BACTERIOLOGICAL STUDY OF PAYRA RIVER: MICROBIAL ISOLATION, IDENTIFICATION, ANALYSIS OF ANTIMICROBIAL RESISTANCE AND PATHOGENICITY

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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 Microbiology

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(December 2022)

Declaration

It is hereby declared that

1. The thesis submitted titled "**Bacteriological Study of Payra River: Microbial Isolation, Identification, Analysis of Antimicrobial Resistance and Pathogenicity"** is our own original work while completing our 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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Abstract

Introduction: Water is the most precious element for all living creatures, being used for drinking, bathing, domestic purposes, etc. Being a riverine country, Bangladesh's people depend heavily on river water. This causes the water to be contaminated in various ways that raise serious public health concerns in terms of infection from antibiotic-resistant bacteria.

Methodology: 21 samples were collected from 7 sampling sites in Patuakhali and processed within 24 hours of collection. The samples were spread on several selective media: following which 47 isolates were selected based on distinct morphologies. Several biochemical tests were employed for initial identification, after which antibiotic susceptibility against Amikacin, Amoxicillin, Amoxyclav, Azithromycin, Cefixime, Ceftriaxone, Colistin, Erythromycin, Levofloxacin, Meropenem, Penicillin, Tetracycline, and Vancomycin was examined. DNAse and coagulase tests were investigated to determine the extent of pathogenicity.

Results: In this study, out of 47 isolates, *Staphylococcus spp* was the most predominant (20%) in the water samples, followed by *Escherichia coli* (16%), and *Bacillus spp.* (14%). Among the isolates, 51% and 68% were proven to be coagulase and DNase positive respectively, confirming the pathogenicity of our organisms. 94% of the isolates were penicillin-resistant followed by 73% amoxiclav-resistant. However, 100% and 95% of the isolates were susceptible to meropenem and levofloxacin respectively.

Conclusion: The results from our study form the baseline data for the bacteriology of Payra river which emphasizes the presence of enteric coliforms, especially *Escherichia coli* followed by *Serratia spp., Streptococcus spp, Salmonella spp*., *Klebsiella spp*., *Enterococcus spp.*,. and *Pseudomonas aeuruginosa.* Pathogenic strains were detected which increase the risk of infections. In addition, the high rate of DNAse and Coagulase positive isolates shows the extent of contamination which can spread several diseases among the dwellers who use water from this river.

Keywords: River Water, Antimicrobial Resistance, Pathogenicity, *Staphylococcus spp. E. coli, Bacillus spp.*

Dedication

Dedicated to each other, for being there through the ups and downs for eight long months and for completing this mammoth of a task, the likes of which we had never encountered before.

Acknowledgment

We would like to express our gratitude to the Chairperson of the Department of Mathematics and Natural Sciences, **Professor A F M Yusuf Haider** for allowing us to complete our undergraduate Thesis. Our sincere obligation and indebtedness go to our respected supervisor **Akash Ahmed**, Senior Lecturer, Department of Mathematics and Natural Sciences, BRAC University for his constant guidance, constructive criticism, and encouragement which allowed us to reach our highest potential. We would also like to express gratitude towards Professor **Mahboob Hossain**, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University for his guidance and assistance.

Our sincere appreciation goes out to Laboratory Officers **Md. Mahmudul Hasan** for his unrelenting support and guidance throughout our thesis journey, and **Shamim Akhter Chowdhury** for her unwavering kindness and advice. We would also like to thank research assistants **Mohammad Aziz Hossain**, **Nishat Tasnim Ananna,** and **Rockybul Hasan** for their incredible kindness and support. Additionally, we would like to thank teaching assistants **Somnath Gupta** for his unshakeable assistance throughout, and **Mahfuzul Hasan**, for his support.

We would also like to extend gratitude towards lab attendants Ashik-E Khuda, Tanzila Alam, and office attendant Nadira Yeasmin for their help throughout the eight months we were in the laboratory.

Finally, we would like to appreciate everyone working beside us in the laboratory and those who assisted us with their services.

Chapter 1: Introduction

1.1 Background (Importance of Water)

Water is a precious resource for living species on earth and without it, survival is impossible. Other planets have no water like earth so living species cannot survive there. 97% of earth's total water is reserved in Seas and Oceans which are saline and unusable normally. Only 3% of water is considered freshwater and out of this 2% is stored as ice and glaciers which is also unavailable and the remaining 1% of water is stored in lakes, canals, and underground which is the only source of consumption (Idowu et al.2011). In developing countries in South Asia, the population depends on untreated surface water for different domestic purposes (Ashbolt 2004; Obi et al.2004; Qadri et al. 2005). Waterborne diseases kill millions of children annually in these nations.

Bangladesh is a river-based country. There are about 700 rivers including tributaries. Most of the rivers come from India and enter through the northern part, flow across the country, and fall in the Bay of Bengal in the southern part of Bangladesh. But both the urban and rural areas in Bangladesh suffer from a shortage of safe drinking water (Chowdhury et al. 2002; Islam et al. 2011). surface water is prone to bacterial contamination as it receives wastes and pollutants from human and animal sources, and contaminated water may expose local populations to health risks.

Also, every year, the coastal regions of Bangladesh get frequently affected by floods. During the floods, human and animal excreta, rubbish, and contaminated soil mix with floodwater and pollute the river water. Due to the pollution, the quality of river water degrades eventually and the possibility of spreading waterborne diseases highly increases (Shimi et al. 2010).

1.2 Possibility of Waterborne Diseases in Bangladesh

Waterborne diseases are spread by directly drinking contaminated water with pathogenic microorganisms. These diseases include illnesses caused by both direct and indirect water exposure, such as consumption or skin contact during bathing or recreational water use (Usharani et al., 2010). The major water-borne bacterial pathogens have been identified as *Aeromonas spp., Enterobacter spp., Enterococcus spp., Escherichia coli,* and *fecal coliforms, Klebsiella spp., Campylobacter spp., Clostridium spp., Listeria spp., Burkholderia pseudomallei, Helicobacter pylori, Pseudomonas spp., Salmonella spp., Shigella spp., Staphylococcus spp.,* and *Vibrio cholerae*

Most waterborne diseases are characterized by diarrhea, which causes excessive stooling and can lead to dehydration and death. Waterborne disease is a global burden that is estimated to cause over 2.2 million premature deaths per year and more cases of illness every day, including diarrhea (including cholera and dysentery), jaundice, respiratory infections, hepatitis A and E,

typhoid fever, gastrointestinal diseases, and systemic illnesses, among other things (Bhuiyan and Noor, n.d.). According to the World Health Organization, diarrheal disease accounts for an estimated 4.1% of the total daily global burden of disease and is responsible for the deaths of 1.8 million people every year (Usharani et al., 2010). Developing countries like Bangladesh are more venturesome to these diseases mainly due to poor hygiene and the lack of proper knowledge and awareness (Bhuiyan and Noor, n.d.). According to the report of UNICEF 1999 (cited in Galway, 2000), diarrhea and dysentery continue to be the major causes of sickness, and 15 percent of all deaths in Bangladesh are due to poor water supply and sanitation facilities (Bhuiyan and Noor, n.d.) Death due to water-borne diseases is widespread in Bangladesh, particularly among children (Usharani et al., 2010). In the case of Bangladesh, roughly 80 percent of all diseases are linked with contaminated drinking water, and some 28 percent of children's death is attributed to waterborne diseases, caused mainly by pathogenic microorganisms (Shittu et al., 2010).

1.3 Geographical Analysis of Payra, Kuakata, Patuakhali

The present study was conducted in the Payra River running through the Patuakhali district of Bangladesh. The river originated from the Tetulia River via the Karkhana River and fell into the Bay of Bengal as the Burishwar River (Ahmed et al. 2021). It is located 8 km south of Barisal town and 13 km north of Patuakhali town of Bangladesh and is planned to convert into a developed area by the Government of Bangladesh (figure 2). Payra River is a reach of a tidally influenced (around 1.0 m tidal amplitude) meandering river about 60 km upstream of the mouth of the Bishkhali/Burishwar estuary (Ahmed et al. 2021).

