PCR Based Detection of *Helicobacter pylori* Compared with CLO in Stomach Biopsy Samples from Patients with Dyspepsia: A Pilot Study

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

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Declaration

It is hereby declared that

1. The thesis submitted is our original work while completing the 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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Ethics Statement

This study was conducted under the ethical clearance granted by the ethical review committee of Sheikh Russel National Gastroliver Institute and Hospital, Dhaka, Bangladesh. The certificate of the approval is attached in the **Appendix A** section of this document.

Abstract

Helicobacter pylori infection can lead to a range of gastrointestinal diseases, including gastritis and peptic ulcers, and can develop into stomach cancer if left untreated. Treating *H. pylori* infection in an early stage is crucial to mitigate these health risks and improve digestive health. The CLO test is the primary method for clinical *H. pylori* detection due to its ease of use and time efficiency. We collected seven biopsy samples through endoscopy from patients exhibiting symptoms of dyspepsia. The samples were subjected to molecular diagnostic procedures in the laboratory of Brac University. DNA extraction was carried out followed by polymerase chain reaction (PCR) using specific primers of two genes, UreA and 23S rRNA. It was conducted in order to isolate and amplify the DNA of *H. pylori.* In CLO testing, 71% of the selected patients tested positive for *H. pylori* infection, whereas only 29% of the patient pool was found to be positive for both the UreA gene and the 23S rRNA gene. Such discrepancies in positivity rate**s** raise questions about the effectiveness of the standard testing method (CLO) for detecting an intensive infection like *H. pylori.*

Keywords: *H. pylori*; Dyspepsia; CLO; PCR; positivity rates

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List of Acronyms

CLO	Campylobacter like organism
PCR	Polymerase chain reaction
RUT	Rapid urease test
FISH	Fluorescent in situ hybridization
NGS	Next generation sequencing
UGIT	Upper gastrointestinal tract
DNA	Deoxyribonucleic acid
gDNA	Genomic deoxyribonucleic acid
rRNA	ribosomal RNA
TBE	Tris-borate-EDTA
EDTA	Ethylene diamine tetraacetic acid

Chapter 1

Introduction

1.1 Background

Helicobacter pylori is a gram-negative flagellated and spiral-shaped bacteria that persist in the foveolar epithelium surface (Bharath et al., 2014) of the gastric cavity and is accountable for a spectrum of gastrointestinal diseases such as chronic gastritis, peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma (Roesler et al., 2014). *H. pylori* infects and colonizes the gastric mucosa, which is the mucous lining of the stomach. The common symptoms after the infection by microaerobic *H. pylori* may vary from bloating, abdominal pain, vomiting, loss of appetite and nausea (Wang et al., 2015) to several chronic damages.

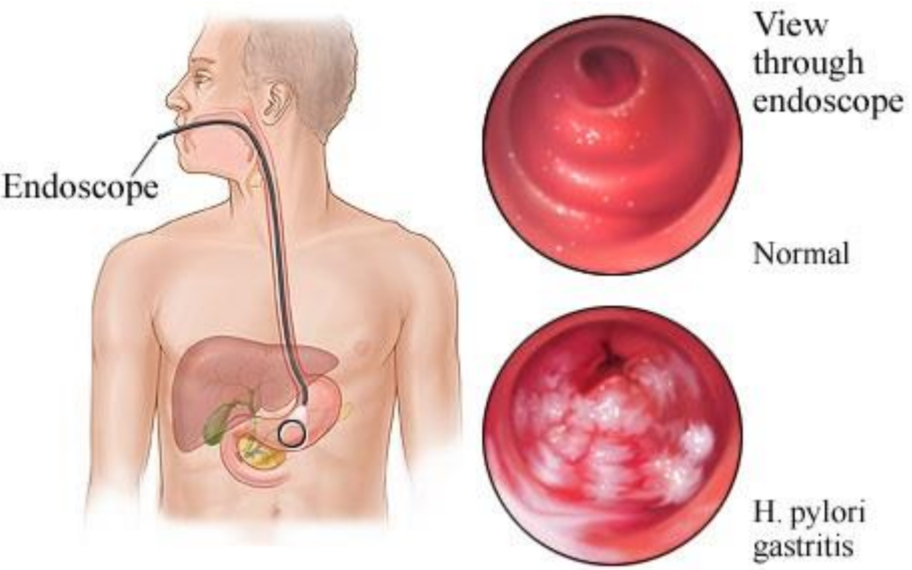

Figure 1 *The stomach tissue of a healthy person compared to the stomach tissue of a patient with H. pylori infection. (Adapted from:* ***SEPA Labs, www.sepalabs.com****)*

As shown, Figure 1 represents the difference between the stomach tissue of a gastritis patient infected with *H. pylori* and a healthy person. Infection of *H. pylori* could be one of the several factors that may cause dyspepsia. In addition to the symptoms involving *H. pylori* infection,

the dyspeptic symptoms might consist of epigastric burning, epigastric pain, early satiety or postprandial fullness. Some possible supportive coexisting symptoms of functional dyspepsia may involve vomiting, nausea plus bloating but are excluded in definition. These criteria have to be fulfilled for the rearmost trimester with the onset of the symptom's minimum of 6 months or more prior to diagnosis (Selgrad et al., 2008).

