A STUDY OF MICROBIAL WATER QUALITY CHECK AND ISOLATION OF *HELICOBACTER PYLORI* FROM WATER SAMPLES TAKEN FROM DHAKA CITY

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology

Department of Mathematics and Natural Science
BRAC University
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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Afra Anika Rafique
Jannatul Sumaiya

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List of Acronyms

H. pylori	*Helicobacter pylori*
E. coli	*Escherichia coli*
CDC	Centers for Disease Control and Prevention
PCR	Polymerase Chain Reaction
dH_2O	Distilled Water
DO	Dissolved Oxygen
MFC	Membrane Fecal Coliform
EMB	Eosin Methylene Blue
TSB	Tryptone Soya Broth
TSA	Tryptone Soya Agar
MIU	Motility Indole Urea
TNTC	Too Numerous To Count
TAE	Tris-Acetate-EDTA
UV	Ultra Violet
g	gram
bp	base pair
ml	milliliter
mg/L	milligram per liter
rpm	rotations per minute
WH	White Hemolytic
WNH	White Non-Hemolytic

Abstract

Water is indispensable for human survival. According to the World Health Organization, around two billion people in the world drink fecal contaminated water. This means there can be higher chances for the presence of the *Helicobacter pylori* in drinking water. *H. pylori* infection is the most common cause for gastric ulcer. In this study, water samples were collected in sterile autoclaved bottles from different areas of Dhaka City. Different media were used to detect fecal coliform, total coliform and differentiated colonies of *H. pylori*. The identification of *H. pylori* was carried out by biochemical tests, and PCR followed by gel electrophoresis. A 520 base pair of *H. pylori* DNA was found in the result of gel electrophoresis. From the result of this study, it is found that the presence of fecal and total coliform is at an alarming rate in the underprivileged areas (Korail slum and Bossila slum) than privileged areas. *H. pylori* has also been successfully isolated from the water samples taken from Korail slum.

Introduction

Chapter 1

1.1 Background of the Study

Water is essential part of human survival. According to Centers for Disease Control and Prevention (CDC), there are 7.8 billion people in the world that does not have access to improved water source. Each of 7.8 billion people has right to drink safe water regardless of socioeconomic condition. But around two billion people in the world drinks fecal contaminated water according to the World Health Organization. *Helicobacter pylori* positive infection happens early in life around 70% –90% of the population in developing countries, (Brain et al., 2014). *H. pylori* infection causing gastric disease is very common in Bangladesh. If there is presence of *H. pylori* in drinking water or food that was washed with water, there is higher rate of *H. pylori* infection causing gastric ulcer in most people (Habib et al., 2016). This would prove that there is a correlation between drinking contaminated water with *H. pylori* and infection of *H. pylori* in the people living around the source of contaminated water. The aim of the study is to find the presence of *H. pylori* in water samples collected from different areas of Dhaka City and to confirm the strain of the species has 16SrRNA gene sequences through genetic analysis (Nguyen et al., 2010). Simultaneously, as part of the study, water quality was determined by colony count of indicator organisms like fecal and total coliform.

1.2 Literature Review

1.2.1 Water Quality

There are three factors that determines water quality: physical, chemical and microbiological quality. Under physical quality, turbidity and colour of water are most vital measurement. Both turbidity and color must have less than five unit of measurement. The color of the water should be agreeable and tastes pleasant. For judging chemical quality of water, the total hardness of

the water should be less than 300mg/L, the amount of chlorides in water should be around 200mg/L and does not exceed 600mg/L, ammonia in water should less than 0.05mg/L, water should be free from nitrites, nitrates in water should not exceed 5mg/L, dissolved oxygen (DO) in the water should be more than 5mg/L and the water should be free of radioactive metals and toxic metals. The criteria to determine the microbiological quality of water are the water must be free of fecal coliform, pathogens and spoilage causing microbes (Walker et al., 2019).

Determining coliform count in scale of:

Class Water 1. *Highly satisfactory*: 0-1 per 100ml

Class Water 2. *Satisfactory*: 1-2 per 100ml

Class Water 3. *Doubtful*: 3-10 per 100ml

Class Water 4. *Unsatisfactory*: >10 per 100ml

1.2.2 Indicator Organisms

Coliforms are known as "indicator organisms". They are the bacteria that are found in the soil, water surfaces, and other parts of environment. Although some presence of coliform are harmless, detection of coliform indicates the presence of potential pathogenic bacteria in water and food sources (Halkman and Halkman, 2014). There are three types of coliforms:

1. Total coliform
2. Fecal coliform
3. *Escherichia coli*

1.2.2.1 Total coliform:

Total coliform are bacteria in the soil, in water that lives freely in surface water, in human faeces and animal waste. Detection of total coliform does not essentially confirm the disease causing bacteria are present (Bartram and Balance, 1996).

1.2.2.2 Fecal coliform:

Fecal coliform are bacteria that are present specifically in the gut and faeces of warm blooded animals. Since the origin of fecal coliform is more specific than total coliform, their detection in a water indicates it is contaminated by sewage and can cause disease. Fecal coliform can cause stomach upset and food poisoning (Bartram and Balance, 1996).

1.2.2.3 Escherichia coli (E. coli):

Escherichia coli is the main species in the fecal coliform group. Since among species of total coliform, only *E. coli* is usually not found growing and reproducing in the environment. *E. coli* is considered to be the species of coliform bacteria that is the best indicator of fecal pollution and the potential presence of pathogens. *E. coli* cannot live long outside the host and their presence in water is evidence that water was contaminated not so long. If contaminated animal meat is consumed it can cause a person to become ill. Rare strains of *E. coli* like 0157:H7 can cause serious outbreak of disease like acute diarrhea (Hachich et al., 2012).

