Divulgence of Bacterial diversity and Corresponding Antibiotic Susceptibility Profile of Coastal Waters in Saint Martin's Island, Bangladesh

By

Sauti Malabika Nadi

ID: 18136015

Marzuq Mohammad Alam

ID: 18136043

Tazrian Jannath Protiva

ID: 18136064

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

Biotechnology Program,

Department of Mathematics and Natural Sciences

BRAC University

Declaration:

It is hereby declared that

1. The thesis titled **"Divulgence of Bacterial diversity and Corresponding Antibiotic Susceptibility Profile of Coastal Waters in Saint Martin's Island, Bangladesh."** is our own 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 that 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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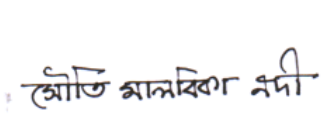

Sauti Malabika Nadi
ID: 18136015

Bachelor of Science,
Biotechnology

Department of Mathematics and Natural Sciences

BRAC University,
Dhaka, Bangladesh

Marzuq Mohammad Alam
ID : 18136043

Bachelor of Science,
Biotechnology

Department of Mathematics and Natural Sciences

BRAC University,
Dhaka, Bangladesh

Tazrian Jannath Protiva
ID : 18136064

Bachelor of Science,
Biotechnology

Department of Mathematics and Natural Sciences

BRAC University,
Dhaka, Bangladesh

Approval

The thesis **"Divulgence of Bacterial diversity and Corresponding Antibiotic Susceptibility Profile of Coastal Waters in Saint Martin's Island, Bangladesh."** submitted by Sauti Malabika Nadi (ID: 18136015) , Marzuq Mohammad Alam (ID : 18136043) and Tazrian Jannath Protiva (ID : 18136064) of Spring 18 , 2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Biotechnology on July 2022.

Examining Committee:

Supervisor: _____

Fahmina Akhtar

Senior Lecturer,

Department of Mathematics and Natural Sciences

BRAC University

Co-supervisor: _____

Akash Ahmed

Senior Lecturer

Department of Mathematics and Natural Sciences

BRAC University

Program Coordinator: _____

Iftekhar Bin Naser

Assistant Professor,

Department of Mathematics and Natural Sciences

BRAC University

Departmental Head: _____

(Chair) A F M Yusuf Haider

Professor and Chairperson

Department of Mathematics and Natural Sciences

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Title: Divulgence of Bacterial diversity and Corresponding Antibiotic Susceptibility Profile of Coastal Waters in Saint Martin's Island, Bangladesh.

Abstract:

Considered to be the largest reservoir of microorganisms, ocean water holds massive potential for scientific research. The Saint Martin's Islands of Bangladesh, located in the north-east part of the Bay of Bengal, is one such reservoir with very limited study having been conducted on its biodiversity. Moreover, the island is a popular tourist destination, so it often faces overpopulation. Hence, it has become a potential home for a diverse culture of bacteria. The purpose of this study was to identify the bacterial diversity of the coastal waters in Saint Martin's Island and to determine the corresponding antibiotic effectiveness profile. The main concern was to identify whether or not the bacterial presence in the island's water could become a public health concern. The samples were collected and analyzed through biochemical testing, antibiotic susceptibility testing, PCR, and gel electrophoresis. The results showed that the marine water stores a significant number of bacteria belonging to the *Enterobacteriaceae*, *Bacillaceae*, *Staphylococcaceae* and *Vibrionaceae* families. Furthermore, at lesser majorities, different species belonging to the *Yersiniaceae*, *Paenibacillaceae*, *Brevibacteriaceae*, *Aeromonadaceae*, and *Morganellaceae* families were also identified. The characteristics of the identities pointed to be mostly rod-shaped (70%) and tested type Gram negative (59.4%). Altogether, 100% of the identifications were tested catalase positive which indicated aerobic nature. Subsequent antibiotic susceptibility profiling yielded in 97.8% effectiveness by Amikacin, 95.7% for Meropenem, 89.4% for Norfloxacin, 85.1% for Amoxicillin + Clavulanic acid, 61.7% for Tetracycline, 57.4% for Azithromycin/Erythromycin, 46.8% for Amoxicillin, 44.6% for Colistin Sulphate, 34% for Vancomycin, and 31.9% for Cefixime. Following PCR runs resulted in the detection of *mcr-1* gene presence in the Colistin Sulphate resistant isolates, justifying the compromised efficacy of the last-resort drug. To this regard, presence of disease causing, antibiotic resistant bacteria in coastal waters indicates declining water quality. This further demonstrates the increasing possibility of severe health risks being linked to interactions with the coastal region if nature is not given sufficient opportunities to heal on its own.

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1.0. Introduction:

1.1. Ubiquitous nature of diversity and importance:

Microbes, explicitly bacteria, are ubiquitous organisms which can be found in most places of the environment. At particular geographic locations, the bacterial diversity can have profound impacts on the regional ecology, affecting local health, food environment, and subsequently, trade and commerce (Horner-Devine et al., 2004). Among the vast assortment of different species, marine bacteria are the type which resides in marine environments, such as regions directly associated with seas and oceans. They have been indicated as being part of the earliest residents of habitable planet Earth (AIMS, n.d.). These bacteria play key roles as primary producers, guiding oceanic cycling of energy and nutrients (Bienfang et al., 2011). Moreover, they also are a vast source of medicinal components, natural-products, genetic resources, and bioremediation (McCarthy et al., 1998; De et al., 2014). Henceforth, the exploration of this diversity is of paramount importance since it directly pertains to the divulgence of vital resources whilst also contributing towards the sustenance of environmental health (Colwell, 1997).

1.2. Saint Martin's Island and its biodiversity:

The location of choice for this study is the Saint Martin's Island, a coral island located at the south-east periphery of Bangladesh. Endowed with a variety of physiographic landscapes, the island boasts a comprehensive distribution of lagoons, marshes, sandy beaches, and coral constellations with a rich co-habitation of species such as corals, seaweeds, algae, and mangroves (Muhibbullah & Sarwar, 2017; UNDP, 2010). Furthermore, as reported by Hossain and Islam (2006), the coastal shores of this region, linked to the Bay of Bengal, provide habitation to around 234 species of fish (Hossain & Islam, 2006). This biodiversity is further enhanced by the tropical climate of the subcontinent along with direct linkage to the Indian Ocean. It is to be noted that this richness not only applies to visible organisms, but also extends to the microbiological world as it has been studied that the environment acts as a viable habitat to a significant variety of bacterial species. As reported in an investigation by Padmanaban et al. (2019), phylogenetic analysis of deep-sea sediments yielded in the identification of a plethora of gram-positive bacteria along with species of *Proteobacteria, Actinobacteria* and many more (Padmanaban et al., 2019). Such findings have been further verified by additional studies. Furthermore, with augmentation of next-generation sequencing techniques, scopes to further characterize these relatively unexplored waters holds massive potential (Akter et al., 2020).

1.2.1. Threats to the island biodiversity:

Due to effects of pollution and increasing anthropological activities, the local biodiversity of the coastal waters reaching the island shores faces significant threats. Due to recent infrastructural

developments, accessibility to the island during the active season has increased significantly (Hasan, 2009). This was proven by a local report which found that the daily tourist-population intake is of around 4000 individuals, which largely surpasses the capacity of 1250 (Ahamed, 2021). Considering this, pollution of all forms has increased hand-in-hand. Consequently, the potential degradation in marine water quality has become a major concern. It is projected that whilst the key focus of the local administration was in facilitating tourism, ignorance may have befallen on managing the waste that is generated during the process. As reported in a study by Muhibullah and Sarwar (2017), the island lacks an organizational drainage-system whilst the land-use pattern follows a random mode of arable and/or free land allocation with a bias towards hotels and resorts (Muhibbullah & Sarwar, 2017). As a result, it is anticipated that the coastline, constituting vicinities with human (or animal) presence, has undergone an upsurge in the population of enteric bacteria. This type comprises of a collection of species, such as *Staphylococcus* and *Vibrio*, which can be generally found in the human (and animal) gut (Donnenberg, 2000). Of further note is that sediments, which are in abundance at these regions, are rich reservoirs of such species (Hassard et al., 2016; Rehmann et al., 2009). Accordingly, the collective health conditions of the locals who are reliant on this ecosystem and the tourists in contact, both become impacted as enteric pathogens can directly cause gastrointestinal infections (Hossain & Islam, 2006; Santamaría, 2003). Antimicrobial agents of different classes are regularly prescribed to mitigate the aforementioned health complications caused by such bacteria. Thus, pairing this situation with the upsurge of antibiotic resistance development, both as a cause and an effect, paints a grim picture in both the tourism and health sectors.

1.3. Overview of antibiotics and antibiotic resistance genes (ARG):

Antibiotics, based on chemical structures and mechanism of action, make up around 16 major antibiotic families, including β-lactams, aminoglycosides, macrolides, tetracyclines, glycopeptides, and many more (Zhuang et al., 2021). Based on their capabilities of particularly neutralizing bacterial cells, these compounds act as a source of significant selective pressure to such populations. Henceforth, the development of spontaneous mutations in the genes of such cells is not an unlikely scenario. In this manner, any alterations to the gene products (proteins or cellular components) that were targeted by a certain class of antibiotics can result in the development of resistance. Furthermore, this may also develop based on bacterial efflux enhancements, target-site modification, and enzymatic degradation of the antibiotic compound (Waglechner & Wright, 2017). Subsequent duplication of such cells further forwards the resistive traits among the species. In this manner, a total of around 3000 antibiotic resistance gene (ARG) subtypes have been elucidated till date (Zhuang et al., 2021). The most significant subtypes are the Polymyxin, Tetracycline, Sulfonamide, and Carbapenem-resistance genes (Zhuang et al., 2021). Thus, with continuing exertion of selective pressure via emerging classes of antibiotics, development of resistance will increase as well, which is further accelerated by a lack of adequate anthropogenic carefulness during usage (Larson, 2007).

1.4. Overview of Polymyxin resistance:

Among the Polymyxin subtypes, a prominent example is the *mcr-1* gene which encodes for a membrane bound protein, phosphoethanolamine transferase (Li et al., 2020). This enzyme subsequently functions to attach a phosphoethanolamine moiety to lipid A molecules situated on the outer bacterial cell membrane of Gram negative cells (Gao et al., 2016). In this manner, the preliminary barrier towards antibiotic entry becomes modified, causing an alteration in the permeability. This facilitates a spill-over effect on the action of the Polymyxin antibiotics which particularly act to alter bacterial membrane permeability by disruption of Ca^{2+} and Mg^{2+} cations (El-Sayed Ahmed et al., 2020). Among these, Colistin Sulphate has been further addressed as a "last-resort drug" against Gram negative cells (Conly & Johnston, 2006). This drug is particularly prescribed to treat multi-drug resistance due to it possessing proven susceptibility patterns against these certain targets (Conly & Johnston, 2006). It is against this much-hailed agent that the *mcr-1* gene poses a significant threat since the gene-product directly alters the target site and entry-pathway of the drug compound, preventing it from reaching a necessary concentration for optimum function. Of greater concern is brought on due to multiple studies having reported that the gene can be transferred among bacterial species via diverse plasmid-mediated pathways (Liu et al., 2016). This was also displayed by the same plasmids having been isolated from the human gut microbiota (Gao et al., 2016). However, an interesting notion has been exhibited in a study by Li et al. (2020) which displayed that upon acquiring plasmid mediated *mcr-1* gene, loss of cell membrane integrity is enhanced (Li et al., 2020). Thus, it can be perceived that Polymixin resistance-development brings on a form of evolution which comes with a hand-off and necessitates further research so that enhanced characterization can be conducted.

1.5. Novelty of research, established techniques and necessity of identification:

It is to be noted that marine bacteria have advantages over terrestrial bacteria in that they can survive in hostile environments. Microorganisms that are thermophiles, halophiles, alkalophiles, and polyextremophiles, coupled with their ability to multiply by using adaptation techniques that include a variety of cellular metabolic systems, are likely to get identified from this Saint Martin's Island (Hossain & Islam, 2006). Thus, based on its geographical location, speculations arise regarding a potentially bacterial-species rich aquatic environment. However, this is still theoretical because such bacterial classification has not been conducted yet. The utilization of biochemical and genetic testing methods to conduct microbiological classification of coastal, beach waters are gaining significant traction in identifying the bacterial biodiversity. This also extends to the associated potential antimicrobial-resistive traits that the organisms might possess. Whilst genetic testing enhances the specificity of detection, biochemical methods also offer a significant degree

of accuracy since they directly test for phenotypic characteristics, such as specific enzymatic presence, whilst being more cost-efficient, attainable, and requiring less specialization to interpret (Franco-Duarte et al., 2019). Subsequently, they enable for rapid identification of potential contamination at risk zones where public health concerns might arise. For instance, a study conducted by Oun et al. (2017) in Michigan using biochemical-based identification of *E.coli* as fecal coliform indicators, discovered that around 50% of the water samples in their study area had surpassed the guidelines provided for standard water quality (Oun et al., 2017). Another study conducted by Pérez et al. (2008) in the Iberian Peninsula, also using fecal coliform (FC) indicators, learned that the majority of the concentrations of FCs were present near river mouths and beach areas (Garrido-Pérez et al., 2008). The utilization of genetic methods to conduct this classification would have been more resource demanding and redundant to a large extent. Moreover, a preliminary investigation in Saint Martin's Island concerning sponge-associated bacteria, conducted by Paul et al. (2001), also utilized biochemical testing methods to identify around 15 bacterial genera (Paul et al., 2021). As a result, the prospect of analyzing the coastal waters of Saint Martin's opens windows for high potential research. Such is the case because the divulgence of the bacterial repository will subsequently enable in further understanding of the region. Consequently, this will facilitate in the innovation of strategies to conserve the natural biodiversity whilst also avoiding a potential public health concern.

1.6. Overview of motive:

To this regard, the following study was conducted with the key motive of identifying the bacterial diversity and potential coliform presence in the coastal waters of the Saint Martin's Island. For this venture, the use of traditional culture methods, biochemical testing, and genetic analysis was employed. Furthermore, we subsequently aimed to determine the presence of antimicrobial-resistance capabilities of the discovered species, and divulge genetic reasoning behind the traits.