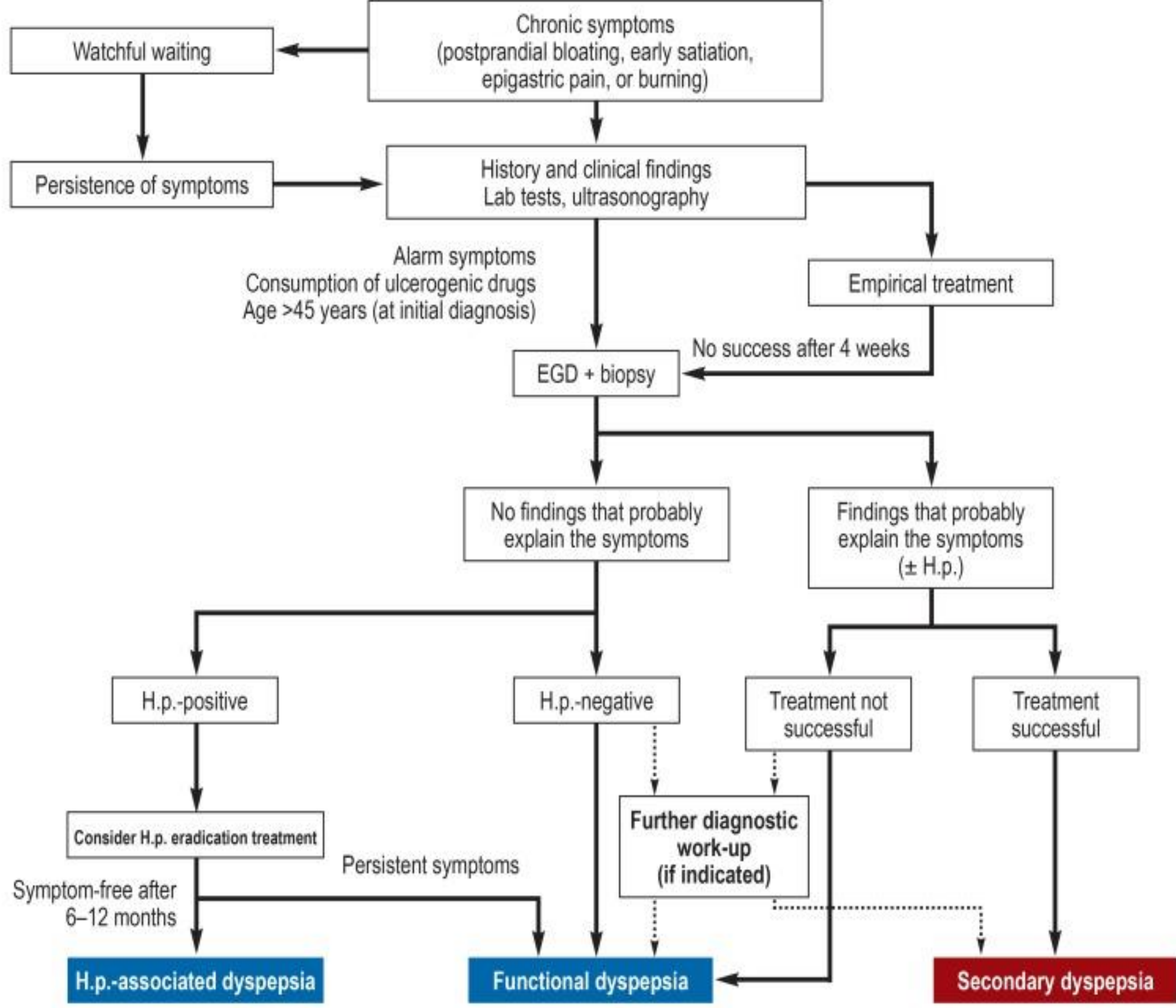

Figure 2 *A simple flow chart differentiating dyspepsia (Adapted from: The Diagnosis and Treatment of Functional Dyspepsia. Deutsches Arzteblatt International by Madisch et al., (2018), DOI: 10.3238/arztebl.2018.0222)*

Figure 2 differentiates between the types of dyspepsia. It summarizes the steps that are needed to diagnose a patient and the following procedures that need to be followed after the completion of appropriate treatments.

Although, the isolation of *H. pylori* in 1983 has commenced understanding of the pathogenesis, etiology and management of diseases and symptoms of diseases that are likely to be caused by it (Warren et al., 1983), the method by which *H. pylori* infection spreads and various other aspects of its epidemiology continue to be uncertain as noted by Tiwari et al. (2005). This endemic microorganism is the most frequent bacteria in developing countries like Bangladesh (Sulami et al., 2008). This is because most proportion of the population live in non-sanitized conditions. Although it is yet to be established, many researchers have suggested that the most likely mode of transmission of *H. pylori* is through contaminated food, water and unclean hands, which enters the body through fecal-oral and oral-oral route (Idowu et al., 2019; Sharndama & Mba, 2022).

1.2 Pathogenesis

H. pylori strains may vary due to their protein expression determining their virulent activity (Enroth et al., 2000). Notably, two of the virulence factors which are vacuolating cytotoxin A (vacA) and cytotoxin-associated antigen A (cagA) are highly associated with causing minute to colossal stomach diseases (Enroth et al., 2000 & Wattanawong Don et al., 2023).

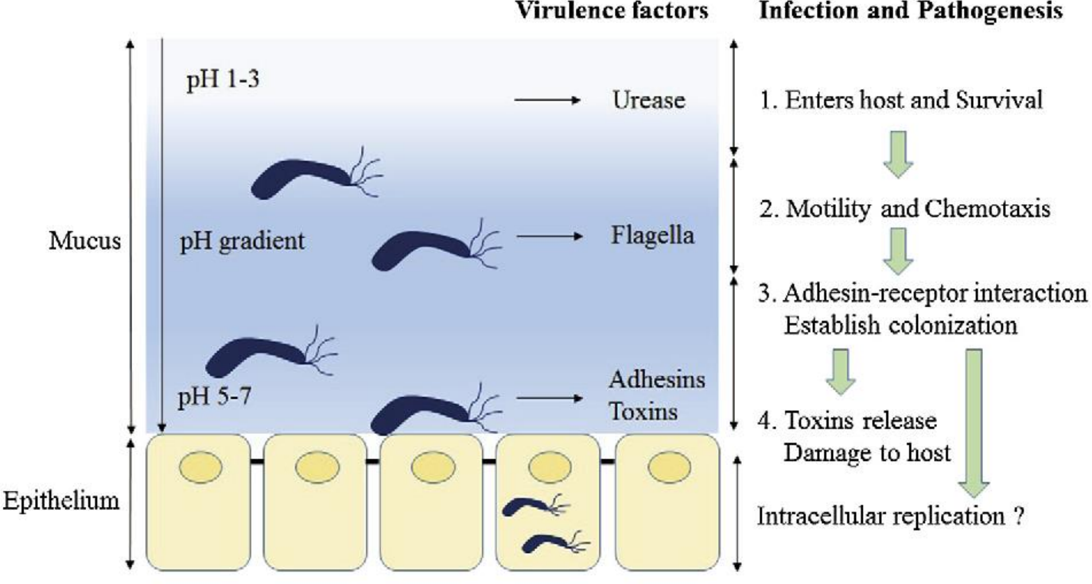

Figure 3 *Schematic diagram of Helicobacter pylori infection.* (Kao et al., 2016)

Toxins, such as cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), contribute to host tissue damage and intracellular replication after successful colonization (Kao et al., 2016).