1.2.3 Helicobacter pylori

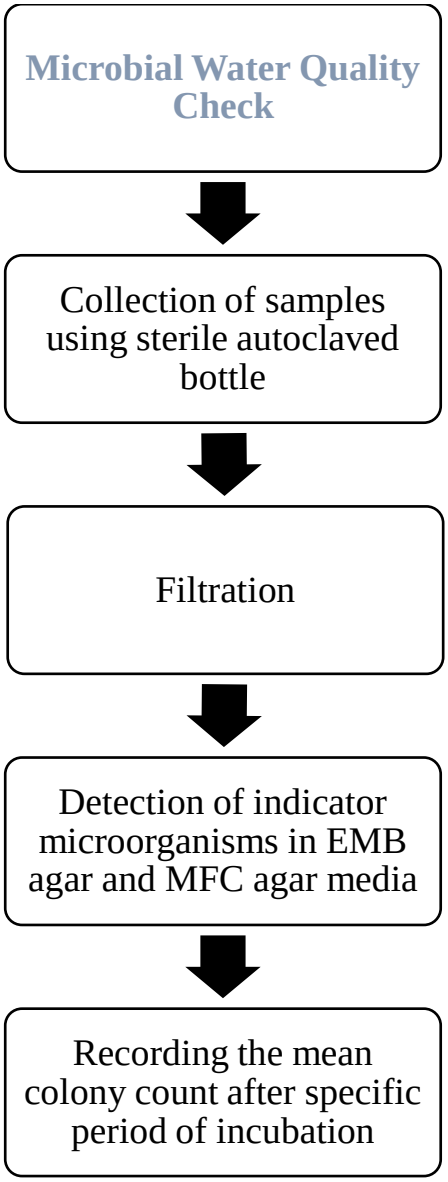
H. pylori is a gram negative, microaerophilic, spiral shaped and flagellated bacteria (Ranjbar et al., 2016). They are adapted in the harsh, acidic environment of the stomach, and can alter the environment by reducing the acidity of the stomach so that they can survive. The spiral shape of *H. pylori* allows it to infiltrate stomach lining where they are protected by mucus. This prevents body's immune cells from reaching them which allows the bacteria to disrupt immune response preventing their own destruction. In the world, 60% of the stomach infection are due to *H. pylori* (Hooi et al., 2017). *H. pylori* is the causes for disease like type B gastritis and gastric adenocarcinoma that leads to stomach ulcer (Shrestha et al., 2012). Contaminated water can play a key role in spreading *H. pylori* to humans through fecal and oral route of transmission (El-Sharouny et al., 2015).