1.6.1. Objectives of the study:

- Identification of bacterial diversity.
- Determination of the potential presence of antibiotic resistance in the identified bacteria.
- Detection of fecal coliform presence in water sources (ocean and community).
- Genetic analysis and classification in terms of *mcr-1* gene presence.
- Determination of the presence of Polymixin resistance in identified bacteria.

1.7. Research Question:

In terms of the bacterial diversity and its corresponding characterization, does the coastal water surrounding the Saint Martin's Island have the potential of becoming a significant public health concern?

2.0. Methodology

2.1. Sample Site and Sample Collection

2.1.1 Description of sample sites

Saint Martin's Island is situated at the northeast side of the Bay of Bengal and is approximately 9 km south of the Cox's Bazaar - Teknaf peninsula. The Island is primarily divided into two sections with a narrow piece of land as the connection point at the center. Furthermore, it lies between 92°18' and 92°21'E longitudes and 20°34' and 20°39'N latitudes with reefs being located around 10-15 km to the west-northwest (Khan & Mamunul, 2010). Moreover, the land is mostly of a flat plane and is situated at 3.6m above sea level. Of further note is that the entire island is surrounded by a continuous beach which allows people to have access to almost all sides of the it, but there is a difference in the intensity of anthropogenic activity at different regions (Khan & Mamunul, 2010). To that regard, more people come in contact with the waters near the jetty for multiple reasons. As the island is only a two-and-a-half-hour boat ride away from the mainland, some tourists come in for a day-long trip. Most of these people opt to stay near the jetty since it is more economically feasible and it also allows easy access to the main market area which is composed of eateries, shops, and all other necessary amenities. Hence, human activity near the jetty is greater compared to the other locations around the island.

The sample sites were selected randomly with significant efforts having been placed to ensure inclusivity of all varieties and intensities of anthropogenic activities and exposures to seawater. Thus, a total of 8 sample sites were selected around the island. The sites include the jetty, the most visited beach on the northwest part, largely rocky areas, reefs, beaches adjacent to farmlands, beaches near fishing area, and so on. The locations were selected once before arrival and then after inspection of the island in person. The coordinates of the samples sites are :- 20.63377052856086, 92.32811941494049 (Jetty) , 20°37'26.4"N 92°19'09.6"E, 20°36'48.6"N 92°19'29.6"E, 20°36'44.3"N 92°19'31.5"E, 20°36'33.3"N 92°19'30.0"E, 20°36'08.4"N 92°19'54.5"E, 20°36'12.8"N 92°19'53.1"E, 20°36'20.6"N 92°19'49.7"E.

Figure 1: Saint Martin's Island Sample Sites, marked with red ink. (Saha, S., Sehrin, S., Ahsan Habib, K., & Abdul Baki, M. (2018). *NEW RECORDS OF TWO LUTJANUS SPECIES (TELEOSTEI: PERCIFORMES: LUTJANIDAE) WITH RE-DESCRIPTION OF SIX LUTJANIDS FROM SAINT MARTIN'S ISLAND OF THE BAY OF BENGAL, BANGLADESH Human health risk assessment of heavy metals in tropical fish and shellfish collected from the river Buriganga, Bangladesh View project DNA barcoding of marine fishes of Bangladesh View project*. https://doi.org/10.3329/bjz.v46i.39056)

2.2. Sample Collection

Water samples were collected during the occurrence of incoming waves, at a height of around 2.5 feet from the depth and at a distance of around 35-40m from dry land. This was done via the use of sterile syringes and the collected samples were stored in sterile falcon tubes. The falcon tubes were then stored in fixed positions within sealed ice boxes to protect the samples from sunlight. This facilitated the prevention of heat shock and minimized the likelihood of mechanical shearing. Also, the tubes were kept upright inside the boxes using Styrofoam frames to avoid sample loss during transportation. To minimize skin exposure to the water as well as the inner surfaces of the tubes, gloves were worn along with synthetic clothing and rubber boots throughout sampling. The samples were subsequently brought to the lab within 24 hours of collection. From every site, 2 sets of samples were collected: - Raw-water sample and sample mixed with Nutrient Enrichment broth (Peptone water). Peptone water is an enrichment medium that helps repair injured cells and assists in protecting the organism volume. It was noted earlier that the loss of organism-volume is inevitable during transportation and sample processing, and therefore, samples were collected in peptone water as reinforcement of the raw sample.

2.3. Sample processing:

2.3.1. Culture in Nutrient Agar and Differential Media

Collected water samples were cultured on selective/differential (SD) media as well as Nutrient Agar (NA) media. With regards to selective/differential media, MacConkey agar, m-FC Agar, UTI agar, Mannitol Salt (MSA) agar, XLD agar, and TCBS agar were used to culture and subsequently make preliminary identification notations of different species of bacterial colonies as each medium selectively promotes the growth of specific bacteria whilst inhibiting others. Furthermore, due to the differential capabilities of the media, the different species growing on the same plate could be differentiated between based on characteristic colony attributes. MacConkey Agar is recommended for the selective isolation and differentiation of lactose fermenting and lactose non-fermenting enteric bacteria (MacConkey Agar M081B Composition, n.d.). m m-FC Agar utilizes comparatively higher temperatures (44.5°C) and the membrane filtration technique to conduct the detection and accounting of faecal coliforms (M-FC Agar Base M1122 Composition, n.d.). UTI is a chromogenic differential medium and is used to identify enteric bacteria found in specimens such as urine, which may contain a large amount of protease and other pathogenic gram–positive organisms (UTI Agar, Modified Composition, n.d). MSA agar is used to identify pathogenic *Staphylococci spp.* from clinical and non-clinical samples (Mannitol Salt Agar Composition, n.d.). XLD agar or Xylose-Lysine Deoxycholate Agar is specifically used to isolate *Salmonella* species. TCBS is used to grow enteropathogenic *Vibrio* species. Nutrient Agar (NA) is a general-purpose media.

2.3.2. Spread Plating, Colony Selection and Isolation

The collected samples were processed to isolate the significant bacterial colonies. At first, raw samples were subjected to serial dilution to avoid lawn growth on the media plates. For this procedure, 0.9% NaCl solutions were used, and the dilutions were conducted on a trial-error basis where dilutions of 10^{-2} and 10^{-3} were determined as being ideal in providing single colonies. Subsequently, the upper limit of the dilution used was 10^{-9}, which was during Peptone water usage for Site 1. The diluted samples were then spread plated on Nutrient agar and other differential media mentioned above. It is to be noted that strict measures were taken to ensure that the plating was being done in a sterile environment to avoid contamination. Upon plating, the culture media plates were incubated in 37° C for 24 hours, except for the m-FC media. At this media, the culture plates were incubated at 44° C, and for 48 hours. After the incubation and following observation with the naked eye, based on their characteristics on different media, single colonies were chosen and sub-cultured on NA media.

2.4. Biochemical Tests

Several biochemical tests were performed on the selected bacterial isolates to characterize their biochemical and enzymatic activities. The results were subsequently used to identify the unknown samples. The tests performed were Gram staining, Catalase test, Oxidase test, TSI test, MIU test, Citrate test, Methyl Red test, and Vogues Proskauer test. To obtain optimum results for all the assessments, fresh cultures (incubated for 24 hours) were used.

2.4.1. Gram Staining

For Gram staining, previously published (Smith, 2019), standard procedures were followed to identify and differentiate between the Gram-positive and Gram-negative bacteria. Single colonies of bacterial isolates were transferred into autoclaved distilled water. The colonies were subsequently smeared over respective glass slides and then heat fixed. Following this, a few drops of Crystal Violet were added onto the area of the smear on the slides with a dropper and then left for 60 seconds. This is the primary dye, which is positively charged, so it adheres to the cell membrane of both Gram positive and negative cells. After 60 seconds, the Crystal Violet stain was carefully washed with distilled water, and Gram's iodine was added. Gram's iodine is a mordant that helps Crystal Violet to adhere to the Gram positive organisms. 60 seconds were again given after the Gram's iodine addition, following which; the slide was washed with distilled water. Then, the organisms were washed with 95% ethanol. This step differentiates the Gram positive and negative organisms. In Gram positive organism, the Crystal Violet- iodine complex is trapped in the cell due to a thicker peptidoglycan cell wall. This shrinks due to alcohol dehydration, thus becoming more constricted and lowering cell wall permeability. In Gram-negative cells however,

the cell boundary contains a thinner peptidoglycan wall and a higher concentration of lipids, which are soluble in alcohol. The ethanol, acting as a decolorizer, dissolves the lipid layer and increases the permeability of the cell membrane of the Gram-negative cells. After the ethanol rinse, a counterstain, Safranin was added to the glass slides which were then washed after 45 seconds.

The Gram-positive bacteria appeared dark purple under the microscope and the Gram-negative bacteria appeared dark pink or red.

2.4.2. Catalase test

For the catalase test, bacterial colonies were smeared onto sectioned filter papers and 3% hydrogen peroxide solution was placed on the respective smears. Following this, observation for bubble formation was conducted. The organisms which contained the catalase enzyme formed bubbles/froth and were classified as catalase positive. On the other hand, the organisms that did not possess the enzyme consequently did not form bubbles/froth. These were classified catalase negative.

2.4.3. Oxidase test

For the oxidase test, a Whatman filter paper was soaked in freshly prepared oxidase reagent (1% tertramethyl-p-phenylene-diamine dihydrochloride). Then, with the tip of a sterilized needle, a single colony of bacteria was smeared on the filter paper and observed for color change. Appearance of a blue color indicated the presence of the cytochrome C oxidase enzyme. Organisms which contain cytochrome C as a part of their respiratory chain are oxidase positive and turn the reagent into dark blue. Organisms devoid of the enzyme do not produce any color.

2.4.4. Triple Sugar Iron test (TSI)

The TSI test was performed by preparing triple sugar iron agar, at a slanted manner, within sterile test tubes. The media were then inoculated with the bacterial isolates, incubated for 24 hours, and then observed according to a pre-determined reference (Aryal, 2019). TSI test differentiates organisms according to their carbohydrate fermentation pattern and hydrogen sulfide production. Carbohydrate fermentation is indicated by the change of color of the pH indicator present in the media which may or may not be associated with gas production.

Expected results chart:

Table 1: TSI result interpretation (*The Triple Sugar Iron (TSI) Test – Principle, Procedure, Uses and Interpretation*. (n.d.).)

Slant	Butt	Predicted Result
Red	Yellow	Dextrose fermentation only
Yellow	Yellow	Dextrose, Lactose ,and/ or sucrose fermentation
Red	Red	No Carbohydrate fermentation
Blackening of medium		Presence of H_2S
Crack in Agar		Production of Carbon dioxide and H_2

2.4.5. (Motility-Indole-Urease) MIU test

MIU test was performed using fresh bacterial culture inoculated in MIU media base for 24 hours at 37° C. Following this, observation was conducted for the presence of bacterial motility and the urease enzyme. If the inoculated colony clouded the stab-line with growth extension, then the organism was motility positive, and if the media base showed color changes to pink/red, then it indicated urease positive activity. The color change would occur due to the presence of Phenol red in the media shifting to an alkaline condition. Upon the addition of Kovac's reagent, the appearance of a pink-red colored ring at the top indicated indole positive organisms.

2.4.6. Citrate test

As for the Citrate test, the organisms were inoculated in Simmons citrate medium and incubated for at least 48 hours at 37°C, and then observed for color change. A Prussian blue color indicated a positive result whilst unchanged color indicated a negative result. It is to be noted that if growth was observed without the media color having been changed to Prussian blue, the result was taken to be positive. Positive result indicates that the bacterial colony possesses the citrate permease enzyme and uses citrate as its carbon and energy source. The presence of a pH indicator (bromothymol blue) facilitates the visible color change when an alkaline by-product of citrate metabolism is generated.

2.4.7. MR - VP test

The Methyl Red (MR) and Vogues Proskauer (VP) tests were performed using Glucose phosphate broth. The samples were incubated for 48 hours, and each sample was incubated in pairs of test tubes, with two test tubes containing broths incubated with the same organism. For the MR test, methyl red reagent was added to the 48 hours-incubated broth, and color change from red to yellow indicated the change of pH. An organism showing positive results can utilize glucose and produce a stable acid that changes the color of methyl red from yellow to red. In the other test tube of the same class, same volume of Barritt's Reagent A and Barritt's Reagent B were added and observed. Positive test was indicated by the appearance of eosin pink color within 30 minutes of periodic shaking. Vogues Proskauer test determines an organism's ability of producing acetylmethyl carbinol from glucose.

2.5. Antibiotic Susceptibility Testing:

2.5.1. Kirby Bauer Disc Diffusion Method:

The Kirby Bauer Disc Diffusion method was utilized to determine the nature of susceptibility or resistance of the isolated aerobic bacterial colonies to a selected collection of antibacterial agents. For the disc diffusion test, MHA (Mueller–Hilton Agar) media and commercial antibiotic discs were used. Moreover, the procedure was run following the guidelines provided by the CLSI (Hudzicki, 2009). The antibiotics that were used are as follows: - Colistin Sulphate (CT) 10, Cefixime (CFM) 5, Vancomycin (VA) 30, Norfloxacin (NX) 10, Amoxicillin + Clavulanic acid (AMC) 30, Meropenem (MRP) 10, Azithromycin (AZM) or Erythromycin 15, Amikacin (AK) 30, Tetracycline (TE) 30, and Amoxicillin (AML) 10.

At first, freshly cultured organisms were inoculated in sterile 0.9 % saline at 0.5 McFarland standard of turbidity. The cultures were then inoculated on MHA Agar using sterile cotton swabs. Following this, different antibiotic discs were placed on the Mueller Hilton agar plates and the media were incubated at 37° C for 24 hours. The antimicrobial agent diffuses out from the disk to spread around the surface of the agar in a relatively circular pattern. According to their respective potencies of being effective, they will inhibit the growth of the organism inoculated on that plate. To this regard, after the incubation time had passed, the zone of inhibition was measured and compared with the CLSI guideline to determine whether the organism developed resistive traits, or whether they were moderately or fully susceptible.

The reference diameters are displayed in Table 1 of the Supplementary section as reference.