During its adherence and colonization, *H. pylori* can cause a variety of genetic changes, express a variety of virulence factors, and activate a variety of adaptive mechanisms. *H. pylori* virulence and pathogenicity are influenced by a complex interplay of virulence factors, hosts, and environmental variables (such as exposure to carcinogens, diet, excessive smoking and alcohol consumption) (Sharndama & Mba., 2022).

Its treatment has become as available as over the counter medicine without patients undergoing any diagnostic test. Treatment that includes widespread use of antibiotics without prior knowledge and abuse of antimicrobial agents by mass people. Besides, the identification and obliteration of *H. pylori* could be used as an approach to eradicate gastric cancer caused by this pathogen that has sparked interest in recent times (Hoo et al., 2017).

1.3 Disease Burden

Helicobacter pylori, an enterobacteria with the ability to cause recurring stomach infections. It is an existing pathogen which has been posing a strong challenge to not just microbiologists but also medical professionals. Currently the rate of *H. pylori* infection is said to be approx. 50% (Salih, 2009) an alarming percentage for developing countries. Among them, a strong percentage either develop peptic ulcers (Salih, 2009) or exhibit dyspeptic symptoms further

hampering lifestyle. This bacterium can adapt itself to the highly acidic environment in the stomach and once infected, can go undetected for a long time until symptoms arise.

It is pathogenic and infects the population of developing countries a maximum of 90% as assumed by Wattanawong Don et al., (2023) and nearly 50% of global inhabitants of the community (Roesler et al., 2013). According to a Bangladeshi study, adults with dyspepsia had an *H. pylori* positivity rate of 47.8% (Saha et al., 2018). In research conducted in the city of Chittagong, 66.67% of a group of dyspeptic patients tested positive for *H. pylori* (Habib et al., 2016). In a study conducted in rural Bangladeshi villages, the prevalence of *H. pylori* positivity among dyspeptic patients was shown to be substantially higher (91.04%) by multiplex PCR (Rahman et al., 2021).

Currently the rate of *H. pylori* infection is seen to be decreased from approx. 58.2% to 43.1% globally. In 2017, it was found to be 50.8% in developing countries and 34.7% in developed countries (Butt & Epplein, 2023).

1.4 *H. pylori* in relation to Dyspepsia:

The eradication of *H. pylori* is fundamental in treatment of functional dyspepsia (Francis & Zavala, 2023). It is because it elevates the symptoms and lowers the risk factors of different stomach malignancies. It was reported that functional dyspepsia persists in approximately 20% patients (Madisch et. al.2018), meanwhile decreasing the patient's quality of life by up to 25%. Its global prevalence is 5% to 11% whereas in Asian countries, it ranges from 5% to 30% and almost 40% in western countries showing a high prevalence rate.

Despite several conflicting results in epidemiological trials regarding the immense prevalence of *H. pylori* in functional dyspeptic patients, meta-analysis has shown dyspepsia pathogenesis to be associated with *H. pylori* infection. Such is possible due to *H. pylori's* instigation of the chemokine network and a complex and intriguing cytokine in the gastric mucosa. If compared, 60% of functional dyspeptic patients had *H. pylori* infection according to intensive study by Suzuki (2011).

H. pylori virulence and pathogenicity are influenced by a complex interplay of virulence factors, hosts, and environmental variables. Since it has numerous genetic variations based on geographical location, it is essential to identify the virulence factors that are disseminated amongst the Bangladeshi population.

1.5 Diagnosis of *Helicobacter pylori*

There are several invasive and non-invasive methods for the detection of *H. pylori.* These methods are based on different principles. All of these tests have some disadvantages and limitations; therefore, it is suggested to use at least two or more methods to confirm the presence of *H. pylori* (Ramis et al., 2012).

1.5.1 Invasive tests

Invasive tests include the collection of biopsy samples from the antrum and/or corpus of the stomach from the patient.

1. For microscopic/histopathological examination, a smear is prepared from a biopsy sample on a slide using Gram stain, Geimsa stain or fluorescent acridine orange. If *H.*

pylori is present, its morphology can be identified under the microscope. The sensitivity of this method is close to 80% (Mégraud & Lehours, 2007).

2. Grinded biopsy samples can be used for bacterial culture on Columbia agar plates. Several growth supplements and selective supplements are used to isolate *H. pylori.* Ideal growth conditions of this bacteria are difficult to achieve. If strict laboratory conditions are met and the samples are handled with extreme care, sensitivity of this method can go up to 95% (Mégraud & Lehours, 2007).
3. Multiple biochemical tests can be performed using the tissue samples and the bacterial isolates obtained from culture, such as rapid urease test (CLO), urease test, oxidase and catalase tests (Abu-Sbeih et al., 2014).

1.5.2 Non-Invasive tests

Among the several non-invasive tests, some can detect active (on-going) infection. While other tests can only detect the exposure to *H. pylori*, which is called passive infection and does not provide evidence of current infection (Ricci C et al., 2007).

1. Stool antigen test (SAT) is a direct method of detecting *H. pylori.* It is designed to detect monoclonal and polyclonal antibodies like Enzyme immunoassay (EIA) and immunochromatic assay (ICA) tests. Different commercial kits of SAT are available (Qiu et al., 2021).
2. The urea breath test detects urease activity in patients with an active infection of *H. pylori.* However, false positive and false negative results are common with this type of test. It has been found to be less accurate among the pediatric population with sensitivity and specificity ranging from 75% - 100% (Sankararaman et al., 2023).
3. For serological tests, blood serum samples are collected and an Enzyme-Linked Immunosorbent Assay (ELISA) is performed. Anti-*H. pylori* antibodies (IgG) are

detected using this method. This test shows a sensitivity of almost 95% (Abu-Sbeih et al., 2014). In low prevalence populations, the use of serology is not recommended by the American College of Gastroenterology. It is due to the findings that the prevalence of increased IgG levels is higher in developing countries compared to developed countries. (Kayali et al., 2018).