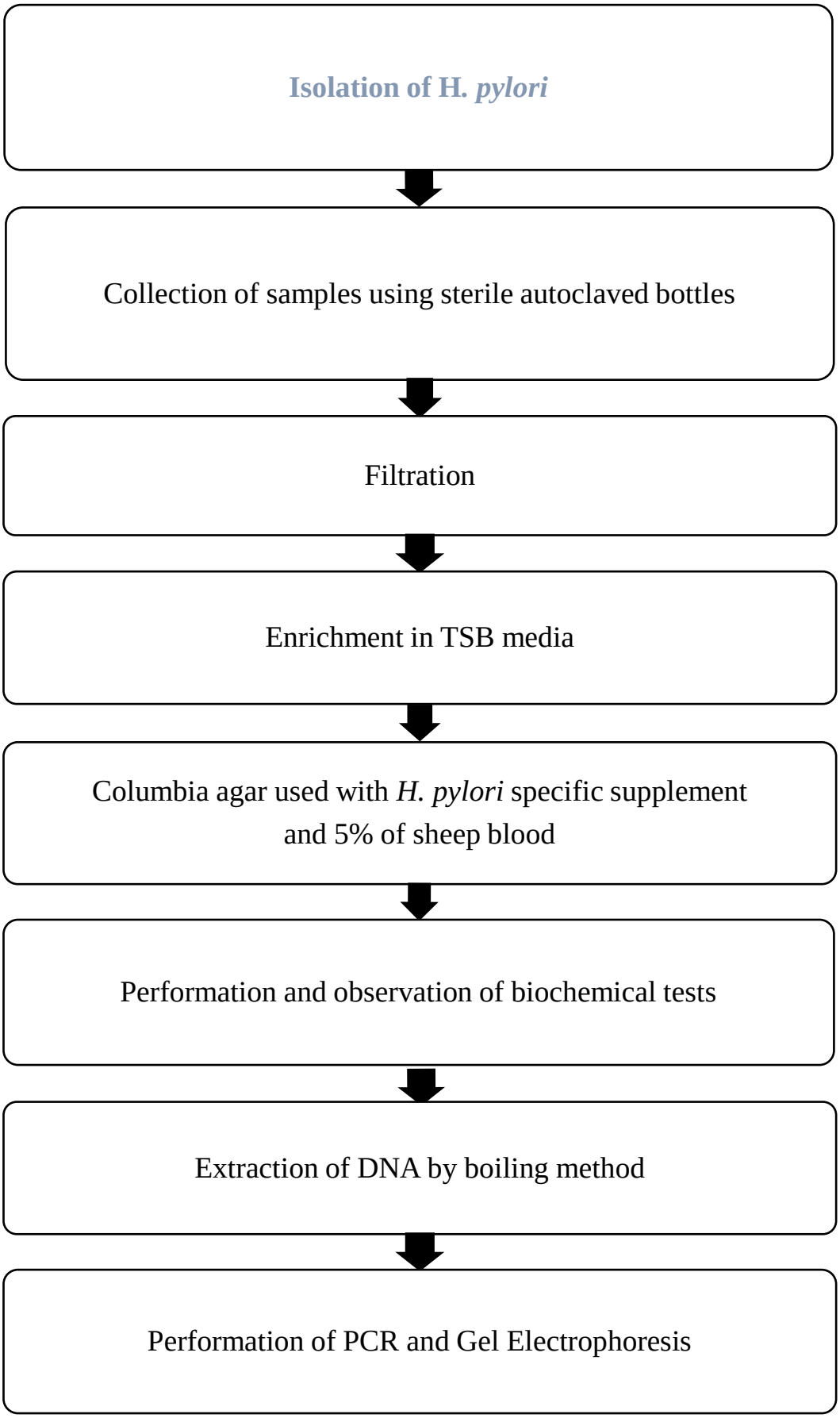
Workflow

Chapter 2

2.1 Experimental Workflow

The flowchart below shows the two workflows; one for the microbial water quality check and the other for isolation of *H. pylori*.




Isolation of H. pylori
Collection of samples using sterile autoclaved bottles
Filtration
Enrichment in TSB media
Columbia agar used with H. pylori specific supplement and 5% of sheep blood
Performation and observation of biochemical tests
Extraction of DNA by boiling method
Performation of PCR and Gel Electrophoresis

Methods

Chapter 3

3.1 Sample Collection

We had collected water samples from different areas of Dhaka city. In total, we had 43 samples from Mirpur, Mohakhali, Mohammadpur, Lalmatia, Dhaka Cantonment, Motijheel, Rampura to conduct our research. We also collected water from two densely populated slum areas; Bossila slum and Korail slum.

3.2 MFC agar for the detection of fecal coliforms

This is a membrane filtration. The m-FC agar contains selective and differential agents. It contains rosolic acid for the inhibition of bacterial growth in general, except for fecal coliforms. The indication of fermenting ability is caused by aniline blue which is responsible for the pH change in the medium. Blue color confirms the identification of fecal coliforms. The membrane filter method was described by James G. Cappuccino and Natalie Sherman in their book "Microbiology: A Laboratory Manual" (2008).

⇒ first, the agar powder was measured and it was put into a conical flask containing dH_2O.

⇒ then the media was heated to dissolve.

⇒ it did not require autoclaving.

⇒ the media was cooled to 45-50℃ .

⇒ after that, the media was poured in number of plates.

⇒ the second phase of this method started with the filtration of water samples where 100ml of water was filtered through the filter paper.

⇒ after the filtration was done, the filter paper was kept in the respective plate.

⇒ the plates were incubated for 24 hours at 45℃ .

⇒ after this incubation period, the colony count was recorded.

3.3 EMB agar for the detection of total coliforms

EMB agar is used for the detection of indicator micro-organism in water quality tests. It is both selective and differential culture medium. It contains Methylene blue and Eosin-y that inhibits gram-positive bacteria, they both serve as differential indicators. Coliforms appear in pink colonies. The membrane filter method was described by James G. Cappuccino and Natalie Sherman in their book "Microbiology: A Laboratory Manual" (2008).

3.3.1 Media preparation and plating:

⇒ the agar powder was measured and transferred to a conical flask containing dH_2o.

⇒ mixed it until the suspension is uniform.

⇒ then the media was heated to dissolve.

⇒ after that, the media was sterilized by autoclaving.

⇒ after the autoclaving was done, the media was cooled to 45-50℃ .

⇒ finally, it was poured in a number of plates.

3.3.2 Filtration and incubation:

⇒ filtration was done where 100ml of water was filtered through the filter paper.

⇒ after the filtration, filter papers were kept in those plates.

⇒ later, the plates were incubated for 24 hours at 37℃.

⇒ after the incubation period, colony count was recorded.

3.4 Detection of Helicobacter pylori

3.4.1 Preparation of Columbia agar:

Columbia agar with 5% sheep blood is considered highly nutritious for both the isolation and cultivation of a wide variety of fastidious microorganisms.

⇒ firstly, the agar powder was measured and transferred to a conical flask containing dH_2o.

⇒ the media was heated to dissolve.

⇒ then it was sterilized by autoclaving.

⇒ after that, the conical flask was put in water bath at 50℃ to prevent it from solidifying.

⇒ after the specific time period, it was taken out from the water bath and supplement was added for the growth.

⇒ then sheep blood was added (for 380 ml of media the required amount of sheep blood was 20 ml.

⇒ after shaking the flask , the media was poured in a number of plates.

⇒ plates were stored.

3.4.2 Preparation of TSB:

TSB (Tryptone Soya Broth) contains pancreatic digest of casein, papaic digest of soybean meal for the growth of bacteria.

⇒ the powder was measured and transferred to a conical flask containing dH_2O.

⇒ it was warmed slightly and later sterilized by autoclaving.

3.4.3 Preparation of TSA:

Tryptic soy agar is recommended for the isolation and growth of microorganisms.

⇒ the agar powder was weighed and then transferred to a conical flask containing dH_2o.

⇒ it was heated and sterilized by autoclaving.

⇒ the media was poured in number of plates.

⇒ finally, the plates were stored for sub-culture.

3.4.4 Filtration and Spread plating:

100 ml water from each sample was filtered through filter papers.

⇒ filter papers were put in conical flasks containing 5ml of TSB, these papers were submerged.

⇒ after that, those conical flasks were kept in the shaking incubator for an hour for enrichment.

⇒ after an hour, the flasks were taken out from the incubator.

⇒ using a micropipette 100 ml of media was taken from each conical flask and released into respective plates.