2.6. Determination of the presence of *mcr-1* Polymyxin Antibiotic Resistance Gene:

2.6.1. DNA extraction by Boiling Method

According to the results of the antibiogram, the isolates showing resistance to Colistin Sulphate were selected for DNA isolation and the PCR procedure. The *mcr-1* gene is a plasmid mediated Colistin resistance (CLR) conferring gene. The presence of it in bacteria threatens the clinical utility of the antibiotic, as Colistin Sulphate is considered as a last-resort antibiotic in treating multi drug resistant bacterial infections. This agent has activity against most Gram negative bacteria. Phenotypic Colistin resistance is related to the presence of plasmid mediated mobile Colistin resistance (*mcr*) gene (Islam, 2020).

The selected organisms for DNA isolation and PCR were as follows :- MSA RS1, MSA RS5 (1), MAC RS1 (2), UTI RS1 (G), MAC RS8 (2), RS7 10^-6, MSA RS5 (2), TCBS RS2 (3), MSA RS5 (1), TCBS RS2 (2), XLD RS1, XLD RS6, XLD RS 8 (2), XLD RS8 (1), MAC RS6, MSA RS3, MSA RS5 (2), MSA RS5 (1), UTI RS4, MFC RS6, TCBS RS1, TCBS RS2, TCBS RS1.

Subsequently, the selected isolates were inoculated in respective test tubes containing sterile LB broth and then subsequently incubated at 37° C for 24 hours in a shaker incubator. The next day, for each isolate, 700µl of the broth was transferred in respective Eppendorf tubes and centrifuged at 13000 rpm for 10 minutes. The supernatants were then carefully discarded and 300µl of PBS (Phosphate Buffer Saline) were added and mixed with slow re-pipetting motions. Following this, the mixtures were again centrifuged at 14000 rpm for 5 minutes. The supernatants were discarded, 200µl of TE (Tris-EDTA) buffer was added, and the mixtures were subjected to boiling at 100°C for 15 minutes inside a water-bath. After this, the mixtures were immediately cooled in a freezer at -20°C for 10 minutes and were then finally centrifuged at 14000 rpm for 5 minutes. The debris were discarded this time and the supernatants were collected in fresh Eppendorf tubes. The extracted DNA collections were securely stored at -20°C until further use.

2.6.2. Performance of the Polymerase Chain Reaction (PCR):

To determine the presence of the CLR gene (*mcr-1*) among the colistin-resistant bacterial colonies, PCR was carried out on the respective samples of DNA which had been previously extracted from the colistin-resistant colonies. A total of 32 pairs of forward and reverse primers were prepared to make up a total volume of 5µl. The sequences of the primers in the pair were as follows: - Forward Primer 5'-CGGTCAGTCCGTTTGTTC-3' and Reverse Primer 5'-CTTGGTCGGTCTGTAGGG-3'. Firstly, 5µl of nuclease-free water was added to each PCR Eppendorf to help avoid DNA

degradation and then 11µl of master mix was added to each tube. The final volume of the total PCR mixes in each Eppendorf totaled to 21µl. As templates for PCR, the previously extracted DNA isolates of colistin-resistant organisms were added to the PCR tubes at a volume of 4µl. All PCR processes were performed in a thermal cycler following a set among a list of previously published, standardized conditions (Yi-Yun Liu , 2015; Salequl Islam, 2020).

The condition for the PCR is given in the table below:

Table 2: PCR conditions utilized for targeted amplification of the *mcr-1* gene.

Primer	**Primer sequence**	**Conditions set for PCR**	**Number of Cycles conducted**	**Target *mcr-1* amplicon Size**
CLR	CLR F 5'-CGGTCAGTCCGTTTGTTC-3' CLR R 5'-CTTGGTCGGTCTGTAGGG-3'	94°C for 7 minutes 94°C for 30 seconds 58° C for 90 seconds 72°C for 60 seconds 72°C for 10 minutes	36	309bp

2.6.3. Gel electrophoresis:

The sizes of the PCR products were resolved by 1.2% agarose gel electrophoresis. A 100 bp ladder was used for the sizing and quantification of the PCR products and the procedure was performed in a Biometra Electrophoresis machine. For visualization under a UV spectrophotometer, 4.5 µl of Ethidium Bromide was added to the loading gel. 1X concentration of TAE buffer was used as the running buffer of the electrophoresis procedure and no extra loading dye was needed as the PCR products already contained green-colored dye from the master mix. The whole process of gel-run was conducted for about 45 minutes and 80 Volts were used. Subsequently, the results were photographed and noted down.

3.0. Results:

3.1. Bacterial identification per sample zones:

The conduction of bacterial identification was one of the major phases of this study. It was conducted via the use of data representing the respective colony characteristics and the biochemical analyses. This data was subsequently cross referenced with use of the ABIS software, standard published online resources, and the Bergey's Manual of Systematic Bacteriology. The results were formulated into Table 3. Here, the first column shows the 8 different zones on the island from which samples were collected whilst the second column demonstrates the divulged identities of the bacteria found with respect to each site. Sites 1 (26%) and 8 (19%) yielded in the largest and second-largest number of bacterial isolates, respectively. On the contrary, site 2 yielded in the least amount. With respect to the identities, bacterial species of the *Enterobacteriaceae* families were found to be present at the highest amount with bacteria of *Staphylococcus spp.* and *Bacillus spp.* occurring most frequently.

Table 2 of the Supplementary section summarizes the identified organisms and their characteristics.

3.2. Cultured colony characteristics on selective/differential (SD) media:

The SD media were utilized in culturing each of the samples upon dilution. For each respective zone, a uniform cohort of the media was allocated so that the conditions and likelihood of growth of all viable organisms were maximized as much as possible. To this regard, the samples were cultured for 24 hours except for the cultures on the m-FC media. That was cultured for 48 hours at 44° C. Upon the completion of culturing, respective tables for each media type were developed with the columns representing the source of the sample plate, the total number of the colonies and the number of visibly different colonies. Along with these, the forms, elevation, color, and surface nature of the colonies were also noted. The results were formulated into tables which are provided in the Supplementary section.

3.3. Results of biochemical testing:

A total of 8 biochemical tests were conducted to check for 15 different attributes of the sampled colony isolates. The results of the tests were formulated into Table 4, 4.1 and 4.2. Here, the columns represent positive or negative test results regarding each respective test while the leftmost and rightmost columns represent the sample names and the associated identified bacteria. The identification was done upon attaining and cross-referencing the respective results between the

ABIS software and the Bergey's Manual of Systematic Bacteriology. It was observed that a majority of the identities were rod shaped (70%) and of the Gram negative type (59.4%). Furthermore, a 100% of the samples tested were catalase positive, indicating an aerobic nature. Also, among the sugar sources, glucose utilization was observed to be widespread throughout the samples. This was indicated in the TSI (Triple Sugar Iron test) results, where 68% of the total tested organisms showed glucose utilization. Moreover, as the Methyl Red test results were observed, 93.6% of the total tested organisms had shown positive results which indicated glucose phosphate utilization in the MR broth. In contrast to the higher percentage occupancies, urease activity, gas production, and H_2S formation were less frequently observed among the samples. Positive and negative results were distributed at moderate frequencies with respect to the other tests. Upon the completion of the investigations, the results were further cross-referenced with established databases for each respective test to strengthen the confidence behind the identity determinations.

The images, given below (Figures 2-6) were obtained during biochemical testing and are presented here as references. They represent the positive and negative results of each respective test which have been conducted.

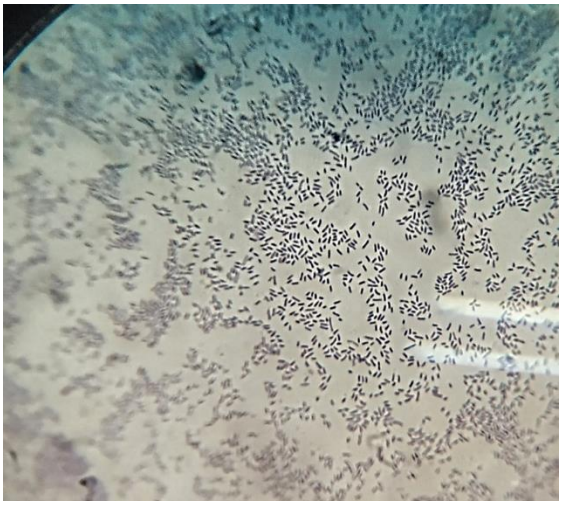

Figure 2: Gram positive bacilli

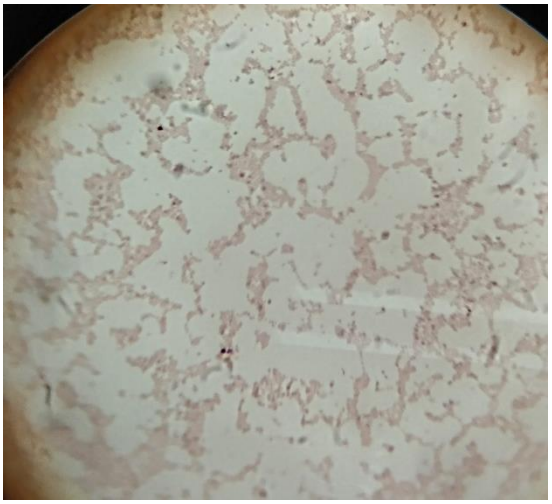

Figure 2.1: Gram negative cocci

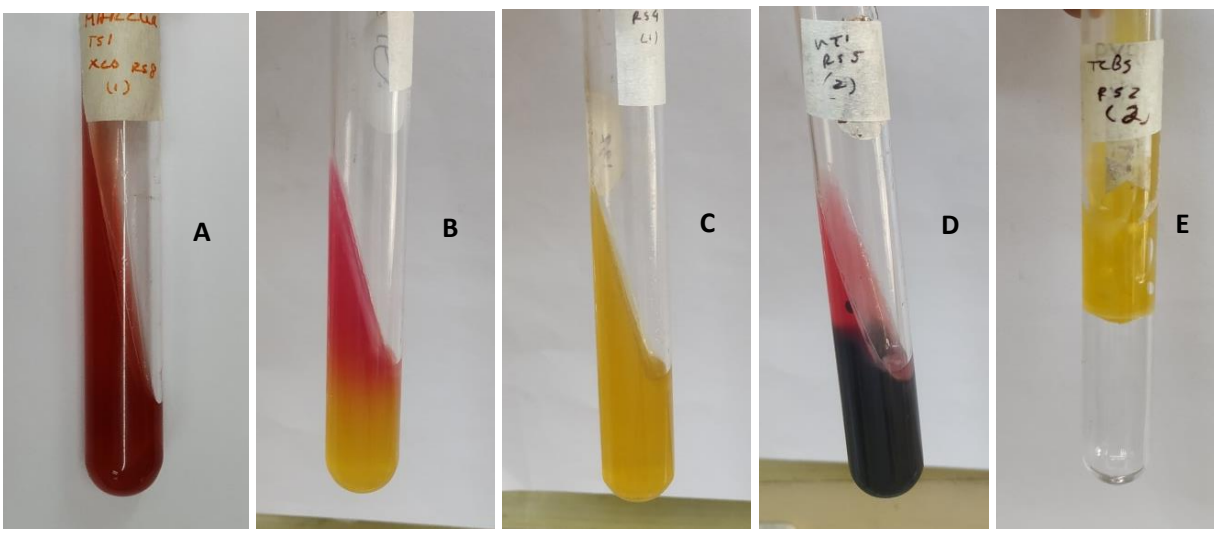

Figure 3: Results of the TSI test (A) No gas (NG), No H_2S (N H_2S), Red butt and slant. (B) NG, NH_2S, Yellow butt and red slant. (C) NG, NH_2S, Yellow butt and slant. (D) NG, H_2S, Yellow butt and red slant. (E) Gas, NH_2S, Yellow butt and slant.

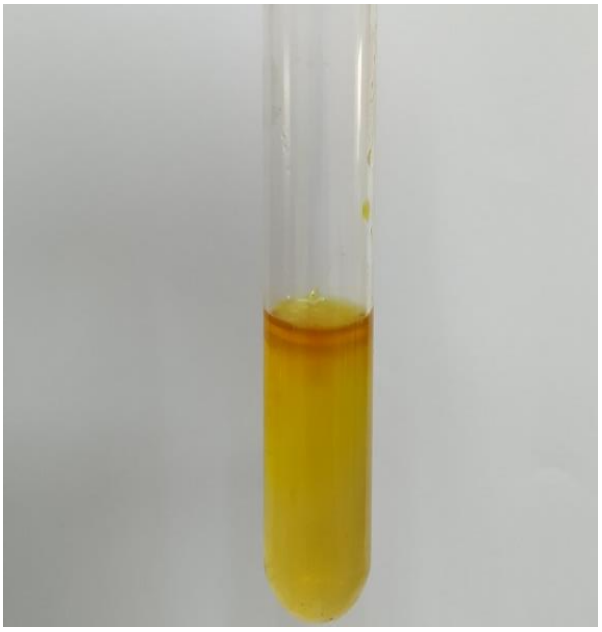

Figure 4: MIU negative (All 3 attributes)

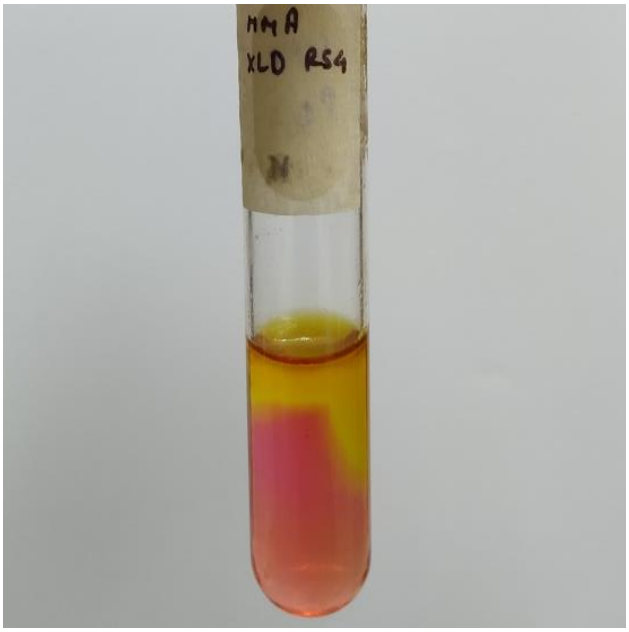

Figure 4.1: MIU positive (All 3 attributes)