1.4 Analysis of Industries near Payra Port(that could be linked to potential water-borne diseases)

The present experiment was conducted on the sample water collected from the Payra river where the seaport Payra is located. Payra port is Bangladesh's third seaport, located on the bank of Rabnabad Channel under Kalapara Upazila of Patuakhali district. Even though the port is responsible for a significant boost in trade and commerce in the southern region of Bangladesh, (Cho et al., 2020) it is a hub of major sources of water pollution as well.

Around the port area, large ships with engines that use the dirtiest fuel available loaded cargo, and thousands of diesel trucks visit several times a day. Such polluting equipment, and other activities at marine ports contribute to river water pollution that can cause serious consequences to the local communities. These have an impact on water quality and contribute to the spread of many waterborne diseases.

1.5 A Review of the Literature

Rivers have played an integral part in human settlements since the beginning of time, forming the backbone of human civilizations. According to (Kubera 2021) rivers have been “Extremely important for agriculture and transport, they were also the recipient of municipal sewage.” The importance of water cannot be downplayed, considering the surface, ground, marine, and coastal waters support all living things (Usharani et al., 2010). Having said that, despite the huge quantity of water available, only 3% of the water in the universe is fresh water. Among fresh waters, only about 5% of them, or 0.15% of the total world waters are readily available for beneficial use (Usharani et al., 2010).

Currently, aquatic ecosystems are utilized for bathing and recreation and hence are exposed to biological, chemical, and physical contamination. There are established water bodies exclusively for bathing which are monitored regularly. As per (Kubera, 2021) “The recreational use of such waterbodies against the official recommendations may lead to their ecological degradation and is an epidemiological threat.” This epidemiological threat is most prevalent in developing countries, where, according to (Afzal et al. 2021), the contamination of water accounts for 90% of mortality in children under five.

Water holds massive public health significance. A plethora of infectious diseases are spread by the fecal-oral route, which kills about 5 million children in a year and accounts for the sickness of 1/6th of the globe’s population (Shittu et al., 2010). Not to mention, waterborne diseases are the leading cause of mortality and morbidity globally, accounting for 22 million deaths annually (Cho et al. 2020). Developed countries have seen some success, (Cho et al. 2020) with upgraded sanitation downsizing the quantity and impact of the worst infections such as cholera and typhoid, especially when compared to developing countries like India, Bangladesh, and Pakistan, where a considerable fraction of the population depend on untreated surface water for drinking, bathing, recreation, etc (Ram et al., 2008).

Bacteriological analyses of river water are fundamental to understanding the widespread distribution of pathogenic and antibiotic-resistant bacteria in the surface waters of developed and developing countries alike. (Cho et al. 2020) Eutrophication of rivers and lakes, sewage, and animal waste from hospitals and industries facilitate the growth of fecal coliforms (Ram et al. 2018). Contaminated water has been known to house disease-causing bacteria like *Vibrio spp., Salmonella spp.,* and certain enteric pathogens (Al Bayetti et al. 2021).

All countries comply with WHO recommendations about water quality, with drinking water standards being held to a higher standard than recreational waters (Idowu et al.2011). The difficulty with assessing water quality is the impossibility of determining the concentration of all pathogens, hence the microbiological quality of water is assessed by certain fecal indicator bacteria (FIB) (Kubera, 2021) like certain strains of *E. coli* (Ram et al., 2008) There is growing public health concern about water quality and antimicrobial resistance of bacteria (Kubera,

2021).

Although antimicrobials have been significantly helpful in combatting bacterial infections worldwide, antimicrobial resistance due to over and misuse of antimicrobial agents soon became a global public health concern, as this renders antimicrobials useless (Cho et al. 2020). This resistance is passed on via antibiotic resistance genes (ARGs) which, according to (Li & Zhang, 2020) "have become a major threat to public health and environmental stability."

The exposure of bacteria to different types and concentrations of antibiotics (Sayah et al., 2005) results in random DNA mutation or horizontal or vertical gene transfers (Stange et al., 2016), which ultimately lead to the propagation of ARGs, especially if the genes are housed in mobile genetic elements like plasmids or transposons (Stange et al., 2016). Research on antimicrobial resistance estimates that in about three decades 300 million premature deaths will be caused by infections originating from antibiotic-resistant pathogenic bacteria (Cho et al. 2020). Studies on river water are crucial because rivers work as a bridge that transmits antibiotic resistance between the environment and humans. Antibiotic residues and bacteria can enter aquatic ecosystems through the discharge of effluents, hospital wastewater, and runoffs (Hanna et al., 2020).

Extended-spectrum beta-lactamase genes are of massive concern, as ESBL enzymes can render beta-lactams (except carbapenems and cephamycins) ineffective (Guyomard-Rabenirina et al. 2017). Among ESBLs, CTX-M enzymes are common in recent years, coming ahead of TEM and SHV ESBLs, with blaCTX-M genes often being associated with other antibiotic resistance determinants (Guyomard-Rabenirina et al. 2017).

Research into the presence of ARGs and ESBL enzymes is crucial in river-based countries like Bangladesh (Uddin and Jeyong 2021). Surface water used for drinking is often contaminated in third-world countries like Bangladesh (Hosen et al. 2021), with frequent cholera epidemics plaguing the nation. *V. cholerae* are largely aquatic microorganisms, hence cholera outbreaks are linked heavily with frequent flooding and lack of access to potable water (Hosen et al. 2021). These frequent outbreaks are also treated with the same antibiotics, leading to an increase in antibiotic resistance (Hosen et al. 2021). Due to factors like accelerated growth of population, and hasty urbanization, the water microbial quality has been severely damaged (Islam et al., 2018). Recent studies have shown widespread fecal contamination in Betna River (Islam et al., 2018). This is of colossal concern, as the water is used for domestic purposes, bathing, and even drinking (Islam et al., 2018).

To identify pathogenic and antibiotic-resistant microbes in water samples, culture-based approaches can be used, where the microbes are grown on a nutrient medium. The medium can be either selective or universal, depending on the specific kinds of bacteria being sought. Then,

isolates can be tested for antibiotic susceptibility by growing them on a separate nutrient medium (often MHA), with antibiotics being supplied to the medium (Nnadozie and Odume 2019). Other approaches include molecular approaches like polymerase chain reaction (PCR), quantitative polymerase chain reaction (Q-PCR), and metagenomics profiling (Nnadozie and Odume 2019).

1.6 Gaps in Previous Studies

Bacteriological analyses of river waters have been heavily studied, however, there have been gaps in previous research.

Although the river waters of the South Asian subcontinent have been studied, Bangladesh has been left understudied in many instances. There is a lot of literature on the rivers and tributaries of India and Pakistan, but less so on Bangladesh, despite Bangladesh being a river-based country with about 700 rivers and tributaries (Uddin and Jeyong 2021). Though many of the rivers originate from India (Uddin and Jeyong 2021), there are unique physical and chemical compositions of said rivers.

The second scope of research to consider is the fact that Patuakhali is often overlooked when it comes to river-based studies. Although there is data on the bacteriological composition of significant rivers like Padma, Meghna, Jamuna, Turag, and Buriganga, there is little to no data on the microbiological contamination of Payra and Andharmanik rivers. This gap in research was ideal for the design of this particular study, which focuses solely on the aforementioned rivers. Not to mention, sea samples are often overlooked in this field of study, as most of the literature focuses on samples from specific rivers, overlooking the eventual passage of ARGs and AMR bacteria into the Bay of Bengal.

There are gaps in previous literature on the specific antibiotic resistance genes (ARGs) that can be found in said rivers. Filling in this space is important to have data on the composition of ABR and AMR bacteria that are found in freshwater surfaces, as these are often used for drinking, bathing, and other domestic purposes.

Additionally, studies in Bangladesh often focus exclusively on cholera outbreaks, hence there is an overemphasis on *V. cholerae* as opposed to a general study on the genus and species of resistant bacteria that cause microbiological contamination.