⇒ with a sterilized spreader, it (the liquid containing the bacteria) was spreaded onto the surface of the agar media.

⇒ the plates were incubated overnight at 37℃.

3.4.5 Subculture:

Subculture is a process through which strains can remain alive by transferring them to fresh growth medium.

⇒ TSA plates were used for the subculture.

⇒ a inoculating loop was taken and it was flamed for the use.

⇒ the loop was cooled.

⇒ it was dipped into the bacterial colony and streaked in the TSA plate.

⇒ the plates were incubated overnight

3.5 Biochemical Test

3.5.1 Gram-staining:

Gram staining is a method which is designed to use crystal violet, gram's iodine, Safranin to differentiate bacterial species into two large groups: gram positive bacteria and gram negative bacteria (Cappuccino and Sherman, 2005).

⇒ firstly, we had prepared smears and fixed in flame.

⇒ stained for 1 minute with crystal violet.

⇒ washed in tap water.

⇒ immersed in gram`s iodine for 1 minute.

⇒ washed in tap water and dried.

⇒ decolorized with 95 % alcohol for 30 seconds.

⇒ counterstain for 5 minutes with safranin.

⇒ washed in tap water.

3.5.2 Urease test:

This is a biochemical test that is performed by growing test organism on urea agar slant or urea broth. The ability of organism to produce urease enzyme is observed with the development of pink color (Cappuccino and Sherman, 2005).

⇒ At first, MIU agar powder was measured and transferred to a conical flask containing dH_2O.

⇒ the solution was heated until all the powder was dissolved.

⇒ later the media was autoclaved and 40% urea solution was prepared.

⇒ 5.4 ml of 40% urea solution from 10 ml of unsterilized 40% urea solution was added.

⇒ a syringe (l0ml) was used while adding the 40% urea solution.

⇒ membraned syringe filter attached tightly at the opening of the syringe.

⇒ the syringe was gently pushed so that the 5.4 ml of 40% urea solution could get sterilized by the filter.

⇒ after that, the urea solution was added into the cooled (55℃.) autoclaved 100 ml MIU basal media.

⇒ it was mixed properly.

⇒ the test organism was inoculated on the prepared urea broth.

⇒ the urea broth was incubated at 37℃ for 24 hours.

⇒ the development of pink color was examined.

3.5.3 Oxidase test:

The oxidase test is performed to determine whether the organism can produce the Cytochrome C oxidase with the development of a dark purple color or not (Cappuccino and Sherman, 2005).

⇒ 0.2g of reagent was added in 20 ml of dH_2O.

⇒ the filter paper was soaked in the solution.

⇒ after that, an isolated colony was picked to be tested with a loop and it was smeared in the filter paper.

⇒ color change was observed within 10-30 seconds.

3.6 T1N1 media

⇒ the media was prepared using the standardized ratio of Tryptone, NaCl and agar.

⇒ it was heated until bubbles appeared and it was dissolved thoroughly using a stirrer until the solution was clear.

⇒ then, 2.5 ml of the T1N1 media was added to each vial using micropipette.

⇒ the vials were prepared and pre-labelled.

⇒ those were autoclaved.

⇒ under the laminar, the hot autoclaved T1N1 media was cooled to solidify.

⇒ using sterile stab, the subculture was touched gently and stabbed 3-5 times into the solidified media.

⇒ the caps of these vials were loosely closed and the vials were incubated for 24 hours in 37℃ incubator.

3.7 DNA extraction and PCR

⇒ At first, pellet was formed by centrifugation (5000 rpm, 2 minute).

⇒ after the pellet formation the soup was discarded.

⇒ 1ml autoclaved distilled water was added in each of the eppendorfs.

⇒ vortexing was done.

⇒ those eppendorfs were taken to the water bath and placed in the water bath for 5 minutes at 95℃. those were stored at 4℃.

⇒ PCR master mix was prepared.

⇒ finally, PCR was performed with 40 cycles.

3.8 Agarose gel preparation and gel electrophoresis

For gel electrophoresis, 0.5 g agarose was added to 50 ml of 1x TAE buffer to prepare a 1.3% agarose gel. Ethidium bromide was then added with care. Later, the gel was carefully placed to the electrophoresis tank and samples were loaded on to the wells. Gel run was carried out with 1x TAE buffer at 70 volts. After the gel run was done, the gel was viewed under UV illumination.

Results

Chapter 4

4.1 Appearance of colonies on EMB and MFC

EMB is the selective media for total coliform and MFC for faecal coliform. Figure 1 shows countable pink colonies of total coliform are grown on EMB media. Figure 2 shows countable blue colonies of faecal coliform are grown on MFC media.

