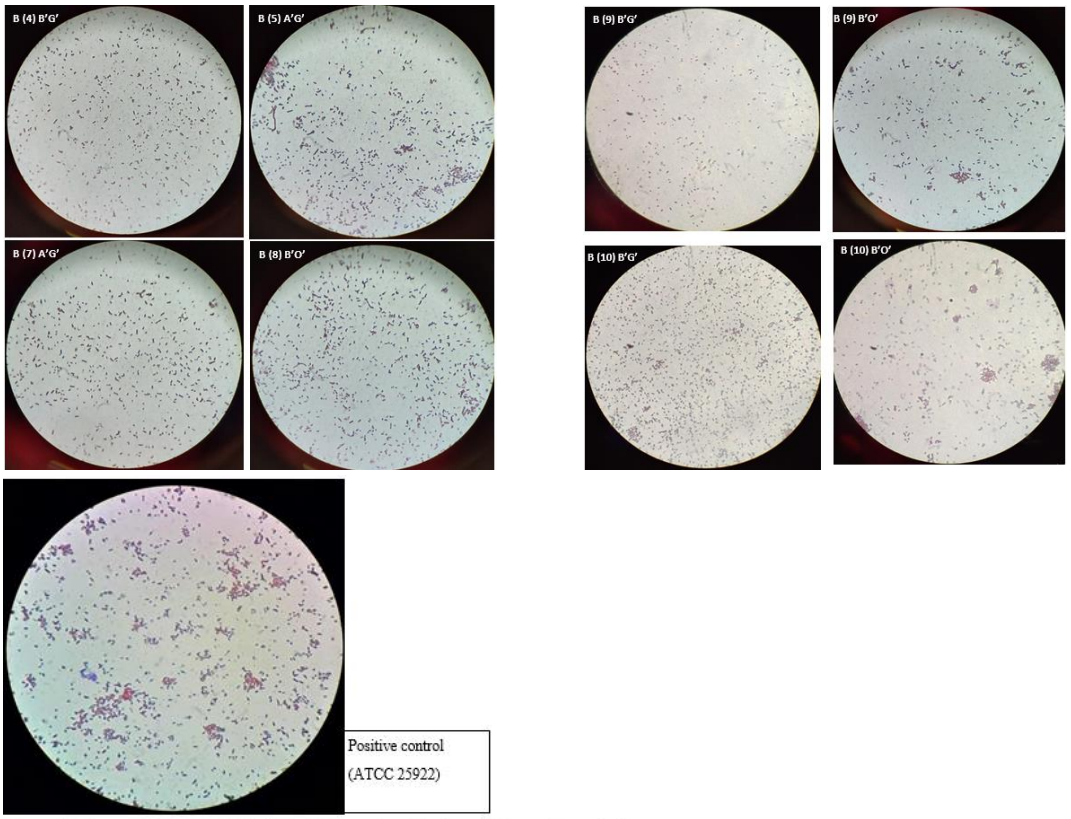


Figure 18. Microscopic images (100X) of pink rods observed samples along with the positive control (ATCC25922)

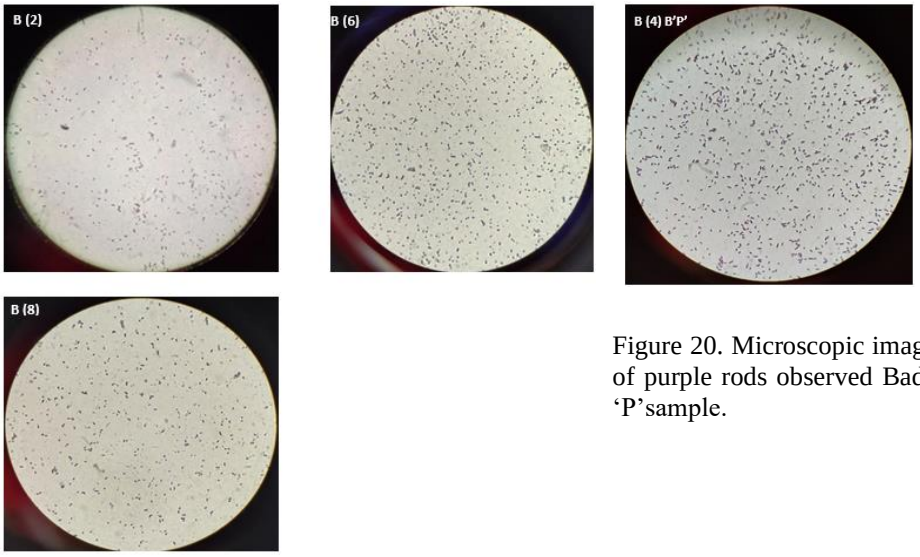


Figure 20. Microscopic images (100X) of purple rods observed Badulla (4) B 'P'sample.

Figure 19. Microscopic images (100X) of pink cocci observed samples

Indole Test

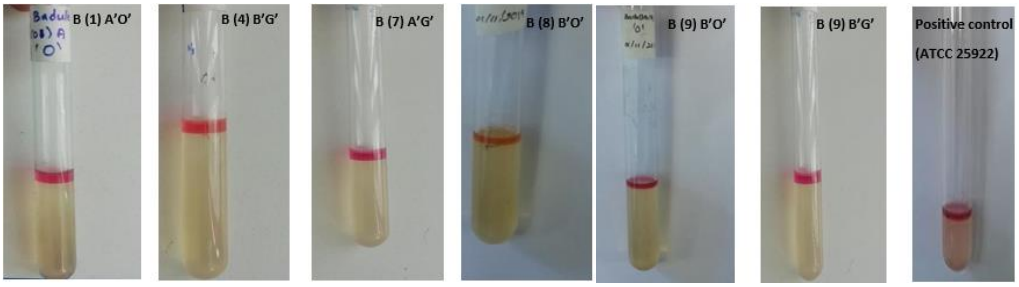


Figure 21. Positive results of indole test, cherry pink color ring was observed in 6samples and in the positive control (ATCC 25922)

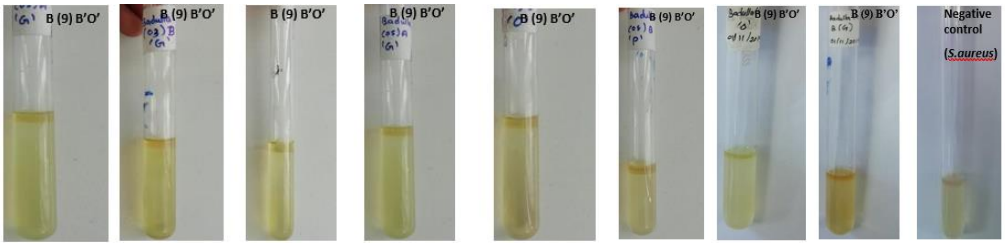
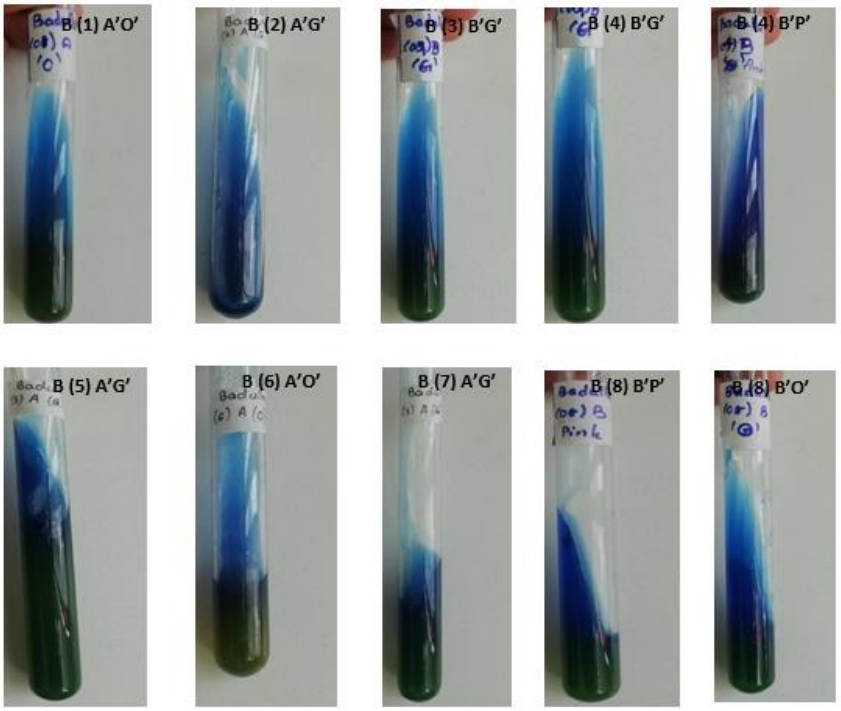