1.6 Molecular detection techniques

For molecular detection, a wide range of clinical specimens can be taken such as, saliva, gastric juice, dental plaque, gastric biopsies, stool and other environmental samples. Depending on the type of sample taken, it can be classified into both invasive and non-invasive procedures (Cardos et al., 2022).

1. Polymerase Chain Reaction (PCR) - Conventional PCR, Real-time PCR and droplet digital PCR (dd-PCR) are a few methods that can be used to detect the presence of *H. pylori.* DNA has to be extracted from the collected samples first in order to amplify it using PCR. These methods showed 80-95% specificity and sensitivity (Gong & El-Omar, 2021).
2. Fluorescent in situ hybridization (FISH) - Assays based on in-situ probe hybridization for *H. pylori* genes are used in this method. 80-95% sensitivity and specificity were found using these techniques (Gong & El-Omar, 2021).
3. Next-generation sequencing (NGS) - 16S rRNA sequencing uses DNA-based NGS assay for the detection of 16S rRNA gene of this specific bacteria. Sensitivity and specificity were found to be almost 95-100%.
Meta-genomic sequencing and meta-transcriptomic sequencing uses DNA-based assay and RNA- based assay respectively for the sequencing of whole meta-genome and

whole meta-transcriptome. Metagenomics also show a high sensitivity and specificity of 95 - 100% (Gong & El-Omar, 2021).

1.7 Previous studies comparing PCR and CLO

In a study carried out in a population from Chittagong, Bangladesh by Habib et al. (2016), the results of CLO positive patients were slightly higher than those of PCR based on 16S rRNA gene. In contrast, a study by Trung et al. (2019) in Vietnam revealed a much higher positivity rate by multiplex PCR than that of CLO.

In light of these contradicting results, we aimed to find the scenario of dyspeptic patients in Dhaka, Bangladesh. Antibiotic treatments in hospitals are given based on the CLO result alone. Determining the accuracy and sensitivity of this is necessary in order to better manage the patients being admitted with dyspeptic symptoms. We aim to find out the impact of geographical location on the presence of *H. pylori* and the relation of dyspepsia with this bacterium.

1.8 Objective of the study

The aim of our study is to compare the results of Campylobacter Like Organism Test (CLO) and Polymerase Chain Reaction (PCR) of *H. pylori* specific genes for the detection of *H. pylori* bacteria in dyspeptic patients.

Chapter 2

Methods and Materials

2.1 Case definition

Consecutive adult dyspeptic patients attending the outpatient department of medical gastroenterology in Sheikh Russel National Gastroliver Institute and Hospital were chosen for this study.

2.2 Patient Screening

Initially, 12 dyspeptic patients were counseled from different backgrounds and different parts of Bangladesh coming to the hospital. They were subjected to a prepared questionnaire to diagnose dyspepsia and then selected based on inclusion and exclusion criteria.

Inclusion:

- Age - 18 years and above
- Rome IV Diagnostic Criteria of Functional dyspepsia - The dyspeptic symptoms are epigastric pain, epigastric burning, postprandial fullness or early satiety. Bloating, nausea and vomiting may co-exist and are supportive symptoms but not included in definitions. Criteria fulfilled for the last 3 months with symptom onset at least 6 months before diagnosis
- Patients free from antibiotics/antisecretory drugs/other eradication therapies for at least 2 weeks.

Exclusion:

- Lactating mother

- Pregnancy
- Significant underlying disease for instance, renal or pulmonary diseases, cardiac, liver or history of gastric surgery or cancer.
- Use of H2 receptor antagonists, PPI, non-steroidal anti-inflammatory drugs or antibiotics during the previous two weeks.

2.3 Sample Collection

Endoscopic biopsy of the upper gastrointestinal tract was performed. Two tissue samples from the antrum of the stomach were collected from dyspeptic patients attending Sheikh Russel National Gastroliver Hospital, Dhaka during the month of January of 2023.

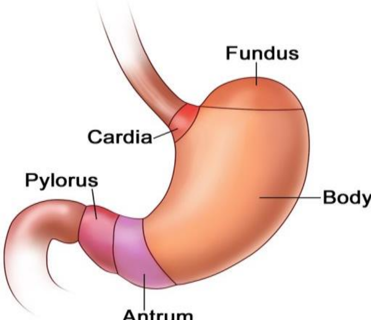

Figure 4. *Collection site of tissue- Antrum. (Adapted from, Incidence of Cancers of the Lower Stomach Increasing among Younger Americans, by NCI staff, 2018, National Cancer Institute)*

2.4 Endoscopic procedure of biopsy samples

Two biopsy samples were taken from the antrum of the stomach. An experienced endoscopist did an endoscopy of upper gastrointestinal tract (UGIT) within two weeks of enrollment.

Subjects who were selected for endoscopy were fasted overnight. Detailed information about the endoscopic procedure was given to all individuals during their personal interviews, and written consent was obtained from each person before they underwent endoscopy. Endoscopic findings were recorded using standard definitions. An active ulcer was defined as an area of