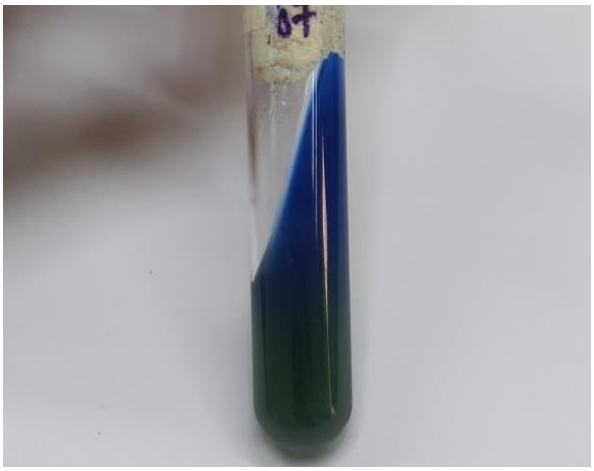

Figure 5: Citrate positive

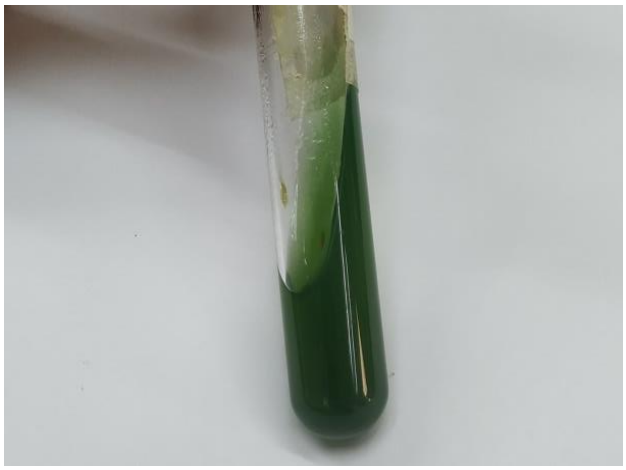

Figure 5.1: Citrate negative

Figure 6: MR negative

Figure 6.1: MR positive

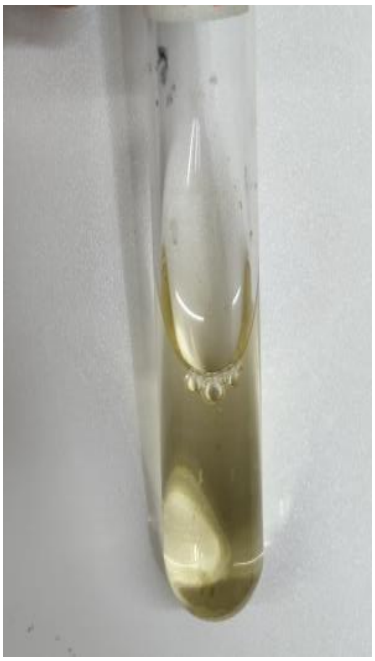

Figure 6.2: VP negative

3.4. Antibiotic susceptibility profiling:

The identified samples were subjected to antibiotic susceptibility testing in order to determine the effectiveness of a selected cohort of modern antibiotics. The data obtained with regards to the zones of inhibition were subsequently compared to the established guideline of CLSI (Clinical and Laboratory Standards Institute). Accordingly, the organisms were classified as being susceptible

"S", intermediate "I", or resistant "R". Tables 5, 5.1, and 5.2 represent the antibiotic susceptibility profile of the samples to 10 antibiotics. It was observed that Amikacin, Meropenem, Norfloxacin, and Amoxicillin + Clavulanic acid possessed susceptibility at 97.8%, 95.7%, 89.4%, and 85.1% respectively. These were at the higher tier of effectiveness. Tetracycline and Azithromycin/Erythromycin were at the middle tier of effectiveness with susceptibility percentages of 61.7% and 57.4% respectively. At the lower tier of effectiveness were the remaining antibiotic agents, namely Amoxicillin, Colistin Sulfate, Vancomycin, and Cefixime with corresponding susceptibility percentages of 46.8%, 44.6%, 34% and 31.9%.

Figure 7 is presented below as a reference.

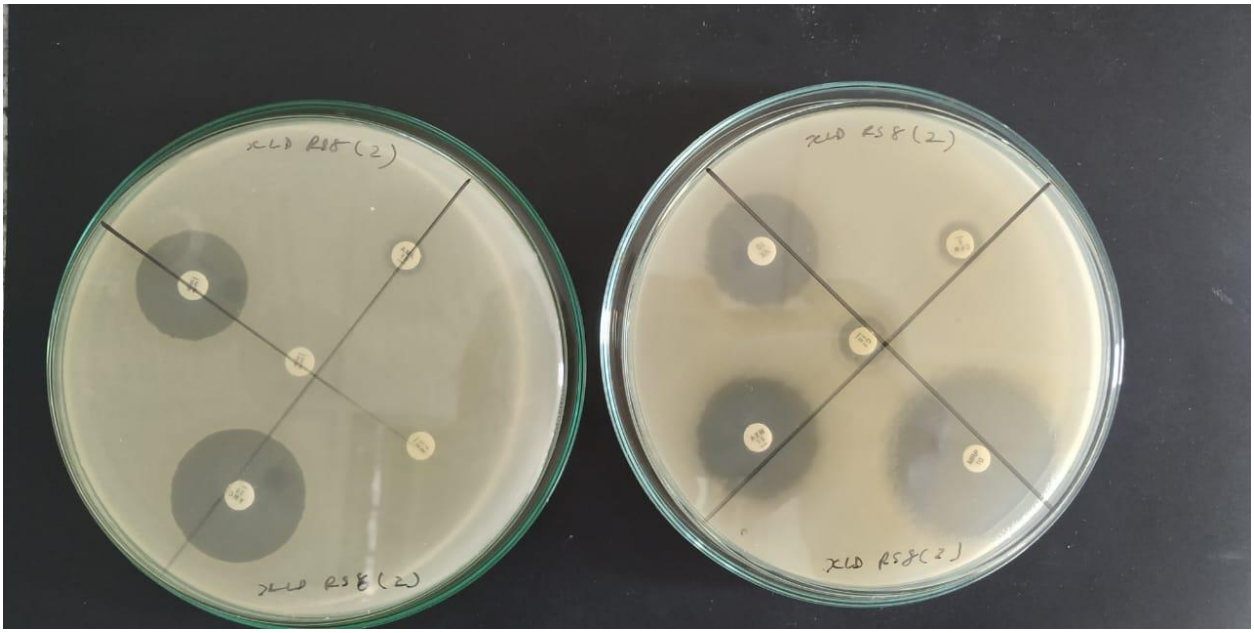

Figure 7: Antibiotic susceptibility profiling on MHA agar.

3.5. Determination of *mcr-1* amplicon presence via Gel electrophoresis:

The process of gel electrophoresis was subsequently conducted after PCR to confirm the prevalence of the targetted *mcr-1* gene among the variety of the samples which possessed resistance to Colistin Sulphate. The figures 8 and 8.1 displayed below represent the results of the gel run. It was observed that a total of 4 isolates possessed the CLR conferring *mcr-1* gene.

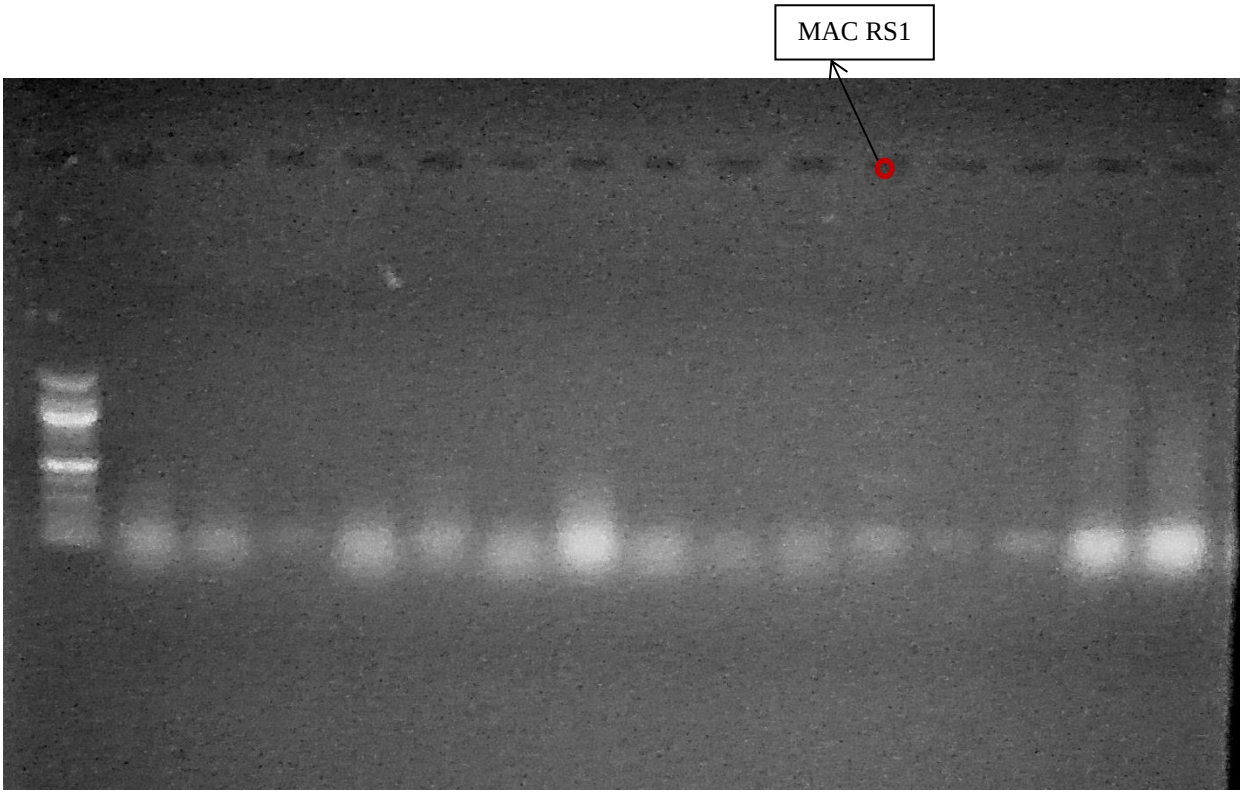

Figure 8: Gel run result Batch 1

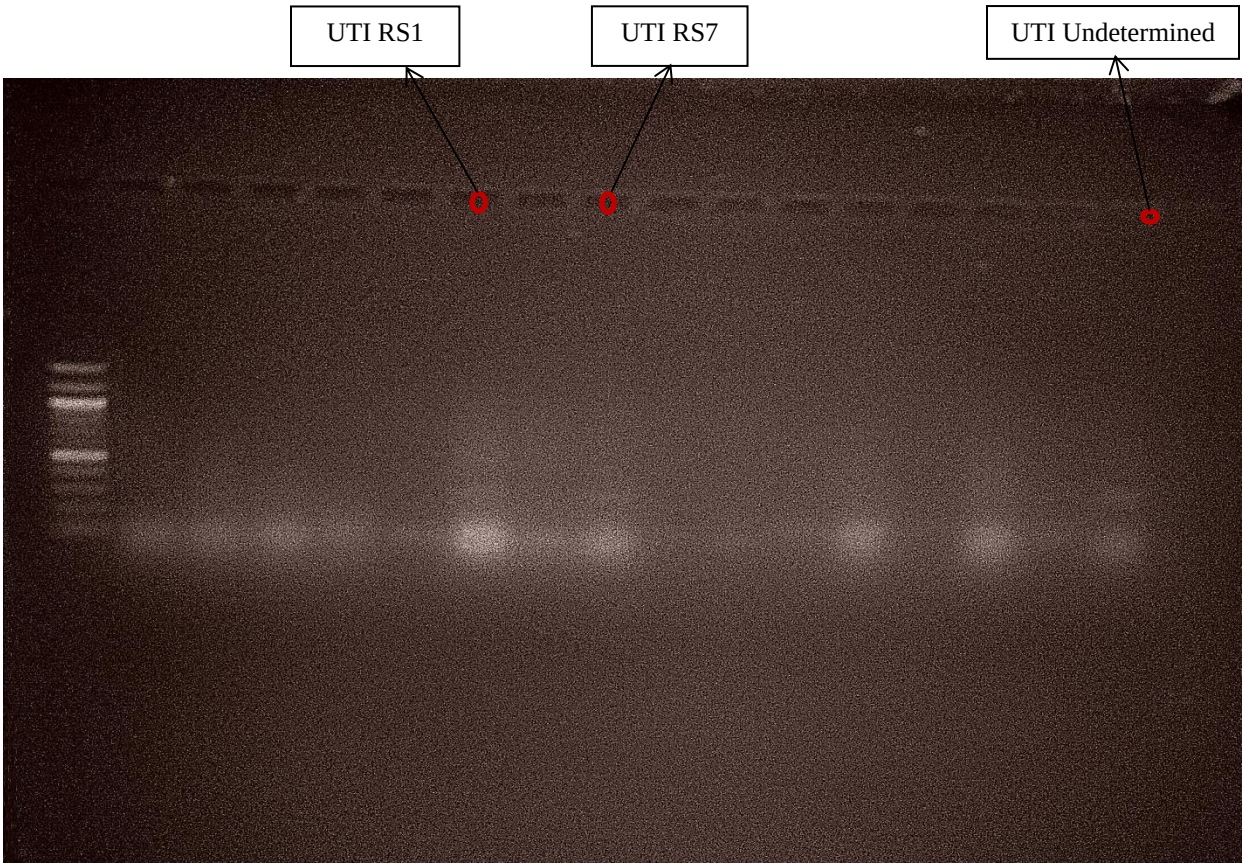

Figure 8.1: Gel run result Batch 2

Table 3: Identified bacteria per site of sampling.