Figure 22. Negative results of indole test, yellow colour ring was observed in 8 samplesand in the negative control (S,aureus)

Citrate test



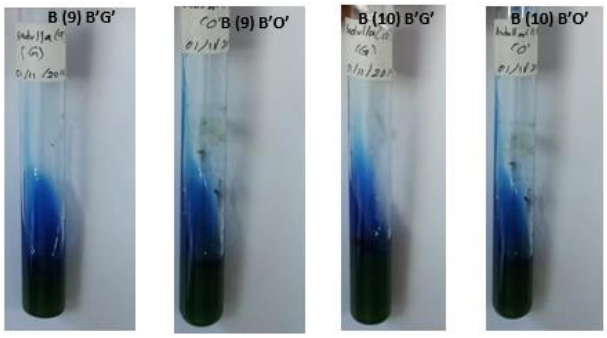


Figure 23. Positive results of citrate test, blue color was observed in all 14 samples

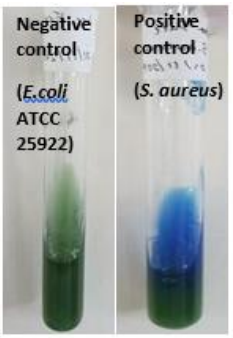


Figure 24. Green color was observed in citrate negative control (E. coli ATCC25922) and blue color was observed in citrate positive control (S.aureus)

Table 7. Summary table of gram staining and biochemical analysis results for 14 samples

Sample Number	Sample label	Gram staining results	Indole test results	Citrate test results
1	Badulla (1) A 'O'	+	+	+
2	Badulla (2) A 'G'	-	-	+
3	Badulla (3) B 'G'	+	-	+
4	Badulla (4) B 'G'	+	+	+
5	Badulla (4) B 'P'	-	-	+
6	Badulla (5) A 'G'	+	-	+
7	Badulla (6) A 'O'	-	-	+
8	Badulla (7) A 'G'	+	+	+
9	Badulla (8) B 'O'	+	+	+
10	Badulla (8) B 'P'	-	-	+
11	Badulla (9) B 'G'	+	+	+
12	Badulla (9) B 'O'	+	+	+
13	Badulla (10)B 'G'	+	-	+
14	Badulla (10)B 'O'	+	-	+

(+) – Positive results

(-) – Negative results

Genomic DNA Gel image for Promega Kit based DNA extraction

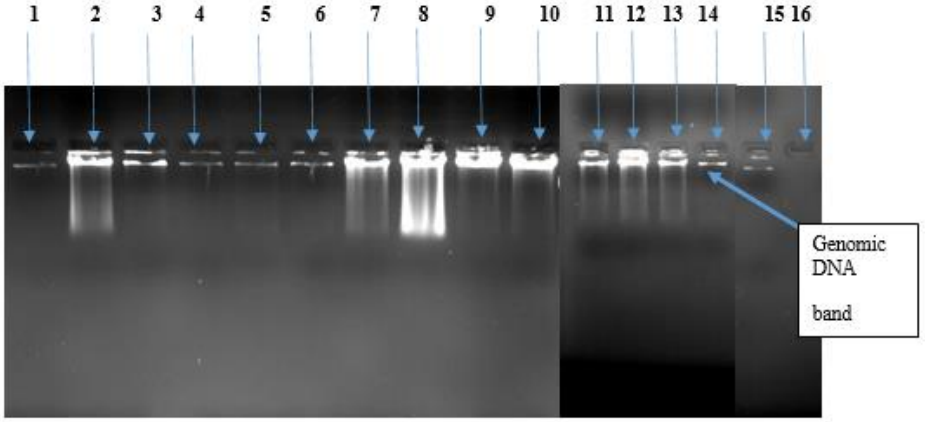


Figure 25. 0.8% Agarose gel genomic DNA image for promega kit-based DNA extraction.

Table 8. Samples loading arrangement and results interpretation of Gel image (figure 25)

Lane Number	Sample/Falcon	Presence ofbands
1	Badulla (1)A 'O'	+
2	Badulla (2)A 'G'	+
3	Badulla (3)B 'G'	+
4	Badulla (4)B 'G'	+
5	Badulla (4)B 'P'	+
6	Badulla (5)A 'G'	+
7	Badulla (6)A 'O'	+
8	Badulla (7)A 'G'	+
9	Badulla (8)B 'P'	+
10	Badulla (8)B 'O'	+
11	Badulla (9)B 'G'	+
12	Badulla (9)B 'O'	+
13	Badulla (10)B'G'	+
14	Badulla (10)B'O'	+
15	Positive control (*E-coli*,ATCC 25922) water)	+
16	Negative control (AutoclavedDistilled	-

(+) – Bands present

(-) – Bands not present

Detection of 16s rRNA Gene (424bp) using 2% Agarose Gel Electrophoresis

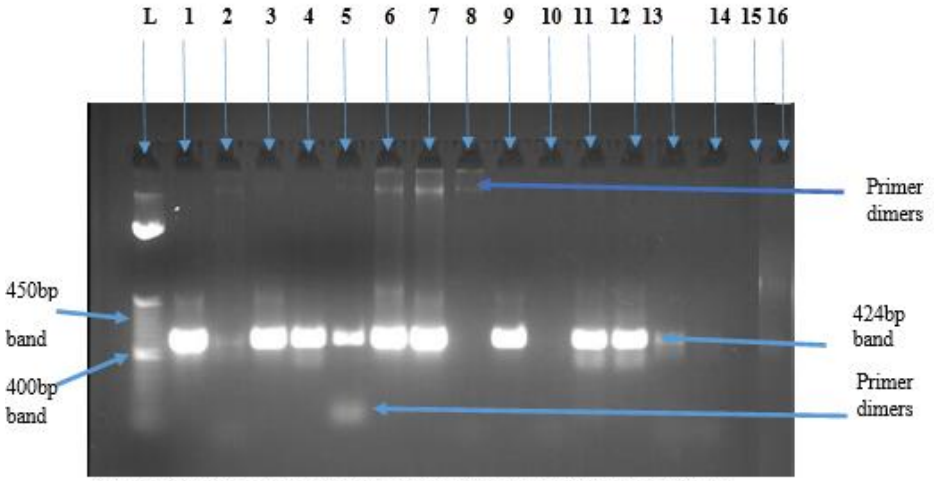


Figure 26. 2% Agarose gel image for detection of 16s rRNA gene (424 bp).