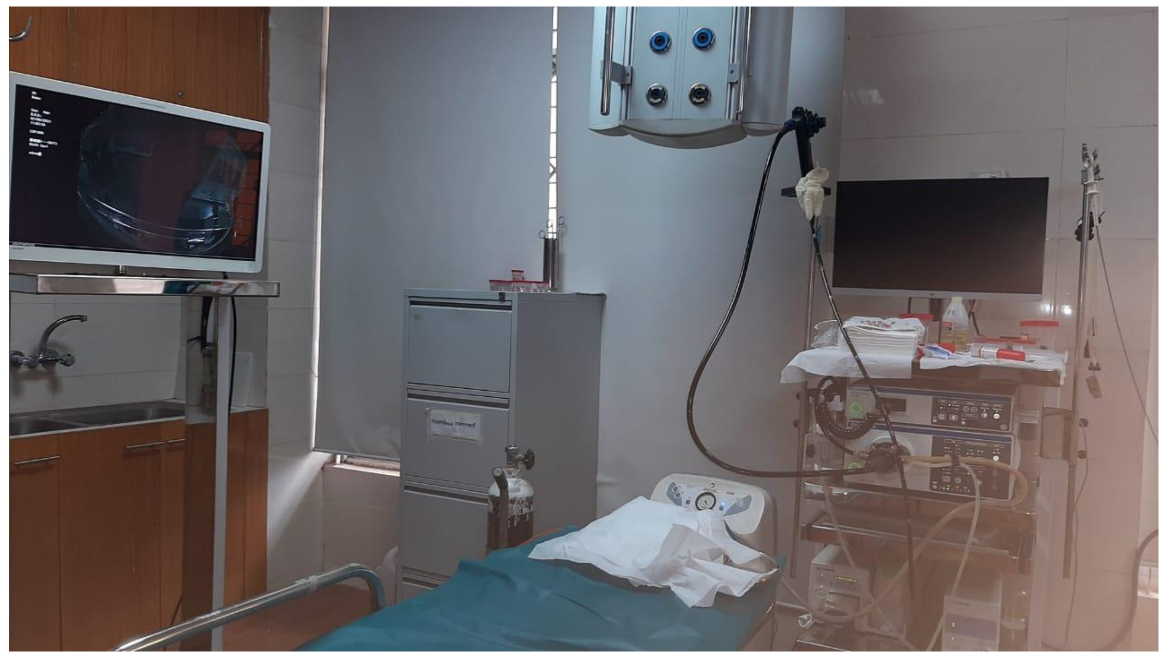

Figure 5 *The operating room with sterilized equipment where the endoscopy takes place*

Figure 6 *Surgeon performing endoscopy at Sheikh Russel National Gastroliver Institute and Hospital*

denuded epithelium of 5 mm or more. Acute gastric or duodenal erosions were defined as mucosal breaks of ≤ 5 mm in diameter without depth in the stomach and/or duodenum. Subjects

with both peptic ulcers and erosions were counted as having a peptic ulcer. An active ulcer was termed acute when there was no evidence of scarring or deformity and chronic when there was scarring or deformity. Past ulceration was inferred if there was definite evidence of scarring or deformity without an active ulcer. Peptic ulcers included duodenal ulcers and benign gastric ulcers. All gastric ulcers detected during endoscopy were biopsied for histological examination. No biopsy specimens were taken from the ulcers in patients with duodenal ulcers unless the endoscopists suspected the possibility of malignancy. If more than one mucosal break was present, at least one had to fulfill this definition for an ulcer to be diagnosed. Deformity of the duodenal bulb was considered to be present when flattening, scars, stenosis, or narrowing of the bulb were seen.

2.5 Sample Transport

After biopsy, samples were collected within 4 hours. They were transported on ice cold sterile saline solution (0.9% NaCl) from the hospital to the laboratory of BRAC University.

2.6 Storage

Samples were stored at -20°C until DNA extraction was performed.

2.7 *H. Pylori* detection by Campylobacter Like Organism Test (CLO):

1. The CLO test is a qualitative RUT assay based on the detection of urease, produced by *H. pylori.*
2. The test system consists of a test well filled with a urea containing gel where the suspected tissue is inoculated and allowed to incubate. If *H. pylori* is present in the patient’s sample, urease will hydrolyze the urea in the gel leading to an accumulation of ammonium ions (NH4+). This causes a rise in pH, which is detected by a pH

indicator in the test system changing from yellow to magenta. Other shades of red such as pink or orange are also considered positive. Yellow is considered a negative screen.

3. Gastric biopsy specimens should be taken preferably from an area that is not as eroded or denuded; *H. pylori* is present in smaller numbers in these areas than in otherwise normal looking tissue.
4. A CLO is reported as negative if there is no change in color until the end of the 24-hour period.

2.8 Polymerase Chain Reaction

2.8.1 Sample pre-processing

- The tissue samples were thawed at room temperature.
- The thawed tissue was homogenized manually using a tissue grinder.

Figure 7 *Fisherbrand™ Pellet Pestles™*

2.8.2 DNA Extraction

DNA from the biopsy samples were extracted using Monarch kit following the manufacturer's instructions:

Figure 8 *Monarch DNA extraction kit*

Figure 9 *Reagents and apparatus inside the kit*

1. Cut tissue into small pieces to ensure rapid lysis and high yields. Weigh the appropriate tissue amount and place in a 1.5 ml microfuge tube.

Table 1 Recommended amount of starting material	
STARTING MATERIAL	**RECOMMENDED INPUT AMOUNT**
Fibrous tissue (muscle, heart)	Up to 25 mg

2. Next, 10 µl Proteinase K and 200 µl of Tissue Lysis Buffer was added to each sample. It was mixed immediately by vortex.
3. The mixture was then incubated at 56°C in a thermal mixer with agitation at full speed (1400 rpm) until tissue pieces had completely dissolved (typically 30-60 minutes).
4. Samples were centrifuged for 3 minutes at maximum speed (> 12,000 x g) to pellet debris and then transferred the supernatant to a fresh microfuge tube.
5. RNase A (3 µl) was added to the lysate, vortexed thoroughly and incubated for 5 minutes at 56°C with agitation at full speed.
6. To the tubes, 400 µl of gDNA Binding Buffer was added to the sample and mixed thoroughly by pulse-vortexing for 5-10 seconds.
7. Then it was transferred the lysate/binding buffer mix (~600 µl) to a gDNA Purification Column pre-inserted into a collection tube, without touching the upper column area. Proceeded immediately to Step 3.
8. The cap was closed and centrifuged: first for 3 minutes at 1,000 x g to bind gDNA (no need to empty the collection tubes or remove from centrifuge) and then for 1 minute at maximum speed (> 12,000 x g) to clear the membrane.
9. The flow-through and the collection tube was discarded. For optimal results, it was ensured that the spin column is placed in the centrifuge in the same orientation at each spin step (for example, always with the hinge pointing to the

outside of the centrifuge); ensuring the liquid follows the same path through the membrane for binding and elution can slightly improve yield and consistency.