1.7 Novelty of Our Study

River water easily gets contaminated via the discharge of industrial effluents, hospital and municipal wastewater, and human and animal discharges as well. Hence, there is a high chance of getting antibiotic residues and antibiotic-resistant microorganisms in river water. Antibiotic

resistance bacteria (ARGs) like Staphylococcus spp, Salmonella/Shigella, and Bacillus spp are often found in river water. Since most of the previous studies are focused on V. cholerae only, this study focuses more on identifying the level of contamination to fill those study gaps. Also, being in a coastal area the water of the Payra river is eventually plunged into the sea and there are rarely found literature reviews about ABR and AMR on Sea. Hence, seawater had been collected for this study as well.Our study will be the baseline study on the water condition of Payra River and it would be helpful for further research purposes.

1.8 Aims, Objectives, and Hypothesis

Having all the above facts in mind, the general aim of this study was to determine the bacteriological pattern and the prevalence of antibiotic-resistant in the Payra River and Kuakata sea. Moreover, this study evaluated the antimicrobial susceptibility patterns of the isolates and their pathogenicity. The authors hypothesized that due to large amounts of pollution from nearby industries, there would be a plethora of antimicrobial-resistant and pathogenic organisms recovered from the waters.

Chapter 2: Methods & Materials

2.1 Study Area/ Location (Sampling Sites)

The study was conducted in the Payra river (21.941°N 90.140°E) located in Kalapara Upazila, Patuakhali district of south-central Bangladesh. The study area covers an area of around 10 km^2. Water samples were collected at the end of March 2022. 5 sampling sites (**P1** Payra Port, **P2** Baliatoli Ghat, **P3** Kalapara Kheya Ghat, **P4** Nobipur-Kolapara Boat Line, **P5** Khepupara launch ghat) from the river were selected considering the pollution and human use of water, and 2 sea sampling sites (**P6** and **P7**) from Kuakata beach were selected randomly. (Fig 2.1.1 and 2.1.2)

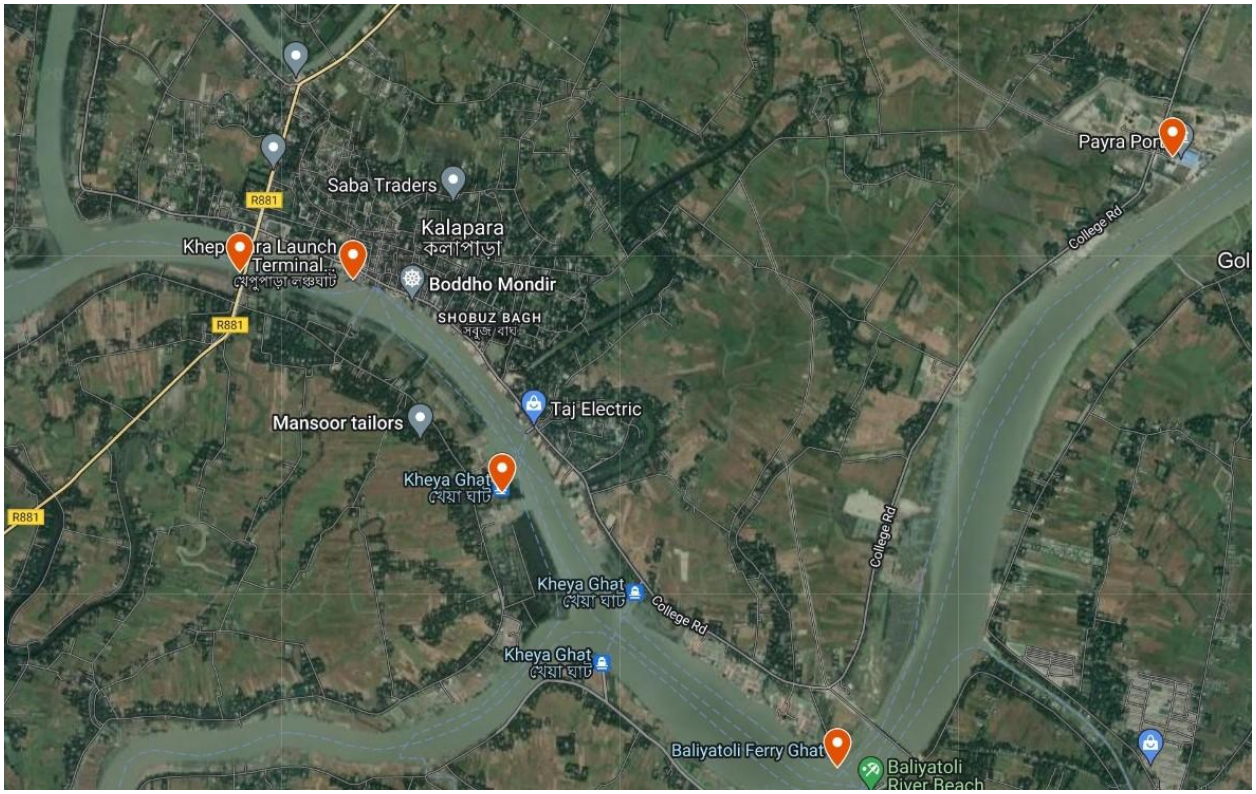


Figure 2.1.1: Sampling sites of Payra River

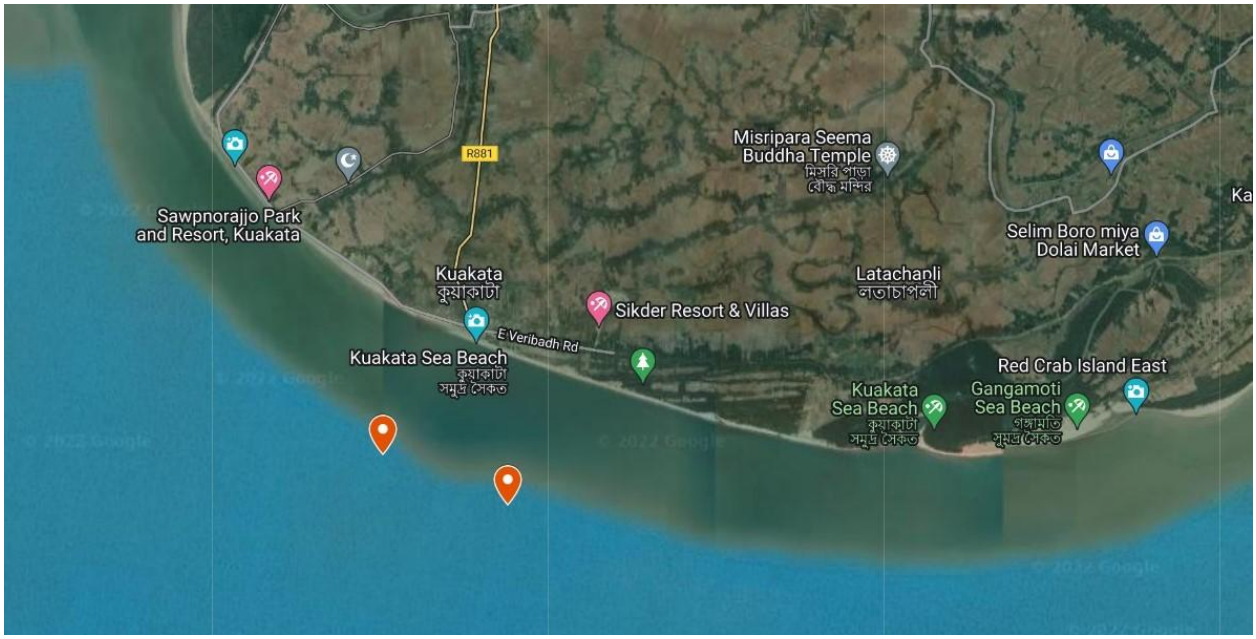


Figure 2.1.2: Sampling Sites of Kuakata Beach

2.2 Sample Collection

A total of 21 water samples in 3 different sets were collected from each sampling site. The raw river water and sea samples were collected from all the aforementioned sampling sites in sterile collection bottles which had been previously autoclaved. Each container had a capacity of 500ml. The container caps were removed aseptically. Following the removal, the bottles were lowered into the sampling sites to depths of about 2 meters. The bottles were then brought back up and sealed shut as soon as they were brought to the surface to minimize the chances of contamination.