Sample zones	Bacterial Identities
Site 01	*Cedecea neteri, Staphylococcus aureus, Yersinia mollaretii, Citrobacter gillenii, Bacillus novalis, Bacillus tequilensis, Paenibacillus macerans, Brevibacterium iodinum, Vibrio pacinii, Aliivibrio logei, Photorhabdus asymbiotica (Emerging Human pathogen)*
Site 02	*Vibrio furnissii, Enterobacter spp., Aeromonas schubertii, Xenorhabdus beddingii*
Site 03	*Staphylococcus epidermidis, Lysinibacillus sphaericus, Escherichia spp.*
Site 04	*Providencia rustigianii, Lysinibacillus sphaericus, Aneurinibacillus aneurinilyticus*
Site 05	*Staphylococcus aureus, Staphylococcus epidermidis, Bacillus carboniphilus, Lysinibacillus sphaericus, Salmonella spp.*
Site 06	*Moellerella wisconsensis, Shimwellia blattae, Serratia fonticola, Viridibacillus neidei, Providencia alcalifaciens*
Site 07	*Bacillus aminovorans, Serratia fonticola, Staphylococcus spp.*
Site 08	*Klebsiella pneumoniae, Shimwellia blattae, Buttiauxella brennerae, Citrobacter youngae, Paenibacillus macquariensis, Shigella dysenteriae, Salmonella spp., Shigella boydii*

Table 4: Representation of biochemical testing results of the identified bacteria

VP	MR	Indole	Citrate	Gas	H_2S	Catalase	Gram Stainin	Colony Morphology	Sample Number
-	+	-	+	-	-	+	-	Bacilli	**XLD RS1**
-	+	-	+	-	-	+	-	Bacilli	**XLD RS6**
-	+	-	-	-	-	+	-	Bacilli	**XLD RS8 (1)**
-	+	-	-	-	-	+	-	Bacilli	**XLD RS8 (2)**
-	-	+	-	-	-	+	-	Bacilli	**XLD RS4**
-	+	-	+	-	-	+	-	Bacilli	**MAC RS6**
-	+	-	+	-	-	+	-	Bacilli	**MAC RS8**
-	+	-	+	-	-	+	+	Staphylococci	**MSA RS1**
-	+	-	-	-	-	+	+	Staphylococci	**MSA RS5 (1)**
-	+	+	+	-	-	+	+	Staphylococci	**MSA RS5 (2)**
-	+	-	-	-	-	+	-	Cocci	**MAC RS1 (1)**
-	+	-	-	-	-	+	-	Cocci	**MAC RS1 (2)**
-	+	-	+	-	-	+	-	Bacilli	**MAC RS8 (1)**
-	+	-	+	-	-	+	-	Bacilli	**MAC RS8 (2)**
-	+	-	+	-	-	+	+	Bacilli	**RS 4 (10^-4)**
-	+	-	-	-	+	+	+	Bacilli	**RS 7 (10^-6)**
-	+	-	-	-	-	+	+	Bacilli	**RS 8 (2)**
-	+	-	-	-	-	+	+	Bacilli	**UTI RS 1 (G)**
-	+	-	-	-	-	+	+	Bacilli	**UTI RS 5 (1)**
-	+	+	+	-	-	+	+	Cocci	**MSA RS 3**
-	+	-	+	+	-	+	-	Bacilli	**TCBS RS2 (2)**
-	+	-	+	+	-	+	-	Bacilli	**TCBS RS2 (3)**

Table 4.1: Representation of biochemical testing results of the identified bacteria

Bacteria	Sucrose Utilization	Lactose Utilization	Glucose Utilization	Urease	Motility	Catalase	Gram Staining	Colony Morpholog	Sample Number
Cedecea neteri	+	+	+	-	+	+	-	Bacilli	**XLD RS8**
Moellerella wisconsensis	-	+	+	-	-	+	-	Cocci	**XLD RS8**
Klebsiella pneumoniae	-	-	+	-	-	+	-	Bacilli	**XLD RS8**
Klebsiella pneumoniae	-	-	+	-	-	+	-	Bacilli	**XLD RS6**
Providencia rustigianii	-	-	+	+	+	+	-	Bacilli	**XLD RS7**
Shimwellia blattae	-	-	-	-	-	+	+	Bacilli	**UTI RS5**
Shimwellia blattae	-	-	-	+	-	+	+	Bacilli	**UTI RS4**
Staphylococcus aureus	+	-	-	+	-	+	+	Bacilli	**UTI RS3**
Staphylococcus aureus	+	-	-	+	-	+	+	Bacilli	**UTI RS5**
Staphylococcus epidermidis	+	+	+	+	+	+	+	Bacilli	**UTI RS1**
Yersinia mollaretii	+	+	+	-	-	+	+	Bacilli	**UTI RS1**
Citrobacter gillenii	+	+	+	-	+	+	+	Cocci	**UTI RS1**
Buttiauxella brennerae	-	-	+	+	-	+	+	Bacilli	**UTI RS4**
Citrobacter youngae	-	-	-	-	-	+	-	Cocci	**XLD RS8**
Lysinibacillus sphaericus	-	-	-	+	-	+	-	Bacilli	**MFC RS6**
Bacillus aminovorans	-	-	+	+	+	+	-	Bacilli	**MFC RS2**
Paenibacillus macquariensis	+	+	+	-	+	+	+	Cocci	**MSA RS7**
Bacillus novalis	-	-	+	-	+	+	-	Bacilli	**MFC RS2**
Bacillus carboniphilus	-	-	-	-	+	+	-	Bacilli	**TCBS RS1**
Staphylococcus epidermidis	+	+	+	+	+	+	-	Bacilli	**TCBS RS2**
Vibrio furnissii	+	+	+	-	+	+	-	Bacilli	**TCBS RS1**
Vibrio furnissii	+	+	+	-	+				

Bacteria	Sucrose utilization	Lactose utilization	Glucose utilization	Urease	Motility	VP	MR	Indole	Citrate	Gas	H_2S
Shigella dysenteriae	-	-	-	-	+	-	+	-	-	-	-
Salmonella spp	-	-	+	-	-	-	+	-	-	-	-
Shigella boydii	-	-	+	-	-	-	+	-	-	-	-
Serratia fonticola	-	-	+	-	+	-	+	-	+	-	-
Serratia fonticola	-	-	+	-	+	-	+	-	+	-	-
Lysinibacillus sphaericus	-	-	-	-	+	-	+	-	+	-	-
Aneurinibacillus aneurinilyticus	-	-	-	-	+	-	+	-	-	-	-
Lysinibacillus sphaericus	-	-	-	-	+	-	+	-	+	-	-
Viridibacillus neidei	-	-	-	-	+	-	+	+	-	-	-
Bacillus tequilensis	+	+	+	-	+	-	+	-	+	-	-
Paenibacillus macerans	+	+	+	-	+	-	+	-	+	-	-
Brevibacterium iodinum	-	-	-	-	+	-	+	-	-	-	-
Lysinibacillus sphaericus	-	-	-	-	+	-	+	-	+	-	-
Salmonella spp	-	-	+	-	-	-	+	-	-	-	-
Providencia alcalifaciens	-	-	+	-	+	-	+	+	+	-	-
Enterobacter spp	-	-	+	-	+	-	+	-	+	-	-
Staphylococcus spp	+	+	+	+	-	-	+	-	+	-	-
Enterobacter spp	-	-	+	-	+	-	+	-	+	-	-
Vibrio pacinii	+	+	+	-	+	-	+	-	-	-	-
Aeromonas schubertii	-	-	+	-	+	-	+	-	+	-	-
Aliivibrio logei	-	-	+	-	+	-	+	-	-	-	-

Table 4.2: Representation of biochemical testing results of the identified bacteria

Sample Number	Colony Morphology	Gram Staining	Oxidase	Catalase	H_2S	Gas	Citrate	Indole	MR	VP	Motility	Urease
MAC RS5	Bacilli	-	-	+	+	-	-	-	+	-	-	-
MSA Unknown3	Cocci	+	-	+	+	+	-	+	+	-	+	-
UTI RS8 (1)	Cocci	-	-	+	-	-	+	-	-	-	+	+
MAC RS2	Cocci	-	-	+	-	-	+	-	-	-	+	-

Glucose Utilization	Lactose Utilization	Sucrose Utilizatio	Bacteria
+	-	-	*Salmonella spp*
+	+	+	*Escherichia spp.*
-	-	-	*Photorhabdus asymbiotica (Emerging) Human pathogen)*
+	-	-	*Xenorhabdus beddingii*

Table 5: Representation of the antibiotic susceptibility status of the identified bacteria

Organism identities	AML 30	TE 30	AK 30	AZM 15	MRP 10	AMC 30	NX 10	VA 30	CFM 5	CT 10	Sample
Staphylococcus aureus	S	S	S	R	S	S	S	S	R	R	**MSA RS1**
Bacillus carboniphilus	S	S	S	S	S	S	S	S	R	S	**UTI RS5 (1)**
Staphylococcus aureus	I	S	S	R	S	S	S	S	R	R	**MSA RS5 (1)**
Citrobacter gillenii	S	S	S	S	S	S	S	S	R	R	**MAC RS1 (2)**
Bacillus novalis	R	S	I	S	S	S	S	R	S	R	**UTI RS1 (G)**
Citrobacter youngae	R	R	S	S	S	S	S	R	R	R	**MAC RS8 (2)**
Lysinibacillus sphaericus	S	S	S	R	S	S	S	S	I	S	**RS4 10^-4**
Bacillus aminovorans	S	S	S	R	S	S	S	R	R	R	**RS7 10^-6**
Staphylococcus epidermidis	S	S	S	R	S	S	R	S	R	R	**MSA RS5 (2)**
Shimwellia blattae	S	R	S	S	S	S	S	R	I	S	**MAC RS8**
Buttiauxella brennerae	R	R	S	S	S	S	S	R	R	S	**MAC RS8 (1)**
Vibrio furnissii	S	S	S	S	S	S	S	S	R	R	**TCBS RS2 (3)**
Staphylococcus aureus	S	S	S	R	S	S	S	S	R	R	**MSA RS5 (1)**
Vibrio furnissii	R	R	S	R	S	S	R	R	S	R	**TCBS RS2 (2)**
Cedecea neteri	S	S	S	S	S	S	S	S	R	R	**XLD RS1**
Moellerella wisconsensis	S	S	S	R	S	S	S	R	R	R	**XLD RS6**
Klebsiella pneumoniae	R	R	S	S	S	S	S	R	R	R	**XLD RS 8 (2)**
Klebsiella pneumoniae	R	R	S	S	S	S	S	R	R	R	**XLD RS8 (1)**
Shimwellia blattae	R	R	S	R	S	S	R	R	R	R	**MAC RS6**
Staphylococcus epidermidis	S	I	S	R	S	S	R	S	R	R	**MSA RS3**
Staphylococcus epidermidis	S	S	S	R	S	S	R	S	R	R	**MSA RS5 (2)**
Providencia rustigianii	R	S	S	S	S	R	S	R	R	S	**XLD RS4**

Table 5.1: Representation of the antibiotic susceptibility status of the identified bacteria

Predicted Organism	AML 30	TE 30	AK 30	E15/ AZM 15	MRP 10	AMC 30	NX 10	VA 30	CFM 5	CT 10	Sample
Shigella dysenteriae	R	R	S	S	S	S	S	R	R	S	**XLD RS8**
Salmonella spp	R	S	S	S	I	S	S	R	S	I	**XLD RS8**
Shigella boydii	R	S	S	S	I	I	S	R	S	I	**XLD RS8**
Serratia fonticola	R	S	S	S	S	R	S	R	S	S	**XLD RS6**
Serratia fonticola	R	R	S	S	S	S	S	R	R	S	**XLD RS7**
Lysinibacillus sphaericus	R	R	S	S	S	S	S	R	R	S	**UTI RS5**
Aneurinibacillus aneurinilyticus	I	R	S	S	S	S	S	R	R	S	**UTI RS4**
Lysinibacillus sphaericus	R	R	S	S	S	S	S	R	R	S	**UTI RS3**
Viridibacillus neidei	I	S	S	R	S	S	S	R	S	S	**UTI RS5**
Bacillus tequilensis	R	S	S	I	S	R	S	R	S	S	**UTI RS1**
Paenibacillus macerans	R	S	S	R	S	R	S	R	S	S	**UTI RS1**
Brevibacterium iodinum	R	R	S	R	S	S	S	R	S	I	**UTI RS1**
Lysinibacillus sphaericus	R	R	S	S	S	S	S	R	S	R	**UTI RS4**
Salmonella spp	S	R	S	S	S	S	S	R	S	S	**XLD RS8**
Providencia alcalifaciens	S	S	S	S	S	S	S	R	S	R	**MFC RS6**
Enterobacter spp	S	S	S	S	S	S	S	R	S	S	**MFC RS2**
Staphylococcus spp	S	S	S	S	S	S	S	S	R	S	**MSA RS7**
Enterobacter spp	S	S	S	S	S	S	S	S	R	I	**MFC RS 2**
Vibrio pacinii	S	S	S	S	S	I	S	S	R	R	**TCBS RS1**
Aeromonas schubertii	S	S	S	I	S	S	S	S	R	R	**TCBS RS2**
Aliivibrio logei	S	S	S	R	S	S	S	S	R	R	**TCBS RS1**

Table 5.2: Representation of the antibiotic susceptibility status of the identified bacteria

Organism Identities	AML 30	TE 30	AK 30	E15/ AZM 15	MRP 10	AMC 30	NX 10	VA 30	CFM5	CT10	Sample
Salmonella spp	R	S	S	I	S	S	S	R	S	S	**MAC RS5**
Escherichia spp.	R	S	S	R	S	R	S	R	S	S	**MSA Unknown 3**
Photorhabdus asymbiotica (Emerging Human pathogen)	R	R	S	S	S	S	S	R	R	S	**UTI RS8 (1)**
Xenorhabdus beddingii	S	R	S	R	S	S	S	R	R	S	**MAC Eco**