10. The column was transferred to a new collection tube and 500 μl gDNA Wash Buffer was added. The cap was sealed closed and inverted a few times so that the wash buffer reaches the cap. Mixture was then centrifuged immediately for 1 minute at maximum speed and the flow through was discarded. The collection tube was tapped on a paper towel to remove any residual buffer before reusing it in the next step. The spin column was inverted with a wash buffer, which aids in preventing salt contamination in the eluate.
11. The column was reinserted into the collection tube. Added 500 μl gDNA Wash Buffer and closed the cap. Centrifuged immediately for 1 minute at maximum speed and discarded the collection tube and flow through.
12. The gDNA Purification Column was placed in a DNase-free 1.5 ml microfuge tube (not included). Add 35-100 μl preheated (60°C) gDNA Elution Buffer, closed the cap and incubated at room temperature for 1 minute. Elution in 100 μl is recommended, but smaller volumes can be used and will result in more concentrated DNA but a reduced yield (20–25% reduction when using 35 μl). Eluting with a preheated elution buffer will increase yields by ~20–40% and eliminates the need for a second elution.
13. Finally, tubes were centrifuged for 1 minute at maximum speed (> 12,000 x g) to elute the gDNA.

2.8.3 PCR

- **Procedure:**

Two targeted regions, UreA and 23S rRNA of *H. pylori,* were considered for detection and confirming the presence of *H. pylori.* The primers F- HPU1 and R - HPU2 were

designed to detect the UreA gene in the extracted DNA template. The 23S rRNA was detected through a nested PCR with the first set of primers Hp23S 1835F and Hp23S 2327R. The second set of primers were Hp23S 1942F and Hp23S 2308R.

For preparing the PCR mixture, the amounts on Table 2 were followed for each sample.

Table 2 Composition of PCR mixture

Reagents	**Amount**
Master Mix	6.5 µl
Forward primer	0.3 µl
Reverse primer	0.3 µl
Nuclease free water	3.9 µl
Sample	2 µl

Table 3 Primers and PCR conditions used					
Primer Name	**Gene**	**Sequence**	**PCR conditions**	**Product Size**	**Reference**
HPU	UreA	HPU1, (5′-GCCAATGGTAAATTAGTT-3′ HPU2, 5′-CTCCTTAATTGTTTTTAC-3′)	94°C, 1 min; 50°C, 1 min; 72°C, 1 min (35 cycles) (**modified**)	**411 bp**	21
Hp23S	23S rRNA	F (5′-GGTCTCAGCAAAG AGTCCCT-3′) R (5′-CCCACCAAGCATT GTCCT-3′)	95°C for 2 min, followed by 5 cycles: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; then 30 cycles: 94°C for 15 s, 57°C for 15 s, and 72°C for 20 s (**modified**)	**367 bp**	16
Hp23S	23S rRNA	F (5′ –AGGATGCGTCAGTCGCAAGAT -3′) R (5′-CCTGTGGATAACACAGGCCAGT-3′)	95°C for 2 min, followed by 25 cycles: 94°C for 10 s and 65°C for 20 s (**modified**)	**367 bp**	16

16S	16S rRNA	Hp1, (5′-CTGGAGAGACTAAGCCCTCC-3′) Hp3, (5′-AGGATCAAGGTTTAAGGATT-3′)	95°C, 30s; 55°C, 30s; 72°C, 30s (30 cycles)	**446 bp**	21

After the PCR with respective outlined conditions, an agarose gel electrophoresis was performed to determine the presence or absence of *H. pylori* thereof.

2.9 Agarose gel electrophoresis

2.9.1 Gel Preparation

The following steps were followed to prepare a 2% agarose gel with a total volume of 50 ml.

i. 1g agarose powder was measured on a weighing scale.

ii. The agarose was transferred to a conical flask.

iii. Then 49 ml distilled water was taken in a measuring cylinder.

iv. 1 ml TBE buffer of 50X concentration was added concentration using a micropipette.

v. TBE buffer solution was added to the conical flask and swirled to mix with the agarose.

vi. The conical flask was heated in a microwave oven until the agarose had completely dissolved.

vii. The mixture was allowed to cool down slightly.

viii. 2 µl ethidium bromide was added to the conical flask and swirl to mix.

2.9.2 Running Buffer preparation

For preparing a 1 liter running buffer of 1X concentration, the following steps were followed.

i. 980 ml distilled water was taken in a measuring cylinder.

ii. 20 ml TBE buffer was added to the measuring cylinder.

2.9.3 Gel electrophoresis

i. The solution was allowed to cool to about 50-55°C, swirling the flask occasionally to cool evenly.
ii. The ends of the casting tray were sealed and the comb placed.
iii. The melted agarose solution was poured into the casting tray and let cool until it was solid.
iv. Carefully the combs were pulled out and the seals removed.
v. The gel was placed in the electrophoresis chamber.
vi. Enough running buffer was added so that there was about 2-3 mm of buffer over the gel.
vii. 6 µl of each sample was carefully pipetted into separate wells in the gel.
viii. 5 µl of the DNA ladder was loaded on one well of the gel.
ix. The lid was placed on the gel box, connecting the electrodes.
x. The electrode wires were connected to the power supply.
xi. The power supply was set to 110 volts and the timer set for 60 minutes.
xii. Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode.
xiii. After 60 minutes, the power was turned off.
xiv. The wires were disconnected from the power supply and the lid removed.
xv. Using gloves, the tray and the gel were removed to observe the result.

Chapter 3

Result

3.1 Study population

Seven patients with dyspeptic symptoms were chosen for this study. Among them four (57%) were males and three (43%) were females. Their age ranged from 21-65 years.