Table 9. Samples loading arrangement and results interpretation of Gel image (figure 26)

Lane Number	Sample/Falcon	Presence of bands	Positive/Negative
L	50 bp ladder	+	Bands were present
1	Badulla (1)A 'O'	+	Positive
2	Badulla (2)A 'G'	+	Positive
3	Badulla (3)B 'G'	+	Positive
4	Badulla (4)B 'G'	+	Positive
5	Badulla (4)B 'P'	+	Positive
6	Badulla (5)A 'G'	+	Positive
7	Badulla (6)A 'O'	+	Positive
8	Badulla (8)B 'P'	-	Negative
9	Badulla (7)A 'G'	+	Positive
10	Badulla (8)B 'O'	-	Negative
11	Badulla (9)B 'G'	+	Positive
12	Badulla (9)B 'O'	+	Positive
13	Badulla (10)B'G'	+	Positive
14	Badulla (10)B'O'	-	Negative
15	Positive control (*E-coli*,ATCC 25922)	-	Negative
16	Negative control (Autoclaved Distilled water)	-	Negative

(+) – Bands present

(-) – Bands not present

Detection of uidA Gene (147 bp) using 2% Agarose Gel Electrophoresis

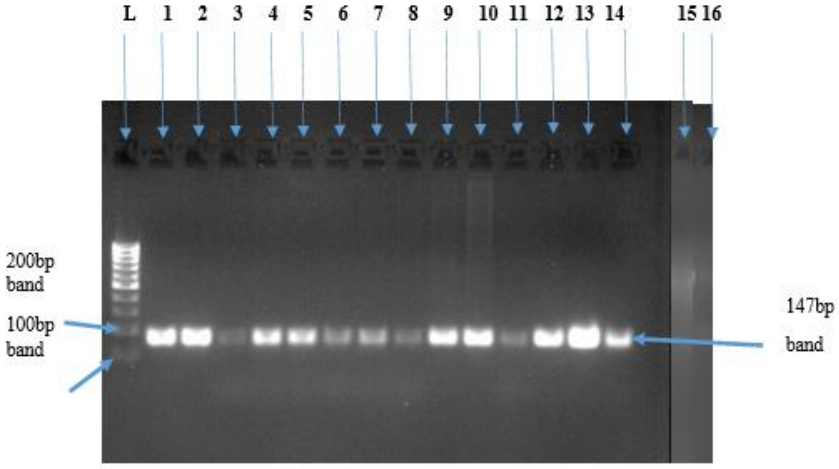


Figure 27. 2% Agarose gel image for detection of uidA gene.

Table 10. Samples loading arrangement and results interpretation of Gel image (figure 27)

Lane Number	Sample/Falcon	Presence of bands	Positive/Negative
L	100 bp ladder	+	Bands were present
1	Badulla (1)A 'O'	+	Positive
2	Badulla (2)A 'G'	+	Positive
3	Badulla (3)B 'G'	+	Positive
4	Badulla (4)B 'G'	+	Positive
5	Badulla (4)B 'P'	+	Positive
6	Badulla (5)A 'G'	+	Positive
7	Badulla (6)A 'O'	+	Positive
8	Badulla (7)A 'G'	+	Positive
9	Badulla (8)B 'P'	+	Positive
10	Badulla (8)B 'O'	+	Positive
11	Badulla (9)B 'G'	+	Positive
12	Badulla (9)B 'O'	+	Positive
13	Badulla (10)B'G'	+	Positive
14	Badulla (10)B'O'	+	Positive
15	Negative control (Autoclaved Distilled water)	-	Negative
16	Positive control (*E-coli*, ATCC 25922)	-	Negative

(+) – Bands present

(-) – Bands not present

Detection of Lac Y Gene (104 bp) using 2% Agarose Gel Electrophoresis

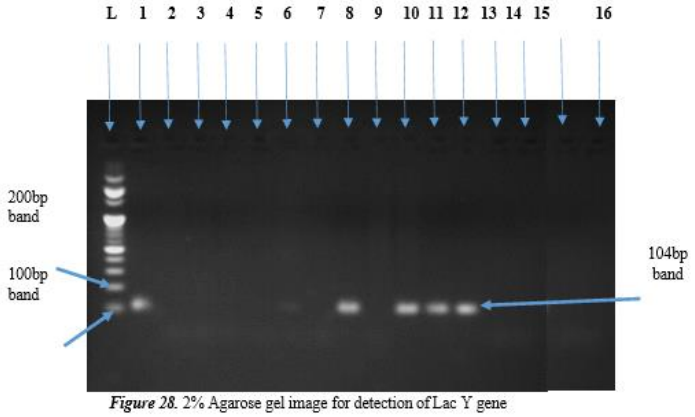


Figure 28. 2% Agarose gel image for detection of Lac Y gene

Table 11. Samples loading arrangement and results interpretation of the gel image (figure28)

Lane Number	Sample/Falcon	Presence of bands	Positive/Negative
L	100 bp ladder	+	Bands were present
1	Badulla (1)A 'O'	+	Positive
2	Badulla (2)A 'G'	-	Negative
3	Badulla (3)B 'G'	-	Negative
4	Badulla (5)A 'G'	-	Negative
5	Badulla (4)B 'P'	-	Negative
6	Badulla (4)B 'G'	+	Positive
7	Badulla (6)A 'O'	-	Negative
8	Badulla (7)A 'G'	+	Positive
9	Badulla (8)B 'P'	-	Negative
10	Badulla (8)B 'O'	+	Positive
11	Badulla (9)B 'G'	+	Positive
12	Badulla (9)B 'O'	+	Positive
13	Badulla (10)B'G'	-	Negative
14	Badulla (10)B'O'	-	Negative
15	Negative control (Autoclaved Distilled water)	-	Negative
16	Positive control (*E-coli*,ATCC 25922)	-	Negative

(+) – Bands present

(-) – Bands not present

Samples selection for Antibiotic Sensitivity Test (ABST)

Table 12. Summary of PCR results and samples selection for ABST

Sample	16s rRNA (424bp)	uidA (147bp)	Lac Y	Selected/ Not selected
Badulla (1)A 'O'	+	+	+	Selected
Badulla (2)A 'G'	+	+	-	Not selected
Badulla (3)B 'G'	+	+	-	Not selected
Badulla (4)B 'G'	+	+	+	Selected
Badulla (4)B 'P'	+	+	-	Not selected
Badulla (5)A 'G'	+	+	-	Not selected
Badulla (6)A 'O'	+	+	-	Not selected
Badulla (7)A 'G'	+	+	+	Selected
Badulla (8)B 'P'	-	+	-	Not selected
Badulla (8)B 'O'	-	+	+	Selected
Badulla (9)B 'G'	+	+	+	Selected
Badulla (9)B 'O'	+	+	+	Selected
Badulla (10)B'G'	+	+	-	Not selected
Badulla (10)B'O'	-	+	-	Not selected

(+) – Bands present

(-) – Bands not present

- Selected (both uid A and Lac Y positive)

Antibiotic Sensitivity Test (ABST) for 6 samples and measurements of zonesof inhibition.

Table 13. Zone of inhibition results of ABST.

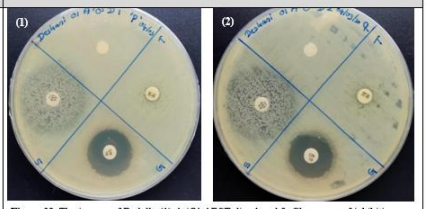
Sample/ Falcon	ABST disc 1 and ABST disc 2
Badulla (1) A 'O'	

Figure 29. The images of Badulla (1) A 'O' ABST disc 1 and 2. Clear zonesof inhibition were observed around Gentamicin discs and faint zones of inhibition were observed around Sulfonamide. Around Tetracycline and filter papers (Control) no zones of inhibitions were observed.

Badulla (4) B 'G'

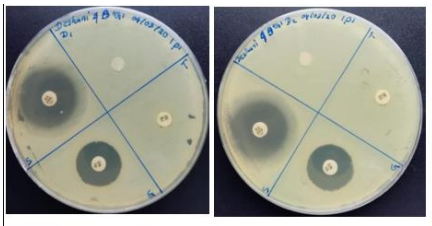


Figure 30. The images of Badulla (4) B 'G' ABST disc 1 and 2. Clear zones of inhibition were observed around Sulfonamide (few numbers of resisted colonies were observed) and Gentamicin discs where there were no zones of inhibitions were observed around Tetracycline and filter papers (Control).

Badulla (7) A 'G'

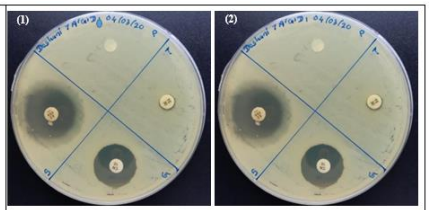


Figure 31. The images of Badulla (7) A 'G' ABST disc 1 and 2. Clear zones of inhibition were observed around Sulfonamide (few numbers of resisted colonies were observed) and Gentamicin discs where there were no zones of inhibitions were observed around Tetracycline and filter papers (Control).