In each sampling site, the same sample collection method was followed.

2.3 Sample Transportation

These samples were then transported at refrigerator temperature to the laboratory and processed within 24 hours of collection.

All of the containers were kept inside an icebox at 4^{o} C to maintain the cold chain. Samples usually need to be processed within six hours of collection. However, the distance from Patuakhali to the lab was about 294km, which meant the tubes would have to be kept in sterile conditions for more than 6 hours. The cold chain helped maintain stable conditions as most microorganisms cannot grow in temperatures that low.[1] Furthermore, the containers were sealed to prevent spillage and as airtight as possible to prevent contamination. The journey from sample collection sites to the laboratory took about 10 hours.

The samples were processed within 16 hours of collection.

2.4 Sample Processing

The collected samples were processed in two main ways: primary and secondary.

1) Primary Processing

The primary processing consisted of three steps. Those were: serial dilution, membrane filtration, and codename generation.

Serial Dilution

For the first step of primary processing, 50µl of each sample was taken without dilution. For this paper, the authors have addressed those undiluted samples as “raw.” The spread plate method

was employed for this particular step. The 50µl raw samples were placed onto Xylose Lysine Deoxycholate Agar, Thiosulfate Citrate Bile Salts agar, Mannitol Salt Agar, and Cetrimide agar. These particular media were chosen for the isolation and identification of *Salmonella spp, Shigella spp, Vibrio spp, Staphylococcus spp,* and *Pseudomonas spp* respectively.

50µl of each sample was taken again, and serial dilution was applied. These samples were diluted to dilution factor 10^5. Using the spread plate method, the diluted samples were placed onto MacConkey agar and Nutrient agar. Nutrient agar was used to observe the general growth of microorganisms while MacConkey was specifically used to identify and enumerate *E.coli.* All the samples, consisting of diluted and raw, were incubated at 37℃ for 24 hours.

Membrane Filtration

The membrane filtration method was utilized in the second step of primary processing to isolate *E. coli* and other total and fecal coliform bacteria. The samples were analyzed for the total heterotrophic count, presence of total coliform, and fecal coliform. MFC agar was used in this process.

In this procedure, 100 ml of each water sample was filtered through a sterile, white, grid-marked filter paper, with a diameter of 47 mm and a pore size of 0.45µm. The membrane filtration unit was set up as per [insert reference].

Each sample was poured on top of the filter paper, which was used to retain bacteria. After filtration, the membrane filter containing the bacteria was placed on a selective differential medium, MFC agar, and incubated at 37℃ for 24 hours. The plates were observed afterward for bacterial growth and subsequent enumeration and identification.

2) Secondary Processing

The secondary processing had three steps: repeat serial dilution, colony selection, and codename generation.

Serial Dilution

Several samples needed to be spread again for bacteria enumeration, and different dilution factors were given for these specific media. Four such specific samples were re-diluted. X3, which had previously been spread on XLD without dilution, was diluted to a factor of 10^2. M6 and M7 were diluted again till 10^4. Lastly, X7 was diluted to 10^3. Subsequently, the spread method was repeated for all of the aforementioned samples.

Colony Selection

After bacterial enumeration took place and the colonies of each sample were counted, colonies with distinct morphologies were selected from each sample for further analysis. A total of 79 colonies were selected. For this paper, the authors refer to those selected colonies as the isolates. Subsequently, codenames were generated for our samples.

2.5 Microbial Identification

2.5.1 Biochemical Tests

The selected isolates were subcultured in nutrient agar for pure culture isolation so that microbial identification through biochemical tests could be carried out. The specific biochemical tests chosen for this particular study were Gram Staining, Oxidase, Catalase, Triple Sugar Iron test (TSI), Motility-Indole-Urease test (MIU), Methyl Red and Voges-Proskauer (MR-VP), and Citrate Utilization.

Gram Staining

Gram staining is used for differentiating the gram positive and gram negative bacteria based on cell wall composition. The basic principle of gram staining involves the ability of the bacterial cell wall to retain the crystal violet dye during the ethanol solvent treatment. In the case of gram positive organisms, initially, after taking up crystal violet dye, with the use of 95% ethanol, their thick peptidoglycan cell wall becomes dehydrated and shrinks, causing the pores to get closed and preventing the stain from exiting the cell. So the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of gram positive bacteria is not removed and appears blue or purple. In the case of gram-negative organisms, due to the thin layer of peptidoglycan, the lipid layer is dissolved, losing the primary stain. So, as the final step, a basic fuchsin stain such as safranin is used to give decolorized gram-negative bacteria pink color for easier identification. (Tripathi and Sapra 2022).

The Gram Staining procedure was followed according to ASM's Gram Staining Protocol and the number of Gram Positive and Gram Negative bacteria were recorded along with their shape.

Oxidase

The oxidase test is used to determine the presence of bacterial cytochrome oxidase enzyme, an enzyme of the bacterial electron transport chain that catalyzes the oxidation of cytochrome c. **Kovacs Oxidase Reagent containing** 1% tetra-methyl-*p*-phenylenediamine dihydrochloride is used which is oxidized to indophenol, a dark purple colored end product in presence of the oxidase enzyme. A positive test(presence of oxidase) is indicated by the development of a dark purple color. No color development indicates a negative test and the absence of the enzyme.

To perform this test, one drop of distilled water was added to Whatman's filter paper, and a sterile toothpick was used to pick a single colony from the nutrient agar plates. Afterward, the colony was placed against the reagent and any color change or lack thereof was recorded.

Catalase

The catalase test is used to determine the presence of catalase, an enzyme that catalyzes the release of oxygen from hydrogen peroxide (H_2O_2). Strict aerobic and facultative anaerobic bacteria can be categorized as catalase positive. Consequently, oxygen is a terminal electron acceptor in all such bacteria. To conduct the catalase test, a small inoculum of the bacterial isolate was mixed into a hydrogen peroxide solution (3%). Then, bubble production or lack thereof was observed.

TSI

The purpose of this experiment is to determine the fermentation of glucose, lactose, or sucrose and H2S production. TSI contains three sugars – glucose, sucrose, and lactose. Phenol Red is the indicator that turns yellow at an acid pH resulting from fermentation. The glucose concentration is 0.1% and the lactose and sucrose concentrations are 1%. If an organism ferments glucose only, fermentation takes place in the butt, which turns the butt yellow. In the slant, aerobic oxidation takes place and it remains red. If sucrose or lactose is fermented, enough acid is produced by fermentation in the butt to lower the pH of both the butt and the slant, turning both yellow. Gas production can also be detected as bubbles trapped in the agar or actually splitting or pushing the agar upwards. H2S production is detected by forming ferrous sulfide (FeS), an insoluble black precipitate where ferrous ammonium sulfate reacts with the H2S gas.

TSI Agar was first prepared according to HiMedia instructions. The tubes were slanted and the media was allowed to solidify. Then, a sterile needle was used to pick a single colony from each sample. Afterward, the needle was stabbed into the slanted media, pulled out, and the slanted

surface was streaked subsequently. The test tubes were incubated for 24 hours at 34℃ and the results were recorded following this incubation period.

MIU

Three tests are performed in a single tube for MIU to help differentiate the organisms based on motility, urease, and indole production.
Motile organisms show growth extending away from the stab inoculation line, while non-motile ones show growth along the stab line.
Urease hydrolyzes urea and releases ammonia and carbon dioxide, which react in solution to form ammonium carbonate, then pH rises and the color of the phenol red yellow to pink-red in alkaline pH, giving a positive urease result. Tryptophan in casein enzymic hydrolysate is converted to indole by the enzyme tryptophanase in indole-positive organisms. The resulting indole reacts with the p-dimethyl amino benzaldehyde in Kovac's reagent to form a quinoidal red-violet compound.
The MIU media was prepared as per the instructions stated in HiMedia. 40% urea solution was separately prepared according to the volume needed. Additionally, Kovac's indole reagent was prepared by following the procedure stated in HiMedia.
Each test tube was incubated for 24 hours at 34°C after inoculation. Results for Motility and urease were recorded first. Upon adding 10 drops of Kovac's reagent to the tube formation of a red-pink ring in the tube was noted and the results were recorded.