The PCR result of the UreA gene is given in **figure 10.**

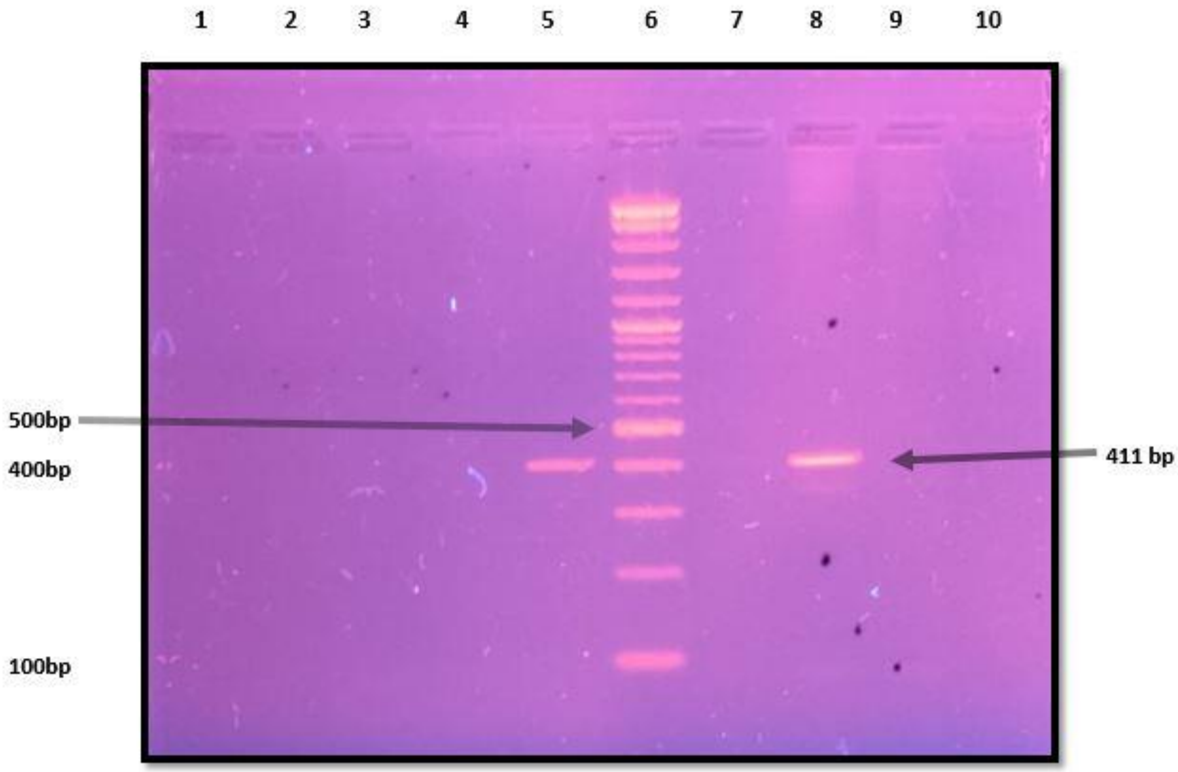

Figure 10 *PCR detection of H. pylori using primer specific for UreA gene*

Lane 1-5 and 7 and 8 contain DNA samples from the patients. Lane 6 contains a ladder of 100 bp-1000 bp. Lane 9 and 10 are negative controls consisting of a different bacterial sample and nuclease-free water respectively. Only lane 5 and 8 showed positive results for the presence of UreA gene.

PCR results of the 23S rRNA gene is given in **Figure 11.**

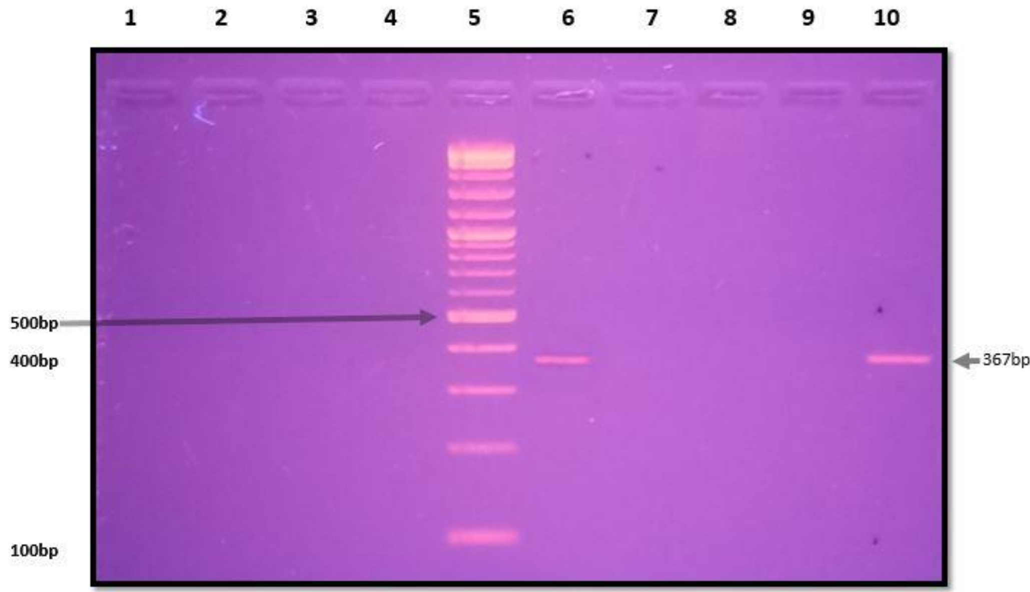

Figure 11 *PCR detection of H. pylori using primer specific for 23S rRNA gene*

Lane 1-4 and 6, 7 and 10 contain DNA extracted from the subjects. Lane 5 contains a ladder of 100 bp-1000 bp. Lane 8 and 9 contain negative control. Lane 8 is loaded with a different bacterial sample and 9 is loaded with nuclease-free water. Lane 6 and 10 were found to be positive for 23S rRNA gene specific for *H. pylori.*

Table 4. Summary of patient information			
Patient ID	**Date of collection**	**Gender**	**Age**
23001	11.01.2023	Male	21
23002	11.01.2023	Male	32
23003	17.01.2023	Male	27
23004	17.01.2023	Female	55
23005	23.01.2023	Female	35
23006	23.01.2023	Male	55

23007	26.01.2023	Female	40

3.2 *H. pylori* detection by CLO and PCR

To check for the presence of *H. pylori* CLO test was carried out first. To confirm the results, PCR of the UreA gene and 23S rRNA specific for *H. pylori* were performed.