Badulla (8) B 'O'

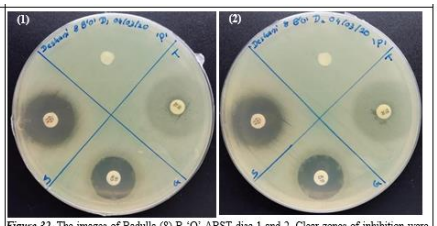


Figure 32. The images of Badulla (8) B 'O' ABST disc 1 and 2. Clear zones of inhibition were observed around Sulfonamide and Gentamicin discs where there were faint zones of inhibitions were observed around Tetracycline. No zones of inhibition were observed around the filter papers (Control).

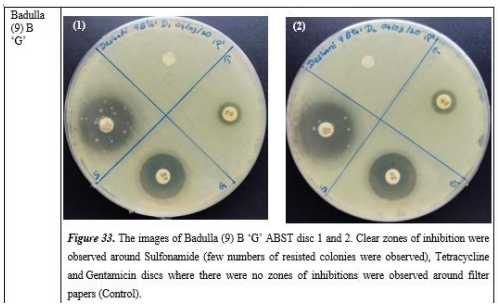


Figure 33. The images of Badulla (9) B 'G' ABST disc 1 and 2. Clear zones of inhibition were observed around Sulfonamide (few numbers of resisted colonies were observed), Tetracycline and Gentamicin discs where there were no zones of inhibitions were observed around filter papers (Control).

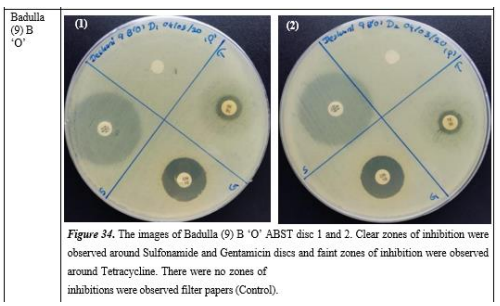


Figure 34. The images of Badulla (9) B 'O' ABST disc 1 and 2. Clear zones of inhibition were observed around Sulfonamide and Gentamicin discs and faint zones of inhibition were observed around Tetracycline. There were no zones of
inhibitions were observed filter papers (Control).

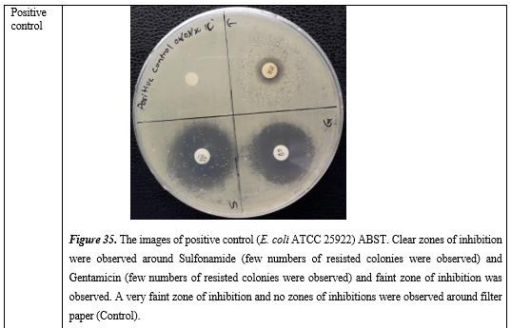


Figure 35. The images of positive control (*E. coli* ATCC 25922) ABST. Clear zones of inhibition were observed around Sulfonamide (few numbers of resisted colonies were observed) and Gentamicin (few numbers of resisted colonies were observed) and faint zone of inhibition was observed. A very faint zone of inhibition and no zones of inhibitions were observed around filter paper (Control).

Table 14. Diameter measurements of zones of inhibitions of 6 samples (mm)

Sample	Tetracycline		Sulfonamide		Gentamicin	
	Disc 1	Disc 2	Disc 1	Disc 2	Disc 1	Disc 2
Badulla (1)A 'O'	8	7	31	32	20	19
Badulla (4)B 'G'	0	0	25	25	19	19
Badulla (7) A 'G'	7	6	13	11	19	17
Badulla (8) B 'O'	14	13	12	12	19	19
Badulla (9) B 'G'	9	10	11	13	18	20
Badulla (9) B 'O'	10	10	32	31	9	8

Table 15. Mean and Standard Deviations of the diameters of zone of inhibitions

Sample	Tetracycline (mm)				Sulfonamide (mm)			
	Disc1	Disc 2	Mean	Standard Deviation	Disc1	Disc2	Mean	Standard Deviation
Badulla(1)A 'O'	8	7	7.5	7.5 ± 0.707	31	32	31.5	31.5±0.707
Badulla(4)B 'G'	0	0	0	0	25	25	25	25±0.00
Badulla (7) A'G'	7	6	6.5	6.5±0.707	13	11	12	12±1.414
Badulla (8) B'O'	14	13	13.5	13.5±0.707	12	12	12	12±0.00
Badulla (9) B'G'	9	10	9.5	9.5±0.707	11	13	12	12±1.414
Badulla (9) B'O'	10	10	10	10±0.00	32	31	31.5	31.5±0.707

Table 16. Tetracycline, Sulfonamide and Gentamicin zones of inhibition diameter CLSIstandards for Enterobacteria (gram negative (Jorgensen and Ferraro, 2009).

Antimicrobial species	Antibiotic	No resistance	Intermediate resistance	Resistance
Enterobacteria	Tetracycline	≥ 15 mm	12- 14 mm	≤ 11 mm
	Sulfonamide	≥16mm	11-15mm	≤ 10mm
	Gentamicin	≥ 15 mm	13 – 14 mm	≤ 12 mm

Then, with the reading of diameters 5 samples showed complete resistance for Tetracycline while Badulla (8)B 'O' samples were showed intermediate resistance (table 14). So, all 6 samples were selected to undergo PCR to detect Tetracycline resistance genes. Diameters of zones of inhibition for Sulfonamide

were observed more than 11mm (table 14). So, sampleswere not showered resistance or intermediate resistance for sulfonamide (table 14) and were not selected for PCR to detect sulfonamide resistance genes. As for Gentamicin 6 samples werenot showed any resistance or intermediate resistance (table 14). All the diameters were categorized according to CLSI standards for Enterobacteria (table 16).

Agarose gel electrophoresis for detection of Antibiotic resistance Genes –Tet A (210bp).

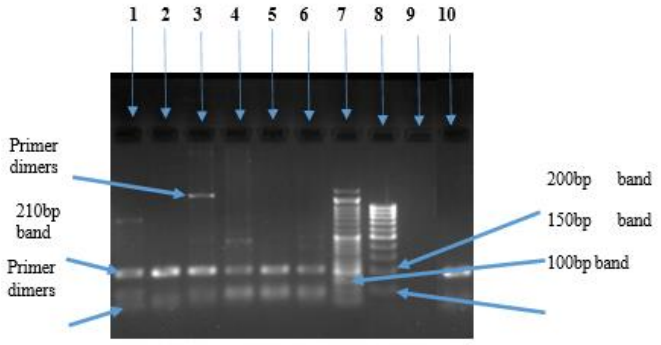


*Figure 36.*2% Agarose gel image for detection of Tet A.

Table 17. Samples loading arrangement and results interpretation of Gel image (figure 36).

Lane Number	Sample/Falcon	Presence of bands	Positive/ Negative
1	Badulla (1)A 'O'	+	Positive
2	Badulla (4)B 'G'	+	Positive
3	Badulla (7) A 'G'	+	Positive
4	Badulla (8) B 'O'	+	Positive
5	Badulla (9) B 'G'	+	Positive
6	Badulla (9) B 'O'	+	Positive
7	50 bp ladder	+	Bands were present
8	100 bp ladder	+	Bands were present
9	Negative control (Autoclaved Distilled water)	-	Negative
10	Positive control (*E-coli*, ATCC 25922)	+	Positive

(+) – Bands present
(-) – Bands not present

Agarose gel electrophoresis for detection of Antibiotic resistancegenes – Tet C (335bp)

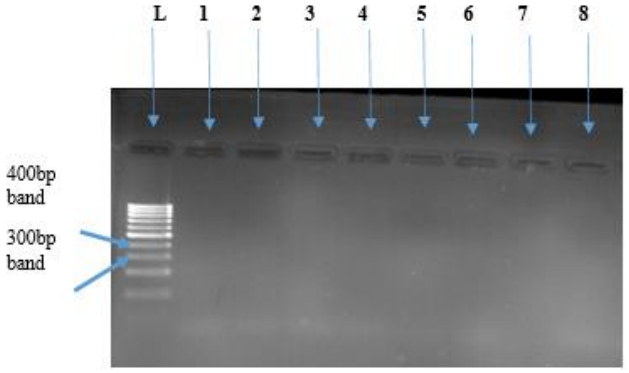


Figure 37. 2% Agarose gel image for detection of Tet-C gene.