MR and VP

The Methyl-Red test was performed on our bacterial isolates to detect the ability of an organism to produce stable acids and end products from supplied glucose. These bacteria metabolize **glucose to pyruvic acid,** subsequently producing **stable acid** via the '**mixed acid pathway**. The acid results in a decrease in pH to 4.5 or below. This change in pH results in a colour change of the indicator methyl red, which turns from **yellow to cherry red**, indicating a positive MR test.
The Voges-Proskauer test identifies bacteria that ferment glucose, leading to 2, 3-butanediol accumulation in the medium. The addition of 40% KOH(Barrit's B) and a 5% solution of alpha-naphthol in absolute ethanol (Barritt's A) is used to detect a precursor in the production of 2, 3-butanediol: acetoin. In the presence of the reagents and acetoin, a cherry-red color develops. The development of a red color in the culture medium 15 minutes following the addition of Barritt's reagents represents a positive VP test; the absence of a red color is a negative VP test.

Citrate

The citrate utilization test determines whether bacteria can use citrate as a sole carbon source. The bacterium converts citrate to pyruvic acid and CO2. Simmons citrate agar slants contain sodium citrate as the carbon source, NH4+ as a nitrogen source, and bromothymol blue. When bacteria oxidize citrate, they remove it from the medium and liberate CO2 which combines with the sodium and water to form an alkaline compound: sodium carbonate. The rise of the pH turns the pH indicator to a blue color- resulting in a positive citrate test. Simmon's Citrate Agar was prepared, poured into sterile vials, and tilted to allow the media to slant. Subsequently, a sterile needle was used to pick out a single colony from each isolate. The solidified media was then stabbed by the needle and the slanted surface was streaked as the needle was pulled out.

The vials were incubated for 24 hours at 34℃ and results were observed.

2.5.2 Pathogenicity Tests

DNAse

The DNAse test was conducted to find out which of the isolates, if any, were capable of degrading DNA by producing DNAse enzyme. To carry out the DNAse test, three preceding steps took place. DNA was extracted, and DNAse media and 1N HCl were prepared.
DNA was extracted using the boiling method as described in (Dashti et al. 2009), from two *Klebsiella* species: *Klebsiella pneumoniae* and *Klebsiella variicola.* Following DNA extraction a preliminary gel run was conducted to confirm the presence of DNA. Once DNA presence had been confirmed, the extracted DNA was stored in sterile microcentrifuge tubes at -20℃.
DNAse agar was prepared following the recipe stated in HiMedia. After the media was autoclaved, the previously extracted DNA was added to the media and then it was poured on large Petri plates. The last step of this step was to streak each isolate onto the DNAse agar. Each DNAse plate was incubated at 34℃ for 24 hours. Post incubation, a few drops of 1N HCL was added to each plate. After the HCl had been absorbed into the media, about five minutes post addition, any clear zones or lack thereof were observed.

Coagulase

A myriad of microorganisms possesses the ability to produce an enzyme called coagulase, which facilitates the conversion of fibrinogen to fibrin. This test is often used to distinguish the coagulase-positive *Staphylococcus aureus* from other *Staphylococcus* species.

Staphylococcus aureus is known to produce two different types of coagulase; free coagulase and bound coagulase. The former is identified using the tube method, while the latter, known as cell wall-bound coagulase, is detected using slides.

For this paper, the authors employed the tube method. Rabbit plasma was diluted using physiological saline, and bacterial suspension from each isolate was prepared in micro-centrifuge tubes. Following these preparations, 100µl of suspension of each isolate and 500µl diluted plasma were added to each test tube. Then the tubes were incubated overnight at 37℃ and observed for signs of clumping or clotting.

2.6 Antibiotic Susceptibility Tests

An antibiogram consists of testing antibiotic susceptibilities for common bacterial pathogens. Recently, many bacterial species have developed resistance due to widely used antibiotics.

The Kirby-Bauer disk diffusion susceptibility test prioritizes McFarland turbidity (Range 0.5 -1.0). Here, a colony was suspended into a test tube filled with saline, and the suspension was matched with McFarland turbidity. **Kirby-Bauer disc diffusion** test method technique was utilized by performing lawn on a Mueller-Hinton agar plate. To determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria, all the Isolates were tested for antibiotic resistance utilizing the following antibiotics:
Azithromycin, Ciprofloxacin/Levofloxacin, Meropenem, Amoxicillin, Amoxyclav, Colistin, Tetracycline, Cefixime, Vancomycin, Ceftriaxone, Cephalosporin, Amikacin. All of these antibiotic-impregnated disks were placed safely on the MHA plates, and according to CLSI guidelines, all the inhibition zones were measured in millimeters to determine the sensitivity or resistance of all the isolates.

Chapter 3: Results

3.1 Organism Identification

Sampling Sites	Name of Organisms
P1	*Streptococcus spp. , Staphylococcus spp., Yersinia spp., Serratia spp., Paenibacillus spp.,*
P2	*E. coli, Bacillus spp.,*
P3	*Hafnia spp., E. coli, Paenibacillus spp., Salmonella spp.*
P4	*Staphylococcus spp., Bacillus spp., Pseudomonas aeruginosa, Klebsiella spp., Micrococcus spp., Staphylococcus aureus, Proteus spp, Enterococcus spp., Staphylococcus spp.*
P5	*Pseudomonas spp., Bacillus spp., Staphylococcus spp., Pseudomonas aeruginosa,*
P6	*E. coli, Bacillus spp., Staphylococcus spp., Enterobacter spp., Staphylococcus aureus, Streptococcus spp., Serratia spp.,*
P7	*Bacillus spp., Yersinia spp., Vibrio cholerae*

Fig 3.1.1: Organisms found in different sites

The distribution of organisms at the 7 different sampling sites varies as shown in Fig 3.1.1. The highest number of pathogenic organisms are present at P4 and at P2, the presence of pathogenic organisms is the lowest. Curiously, *Vibrio cholerae* was found in the sample water collected from P7 in Kuakata Sea Beach.

Out of 47 isolates, *Staphylococcus spp* was the most predominant (20%) in the water samples, followed by *Escherichia coli* (16%), and *Bacillus spp.* (14%), *Pseudomonas spp* (9%), *Serratia spp. (7%), Streptococcus spp(7%), Yersinia spp(5%), Paenibacillus spp.(5%) Salmonella spp*. (2%), , *Klebsiella spp.* (2%)*,, Micrococcus spp.*(2%)*, Staphylococcus aureus*(2%), *Proteus spp*(2%), *Enterococcus spp.*(2%), *Vibrio cholerae*(2%), *Hafnia spp.*(2%) fig 3.1.2.