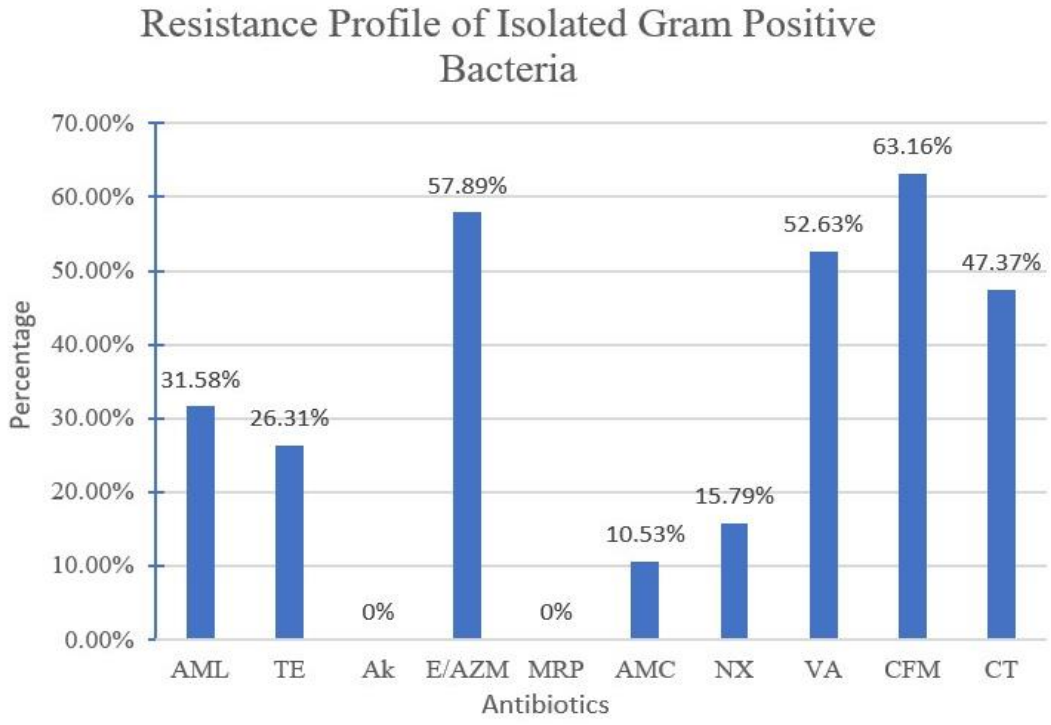

Figure 9: Representation of antibiotic resistance profile against Gram positive bacteria

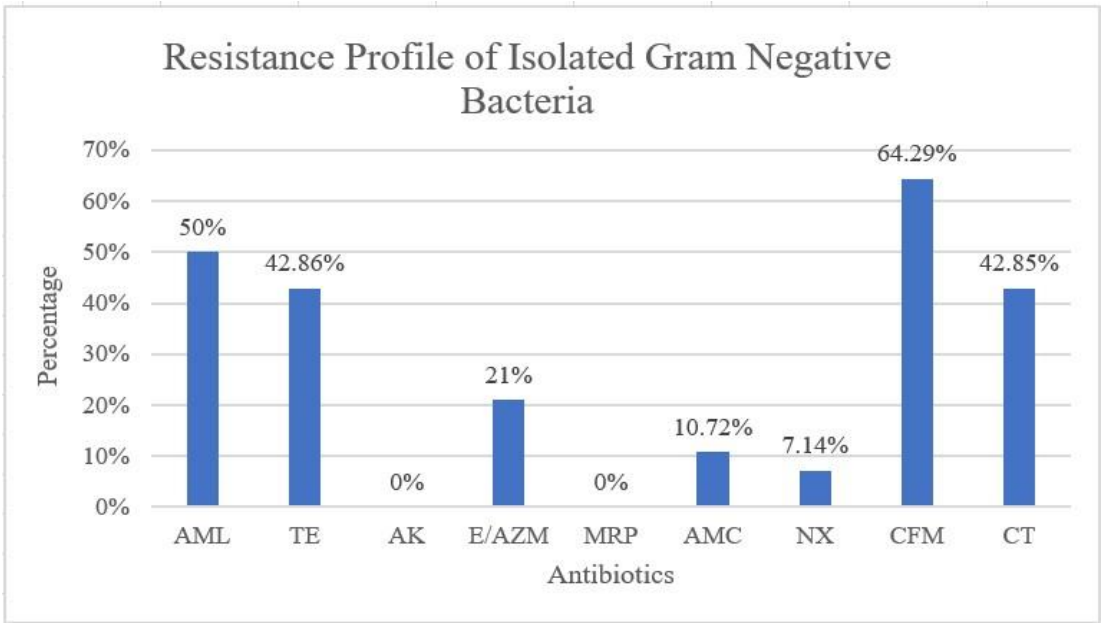

Figure 9.1: Representation of antibiotic resistance profile against Gram negative bacteria

The total number of identified bacteria were 47 and figures 9 and 9.1 display the overall resistance profile for Gram positive and negative types, respectively. For the purpose of better representation, abbreviated names of the antibiotics are used.

Gram positive bacteria: For Gram positive cells, the highest resistance was observed against Cefixime (63.16%) and the second highest against Azithromycin/Erythromycin (57.89%). 47.37% bacteria had shown resistance to Colistin Sulfate and 31.58%, to Amoxicillin. Tetracycline had a bacterial resistance percentage of 26.31% and Norfloxacin, 15.79%. For Amoxicillin + Clavulanic acid, 10.53% of the bacteria were resistant. 52.63% of the bacteria were resistant to Vancomycin. From the chart we can see that Amikacin and Meropenem were the most effective antibiotics as none of the organism had shown any resistance towards these two antibiotics.

Gram negative bacteria: For Gram negative cells, Amikacin and Meropenem were the most effective with no resistant bacteria. Moreover, Amoxicillin + Clavulanic acid and Norfloxacin were largely effective, with only 10.72% and 7.14% of the bacteria being resistant against them. Without Clavulanic acid, resistance against Amoxicillin rose to 50%. In terms of the higher tier, the highest amount of resistance falls against Cefixime at 64.29%. Resistance against Colistin Sulphate and Tetracycline were near about the same value, at 42.85% and 42.86%, respectively. Lastly, the antibiotic Azithromycin/Erythromycin proved to be relatively effective with 21% of the Gram negative bacteria being resistant against it.

4.0. Discussion:

4.1. Overview of bacterial identification determination:

The utilization of a predetermined set of selective and differential (SD) media facilitated in the obtaining of 116 isolates among which, 85 isolates were forwarded to further processing, classification, and identification. Among this selection, 47 isolates were noted based on the uniqueness of their corresponding data. The growth characteristics of the bacterial colonies on the respective media types enabled in the conduction of preliminary identifications. For this, standard, pre-published databases were used whilst the information obtained from biochemical testing allowed for enhanced cross-referencing. Furthermore, the ABIS software and the Bergey's Manual of Systematic Bacteriology were used to integrate the data and determine the respective identities of the isolates.

4.2. Growth summary:

Upon the completion of cross-referencing, a total of 26 genera were identified. Of these, the major proportion belonged to the family of *Enterobacteriaceae* with the families of *Bacillaceae*, *Staphylococcaceae* and *Vibrionaceae* making up the second, third, and fourth largest majorities, respectively. This was largely in parallel with the preliminary assumptions as bacterial species of the *Enterobacteriaceae* and *Staphylococcaceae* families are known habitants of the soil and marine environments along with the gastrointestinal tract of humans and animals (Patel et al., 2014). Thus, to mimic the saline and alkaline conditions of the marine environment to the farthest extent, the choices of the SD media used were conducted accordingly. Colonies were cultured on each respective media type per sample-site where designated inoculum amounts were taken from both the raw and peptone water samples upon serial dilution. This was done to ensure uniformity and facilitate opportunities of double verification.

4.3. Recognized habitats of the identified bacterial families:

Certain genera of the *Enterobacteriaceae* and *Staphylococcaceae* families which have been identified from the samples, namely the *Staphylococcus*, *Enterobacter*, *Citrobacter*, *Serratia*, and *Klebsiella* genera, are a part of the normal flora of human and animal skin (De et al., 2014; Muhibbullah & Sarwar, 2017). Alongside these, members of the *Bacillaceae* family are known to survive as soil bacteria which are also halophilic in light of endospore-forming capabilities (Mandic-Mulec et al., 2015). Thus, shifting sediments enable the easy entry of these bacteria to marine environments. The family incorporating the *Vibrio* genera, the *Vibrionaceae* family, can also be regularly found in marine environments among which, *Aliivibrio spp.* and *Vibrio furnissii* are ubiquitous (Lee & Ruby, 1994; Ballal et al., 2017). Moreover, *Vibrio cholerae* are fecal coliforms which can be introduced into the environment through exposure to infected fecal matter (Cottingham et al., 2003).

4.4. Facilitation of bacterial spread across the marine environment:

It is to be noted that the Saint Martin's Island is already accommodating an above-capacity intake of tourists during the on-season with land-use and drainage patterns that have been suspected of being unplanned and dysregulated (Ahamed, 2021; Muhibbullah & Sarwar, 2017). Moreover, the population of dogs on the island has considerably increased which was also corroborated by the sampling team during their expedition (Yousuf, 2020). As previously stated, the intestinal and skin floras of these animals are known habitats of the *Enterobacteriaceae* and *Staphylococcaceae* families. Thus, contact of the beach sediments and water to untreated sewage and anthropological activities regularly facilitates in the transfer of the aforementioned bacteria to the marine environment. Upon identification, it was observed that a large proportion of the sampled species were sourced from the Northern regions of the island. Subsequently, it was also observed that these regions underwent regular interactions with humans since these were either near to the jetty (or mooring points), markets, resorts, or the farmlands. Henceforth, the findings further corroborated the initial predictions. Moreover, due to frequent agitation by shifting waves and tides, sediments which can particularly house species of the *Staphylococcus* and *Vibrio* genera are shifted throughout the region, thus further distributing the cells to areas with low anthropogenic exposure (Rehmann et al., 2009). Since a percentage of these bacterial species already possess halophilic properties, the highly compatible, tropical environment of the waters surrounding the island with average salinity of around 32.84 ppt and temperatures ranging between 25 to 30°C, allowed the cells to thrive and repopulate (Mehedia et al., 2000).

4.5. Cross referencing with Gram stain results and known habitation:

The findings were further corroborated by the Gram staining technique which enabled in the determination of the type of the cells along with their respective morphologies. It was observed that majority of the samples were Gram negative (59.4%) and of the rod shape. This was in accordance with the findings which showed majorities among the *Enterobacteriaceae* and *Vibrionaceae* families. Moreover, this conclusion was further strengthened by a previous study conducted by Paul et al. (2021) to determine the identities of sponge-associated bacteria surrounding the island of concern, which found the *Bacillaceae* and *Vibrionaceae* families to occur in significant abundances (Paul et al., 2021). In this manner, the finding of the families in the coastal, marine environment of Saint Martin's Island was largely in tune with preliminary identification analyses as well as literature reviews. Furthermore, bacterial families that were in lesser majorities, namely of the *Yersiniaceae*, *Paenibacillaceae*, *Brevibacteriaceae*, *Aeromonadaceae*, and the *Morganellaceae* families, were also among the list of typical bacteria which are associated with such environments. This is as such because the *Yersiniaceae*, *Paenibacillaceae* and *Morganellaceae* families have been frequently found from soil and fecal sources whilst *Aeromonadaceae* and *Brevibacteriaceae* are common inhabitants of aquatic

habitations with the latter being especially halophilic (Adeolu et al., 2016; Mayilraj & Stackebrandt, 2014; Forquin-Gomez et al., 2014; Pereira et al., 2008; Kim et al., 2007).

4.6. Pathogenic capabilities of the identified species:

Based on the findings, it is evident that the coastal water of Saint Martin's Island is rich in bacterial diversity. However, there lies a significant cause for concern due to the water essentially being contaminated by pathogenic and potentially opportunistic bacteria which can cause various gastrointestinal diseases under the appropriate conditions. Among the findings, the *Morganellaceae* and *Aeromonadaceae* families, the *Yersinia spp*., and certain species under the *Enterobacteriaceae* family, namely the *Enterobacter spp*., *Klebsiella spp*., *Citrobacter spp*., and *Shimwellia spp*., can lead to acute diarrhoea, inflammatory bowel diseases (IBDs), urinary tract infections (UTIs), sepsis, and diseases alike (Janda 2021; Pereira et al., 2008; Chander et al., 2006). Of these, the *Klebsiella spp*., *Shimwellia spp*., and *Citrobacter spp*. are notable indicators of fecal contamination. This further corroborates the already existent issues of the island infrastructure with respect to improper drainage and leaking sewage matter (Halkman & Halkman, 2014). Supplementing this concern is the presence of *Staphylococcus aureus* in the waters which are regularly used for recreational purposes by tourists as well as the locals. Staph infections can be deadly, ranging from disorders of the skin to gastroenteritis, pneumonia, and toxic shock syndrome (Lowy, 1998). In addition to this, the presence of bacteria of the *Vibrionaceae* family, particularly *Vibrio pacinii (cholerae)* and *V. furnissi*, poses high risk of gastro-enteric diseases in occurring upon simple exposure (Farmer et al., 2015). Of significant note was a rather unexpected finding attributed to the originally nematode symbiont, *Photorhabdus asymbiotica*, being found from zone 8. This bacterium has been classified as an emerging pathogen that has recently been studied to cause bacteremic and invasive soft tissue infections (Gerrard et al., 2006). Thus, presence of it in coastal waters is alarming. Also, whilst the other identified bacteria have not known to be significantly pathogenic towards humans, the presence of opportunistic traits is fairly common which is particularly concerning due to the lack of strict preliminary checking and regulations with regards to travel, habitation, and recreation on the island.

4.7. Poor healthcare condition on the island:

It is to be noted that the healthcare condition on the island is of a grim state. Only a single 10-bed hospital is currently present there to provide support should it be required. However, the same facility has been reported to be understaffed and underdeveloped with most patients having to travel to the Cox's Bazaar town, a good 4–5-hour journey away, to seek medical attention (Aziz, 2017). To this regard, the identification of the bacterial species available and in contact with the human population in the region holds potential of becoming a severe situation. It is of further note that during the sampling expedition, the team has engaged in casual dialogue with other tourists. This was done to gain a general idea regarding the overall health status of the inhabitants.