Table 18. Samples loading arrangement and results interpretation of Gel picture (figure 37)

Lane Number	Sample/Falcon	Presence of bands	Positive/ Negative
L	100 bp ladder	+	Bands were present
1	Badulla (1)A 'O'	-	Negative
2	Badulla (4)B 'G'	-	Negative
3	Badulla (7) A 'G'	-	Negative
4	Badulla (8) B 'O'	-	Negative
5	Badulla (9) B 'G'	-	Negative
6	Badulla (9) B 'O'	-	Negative
7	Negative control (Autoclaved Distilledwater)	-	Negative
8	Positive control (*E-coli*, ATCC 25922)	-	Negative

(+) – Bands present

(-) – Bands not present

Summary of the results.

Table 19. Summary table of all the results

Sample	Gram staining (Pink rods)	Indole	Citrate	16s rRNA	uidA	LacY	TetA	TetC	Sul 1
Badulla (1)A 'O'	+	+	+	+	+	+	+	-	×
Badulla (2)A 'G'	-	-	+	+	+	-	×	×	×
Badulla (3)B 'G'	+	-	+	+	+	-	×	×	×
Badulla (4)B 'G'	+	+	+	+	+	+	+	-	×
Badulla(4)B 'P'	-	-	+	+	+	-	×	×	×
Badulla (5)A 'G'	+	-	+	+	+	-	×	×	×
Badulla (6)A 'O'	-	-	+	+	+	-	×	×	×
Badulla (7)A 'G'	+	+	+	+	+	+	+	-	×
Badulla(8)B 'P'	-	+	+	-	+	-	×	×	×
Badulla (8)B 'O'	+	+	+	-	+	+	+	-	×
Badulla (9)B 'G'	+	_	+	+	+	+	+	-	×
Badulla (9)B 'O'	+	+	+	+	+	+	+	-	×
Badulla (10)B'G'	+	-	+	+	+	-	-	×	×
Badulla (10)B'O'	+	-	+	-	+	-	-	×	×

(+) – Positive

(-) – Negative

(×) - Not tested

DISCUSSION

Drinking water is a crucial substance for each living organism (WHO, 2019). But In day today life water pollution has become a major issue, especially contamination by pathogenicbacteria which causes antibiotic resistances (Ashbolt,2004) and this study was conducted checkwhether water sources around Badulla Municipal Council Area has contaminated with Escherichia coli. Then 10 places were chosen, both protected and unprotected water sources. Samples were collected and physiochemical parameter were measured (table 4) (Priyanka, 2017).

Membrane filtration was carried out as triplicates to increase the probability of getting more colonies (Mahfouz, Shaieb and Elzen, 2008) and cellulose nitrate filter papers (0.45µm) (GK, 2020) were used because E-coli itself is approximately about 0.5 µm in width and to retains on the paper. Hi- crome Agar was used as the culture media. Most of the E. coli strains have glucuronidase enzyme (Caruso, 2017) which helps to differentiate them from other coliforms (Garcia-Armisen, Lebaron and Servais, 2005). Glucuronidase enzyme splits the bond between the chromophore and the glucuronide in the culture media and released chromophore produce bluish green color (Akter, Haque and Salam, 2020). While bile salts inhibit gram-positive organisms by causing extensive protein aggregation (Urdaneta and Casadesus, 2017) inside thebacteria. Tryptone and peptone provides essential growth nutrients to the organisms (HiMedia, 2019). Pink color colonies usually occurs due to ß- galactosidase enzyme activity on salmon-gal(Merkwitz et al., 2017) by some strains of E-coli (Garcia-Armisen, Lebaron and Servais, 2005).But in here pink color could be initially the colonies' color because hi-crome agar media doesnot contain salmon-gal (HiMedia, 2019). Opaque colonies were present in all 10 samples (figure 6), green colonies were present in 8 samples (figure 12) while pink was present in 2 samples (table 9) were opaque color colonies might be some strain of E.coli which do not express the enzyme activity such as Shiga toxin producing E.coli strain 0157:H7 (Favier et al, 2014) So, because of that not only green but also opaque and pink colonies were sub cultured inLB (table 4.2.1). Main Benefit of LB is the high reproducibility of the bacteria where the bacteriafrom the same stain will achieve a same state of growth at a given time (Caruso, 2017). The carbon sources for E. coli in LB are catabolizable amino acids (tryptone which extracted from casein in cow's milk) (Sezonov, Joseleaou-Petit and D-Ari, 2007).

Gram staining involves three processes: staining with a water-soluble dye called crystal violet, decolorization, and counterstaining, usually with safranin. Due to differences in the thickness of a peptidoglycan layer in the cell membrane between Gram positive and Gram negative bacteria(Gan, Chen and Jensen, 2008), Gram positive bacteria (with a thicker peptidoglycan layer) retaincrystal violet stain during the decolorization process, while E –coli is a gram negative rod shapedbacteria which loses its crystal violet stain (figure 18) due to thin layer of peptidoglycan and remain safranin (pink color) (Thairu, Usman and Nasir, 2014). Pink, cocci shaped (figure 19)organism could be species of Veillonella which is an organism can be found in normal flora of warm animal's gut systems. But the sample which was showed purple color and rod in shaped (figure 20) can be either Actinomyces which can find in soil or Bifidobacterium which can find in human gut. However, according to the HiMedia hi-chrome protocol (HiMedia, 2019) gram positive organisms should have inhibited (Urdaneta and Casadesus, 2017). So, it could be a staining error such as over stained with crystal violet or that organism

(especially Bifidobacterium) might have avoided the bile salt activity by producing antimicrobial substancesagainst bile's toxicity (Begley, Gahan, and Hill, 2005). And there was a possibility that gram negative rod shaped (figure 18) all samples might be not carrying only E-coli but also some species of Shigella, Salmonella and other coliform bacteria.

When comes to biochemical analysis Indole test was chosen because E-coli poses Tryptophanase enzyme which deaminate tryptophan and produce cherry red color ring by reacting with Kovac's reagent (P- dimethylaminobenzaldehyde) (figure 21) which takes as positive result while negative result gives a yellow color band (figure 22) (Aryal, S., fuaf, s. and Ahmed, T. (2019). In this study according to gram staining results there were 10 samples which were gram negative, and rod shaped. But according to indole results only 6 samples and the positive control (E. coli strain ATCC 25922) were positive (table 21). So, that was provedthe previous argument about other possible organisms because they do not have the Tryptophanase enzyme (Aryal, S., fuaf, s. and Ahmed, T. (2019). However, some of the E. coli samples also might not have showed as indole positive because they might have mutation in their gene which code for Tryptophanase enzyme (Li and Young, 2013). And other thing is that all indole positivesamples might be not only positive for E. coli but also some strains of Klebciella (most are grampositive), Aeromonas hydrophila and Plesiomonas shigelloides which are also have this enzyme(Darkoh, et al, 2015).

As for the Citrate utilization test, citrate agar is used to test an organism's ability to utilize citrateas a source of energy (Aryal, S. and Jennifer, T. (2019). The medium contains citrate as the onlycarbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the only source of nitrogen. Bacteria that can grow on this medium has an enzyme, Citrate-permease, which have the abilityof converting citrate to pyruvate (Brocker et al, 2020). When the bacteria metabolize citrate, theammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turnsthe bromothymol blue indicator in the medium from green to blue above pH 7.6 (Hofwegen, Hovde and Minnich, 2020). Most strains of E. coli don't have this enzyme and can't utilize citrate agar and increase the pH to change the color from green to blue.