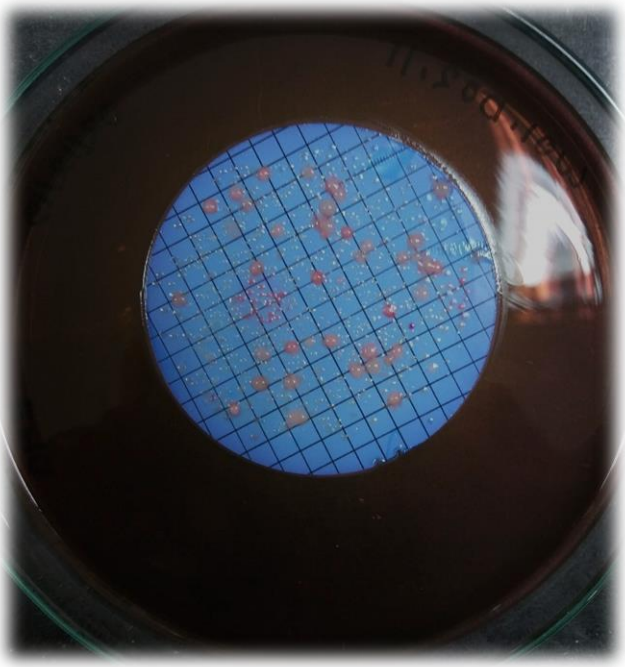


Figure 1: Total coliform grown on EMB media

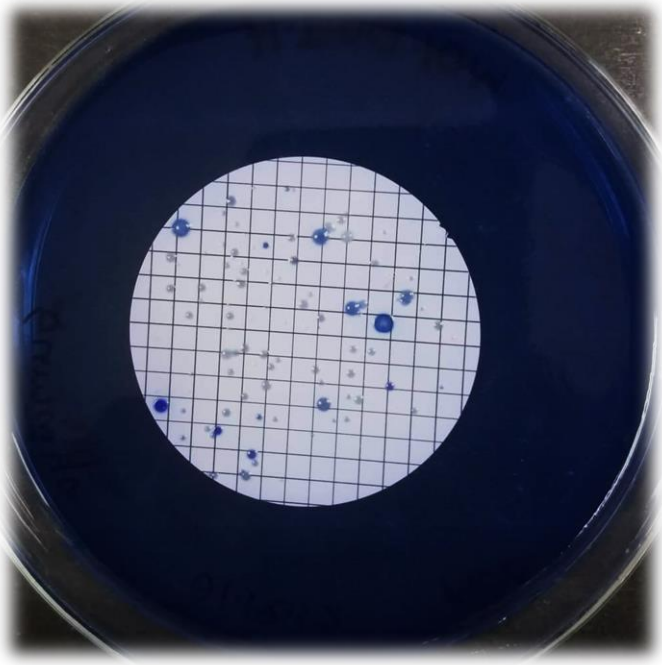


Figure 2: Faecal coliform grown on MFC media

4.2 Colony count of total coliform and faecal coliform

There are 10 areas from where water samples were collected. Mean colony count per 100ml water was taken for both total and faecal coliform. In some media, uncountable colonies were grown which was labelled as TNTC (Too Numerous to Count). Table 1 shows mean colony count of total coliform is more than that of faecal coliform.

Sample ID	No. of samples tested	Total Coliform		Faecal Coliform	
		Mean Colony Count/100ml Water	TNTC	Mean Colony Count/100ml Water	TNTC
B	3	70	2	18	
DC	2		2		2
LMT	3	31		14.3	
M	1	33		7	
MIR	4	29	2	0.5	
MK	4	19.8	1	11.3	
MTJ	2		2	30	
NKT	2		2	48	
RMP	3	19		13.3	
K	18	41.2	8	17	

Abbreviation

1. B= Bossila Slum
2. DC= Dhaka Cantonment
3. LMT= Lalmatia
4. M= Mohammadpur
5. MIR= Mirpur
6. MK= Mohakhali
7. MTJ= Mothijeel
8. NKT= Niketon
9. RMP= Rampura
10. K= Korail Slum

Table 1: Areawise occurrence of total coliform and faecal coliform in water samples taken from different places of Dhaka City.

4.3 Growth and Appearance on Columbia Blood Agar

Columbia Blood agar is a selective media for Campylobacter bacteria. There are two types of colonies seems to grow on Columbia Blood agar. One is white non-hemolytic colony, which is small in diameter and translucent and another is white hemolytic, which is larger in diameter and dull in color. The area around hemolytic colonies seem to be cleared out because of hemolysis of blood by the bacteria. *H. pylori* colony in Columbia Blood agar has characteristic similar that of white non-hemolytic colony.

Figure 4: White non-hemolytic colonies grows on Columbia Blood agar from a drinking water of Korail slum.

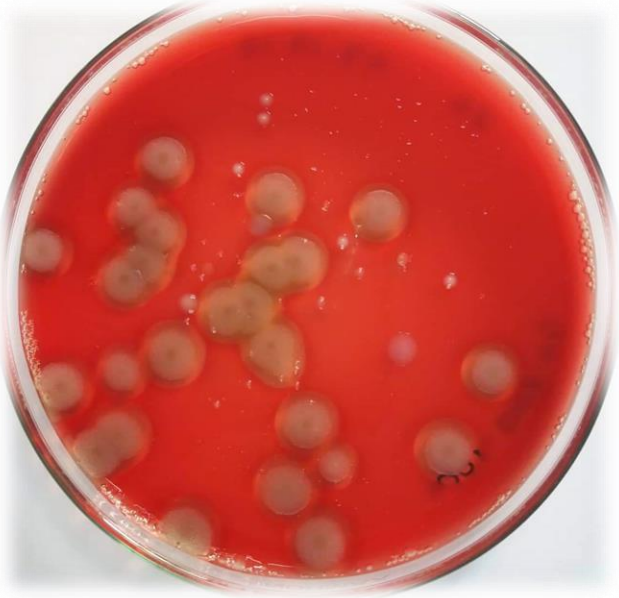


Figure 3: White hemolytic colonies grows on Columbia Blood agar from a drinking water of Korail slum.

4.4 Biochemical Test

There were total of 20 hemolytic and non-hemolytic colonies grown in Columbia Blood agar from 18 different water samples. Biochemical tests were performed to determine if the species growing in these colonies is *H. pylori*.

4.4.1 Gram Staining:

In addition to gram staining, the morphology of the differentiated colonies grew on Columbia Blood agar were checked under the microscope. All the 20 samples exhibited both gram positive and gram negative results. The gram positive bacteria shows purple stain and gram negative bacteria shows pink stain. Morphologically, there were circular and rod shaped bacteria found. On gram staining, *H. pylori* gives pink stain.