Among the seven patients, 5 tested positive in CLO, which is 71%. Only 2 patients tested negative in CLO, which is 29% of the total selected patient population. UreA gene, which is specific for *H. pylori*, was checked in the samples and the positivity rate was 29%. PCR of 23S rRNA gene also gave the same result with 2 (29%) patients being positive for *H. pylori.* The results are summarized in **Table 5.**

Table 5 Comparative representation of CLO and PCR results with respect to patient samples

Patient ID	**CLO Test**	**PCR**	
		UreA	**23S rRNA**
23001	Positive	Negative	Negative
23002	Positive	Negative	Negative
23003	Negative	Negative	Negative
23004	Negative	Negative	Negative
23005	Positive	Positive	Positive
23006	Positive	Negative	Negative
23007	Positive	Positive	Positive

The PCR conditions for both UreA and 23S rRNA were optimized. According to Singh (2008), the annealing temperature of UreA primer was 45°C. At this temperature, multiple non-specific binding of DNA and primer took place and hence the annealing temperature was changed to 50°C. At this temperature, specific bands for *H. pylori* were observed.

Nested PCR using two sets of primers specific for 23S rRNA was carried out for detection of *H. pylori*. Rimbara (2013) stated the PCR conditions, where the annealing temperature was 57°C for primer set 1 and 63°C for primer set 2. Multiple non-specific binding took place at this temperature and hence the temperatures were changed to 60°C and 65°C respectively for primer sets 1 and 2. Specific binding and single bands on the gel indicated the presence of *H. pylori*.

Chapter 4

Discussion

In our study, we found the positivity rate of CLO to be higher than that of PCR. Only 29% of the patient pool was found to be positive for both UreA gene and 23S rRNA gene specific to *H. pylori.* In contrast, the positivity rate of CLO was 71% of the total selected patients.

The first paper in Bangladesh that compared CLO vs PCR was by Habib et al. (2016). It stated that among 111 patients, 48.65% were PCR positive and 54.05% were CLO positive where *H. pylori* specific 16S rRNA primers were used. However, Trung et al. (2019) suggested that in the Vietnamese population, for biopsy samples taken from the antrum of the patients, multiplex PCR of 100% sensitivity had a 72.1% positivity rate. Although this method is difficult to apply in routine clinical practice due its high expense and lack of skilled personnel. In comparison, the CLO test gave 38.3% positive results.

In 2019, an article showed that CLO has a sensitivity and specificity of 71.96% and 100% respectively; however, the CLO test should be combined with the other tests to reduce false-negative results. Despite its accuracy of 85.07%, positive results were only 38.3% due to prior use of antibiotics or PPI drugs when the test was run (Trung et al.,2019).

The result of the study in Chittagong, Bangladesh by Habib et al. (2016) had a higher percentage of CLO positive samples compared to PCR positive results. This aspect of the study was similar to our findings, as our results also showed a higher percentage of CLO positive. However, the results of the Vietnamese study conducted by Trung et al. (2019) were contrasting to ours. Their study showed a higher percentage of patients positive for *H. pylori* using PCR compared to the CLO test. The reasons for this can be the difference in primer sequences used by these researchers and the change of geographical locations.

The results of our study may not be entirely conclusive because the sample size is small. For PCR detection, we took biopsy samples from the antrum only. *H. pylori* bacteria is unevenly distributed in the gastric mucosa (Habib et al., 2016). Taking more tissue samples from the same patient's corpus and repeating the tests might have increased the accuracy of our results.

The high positivity rate of the CLO test might be due to false positive results. The presence of other bacteria in the samples that produce urease enzymes might cause a false positive in both CLO (Allahverdiyev et al., 2015).

Our conducted study helped us to understand the importance of molecular techniques for accurate diagnosis. A larger study population will give a better idea of the prevalence of *H. pylori* in dyspeptic patients in Dhaka, Bangladesh.

Chapter 5

Future Prospects

A small sample size was taken for this research as it is only a pilot study. This study may be expanded with a larger sample size and taking more tissue samples from the corpus of the stomach. This will ensure more accurate and reliable results.

As the infection rate of such bacteria is high in Bangladesh, antibiotics are prescribed over the counter without any predisposed diagnostic tests. Therefore, antibiotic consumption across the country increases significantly over time, which leads to the emergence of nation-wide multidrug resistant strains of *H. pylori*. As a result, the existing generalized treatment is gradually becoming ineffective towards *H. pylori*, which poses a catastrophic complication for doctors all over the country.

A global discrepancy has been observed between the results obtained by CLO test and PCR to confirm the presence of *H. pylori*. It has been stated by Sharndama and Mba (2022) that the possibility of *H. pylori* causing a disease depends on the local climate and geographical location. Therefore, it is important to find the accurate number of *H. pylori* infections leading to severe gastrointestinal diseases in Bangladesh.

Hence, an elaborate study will make it easier to determine the antibiotic resistance and virulence factors of *H. pylori*. This method is reliable and efficient to isolate and study these particular fastidious bacteria. Our results will not only enable swift and accurate results but also improve our understanding of pathogenesis of *H. pylori*, specific virulence factors and antibiotic resistance mechanisms. Thus, it will allow for establishment of targeted therapies and strategies to combat *H. pylori* infection.

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Appendix A

গণপ্রজাতন্ত্রী বাংলাদেশ সরকার
শেখ রাসেল গ্যাস্ট্রোলিভার ইনস্টিটিউট ও হাসপাতাল
Sheikh Russel Gastroliver Institute & Hospital

Date: 14/03/2023

Memo No. SRGI&H/ERC/2023-2024/021...............

Ethical Clearance Certificate

The Ethical Review Committee of Sheikh Russel National Gastroliver Institute and Hospital
Approved the Following Research Protocol

Title of the Research Work : **The analysis of antibiotic resistance pattern, resistance-associated genes and virulence factors of *Helicobacter pylori* strains isolated from patients with dyspeptic symptoms in Bangladesh.**

Principal Investigator : Dr. Fahim Kabir Monjurul Haque
Assistant Professor
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Co-Investigators : Prof (Dr.) Touhidul Karim Majumder
Sheikh Russel National Gastroliver Institute and Hospital
Dr. Mohammed Masudur Rahman
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Place of Study : BRAC University, 66 Mohakhali, Dhaka-1212
Sheikh Russel National Gastroliver Institute and Hospital. Mohakhali, Dhaka-1212.

Duration : 18 Months

Date of Commencement : February 2023

Date of Completion : July 2024

SHEIKH RUSSEL GASTROLIVER INSTITUTE & HOS

Prof (Dr.) Touhidul Karim Majumder
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