However, in this study both indole positive and negative all 14 samples were showed positive results for citrate test (figure 23). That could be sub culturing error such as the inoculum loopmight have touch colonies which are not E. coli also (Aryal, S. and Jennifer, T. 2019). That meansin that subcultures (green colonies) not only the suspected E-coli colonies but also citrate positive bacteria species were there. Not only E-coli but also some Shigella spp, Salmonellatyphi and Salmonella paratyphi A are citrate negative while Salmonella other than typhi and paratyphi A and Klebsiella pneumonia show positive results. Most especially citrate agar itselfproduces an anaerobic condition for E-coli which is known as a facultative anaerobic organism but environmental conditions such as high room temperature might have caused differences in the media to show positive results (Hofwegen, Hovde and Minnich, 2020). And the other thing is there are some E. coli strains such as E. coli K-12 which can be found in waste products of pigsand pigeons has showed positive results for citrate test (Ishiguro, 1978).

Molecular analyses were fully based on DNA extraction, PCR, and agarose gel electrophoresis. Sample's DNAs were extracted using Promega kit. Kit based extraction DNA showed thick/high intense bands (figure 25) in the gel picture. Band was not present in negative control which gives the idea there was no contamination. Band was present in positive control that

means the reagents have done their role. Low dense bands could bedue to DNA fragmentation or extraction and PCR error such as poor performance of reagents.

16s Ribosomal RNA (16s rRNA) gene is a universal gene which is involving in the formation ofthe bacterial ribosomes structure (Clifford et al. 2020). and which use for detection of bacteria,and it has many variable regions. According to the type of bacteria these variable regions have classified. Here a special primer sequence which present in coliforms, and which is 424bp in length was used to detect coliforms in the samples (Janda and Abbott, 2007).

In this study 1 set of (reverse and forward) 16s rRNA primer (424bp) (Clifford et al., 2020) wasused and 11 samples such as Badulla (1) A 'O' and (2) A 'G' were showed bands between 400bpand 450bp ladder bands level (figure 26). uidA gene which encodes ß-glucuronidase enzyme is a specific enzyme which can be found in E. coli as before mentioned. So, detecting that gene has a high possibility of that sample to be E coli. But E. coli are not the only species that contain uidA gene in their genome (Zhang et al, 2015). But some of Shigella spp also have shown positive results for uidA. In this study all 14 were showed bands in the gel image of uidA gene detection (figure 27). There are several possibilities to get that results such as all samples could be either Shigella spp or E-coli spp (Suardana, 2020) or handling errors such as contamination while doing PCR could be also the reasons. Lactose operon (Lac Y) is an operon which required for the transport and metabolism of lactose in bacteria including E-coli (Kuhlman et al, 2007). Even though glucose is the favored carbon source for most bacteria, the lac operon lets for effective digestion of lactose when glucose is not available through the activity of beta-galactosidase (Ullmann, 2009). As mentioned before that enzyme is present in E-coli which means it also should be positive for LacY gene (Horakova, Mlejnkova and Mlejnek, 2008) and in this study 6 samples were positive forLac Y gene (figure 28). Even Shigella spp could show positive for uidA but not for Lac Y because even they have Lac operon in their genome, but they lack the promoter region (Ud-Din and Wahid, 2014). So, they can't express the gene. Throughout the PCR steps positive control was not given a band in gel images (figure 29). Low intensity of the bands, poor amplification of DNA and DNA fragmentation could be due to low concentration of DNA. When the primersare not very specific to the target the primer dimers occur (Lorenz, 2012). Both Lac Y and uidAgenes positive samples were chosen to undergo ABST. Because that increases the possibility ofthe samples to be E-coli.

Then, 6 samples were subjected to ABST (table 12) as duplicate to increase the efficacy of the test (Jorgensen and Ferraro, 2009) and disk diffusion method was used rather than using welldiffusion because the antibiotics were not in liquid form (Balouiri, Sadiki and Ibnsouda, 2016).Tetracycline (30µg) (Zhang et al, 2014), Sulfonamide (300µg) and Gentamicin (10µg) were chosen as the antibiotics. Detection of Tetracycline and Sulfonamide resistance were the majoraim of this study because they are common antibiotics which are used to treat E-coli infections (Pallecchi et al., 2007). Gentamicin has shown good results in treating these kind of diseases (Roldan-Masedo et al., 2019) and that can be proved by the results of this study also where it showed no resistance (table 13). As for negative control autoclaved filter papers was used to justify the results (Lanz, Kuhnert and Boerlin, 2003).

Diameters were measured and categorized in to either no resistance, intermediate resistance, or complete resistance according to standard diameters

provided by the CLSI (Jorgensen and Ferraro, 2009). All 6 samples were showed complete or intermediate resistance for Tetracycline,and they were selected to detect resistance genes (Tet A and Tet C) (Hu et al., 2008). But for Sulfonamide all the sample's diameters were recorded as no resistance (table 14). So, they were not selected to detect Sul 1 gene. But when it come Tetracycline resistance gene detectionall 6 samples were positive for Tet A gene along with positive control (figure 17) and all 6 samples were negative for Tet C along with the positive control (figure 18). That result couldbe concluded as all 6 sample's showed Tetracycline resistance in ABST due to Tet A gene (table 19).

CONCLUSION

Even though 10 samples were collected from different water sources in Badulla Municipal council Area (figure 4) 6 out of 14 colonies(samples) from 5 sources of water were detectedfor E. coli strains. Tet A resistance genes in their genome which has grown under thephysiochemical conditions in the table 4 were detected. From them 2 of the water samples were collected from protected wells, 3 of them were unprotected common wells.

Future work

For the biochemical analysis Methyl red test can be used because it is more sensitive than citrate.To identify Shigella spp from E. coli motility test and Acetate Utilization test can be used becauseE. coli shows positive results for both tests while Shigella spp shows negative results for both (Chattaway et al., 2017). As for 16s rRNA detection to confirm coliforms another several set of primers tests can be used. ABST can be used to check antibiotics resistance of E. coli for other common antibiotic such as Ampicillin, Co-trimaxazole and Ciprofloxacin including Gentamicin (using different milligrams) (Alanazi, Alqahtani and Aleanizy, 2018). Strain specific PCR should be carried out to find what are the E. coli strains that present in Tet A detected samples. Not only thatbut also even all 6 samples were positive for Tet A and negative for Tet C they could be positivefor other Tet genes such as Tet B, E, M and O. They can detect by performing PCR using specific primers sets (Hu et al., 2008). Shigella, Salmonella and other possible organism specificPCR can be carried out to find out the organisms in the other 8 samples. E. coli strain specific PCR also can be carried out (Molina et al., 2015) to find whether the water has contaminated bysevere pathogenic strains such as Shiga toxin producing 0157:H7 strain (Favier et al, 2014). PCRconditions can be furfure optimize to avoid primer dimers and to increase the intensity of the bands (Lorenz, 2012).

Acknowledgement

I would like to thank my principal supervisor Ms. Uthpala Amarasekara for guiding me throughout the project. All the academic staff including the supervisor Ms. Punsisi Weerasooriyaand the laboratory in charge Mr. Ominda Perera, Ms. Himashi Gurudeniya and Mr Nishantha Karunaharan for extending their support in providing me all the necessary equipment, workplaces, and reagents to conduct my research.

REFERENCES

Aditi, F., Rahman, S. and Hossain, M., 2017. A Study on the Microbiological Status of MineralDrinking Water. The Open Microbiology Journal, [online] 11(1), pp.31-44. Available at:<https://pubmed.ncbi.nlm.nih.gov/28603564/> [Accessed 7 May 2020].