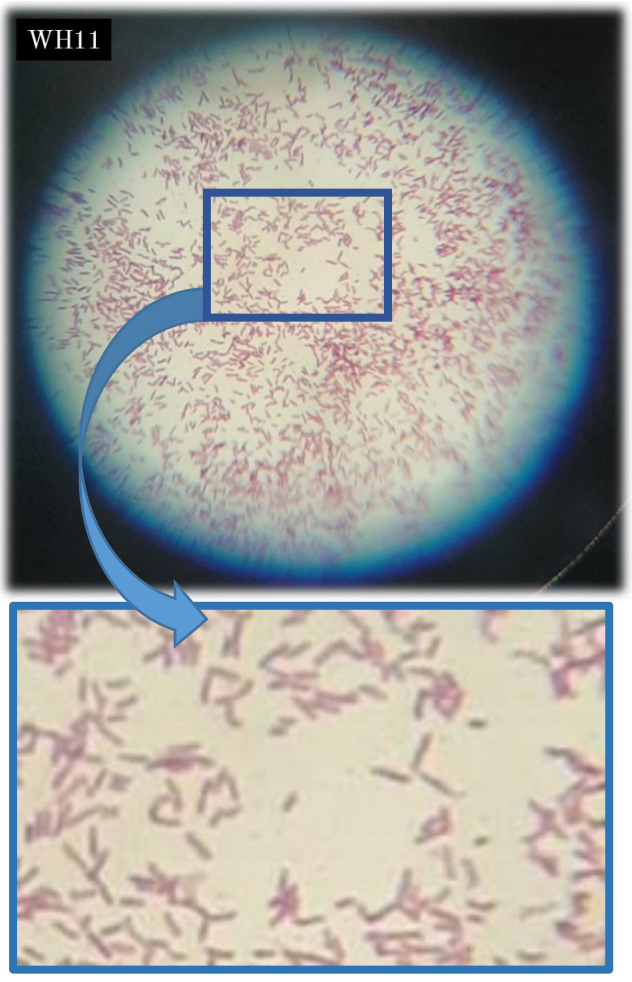


Figure 6: Gram staining showing the pink stain of gram negative bacteria and rod shaped morphology.

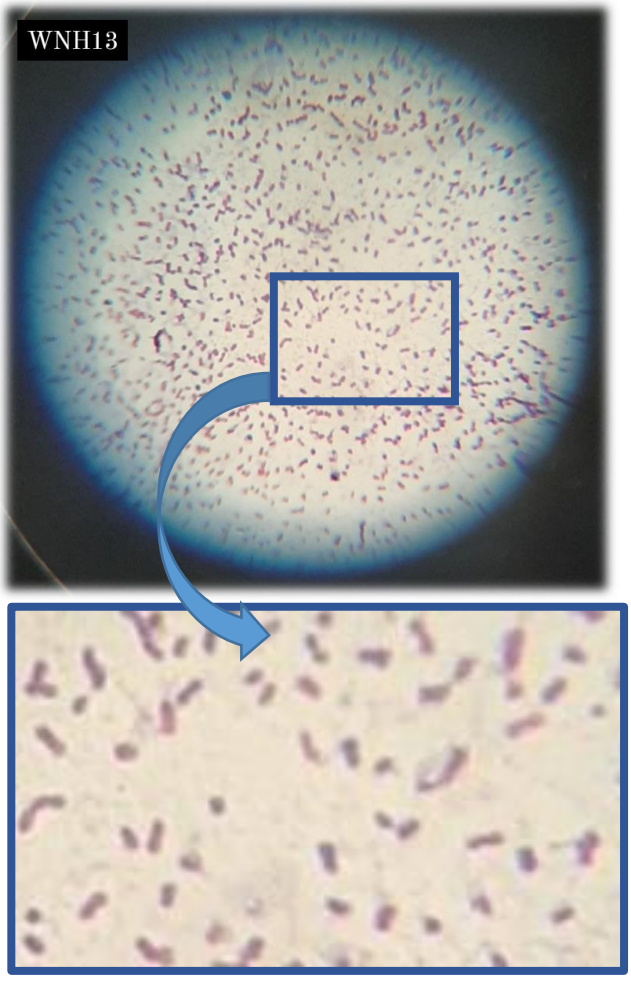


Figure 5: Gram staining showing the purple stain of gram positive bacteria and circular shaped morphology.

4.4.2 Oxidase Test:

Oxidase test was carried out on a filter paper where drops of oxidase reagent was used. Using a sterile loop, when a loop of differentiated colony sample is rubbed the light blue filter paper either turns dark blue or cloudy white. Oxidase positive gives dark blue color and oxidase negative gives cloudy white color. *H. pylori* gives oxidase positive result. The result is then been noted in a table.

Figure 8: Positive oxidase test where filter paper turns dark blue.

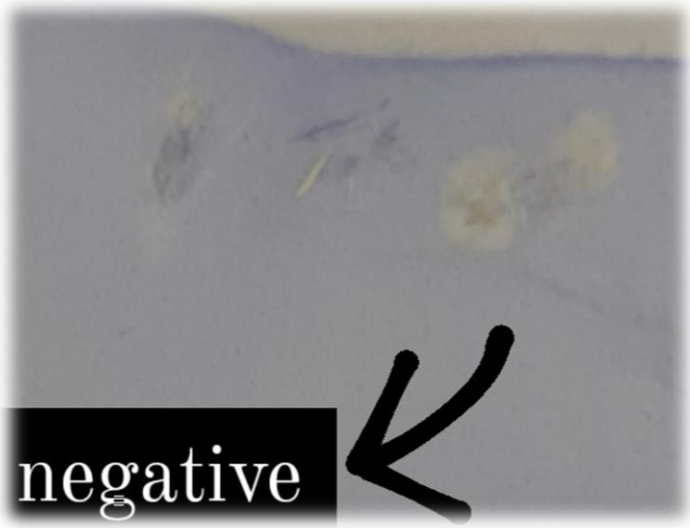


Figure 7: Negative oxidase test where the filter paper turns white.