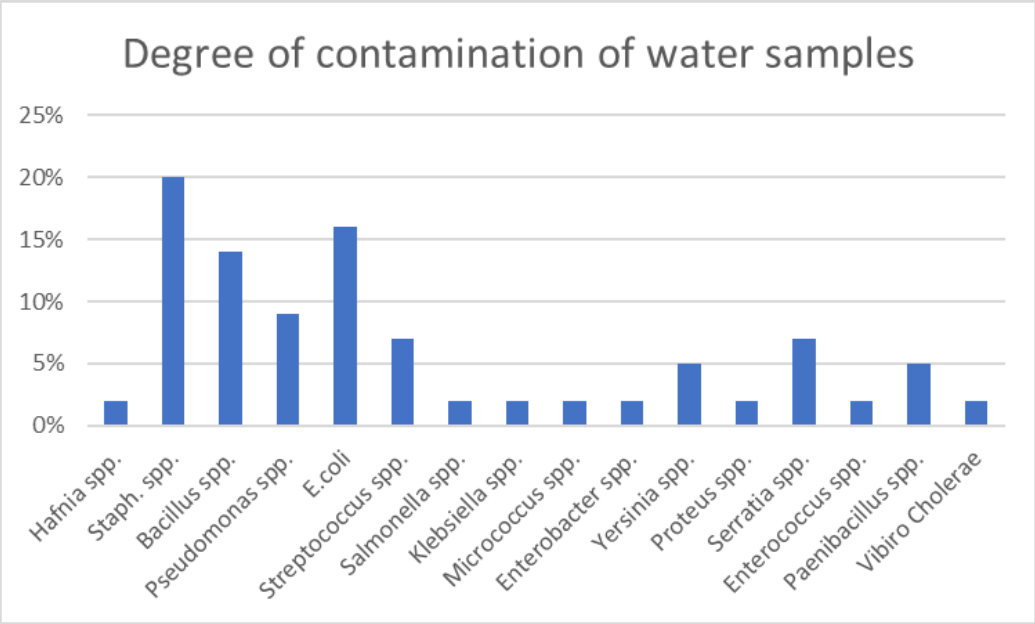


Fig 3.1.2: Degree of Contamination

3.2 Pathogenicity Tests

Among the 47 isolates 32 of them, roughly 68%, tested to be DNAse positive, and 15, roughly 32% were DNAse negative. This data set proves that most of the isolates recovered from the sampling sites had the capacity to degrade DNA, and therefore are pathogenic.

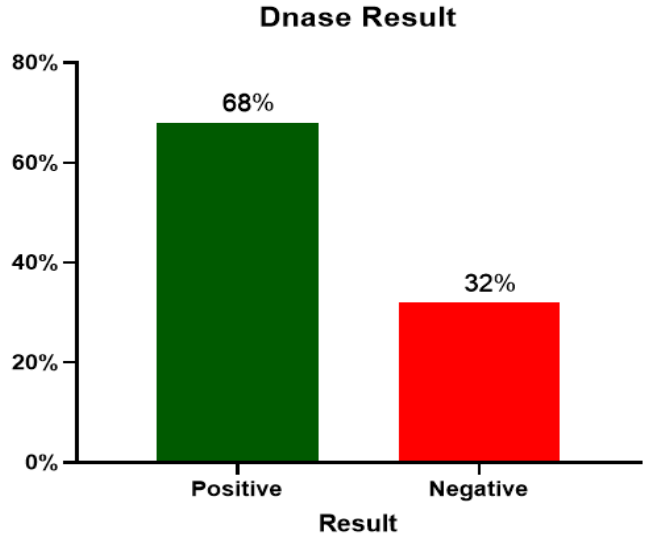


Fig 3.2.1: Graph of Dnase Test Result

The coagulase test results had an even distribution, with 24, almost half of them testing positive for coagulase enzymes, while the other half, 23, testing negative.

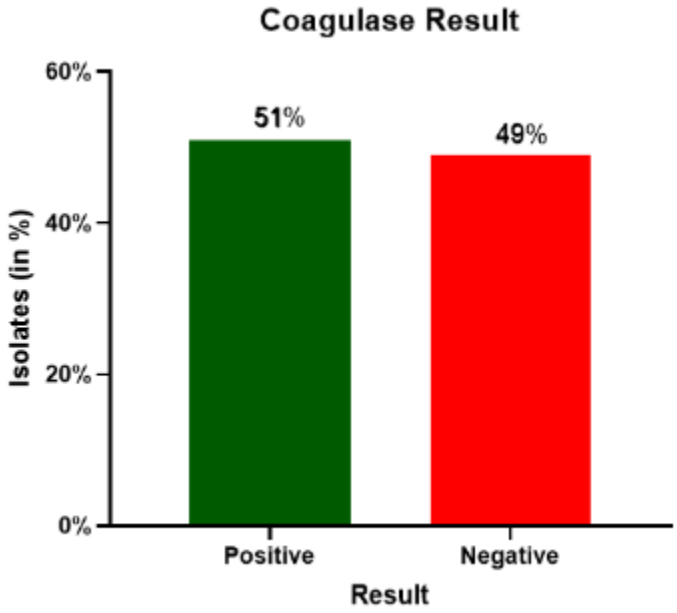
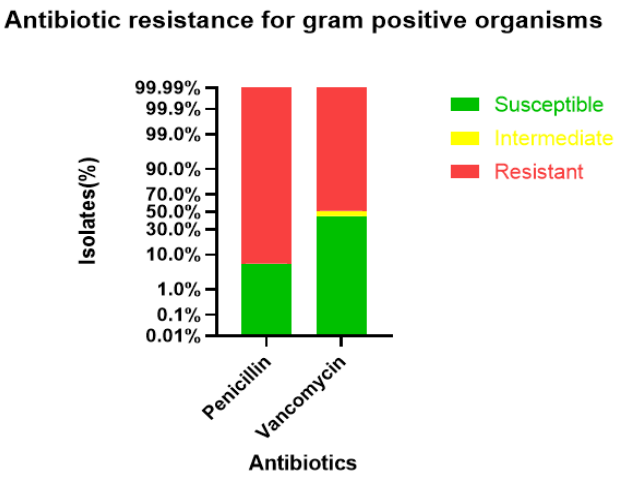


Fig 3.2.2: Graph of Coagulase Test Result

3.3 Antibiotic Susceptibility Tests



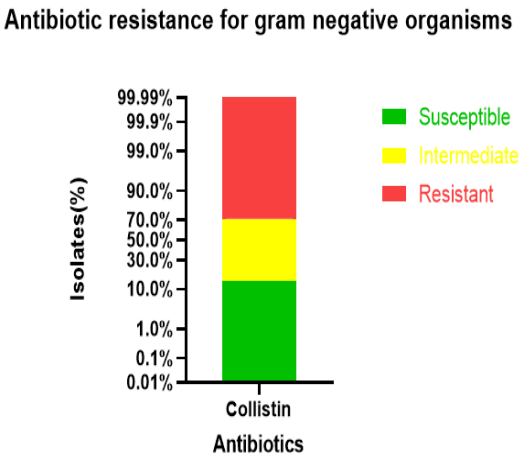


Fig 3.3.1: ABR for G+ organisms

Fig 3.3.2: ABR for G- organisms

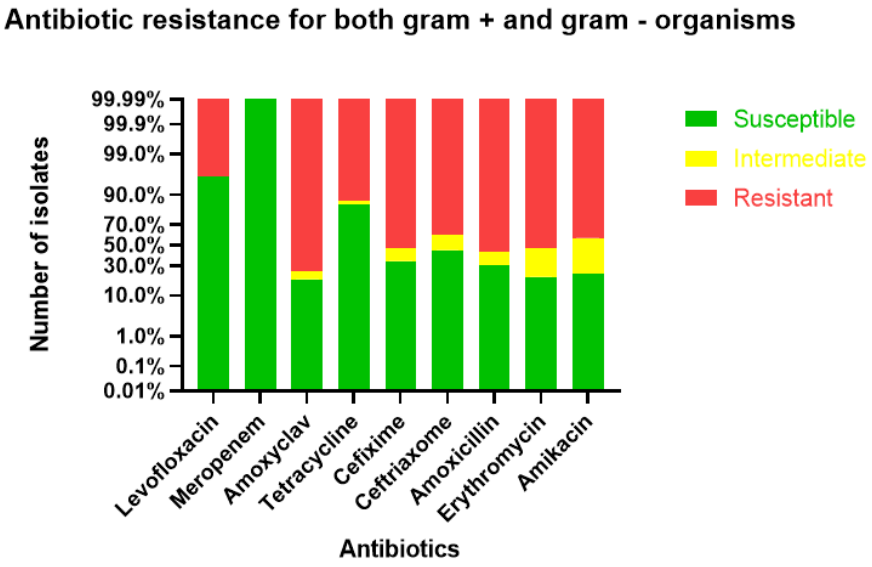


Fig 3.3.3: ABR for both G+ and G-organisms

Antimicrobial agent resistance was detected in all types of samples collected shown in fig 3.3.1,fig 3.3.2 and fig 3.3.3. The most frequently encountered form of resistance in all samples was resistance to Penicillin(P) and Amoxiclav(AMC). However, all the samples were susceptible to Meropenem(MRP) and most of them were susceptible to levofloxacin.