Akter, L., Haque, R. and Salam, M., 2020. Comparative Evaluation Of Chromogenic Agar Medium And Conventional Culture System For Isolation And Presumptive Identification Of Uropathogens | Akter | Pakistan Journal Of Medical Sciences Old Website. [online] PMC. Available at: <https://dx.doi.org/10.12669%2Fpjms.305.5243> [Accessed 16 March 2020].

Alanazi, M., Alqahtani, F. and Aleanizy, F., 2018. An evaluation of E. coli in urinary tract infection in emergency department at KAMC in Riyadh, Saudi Arabia: retrospective study. Annals of Clinical Microbiology and Antimicrobiology, [online] 17(3). Available at:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5806437/> [Accessed 5 May 2020].

Aryal, S., fuaf, s. and Ahmed, T. (2019). Indole Test- Principle, Reagents, Procedure, ResultInterpretation and Limitations. [online] Microbiology Info.com. Availableat: https://microbiologyinfo.com/indole-test-principle-reagents-procedure-result-interpretation- and-limitations/ [Accessed 24 Nov. 2019].

Aryal, S. and Jennifer, T. (2019). Citrate Utilization Test- Principle, Media, Procedure and Result. [online] Microbiology Info.com. Available at: https://microbiologyinfo.com/citrate - utilization-test-principle-media-procedure-and-result/ [Accessed 24 Nov. 2019].

Ashbolt, N. (2004). Microbial contamination of drinking water and disease outcomes in developing regions. Toxicology, [online] 198(1-3), pp.229-238. Available at: https://www.sciencedirect.com/science/article/pii/S0300483X04000952.

Balouiri, M., Sadiki, M. and Ibnsouda, S., 2016. Methods for in vitro evaluating antimicrobialactivity: A review. Journal of Pharmaceutical Analysis, [online] 6(2), pp.71-79. Available at:<https://www.sciencedirect.com/science/article/pii/S2095177915300150> [Accessed 5 May2020].

Begley, M., Gahan, C. and Hill, C., 2005. The interaction between bacteria and bile. FEMS Microbiology Reviews,[online] 29(4),pp.625-651.Available at: <https://academic.oup.com/femsre/article/29/4/625/492093> [Accessed 4 May 2020].

Blanco, P., Hernando-Amando, S., Antonio Reales-Calderon, J., Corona, F., Lira, F., Alcalde- Rico, M., Bernardini, A., Sanchez,

M. and Martinez, J. (2016). Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants.

Microorganisms, [online] 4(1), p.14.Available at:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5029519/ [Accessed 7 Dec.2019].

Blount, Z. (2015). The unexhausted potential of E. coli. eLife, [online] 4. Available at:https://elifesciences.org/articles/05826 [Accessed 3 Dec. 2019].

Brocker, M., Schaffer, S., Mack, C. and Bott, M., 2020. Citrate Utilization By CorynebacteriumGlutamicum Is Controlled By The Citab Two-Component System Through Positive Regulation Of The Citrate Transport Genes Cith And Tctcba. [online] PMC. Available at:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2698389/> [Accessed 14 March 2020]

Byrne-Bailey, K., Gaze, W., Kay, P., Boxall, A., Hawkey, P. and Wellington, E. (2009). Prevalence of Sulfonamide Resistance Genes in Bacterial Isolates from Manured Agricultural Soils and Pig Slurry in the United Kingdom ▿. [Online] NCBI. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2630619/ [Accessed 5 Dec. 2019].

Caruso, G., 2017. β-glucuronidase activity determination as an indirect estimate of Escherichia coli: development of a miniaturized assay and its

application to seawater samples. Journal of Clinical Microbiology and Biochemical Technology, [online] pp.046-050. Available at:<https://www.peertechz.com/articles/doi10.17352-jcmbt.000027.php> [Accessed 19 March2020].

Chattaway, M., Schaefer, U., Tewolde, R., Dallman, T. and Jenkins, C., 2017. Identification of Escherichia coli and Shigella Species from Whole-Genome Sequences. Journal of Clinical Microbiology, [online]55(2), pp.616-623.Available at:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5277532/> [Accessed 3 May 2020].

Chen B, Liang X, Huang X, Zhang T, Li X (2013) Differentiating anthropogenic impacts on ARGs in the Pearl River Estuary by using suitable gene indicaters Water Res 47:28111-2820 doi:10.1016/j.wateres.2013.02.042.

Darkoh, c., Chapelle, C., Gonzales, C. and Okhuysen, P., 2015. A Rapid And Specific Method For The Detection Of Indole In Complex Biological Samples. [online] PMC. Available at:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4651089/> [Accessed 8 March 2020].

Edberg, S., Rice, E., Karlin, R. and Allen, M. (2000). Escherichia coli: the best biologicaldrinking water indicator for publichealth protection. Journal of Applied Microbiology, [online] 88(S1),pp.106S-116S. Availableat: https://sfamjournals.onlinelibrary.wiley.com/doi/abs/10.1111/j.1365-2672.2000.tb05338.x [Accessed 6 Dec. 2019].

Favier, G., Lucero Estrada, C., Cortiñas, T. and Escudero, M., 2014. Detection and Characterization of Shiga Toxin ProducingEscherichia coli,Salmonellaspp.,andYersiniaStrainsfrom Human, Animal, and Food Samples in San Luis, Argentina. International Journal of Microbiology, [online] 2014, pp.1-11. Available at: <https://www.hindawi.com/journals/ijmicro/2014/284649/> [Accessed 5 May 2020].

Fernando, D., Tun, H., Poole, J., Patidar, R., Li, R., Mi, R., Amarawansha, G., Fernando, W., Khafipour, E., Farenhorst, A. and Kumar, A. (2016). Detection of Antibiotic Resistance Genes in Source and Drinking Water Samples from a First Nations Community in Canada. [Online] Applied and Enviromental Microbiology Available at: https://aem.asm.org/content/82/15/4767/article-info [Accessed 2 Dec. 2019].

Gan, L., Chen, S. and Jensen, G., 2008. Molecular organization of Gram-negative peptidoglycan.Proceedings of the National Academy of Sciences, [online] 105(48), pp.18953-18957. Available at: <https://www.pnas.org/content/105/48/18953.short> [Accessed 8 May 2020].

Garcia-Armisen, T., Lebaron, P. and Servais, P., 2005. beta-d-glucuronidase activity assay to assess viable Escherichia coli abundance in freshwaters. Letters in Applied Microbiology, [online] 40(4), pp.278-282. Available at: <https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/j.1472-765X.2005.01670.x> [Accessed 13 March 2020].

GK, S., 2020. Detection And Identification Of Groundwater Bacteria Capable Of Escaping Entrapment On 0.45-Micron-Pore-Size Membrane Filters. - Pubmed - NCBI. [online] Ncbi.nlm.nih.gov. Available at: <https://www.ncbi.nlm.nih.gov/pubmed/1768096> [Accessed 16 February 2020].

Griffin, M., Ray, W., Livengood, J. and Schaffner, W. (1988). Risk of Sudden Infant Death Syndrome after Immunization with the Diphtheria–Tetanus–Pertussis Vaccine. New England Journal of Medicine, [online] 319(10), pp.618-623.

Available at: https://www.nejm.org/doi/full/10.1056/NEJM198809083191006 [Accessed 6 Dec. 2019].

Hasegawa, H., Suzuki, E. and Maeda, S. (2018). Horizontal Plasmid Transfer by Transformation in Escherichia coli: Environmental Factors and Possible Mechanisms. Frontiers in Microbiology. [Online] Available at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02365/full [Accessed 1 Dec. 2019].

Himedialabs.com. 2019. [online] Available at: <http://himedialabs.com/TD/M1297A.pdf> [Accessed 5 January 2020].

Hu, J., Shi, J., Chang, H., Li, D., Yang, M. and Kamagata, Y., 2008. Phenotyping andGenotyping of Antibiotic-Resistant Escherichia coli Isolated from a Natural River Basin. Environmental Science & Technology, [online] 42(9), pp.3415-3420. Available at:<https://pubs.acs.org/doi/abs/10.1021/es7026746> [Accessed 5 March 2020].