4.4.3 Urease Test:

On MIU agar, urease test was carried out which gave both positive and negative result. Urease positive sample turns orange MIU agar into reddish-pink color. Urease negative sample turns orange MIU agar into yellow color. In urease test, *H. pylori* gives urease positive.

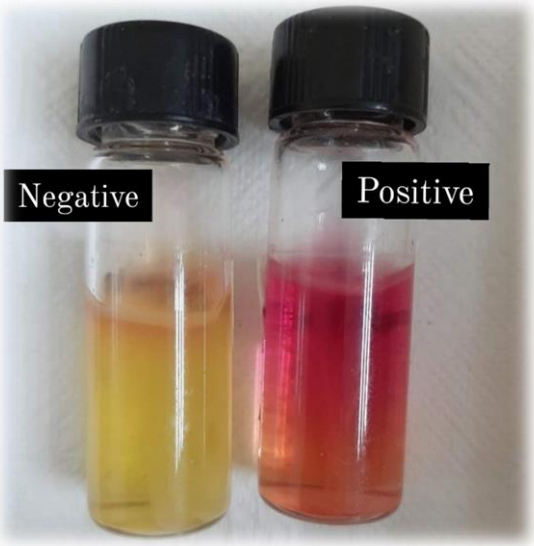


Figure 9: Urease test where yellow is negative result and pink color is positive result.

Among 20 samples, there are 6 samples containing oxidase positive, urease positive and gram negative which are most likely to be *H. pylori*.

Serial #	Name of the samples	Oxidase Test	Urease Test	Gram Staining	Shape	Probable Organism Interpretation
1.	WH01	+	+	-	rod	*H. pylori* might be present
2.	WH02	+	+	-	rod	*H. pylori* might be present
3.	WH08	+	+	-	rod	*H. pylori* might be present
4.	WH11	+	+	-	rod	*H. pylori* might be present
5.	WNH14	+	+	-	rod	*H. pylori* might be present
6.	WH15	+	+	-	rod	*H. pylori* might be present

Table 2: Combined biochemical test results and their interpretation.

4.5 Gel Electrophoresis

The samples were amplified through PCR and gel electrophoresis, with 1000bp of DNA ladder, was carried out. Under UV illumination, only WNH14 shows clear band of 520bp. This confirms that only WNH14 contains *H. pylori*.

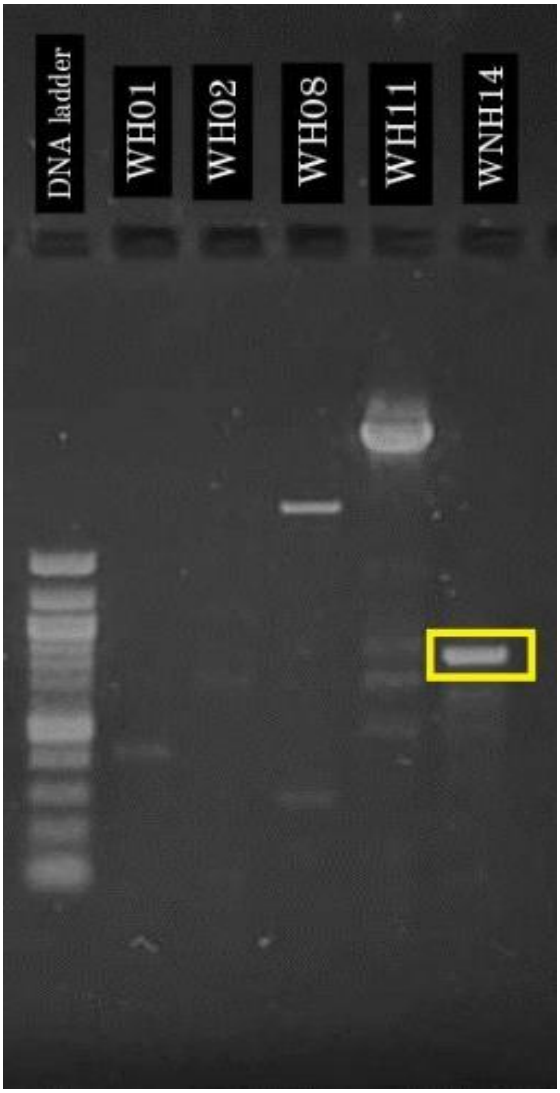


Figure 10: Gel electrophoresis of the probable samples. Only lane 5 (marked in yellow) shows a distinct band of 520bp that confirms the presence of H. pylori in sample WNH14.

Discussion

Chapter 5

5.1 Discussion

This study aims to examine the water quality of different areas of Dhaka city focusing on the presence of fecal indicator bacteria and the presence of *H. pylori.* The gram negative enteric bacterium, *H. pylori* is a frequent colonizer of the human stomach and is recognized as the causative agent of chronic gastritis and peptic ulcer.