Chapter 4: Discussion

4.1 Analysis of Results

Fig 3.1.1 shows that the sample water contains high amounts of microbial contaminants which can be a dangerous threat to public health. The values obtained for the samples from the sampling sites P1, P4, and P6 were higher than other sampling sites. This is expected possibly because of the water pollution from the surroundings compared to the other sites. It was observed that sampling sites P1 and P4 are highly dense areas inhabited by people of lower socioeconomic status who do not maintain sanitation practices. Also, the location of P6 was Kuakata Sea beach where people of different ages pollute the beach area in many ways and also swim in the water for recreational purposes. However, other sampling sites recorded a lower microbial count than the other locations which indicates that maybe the environmental condition was better as well as reduced pressure of use may reduce the degree of contamination. Characterization of the isolates from the water samples from the locations under study was highly contaminated with many bacterial pathogens namely, *Staphylococcus spp*, followed by *Escherichia coli*, *Bacillus spp.*, *Pseudomonas spp*., *Serratia spp., Streptococcus spp, Yersinia spp., Paenibacillus spp., Salmonella spp*., *Klebsiella spp.*, *Micrococcus spp., Staphylococcus aureus*, *Proteus spp*., *Enterococcus spp.*, *Vibrio cholerae*, *Hafnia spp.* These are mostly pathogenic organisms mainly of fecal origin. Any water source used for drinking or cleaning purposes should not contain any organism of fecal origin. The presence of enteric coliforms especially *Escherichia coli* makes the water samples unsuitable for human consumption according to the guidelines set by WHO for the evaluation of the bacteriological quality of drinking water (Idowu et al., 2011) At least six different pathotypes of E.coli can cause enteric diseases, such as diarrhea or dysentery, and other pathotypes cause extra-intestinal infections, including urinary tract infections and meningitis(Kaper et al.,2004). *Staphylococcus aureus* is known to produce enterotoxins if entered into the bloodstream that can cause life-threatening infections. (Bennet and Lancette, 1992). Vibrio cholerae, commonly found in seawater, is also a toxin-producing pathogenic bacterium that can cause enteric diarrhoeal diseases(Maheshwari et al.,2011) Other bacteria isolated from all water samples such as *Serratia spp., Streptococcus spp, Yersinia spp., Paenibacillus spp., Salmonella spp*., *Klebsiella spp.*, *Micrococcus spp., Proteus spp*., *Enterococcus spp.*, *Hafnia spp. Pseudomonas aeuruginosa* are also of public health significance as they may contain pathogenic strains.

This study enlarges the knowledge of the spread and the antibiotic resistance profile of the emerging various pathogenic ABR in water samples. Our results showed a higher prevalence of *ABR and ARG* in River Payra and seawater is also mentioned as a potential source. 100% of the

isolates were susceptible to Meropenem, and 95% to Levofloxacin. Only two of the isolates (6%) were susceptible to Penicillin, with most of the others being in the ranges of 15-40% for the other antibiotics. On the other hand, 94% were resistant to Penicillin, and 75% to Amoxiclav. The resistance to Cefixime and Vancomycin was evenly spread at 53% for each, while Amoxicillin and Erythromycin saw a resistance rate of 57% and 53% respectively.

In the water samples, *Serratia spp., Streptococcus spp., E. coli, Staphylococcus aureus, Enterobacter spp., Staphylococcus spp., Bacillus spp.* have been detected as ABR. Here, the high number of antibiotic resistance and ARG indicates that the river and sea serve as a significant reservoir for the spread of antibiotic resistance to opportunistic pathogens. The high concentration of bacteria that are resistant to penicillin, vancomycin, erythromycin, amikacin, amoxyclav, amoxicillin, colistin and ceftriaxome in rivers indicates that these antibiotics are present in freshwater systems. In addition, the most frequently encountered form of resistance in all samples was resistance to Penicillin (P) and Amoxiclav (AMC). However, all the samples were susceptible to Meropenem (MRP). Additionally, most of the samples were susceptible to Levofloxacin. Previous literature has shown that antibiotic-resistant bacteria and genes can exist in freshwater environments over long periods, which means there is an increased risk of waterborne diseases caused by these pathogenic bacteria. The source of the problem is the mixing of sewage with freshwater; the same body of freshwater that individuals living near Payra port use for consumption and domestic purposes are also collecting waste from nearby hospitals, industries, and ships. As a result of this persistence of ARGs and antibiotic-resistant bacteria, there is a rapid transfer of ARGs between bacteria, resulting in the creation of emerging pathogens.

The Payra River zone stands as a rural area. Hence, the people from nearby areas of Payra river depend on it for basic needs such as, for drinking, bathing, domestic purposes and so on. So, the high number of DNAse and coagulase-positive isolates collected from the river samples are very alarming and the water quality is not in agreement with WHO drinking-water standards. Additionally, the waters don't meet the EPA criteria for recreational use either, since pathogenic organisms are strictly prohibited from being present in water used for domestic and recreational purposes.

Coagulase positivity is attributed chiefly to *Staphylococcus aureus,* which is of massive public health significance. Coagulase-positive *Staphylococci* have been associated with various human and animal infections, such as UTIs, bacteremia, abscesses, and wound infections. Coagulase can clot plasma by converting fibrinogen to fibrin. Consequently, these bacteria have been associated with intrarenal abscesses in humans, often as a result of bacteremia.

Just like coagulase, DNAse is an enzyme secreted by bacteria to survive in the host cell. Dnase activity can destroy neutrophil extracellular traps (NETs) that are composed mainly of DNA and

can prevent bacterial spreading, released by neutrophils when there is a microbial infection. (Silvestre et al. 2021). *Staphylococcus aureus* is one of the most common pathogenic bacteria that is Dnase positive. It causes diseases from minor skin disorders to major life-threatening diseases like septicemia and meningitis (Zaringhalam et al. 2012).

This discovery is alarming due to a large number of the local population using the river and sea waters for all different domestic and recreational activities, who are in constant danger of developing severe infections.

4.2 Recommendations

From previous studies conducted on the microbiological content of rivers and seawaters, it becomes evident that waterborne diseases often emerge and spread due to improper waste disposal, and contamination of water by sewage, surface runoff, etc. It is imperative for more awareness and educational programs to be organized which can work as educational tools for raising awareness among the general funding. There need to be large amounts of budget allocated towards sewage treatment to ensure the water ending up in rivers and seas has been purified. This means, more research, development, and investment needs to go towards wastewater treatment in order to make the water in rivers match the WHO standards for drinking water. Various governmental and non-governmental organizations should carry out educational initiatives in rural areas which lack tap water and hence rely on surface water for drinking, on ways to purify surface water properly.
Additionally, in order to reduce the chances of waterborne disease transmission, such as cholera and typhoid, it would be advisable to screen water sources before they are used for drinking or domestic purposes, against specific pathogenic bacteria such as *Vibrio cholerae, Salmonella spp., Pseudomonas spp., E.coli,* etc. This would also require a sufficient amount of budgeting and investments put into it in order to set up routine checks.

Chapter 5: Future Prospects

5.1 Limitations

Due to the sampling sites being in Patuakhali, a district quite far away from the capital city of Dhaka where the laboratory is situated, the samples could only be collected once. This limited the scope and possible data set of the study since there was no data on seasonal variation. The corollary of this distance meant that the number of sampling sites was also limited to 7. More sampling sites would have provided the authors with a larger range of data to work with. The temperature and pH of the water were not recorded during sample collection, and the turbidity was not measured, which reduced the scope of information about the physical attributes of the

samples. Additionally, though there were initially 79 isolates selected, due to unfortunate circumstances of contamination and merging, the isolate numbers eventually reduced to 47, further reducing the amount of data available. Furthermore, due to the lack of availability of certain primers, there was limited scope of molecular research, especially on vancomycin-resistant genes found in multiple isolates.