Unsurprisingly, the participants have reported facing diarrhoeal symptoms during their initial days of living on the island.

4.8. Overview of antibiotic susceptibility testing:

Based on the identities of the sampled bacteria along with their abilities of causing disease, it was necessary to delineate the proficiencies of modern antibiotics in tackling and neutralizing the different species. Since the study was solely focused on bacterial cells, antiviral or antifungal drugs were not included in the list of concerns. To this regard, a collection of 10 antibiotics were selected whilst keeping inclusivity and diversity in focus. Azithromycin/Erythromycin, Amikacin, and Tetracycline were selected to include a well-rounded set of antibacterial agents which functioned to inhibit or disrupt protein synthesis via ribosomal structure disruption (Parnham et al., 2014; Craig et al., 1991; Chopra & Roberts, 2001). Furthermore, the antibiotics Cefixime, Meropenem, Vancomycin, and Amoxicillin (standalone and conjugated with clavulanic acid) were employed to neutralize cells via bacterial cell wall inhibition (NCBI, 2022; Patel et al., 2022; Evans et al., 2022). Additionally, Norfloxacin was used considering it being able to disrupt bacterial DNA replication (NCBI, 2022). Lastly, Colistin Sulphate was utilized to include the Polymyxin group which functions to disrupt bacterial cell membranes via the displacement of Ca^{2+} and Mg^{2+} cations from the membrane lipids (El-Sayed Ahmed et al., 2020).

4.9. Meropenem susceptibility pattern and reasoning:

The susceptibility and resistance patterns of the different isolates are displayed in table 5. Meropenem was observed to be the second most potent (95.7%) antibiotic agent in neutralizing bacterial cells regardless of their identity. This is a 4^{th} generation, broad spectrum Carbapenem that is useful against both Gram positive and negative cell types considering its active penetration capabilities (Li et al., 2007). Furthermore, it has also shown significant efficacy against extended-spectrum beta-lactamase producing *Enterobacteriaceae*. It is thus prescribed during treatment of moderate to severe bacterial meningitis, UTIs as well as infections of the lower gastrointestinal tract only, facilitating in limited usage (Wiseman et al., 1995). To this regard, the susceptibility pattern is significantly greater compared to the minimum inhibition zone diameter as can be seen with respect to all the samples tested. Moreover, it is of note that Meropenem has been studied to have limited to no activity against methicillin-resistant *Staphylococcus aureus* (MRSA) but has shown to be effective against the same bacteria identified from the water samples (Zhanel et al., 2007). This enables in the conclusion that the bacteria occurring in this region has not developed multidrug resistance at the time of testing.

4.10. Amikacin susceptibility pattern and reasoning:

Amikacin was the drug with the highest susceptibility pattern (97.8%). This is a 3^{rd} generation Aminoglycoside with particular potency towards Gram negative cells since the drug compound possesses the greatest resistance against the action of aminoglycoside modifying enzymes

(Ramirez & Tolmasky, 2017). However, it is to be noted that *Bacillus novalis*, a bacteria found to occur at site 1, showed intermediate susceptibility to the drug. It was speculated that these bacteria fell under the category of Gram positive bacteria possessing plasmid-transferred AAC (6′)-Ib (6′-*N*-acetyltransferase) (Ramirez & Tolmasky, 2017). This had most likely conferred the cells with the capability of modifying the drug compound to consequently reduce effectiveness.

4.11. Amoxicillin and Vancomycin susceptibility pattern:

With respect to Amoxicillin, effectiveness was observed to range at the moderate tier in terms of Gram positive cells, such as the *Staphylococcus spp*. Furthermore, resistance patterns were observed for both Gram positive and negative (more common) cellular types. Due to the antibacterial agent being a β-lactam, resistance by other Gram positive types, such as *Bacillus novalis*, *Lysinibacillus sphaericus*, and *Paenibacillus macerans*, has likely developed due to chromosomal alterations leading towards modified penicillin-binding proteins (PBPs) (Malhotra-Kumar et al., 2016). Moreover, this may also arise based on plasmid-mediated acquisition of resistance conferring genes such as the commonly known *mec-A* (Yao et al., 2019). This, though, can be significantly concluded upon further genetic-level testing. However, with respect to the findings, Gram negative cells show majority resistance towards this 2nd generation antibacterial agent due to these cells possessing a protective, outer cell membrane comprised of lipopolysaccharides (LPS) (Miller, 2016). Similar reasoning can be extended to Vancomycin which displayed effectiveness at a mixed pattern against both Gram positive and negative cell types such as *Cedecea neteri*, *Vibrio pacinii*, and *Aeromonas schubertii*. To this regard, the presence of susceptibility against a significant population of Gram negative samples was uncommon due to the double cell-membrane configuration. With respect to this unusual susceptibility, it could be speculated that the cells possessed compromised outer cell membranes which allowed entry of the drug compound. Moreover, the Disk Diffusion environment may have also possessed an unknown compound which worked as a conjugate to the drug compound, enhancing its potency (Antonoplis et al., 2019). However, this requires further, focused investigation to be firmly established.

4.12. Use of adjuncts, Clavulanic acid, Cefixime, Azithromycin/Erythromycin, and Tetracycline susceptibility patterns and reasoning:

To increase effectiveness of antibacterial agents in general, attachment of adjuncts has been proven to be fruitful. To this regard, Clavulanic acid is conjugated to the Amoxicillin compound. Here, the adjunct acts to irreversibly bind to and inhibit β-lactamases, thus increasing the effectiveness of the combined antibacterial agent (Uto LR & Gerriets, 2022). This is further corroborated by the findings where resistance is shown by only 5 bacteria, *Providencia rustigianii, Serratia fonticola, Escherichia spp., Bacillus tequilensis*, and *Paenibacillus macerans*. The former three are of the Gram negative type. However, the latter are Gram positive, thus raising an interesting situation with regards to the resistance development. This phenomenon has likely either developed based

on aforementioned chromosomal modifications or plasmid-mediated acquisition of β-lactamases that are less sensitive to Clavulanic acid binding (Stapleton et al., 1995). Among the β-lactam agents, Cefixime was observed to show the greatest pattern of resistance with susceptibility to some Gram positive exceptions. However, it must be stated that this pattern could be attributed to comparatively lower concentrations of the drug agent being used. Alongside the compounds, the cells showed moderate susceptibility to Azithromycin/Erythromycin and Tetracycline with the latter being more effective in comparison. Since these compounds target bacterial ribosomes, they are both broad spectrum antibiotics with no particular cell types possessing intrinsic resistance. However, with Erythromycin and Azithromycin being 1st and 2nd generation Macrolides, they have been in more frequent use compared to 3rd generation Tetracycline, and thus, possess a greater likelihood of being ineffective as can be observed from the results. Proposed pathways of resistance development by cells to these agents include the occurrence of spontaneous mutations resulting in modifications to ribosomal target sites, efflux-pump overexpression, and/or the presence of genes such as *mphA* (most probable cause) or *erm* which encode for macrolide resistance via phosphotransferase or methyltransferases (Xiang et al., 2020). Furthermore, it is to be noted that these cells were sourced from the same environment. Thus, it would be plausible to speculate that gene transfer via plasmid mediated pathways may have occurred at some point, therefore transferring, and further distributing resistance capabilities.

4.13. Norfloxacin susceptibility patterns and reasoning:

Apart from the protein-synthesis inhibitors and the β-lactams, the DNA synthesis-inhibiting properties of Norfloxacin appeared to be significantly potent. This can be attributed to it being a broad-spectrum Fluoroquinolone. As mentioned earlier with respect to Azithromycin and Tetracycline, resistance to Norfloxacin may also arise due to mutations causing target-site alterations, efflux-pump enhancements, enzymatic modification, or the acquisition of plasmid mediated resistance genes (*qnr* gene in particular) (Fàbrega et al., 2009). Interestingly, resistance was only observed among the *Staphylococcus spp*. Thus, it can be speculated that due to being of the same genera, horizontal or plasmid mediated resistance gene transfer may have been facilitated with enhanced simplicity at some point, thus distributing the resistance capabilities.

4.14. Effect of the *mcr-1* gene in facilitating resistance against Colistin Sulphate:

A major cause for concern arose when observations were made with respect to the widespread nature of resistance against the Colistin Sulphate antibacterial agent. This is unique in comparison to the other antibiotic choices of the set by way of its mode of action. The drug compound utilizes hydrophobic and lipophilic groups to interact with and disrupt bacterial cell wall permeability (NCBI, 2022). It was observed that a significant amount of the samples possessed resistance to this agent despite it being known to be effective as a “last-resort” drug, particularly against multi-drug resistant Gram negative bacteria (Conly & Johnston, 2006). Preliminary literature review had yielded in information displaying the significant effect of the *mcr-1* gene in conferring Polymixin

resistance via modification of the outer cell membrane of Gram-negative cells (Gao et al., 2016). Furthermore, it was also known that the gene could be transferred horizontally via plasmids, thus facilitating in resistance distribution (Li et al., 2020).

4.15. Genetic testing to determine *mcr-1* presence in CLR bacteria:

In order to observe and determine the effect of the *mcr-1* gene in conferring Colistin resistance (CLR), the samples displaying resistance in the Disk Diffusion assay were isolated and tested for the presence of the gene via the PCR process. To this regard, DNA extraction was conducted and each of the DNA extracts of the respective CLR isolates were subjected to targeted amplification via PCR. Here, specific primer-sequence design enabled in targeted amplification of the gene. Absence of it would not produce any amplicons and thus, no bands would subsequently appear during detection via Gel electrophoresis. In this manner, upon the completion of PCR, the respective isolates were placed on a gel with 1.2% agarose concentration and the bands were observed under UV light. Here, 1.2% of the gel was used based on the moderate size of the desired amplicon which was 309bp. This provided a matrix of optimum resolving density to subsequently attain high resolution amplicon separation. The results are displayed in figure 7 and 7.1 It is to be noted that due to total genomic DNA extraction in the prior step, undesired sequences were also present in the original master mix. These produced slight smearing and undesired bands on the gel that were ultimately disregarded during analysis. After refining, 4 of the lanes were determine as displaying targeted amplicons, thus indicating the positive presence of the *mcr-1* gene. Further corroborating this is the fact that the bands were placed at relatively similar positions, signifying that the product sizes were similar as well. These were determined upon referring to the desired ladder-band size.

An interesting notion was observed where certain lanes did not produce any targeted bands in spite of being Colistin Sulphate resistance, thus indicating that no products had formed. This would raise a question regarding the source of the resistance. However, upon literature review, it was noted that apart from the *mcr-1* gene, presence of other genes ranging from *mcr-2* to *mcr-8*, *pmrC*, *pmrE*, and mutated genes controlling the efflux pumps are also known to confer resistance (Aghapour et al., 2019). Moreover, the *mcr-1* gene can be transferred via plasmids (Li et al., 2020). These plasmids were not isolated as the initial DNA extraction process utilized a protocol which focused on genetic DNA isolation. It is possible that if plasmid isolation protocols were utilized and the PCR process was repeated that amplicons might be attained in the lanes which did not show bands during the first run. Furthermore, primers targeting the other CLR conferring genes could also be used during additional PCR runs. Thus, scope for further research is of significant potential.

5.0. Conclusion:

The coastal water of Saint Martin's Island is at significant risk of developing into a public health concern. Furthermore, this is particularly elevated during the on-season when the island reaches peak activity. The findings obtained from this study have yielded results that confirm the presence of bacteria belonging to the *Enterobacteriaceae*, *Bacillaceae*, *Staphylococcaceae* and *Vibrionaceae* families in a majority. Most species of these families possess disease-causing capabilities both as direct and opportunistic means. The study samples were collected only from shallow water and the results indicate that extended study with the deep seawater will present a rich reservoir of microbes with clinical potential. Moreover, the results also show that while the hard antibiotics remain effective, most of the other agents possess effectiveness at the medium to lower tier. The detection of Colistin Sulphate being in the lower tier of susceptibility was of particular concern due to it generally being used as a last resort drug. Additionally, the presence of the *mcr-1* gene in those organisms indicates greater public health concerns. It is paramount that the healthcare situation of the island be developed with greater attention toward active medical personnel being present. The island requires enhanced governance during the active season to ensure that capacity limits are upheld whilst the safety standards regarding recreational water usage and waste disposal are met. Alongside these, greater attention needs to be provided to conserve the existent biodiversity, particularly during the off-season, so that nature can heal itself when human presence becomes minimal.