Samples were collected from Mirpur, Mohakhali, Mohammadpur, Lalmatia, Dhaka Cantonment, Motijheel, Rampura. We had an aim to enrich our data through the research on the water quality of privileged and under privileged areas of Dhaka city. The aim was to find out the pathogenic microorganisms; fecal and total coliforms as well as the presence of *H. pylori* in those areas. We had sectioned Dhaka city by privileged and under privileged zones. We had collected samples from the zones and tested in three different kinds of media. They were M-FC agar, EMB agar and Columbia agar for the growth of indicator microorganisms and *H. pylori.*

With the specified colonies on Columbia blood agar, we distinguished hemolytic and non-hemolytic colonies. Later, with these distinguished colonies we carried out our biochemical tests to assume which samples could have the probability of having *H. pylori.* In our gram staining results. We got to see both pink and purple color. We got samples (WH01, WH02, WH08, WH13, WH11, WNH10, WNH14 and WH15) which resulted negative with the morphology of being rod shaped. We got samples which were oxidase and urease positive. After the biochemical tests were done, we carried out gel electrophoresis. According to our gel electrophoresis result, we could conclude at this point that the WNH14 which was earlier assumed to have *H. pylori.* Since it was gram negative urease positive and oxidase positive,

gave a clear band while the gel was viewed under UV illumination. With this evidence, it was proved that the water sample had the presence of *H. pylori.*

According to our established data, on the count of indicator microorganism. We can see that the total coliform count is higher than the fecal coliform count. We had used EMB agar media for the growth of total coliform and MFC agar media for the growth of fecal coliform. After the 24 hours incubation. We observed the growth of both fecal and total coliforms. Through this research, we can say the water of different areas of Dhaka city include the contamination of indicator micro-organisms. There are areas such as Dhaka cantonment where the water quality is poor due to the highest number of fecal and total coliforms. Analyzing from our data, we have also found out the presence of indicator microorganisms at an alarming rate in the underprivileged areas (Korail slum and Bossila slum). The water of these slum areas is highly contaminated by the presence of fecal and total coliforms. Also, we have successfully isolated *H. pylori* from one of the water samples taken from Korail slum. Moreover, the water from Mirpur, Motijheel and Niketon has enriched our data because some samples of these areas gave uncountable colonies on EMB agar media. Due to time constraints and the unprecedented pandemic situation, sufficient analysis of the water sample from all the sites could not be done. Hence, further study is required to state definite remarks on the findings of this study.

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Appendix A

Media Composition

The following media was used during the study. All components were autoclaved at 121℃, 15 psi for 15 minutes.

Eosin Methylene Blue Agar

Ingredients	**Gms / Litre**
Peptone	10.000
Dipotassium hydrogen phosphate	2.000
Sucrose	5.000
Lactose	5.000
Eosin-Y	0.400
Methylene blue	0.065
Agar	13.500
Final pH at 25℃	7.2±0.2

m-FC Agar

Ingredients	**Gms / Litre**
Tryptose	10.000
Proteose peptone	5.000
Yeast extract	3.000
Lactose	12.500
Bile salts mixture	1.500

Sodium chloride	5.000
Aniline blue	0.100
Agar	15.000
Final pH at 25℃	7.4±0.2

Columbia Agar

Ingredients	**Gms / Litre**
Pancreatic digest of casein	10.000
Meat peptic digest	5.000
Heart pancreatic digest	3.000
Yeast extract	5.000
Maize starch	1.000
Sodium chloride	5.000
Agar	15.000
pH after sterilization	7.3±0.2

Tryptone Soya Agar

Ingredients	**Gms / Litre**
Tryptone	15.000
Soya peptone	5.000
Sodium chloride	5.000
Agar	15.000
Final pH at 25℃	7.3±0.2

Tryptone Soya Broth

Ingredients	**Gms / Litre**
Tryptone	17.000
Soya peptone	3.000
Sodium chloride	5.000
Dextrose (Glucose)	2.500
Dipotassium hydrogen phosphate	2.500
Final pH at 25℃	7.3±0.2

Appendix B

Reagents

The following reagents were used in the completion of our study:

1. **Crystal violet stain (2%)**

2g of crystal violet was dissolved in 20mL of 95% ethyl alcohol. 0.8g of ammonium oxalate monohydrate was then dissolved in 80mL distilled water. Both the two solutions were mixed and filtered into sterile reagent bottle.

2. **Gram's iodine solution**

6.7g potassium iodide was dissolved in 100mL of distilled water. Later, 3.3g of iodine was added, stirred, and the solution made up to 1 liter with distilled water. The reagent bottle was covered in aluminium foil and stored at room temperature.

3. **Safranin**

0.1g of safranin was dissolved in 75mL of distilled water. The solution was diluted to 100mL filtered and stored in clean reagent bottle.

4. **Oxidase reagent**

100mg of N, N, N^1, N^1- tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10mL distilled water. The solution was covered with aluminum foil and stored at 4°C.

5. **Urea solution**

5.4mL of 40% urea solution was prepared using MIU agar, membraned syringe filter and a syringe.

Appendix C

Instruments

Instrument	Company
Autoclave	SAARC
Micropipettes	Eppendorf, Germany
Cellulose filter paper	Whatman
Freeze (-20°C)	Siemens, Germany
Centrifuge, *Model No: Code: 5433000.011*	Eppendorf, Germany
Incubator	UK
Laminar air flow	UK
Pipette (5 mL, 10 mL)	Eppendorf, Germany
Refrigerator (4℃), *Model: 0636*	Samsung
Microscope	Optima
Weighing balance	ADAM, UK
Shaking Incubator, *Model: WIS-20R*	Daihan Scientific, Korea
Oven (Universal drying oven) *Model: LDO-060E*	Lab Tech, Singapore
Vortex Mixture	VWR International
Hotplate stirrer	Lab Tech
Surgical Millipore syringe filter	Millex-GS
pH meter, *Model: E-201-C*	Shanghai Ruosuaa Technology Company, China