5.2 Future Prospects

In order to achieve an overarching view of the water quality of the Patuakhali district, the authors solely focused on the microbiological contamination of water. The chemical and physical properties of the water also can be thoroughly examined in the future. These include but are not limited to the temperature, pH, dissolved oxygen (DO), BOD, COD, chloride, phosphate, sodium, potassium, and total nitrogen contents of the water. Since Bangladesh is home to several tanneries, there is a possibility of massive physico chemical contamination, which is also a massive public health concern for water that is used for domestic purposes. Additionally, there are several rivers in the district as well. The data sets can be more statistically significant if future experiments are done that focus on multiple rivers. Not to mention, the seasonal variation between microorganisms can also be examined in order to explain the seasonal variation in waterborne diseases such as cholera and typhoid. Furthermore, the authors found several vancomycin-resistant isolates but were unable to conduct molecular analysis due to a lack of primers specific to vancomycin resistance. Additional molecular work related to resistance, and further genome sequencing would be advisable.

Chapter 6: Conclusion

This study forms the baseline study on the water quality of Payra River., being the first of its kind in the country. It focuses mostly on identifying the level of contamination and to identify antibiotic resistant bacteria and antibiotic resistant genes in Payra river.

The results of this study showed severe microbial contamination due to *Staphylococcus spp*, followed by *Escherichia coli*, *Bacillus spp.*, *Pseudomonas spp*., *Serratia spp., Streptococcus spp, Yersinia spp., Paenibacillus spp., Salmonella spp*., *Klebsiella spp.*, *Micrococcus spp., Staphylococcus aureus*, *Proteus spp*., *Enterococcus spp.*, *Vibrio cholerae*, and, *Hafnia spp.* These organisms also showed a high degree of pathogenicty, with 51% and 68% being coagulase and DNAse positive respectively. Additionally, the bacteria were proven to be resistant to multiple antibiotics, with the most being resistant to Penicillin (94%), followed by Amoxiclav (73%). There is plenty of scope for further research on understanding the mechanisms of

resistance, investigating the presence of certain resistance causing genes such as ESBL producing genes, and the extent of physical, chemical and microbial contamination of Payra and other adjacent rivers to understand the degree of public health burden this poses.

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Chapter 8: Appendix

Media compositions

The compositions of all the media (used from HiMedia), employed in the study are given below:

XLD Agar:

Composition	Amount (g/L)
Yeast Extract	3
L-Lysine	5
Lactose	7.5
Sucrose	7.5
Xylose	3.5
Sodium chloride	5
Sodium deoxycholate	2.5
Sodium thiosulfate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.8
Agar	15
Final pH at 25^0C	7.4±0.2

Nutrient Agar

Composition	Amount (g/L)
Peptone	5
Sodium Chloride	5
Beef extract	3
Agar	15
pH	7

Nutrient Broth

Component	Amount (g/L)
Peptic digest of animal tissue	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5
Final pH at 25^{0}C	7.4±0.2

Luria Bertani Broth:

Component	Amount (g/L)
Casein enzymic hydrolysate	10
Yeast extract	5
Sodium Chloride	10.0
Final pH at 25^{0}C	7.5±0.2

Mueller- Hinton Agar:

Component	Amount (g/L)
Beef, dehydrated infusion form	300
Casein hydrolysate	17.5
Starch	1.5
Agar	17
Final pH at 25^{0}C	7.3± 0.1

Saline:

Component	Amount (g/L)
Sodium chloride	9

Motility, Indole, Urease Agar:

Component	Amount (g/L)
Peptone	3%
Sodium chloride	0.50%
Urea	2%
Mono Potassium Phosphate	0.20%
Phenol Red	0.00%
Agar	0.40%
pH	7

Simmons Citrate Agar:

Component	Amount (g/L)
Magnesium Sulfate	0.02%
Sodium chloride	0.50%
Sodium Citrate	0.20%
Di potassium Phosphate	0.10%
Mono potassium phosphate	0.10%
Bromothymol Blue	0.01%
Agar	2%
pH	7

Triple Sugar Iron (TSI):

Component	Amount (g/L)
Beef extract	3
Peptone	20
Yeast extract	3
Lactose	10
Sucrose	10

Dextrose monohydrate	1
Ferrous sulfate	0.2
Sodium chloride	5
Sodium thiosulfate	0.3
Phenol red	0.024
Agar	12

Indole

Component	**Amount (g/L)**
Peptone	10
Sodium chloride	5

Methyl Red and Voges Proskauer Media (MR-VP):

Component	**Amount (g/L)**
Peptone	7
Dextrose	5
Dipotassium hydrogen phosphate	5
Final pH	7

MacConkey Agar:

Component	**Amount (g/L)**
Peptone (Pancreatic digest of gelatin)	17 gm
Proteose peptone (meat and casein)	3 gm
Lactose monohydrate	10 gm
Bile salts	1.5 gm
Sodium chloride	5 gm
Neutral red	0.03 gm

Crystal Violet	0.001 g
Agar	13.5 gm

Thiosulfate Citrate Bile Salt Agar:

Component	**Amount (g/L)**
Proteose peptone	10
Yeast Extract	5
Sodium Thiosulfate	10
Sodium Citrate	10
Bile	8
Sucrose	20
Sodium Chloride	10
Ferric Citrate	1
Bromothymol Blue	0.04
Thymol Blue	0.04
Agar	15
Final pH at 25^0C	8.6± 0.2

Mannitol Salt Agar:

Component	**(Amount g/L)**
Peptone	5
Tryptone	5
HM Peptone B	1
Sodium Chloride	75
D-Mannitol	10
Phenol Red	0.025

Agar	15
pH after sterilization at 25^0C	7.4± 0.2

Brain Heart Infusion Broth:

Component	**(Amount g/L)**
HM Infusion Powder	12.5
BHI Powder	5
Proteose Peptone	10
Dextrose (Glucose)	2
Sodium Chloride	5
Disodium Hydrogen Phosphate	2.5
pH after sterilization at 25^0C	7.4± 0.2

T1N1 (Trypton-NaCl) Agar:

Component	**(Amount g/L)**
Casein Enzymic Hydrolysate	10
Sodium Chloride	10
Agar	15
pH after sterilization at 25^0C	7.2± 0.2

Reagents and Buffer

Gram's iodine (300 ml)

To 300 ml distilled water, 1 g iodine, and 2 g potassium iodide were added. The solution was mixed on a magnetic stirrer overnight, transferred to a reagent bottle, and stored at room temperature.

Crystal Violet (100 ml)

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. In 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

Safranin (100ml)

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of pdimethylaminobenzaldehyde (DMAB), and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with aluminum foil to prevent reagent exposure to light and stored at 4°C.

Methyl Red (200 ml)

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of distilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

Barrit's Reagent A (100 ml)

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

Barrit's Reagent B (100 ml)

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

Catalase Reagent (20 ml 3% hydrogen peroxide)

From a stock solution of 35 % hydrogen peroxide, 583 µl solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

Urease Reagent (50 ml 40% urea solution)

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

1M Tris HCl:

In a McCartney bottle, 1.576g Tris HCl was added. Then 10 ml distilled water was added to prepare 10 ml 1M Tris HCl. After that pH was adjusted to 8. Then it was stored at 4ºC.

0.5M EDTA:

In a McCartney bottle, 1.861 g EDTA was added. Then 10 ml distilled water was added to prepare 10 ml 0.5M EDTA. After that pH was adjusted to 8. Then it was stored at room temperature.

1X TBE Buffer:

In a Durham bottle, 5.4 g of Tris base, 2.75 g of Boric Acid, and 2ml of 0.5M EDTA were added. Then 500 ml distilled water was added to prepare 500 ml 1X TBE Buffer. After that pH of the buffer was adjusted to 8. Then it was autoclaved at 15psi 121ºC. After the buffer was autoclaved, it was stored at room temperature.