Janda, J. and Abbott, S., 2007. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. Journal of Clinical Microbiology, [online] 45(9), pp.2761-2764. Available at:<https://jcm.asm.org/content/45/9/2761> [Accessed 8 March2020].

Kaper, J., Nataro, J. and Mobley, H. (2004). Pathogenic Escherichia coli. Nature Reviews Microbiology,[online] 2(2) pp.123-140. Available at: https://www.ncbi.nlm.nih.gov/pubmed/15040260 [Accessed 4 Dec. 2019].

Kapoor, G., Saigal, S. and Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. Journal of Anaesthesiology Clinical Pharmacology, [online] 33(3), pp.300-305. Available at: http://www.joacp.org/downloadpdf.asp?issn=0970-9185;year=2017;volume=33;issue=3;spage=300;epage=305;aulast=Kapoor;type=2 [Accessed4 Dec. 2019].

Karami, N., Nowrouzian, F., Adlerberth, I. and Wold, A. (2006). Tetracycline Resistance in Escherichia coli and Persistence in the Infantile Colonic Microbiota. [Online] NCBI. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1346771/ [Accessed 6 Dec. 2019].

Kuhlman, T., Zhang, Z., Saier, M. and Hwa, T., 2007. Combinatorial transcriptional control of the lactose operon of Escherichia coli. Proceedings of the National Academy of Sciences, [online] 104(14), pp.6043-6048. Available at: <https://www.pnas.org/content/104/14/6043>[Accessed 5 May 2020].

Lanz, R., Kuhnert, P. and Boerlin, P., 2003. Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland. VeterinaryMicrobiology, [online] 91(1), pp.73-84. Available at: <https://www.sciencedirect.com/science/article/pii/S0378113502002638> [Accessed 11 March2020].

Li, G. and Young, K., 2013. Indole production by the tryptophanase TnaA in Escherichia coli isdetermined by the amount of exogenous tryptophan. Microbiology, [online] 159(Pt_2), pp.402-410. Availableat:<https://www.microbiologyresearch.org/content/journal/micro/10.1099/mic.0.064139-0> [Accessed 5 May 2020].

Lorenz, T., 2012. Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting andOptimization.Journal of Visualized Experiments, [online] 63. Available at:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4846334/> [Accessed 6 May 2020].

Mahfouz, S., Shaieb, F. and Elzen, A., 2008. Detection And Identification Of GroundwaterBacteria In Sebha City, Libya. [online]

https://academicjournals.org/ajmr. Available at: <https://www.researchgate.net/publication/255964061_Detection_and_Identification_of_Grou ndwater_Bacteria_in_Sebha_City_Libya_BY> [Accessed 17 February 2020].

Molina, F., López-Acedo, E., Tabla, R., Roa, I., Gómez, A. and Rebollo, J., 2015. Improved detection of Escherichia coli and coliform bacteria by multiplex PCR. BMC Biotechnology, [online] 15(1). Available at: <https://bmcbiotechnol.biomedcentral.com/articles/10.1186/s12896-015-0168-2#citeas> [Accessed 31 March 2020].

N Ishiguro, G., 1978. Isolation Of Citrate-Positive Variants Of Escherichia Coli From DomesticPigeons, Pigs, Cattle, And Horses.. [online] PubMed Central (PMC). Available at:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC291204/> [Accessed 5 May 2020].

Olson, N. and Morrow, J., 2012. DNA extract characterization process for microbial detection methods development and validation. BMC Research Notes, [online] 5. Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3599793/> [Accessed 3 May 2020].

Pallecchi, L., Lucchetti, C., Bartoloni, A., Bartalesi, F., Mantella, A., Gamboa, H., Carattoli, A.,Paradisi, F. and Rossolini, G., 2007. Population Structure and Resistance Genes in Antibiotic- Resistant Bacteria from a Remote Community with Minimal Antibiotic Exposure. AntimicrobialAgents and Chemotherapy, [online] 51(4), pp.1179-1184. Available at: <https://aac.asm.org/content/51/4/1179.short> [Accessed 11 March 2020].

Priyanka., 2017. Physicochemical And Microbiological Qualityassessment Of Drinking Water From Commercial Suppliers At Selected Locations In Dhaka, Bangladesh. International Journal of Research in Engineering and Technology, 06(01), pp.149-153.

Roldan-Masedo, E., Sainz, T., Gutierrez-Arroyo, A., Gomez-Gil, R., Ballesteros, E., Escosa, L.,Baquero-Artigao, F. and Méndez- Echevarría, A., 2019. Risk factors for gentamicin-resistant E.coli in children with community-acquired urinary tract infection. European Journal of Clinical Microbiology & Infectious Diseases, [online] 38(11), pp.2097-2102. Available at:<https://link.springer.com/article/10.1007/s10096-019-03643-7#citeas> [Accessed 20 March 2020].

Ruddell, K. and Anselmo, C., 2020. Antibiotic Susceptibility Testing Of Gram-Negative NonfermentativeBacilli. [online]PMC. Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC429152/> [Accessed 9 March 2020].

Sezonov, G., Joseleaou-Petit, D. and D-Ari, R., 2007. Escherichia Coli Physiology In Luria- Bertani Broth. [online] PMC. Available at:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2168924/> [Accessed 13 March 2020].

Thairu, Y., Usman, Y. and Nasir, I., 2014. Laboratory perspective of gram staining and its significance in investigations of infectious diseases. Sub-Saharan African Journal of Medicine, [online] 1(4), p.168. Available at: <http://www.ssajm.org/article.asp?issn=2384-5147;year=2014;volume=1;issue=4;spage=168;epage=174;aulast=Thairu> [Accessed 4 May 2020].

Ud-Din, A. and Wahid, S., 2014. Relationship among Shigella spp. and enteroinvasive Escherichia coli (EIEC) and their differentiation. Brazilian Journal of Microbiology, [online] 45(4), pp.1131-1138. Available at: <https://www.scielo.br/scielo.php?s

cript=sci_arttext&pid=S1517-83822014000400002> [Accessed 4 May 2020].

Ullmann, A., 2009. Escherichia coliLactose Operon. Encyclopedia of Life Sciences, [online] Availableat:<https://onlinelibrary.wiley.com/doi/full/10.1002/9780470015902.a0000849.pub2> [Accessed31 March 2020].

Urdaneta, V. and Casadesus, J., 2017. Interactions between Bacteria and Bile Salts in the Gastrointestinal and Hepatobiliary Tracts. Frontiers in Medicine, [online] 4(153). Available at:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5632352/> [Accessed 4 March 2020].

Van Hofwegen, D., Hovde, C. and Minnich, S., 2020. Rapid Evolution Of Citrate Utilization ByEscherichia Coli By Direct Selection Requirescittanddcta. [online] PMC. Available at:<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4800869/> [Accessed 14 March 2020].

Wassenaar, T. (2016). Insights from 100 years of research with probiotic E. coli. European Journal of Microbiology and Immunology, [online] 6(3), pp.147-161. Available at: https://akademiai.com/doi/abs/10.1556/1886.2016.00029 [Accessed 4 Dec. 2019].

WHO (2019). Drinking-water. [Online] Available at: https://www.who.int/en/news-room/fact- sheets/detail/drinking-water [Accessed 4 Dec. 2019].

Zhang, Y., Hong, P., LeChevallier, M. and Liu, W., 2015. Phenotypic and PhylogeneticIdentification of Coliform Bacteria Obtained Using 12 Coliform Methods Approved by the U.S.Environmental Protection Agency. Applied and Environmental Microbiology, [online] 81(17), pp.6012-6023. Available at: <https://aem.asm.org/content/81/17/6012> [Accessed 8 March 2020].

Zhang, Y., Boyd, S., Teppen, B., Tiedje, J. and Li, H., 2014. Role of Tetracycline Speciation inthe Bioavailability to Escherichia coli for Uptake and Expression of Antibiotic Resistance. Environmental Science & Technology, [online] 48(9), pp.4893-4900. Available at:<https://pubs.acs.org/doi/abs/10.1021/es5003428> [Accessed 6 March 2020