6.0. Supplementary Tables:

Table 1: Antibiotic Susceptibility reference table

Antibiotic (Quantity µg per disk)	Diameter of inhibition zone (mm)		
	Resistant	Intermediate	Sensible
Colistin sulphate	<<<	9-10	>>>
Cefixime	<<<	16-18	>>>
Vancomycin	<<<	15-15	>>>
Norfloxacin	<<<	13-16	>>>
Amoxicillin+ Clavulanic acid	<<<	14-17	>>>
Meropenem	<<<	14-15	>>>
Azithromycin	<<<	14-17	>>>
Erythromycin	<<<	14-22	>>>
Amikacin	<<<	15-16	>>>
Tetracycline	<<<	15-18	>>>
Amoxicillin	<<<	12-19	>>>

Table 2: Identified bacteria and their characteristics

	Identified Bacteria	Characteristics	Reference
1	*Cedecea neteri*	• A Gram negative bacillus, rare, urinary tract pathogen. • Family : *Enterobacteriaceae* • Has been reported as emerging opportunistic pathogen.	(Ahmad, 2021)
2.	*Staphylococcus aureus*	• A Gram positive coccus • Family : *Staphylococcaceae* • It colonizes nasal mucosa and skin, can cause a wide range of skin and soft tissue infection. • Has biofilm formation ability.	(Sato, 2019)
3.	*Yersinia mollaretti*	• A Gram negative, non-pathogenic bacterium. • Family : *Enterobacteriaceae* • A common human pathogen that causes intestinal and extra-intestinal syndromes.	(Sulakvelidze, 2000)
4.	*Citrobactor gillenii*	• A facultative anaerobic Gram negative bacterium. • Family : *Enterobacteriaceae* • Normally found in water soil food and intestinal tract of human and animals. • Can be a source of several type of infections: - urinary tract, respiratory, intra-abdomal , wound, bone, blood stream and central nervous system.	**(**Samonis, 2009)
5.	*Bacillus novalis*	• Gram positive non-pathogenic bacteria. • Family: *Bacillaceae* • No known pathogenic activity on humans.	(Heyrman et al., 2004)
6.	*Bacillus tequilensis*	• Gram positive non-pathogenic bacteria. • Family: *Bacillaceae* • No known pathogenic activity on humans.	(Xu et al., 2018)
7.	*Paenibacillus macerans*	• Gram variable bacteria showing Gram-positive bacilli or Gram-variable sporulated bacilli in Gram staining. • Family: *Paenibacillaceae*	(Noskin et al., 2001)

		• Unusual pathogen which is able to cause bacteremia in neonates.	
8.	*Bravibacterium iodium*	• Gram positive soil bacteria that is part of normal human microflora. • Family: *Brevibacteriaceae* • Can rarely cause opportunistic infections in immunosuppressed individuals.	(Heininger et al., 2000)
9.	*Vibrio pacinii*	• Gram negative rods. • Family: *Vibrionaceae* • Associated with gastroenteritis, can infect open wound.	(Gomez-Gil, 2003)
10.	*Aliivibrio logei*	• Gram negative marine bacteria found on fish skin. • Family: *Vibrionaceae* • No known pathogenic activity on humans.	(Willassen et al., 2021)
11.	*Photorhabdus asymbiotica*	• A Gram negative entomopathogenic • Family : *Enterobacteriaceae* • Pathogenic to human and also retains the ability to infect insect. • It has host switching ability and undergoes large metabolic shift at the human host temperature. • Emerging human pathogen.	(Hapeshi, 2016)
12.	*Vibrio furnissi*	• A Gram negative, halophilic bacterium. • Ubiquitously present in marine environment. • Non-cholera vibrio species that can cause human gastroenteritis and extra intestinal infections.	(Ballal, 2017)
13.	*Aeromonas schubertii*	• Autochthonous inhabitants of aquatic environment where natural salinity is 0%-3%. • Facultative anaerobic Gram-negative rods. • So far, has been isolated from abscesses, wounds, skin, pleural fluid, and blood. • Have been implicated as the cause of human wound infections.	(Carnahan, 1989)

14.	*Staphylococcus epidermidis*	• A Gram positive bacterium. • Family : *Staphylococcaceae* • Commonly colonizes skin and mucus membrane of human and mammals. • Most frequent cause of nosocomial infections. • Can cope with extreme salt and osmotic pressure. • Causes vascular catheter related bloodstream infection.	(Otto, 2009)
15.	*Lysinibacillus sphaericus*	• A rod shaped Gram positive bacteria. • Famil: *Bacillaceae* • Commonly found in plant,soil and animals. • Reported to have antimicrobial potential and its bacteriocin has been reportedly used to combat against food borne bacterial and fungal pathogens.	(Seelam, 2017)
16.	*Escherichia spp.*	• Gram-negative bacteria. • Family: *Enterobacteriaceae* • Common inhabitant of the healthy human intestinal tract. • Limited number of clones is responsible for acute diarrhea and extra-intestinal infections.	
17.	*Providencia rustigianii*	• Formally known as *Providencia alcalifaciens* biogroup. • Family : *Enterobacteriaceae* • Reservoir includes water, waste water and soil. • Role in causing human disease is uncertain.	(Hickman-Brenner,1983)
18.	*Aneurinibacillus aneurinilyticus*	• Family : *Paenibacillaceae* • This bacterium can be found in the soil, water bodies, and in sites contaminated with heavy metals.	(Balan, 2017)
20.	*Bacillus carboniphilus*	• A rod shaped Gram positive bacteria. • This bacterium is normally found on air or soil and has no pathogenic activity on humans.	(Fujita et al., 1996)

21.	*Salmonella spp*	• A rod shaped Gram-negative bacteria that belongs to the. *Enterobacteriacea* family. • It is found in different food sources and known to cause salmonellosis in humans	(Gabriela Loredana Popa1 & Mircea Ioan Papa, 2021)
22.	*Moellerella wisconsensis*	• A Gram negative rod shaped a bacterium that belongs to the *Enterobacteriacea* family. • It is normally found in human stool samples and no pathogenic activity on humans recorded till now.	(Brenner et al., 1984)
23.	*Shimwellia blattae*	• Gram-negative bacteria • Family: *Enterobacteriaceae*. • This bacterium is normally found in cockroach intestine and has no pathogenic activity on human.	(Schoch CL et al., 2020)
24.	*Serratia fonticola*	• Rod-shaped Gram-negative bacteria. • Family: *Yersiniacea*. • It is a normal part of water microflora and known to cause unusual skin and soft tissue infections in humans.	(Jump et al., 2016)
25.	*Viridibacillus neidei*	• Rod-shaped Gram-positive bacteria. • Family: *Bacillaceae*. • It is normally found in soil and has no known pathogenic activity on humans.	(Albert et al., 2007)
27.	*Bacillus aminovorans*	• Rod-shaped Gram-positive bacteria. • Family: *Bacillaceae* • It is normally found in ocean sands and has no known human pathogenic activity.	(Verma et al., 2017)
28.	*Klebsiella pneumoniae*	• Rod-shaped Gram-negative bacteria. • Family: *Enterobacteriaceae*. • It is normally found in the environment and causes pneumonia in humans.	(Ashurst & Dawson, 2022)
29.	*Buttiauxella brennerae*	• Rod-shaped Gram-negative bacteria. • Family: *Enterobacteriacea.*	(Morales et al., 2020)

		• It is normally found in snail and has no known pathogenic activity on humans.	
30.	*Citrobacter youngae*	• Gram-negative bacteria. • Family: *Enterobacteriacea*. • It is normally found in the intestinal tract of human and animals and can rarely cause intra-abdominal or urinary tract infection in immunosuppressed individuals.	(Chen & Sue, 2013)
31.	*Paenibacillus macquariensis*	• A Gram positive bacterium. • Family : *Paenibacillaceae* • Secretes extra cellular xylanase which can be exploited for a range of industrial and environmental application such as the production of biofuels.	(Sharma, 2013)
32.	*Shigella dysenteriae*	• Rod-shaped Gram-negative bacteria. • Family*: Enterobacteriacea* • It is normally found in the intestinal tract of humans and other primates and leading cause of Shigellosis.	(Hale & Keusch, 1996)
33.	*Shigella boydii*	• Rod-shaped Gram-negative bacteria. • Family: *Enterobacteriacea* • It is normally found in intestine and rectum of human and other primates and cause Shigellosis diarrhea.	(Hale & Keusch, 1996)
34.	*Xenorhabdus beddingii*	• Gram-negative rod-shaped bacteria. • Family: *Enterobacteriacea* • Naturally found in the stomach of nematodes in the genera. *Heterorhabditis* and *Steinernema.* • May have antibacterial and antifungal activity.	(AKHURSTt, 1986)
35.	*Bacillus aminovorans*	• Gram-positive bacilli. • Family: *Bacillaceae* • Naturally found in sediments and marine environments after displacement	(Rüger, 1983)

Table 3: Colony characteristics based on growth on different selective media

MFC media growth observation

Plate	Different colony type (visible)	Number of colonies (visible)	Form	Surface	Color	Margin/Boundary	Elevation	Opacity	Comments
RS1	1	1	Circular	Matte glisten + Rough	Olive green	Circular but not clear, slightly lobed and spiked	Slightly raised	Opaque	
RS2	2	26	Circular	Glistening & Rough	Dark green + Light brown	Clearly circular + Hazy	Slightly raised at centre	Opaque	
RS3	Difficult to judge (DTJ)	>30	DTJ	DTJ	DTJ	DTJ	DTJ	DTJ	Media changed color
RS4	No growth								
RS5	1	5	Circular and varying	Matte glisten + Rough	Olive green	Clear margin	Slightly raised	Opaque	
RS6	2	7 (countable) + 132 (boxes)	Circular smaller + Oval and Irregular	Glistening & Rough	Olive green + Pale yellow/light brown	Clear margin + Slight dark, lobed bordering	Slightly raised + flat	Opaque	Media changed color
RS7	TNTC	TNTC	Irregular DTJ	Overgrowth	Pale yellow/light brown	Clear bordering for countable colonies	Slightly raised on the sides but flat at central	Opaque	Media changed color
RS8	2	1 (countable) + 139 (boxes)	White circular + irregular	Opaque Rough (No light reflection or glistening)	White + Dark blue	Clear	Slightly raised	Opaque	

MAC media growth observation

Plate	Different colony type (visible)	Number of colonies	Form	Surface	Color	Margin/Boundary	Elevation	Opacity	Comments
RS1 (10^-6)	TNTC		Circular	Smooth with slight glisten	Red	Clear with no lobing	Slightly raised	Translucent	Media has changed color to becoming pale red
RS2	No growth								
RS3									
RS4									
RS5	6	14	Circular	Smooth with slight glisten	Red	Clearly circular with no lobing	Slightly raised	Translucent	Media has retained color but in some places, under some colonies, color shift has occured to orange
RS6	2 similar + Remaining two are of different sizes	4	Relatively circular + Irregular	Smooth and glisten + Rough with no glisten	Dark red	Clear + Clear & Lobed + Clear & circular + Lobed with clear outline	Slightly elevated + Significantly elevated	Translucent	
RS7	No growth								
RS8	3 (Size variation)	3	Circular (All)	Smooth with slight glisten	Red	Clearly circular with no lobing	Slightly raised	Translucent	

MSA media growth observation

Plate	Different colony type (visible)	Number of colonies	Form	Surface	Color	Margin/Boundary	Elevation	Opacity	Comments
RS1		10	Circular (Slightly irregular)	Rough and Matte	Light reddish	Clear	Slightly raise	Opaque	2 colonies comparatively larger + 2 layer
RS2		1	Circular (Slightly irregular)	Rough and Matte	Light reddish	Clear	Slightly raise	Opaque	2 layer
RS3	Small + Larger (Size variation)	4	Irregular circular	Smooth	Large is reddish + Lighter red (Slightly		Slightly raise	Translucent	
RS4	No growth								
RS5	7 + 1	8	Circular	Smooth and glistening	Slightly yellow	DTD	Slightly raise	Translucent	
RS6									
RS7									
RS8	No growth								

TCBS media growth observation									
Plate	**Different colony type**	**Number of**	**Form**	**Surface**	**Color**	**Margin/Boundary**	**Elevation**	**Opacity**	**Comments**
RS1	1 large + 12 similar	13	Relatively circular	Matte glisten + Smooth	Dark green + Yellow/Brownish	Mostly clear + Slightly lobed (Irregular circular)	Elevated at boundary	Opaque	Regions of yellow colonies have changed media color from green
RS2		1	Circular, irregular and lobed	Matte	Yellow/Brown	Clear, lobed and irregular	Elevated	Opaque	Regions of yellow colonies have changed media color from green to yellow + 3 layer
RS3									
RS4					No growth				
RS5		Difficult to determine	Highly irregular, lobing, and branching		Yellow/Brown	Clear	Prominent	Translucent	Regions of yellow colonies have changed media color from green to yellow + 2 layer
RS6									
RS7					No growth				
RS8		1	Circular	Smooth and glistening	Yellow/Brown	Clear, circular	Slightly raised	Opaque	Form is almost like a perfect circule

UTI media growth observation									
Plate	**Different colony type (visible)**	**colonies (visible)**	**Form**	**Surface**	**Color**	**Margin/Boundary**	**Elevation**	**Opacity**	**Comments**
RS1 (10^-6)	TNTC				Dark blue				
RS1 (10^-9)	100+	2 (Green and dark blue)	Circular irregular	Matte glisten	Green and dark blue	Clear (sometimes fully circular, sometimes irregular	Slightly elevated	Opaque	
RS2					No growth				
RS3	1	1	Circular	Rough glisten	Dark blue	Irregular circular	Slightly elevated	Opaque	
RS4	1	1	Circular	Smooth glisten	Yellow	Clear	Slightly elevated	Opaque	2 layered with intensity increasing towards inside
RS5	13	2 (Yellow and off-white)	Circular (2)	Rough glisten + Rough matte (2 OW)	Off-white/peach	Irregular circular (Y) + Clear	Slightly elevated	Translucent	
RS6									
RS7					No growth				
RS8	1	70-80	Circular	Rough matte	Off-white/pale yellow	Clear	Slightly elevated	Translucent	2 layered

Appendix-I Media compositions:

All media used in this research was prepared according to the manual. The composition of all media used in the study is given below

Nutrient Agar:

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Nutrient broth:

Component	Amount (g/L)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH	7.4±0.2 at 25ºC

Mannitol Salt Agar:

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0

Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

MacConkey Agar:

Component	Amount (g/L)
Peptic digest of animal tissue	1.5
Casein enzymatic hydrolysate	1.5
Pancreatic digest of gelatin	17.0
Lactose	10.0
Bile salt	1.50
Crystal violet	0.001
Neutral red	0.03
Agar	15.0
Final pH	7.1 ± 0.2 at 25°C

TCBS Agar:

Component	Amount (g/L)
Proteose peptone	10.0
Yeast extract	5.0
Sodium thiosulphate	10.0
Sodium citrate	10.0
Oxgall	8.0
Sucrose	20.0
Sodium chloride	10.0
Ferric citrate	1.0

Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.0
Final pH	8.6± 0.2 at 25°C

m-FC Agar:

Components	**Amount g/L**
Tryptose	10.0
Proteose peptone	5.0
Yeast extract	3.0
Lactose	12.5
Bile salts mixture	1.5
Sodium chloride	5.0
Aniline blue	0.10
Agar	15.0
Final pH	7.4±0.2

Xylose-Lysine Deoxycholate Agar (XLD Agar):

Components	**Amount g/L**
Yeast extract	3.0
L-Lysine	5.0
Lactose	7.5
Sucrose	7.5
Xylose	3.5
Sodium chloride	5.0
Sodium deoxycholate	2.5
Sodium thiosulphate	6.8
Ferric ammonium citrate	0.80

Phenol red	0.08
Agar	15.0

Hi Chrome UTI Agar:

Components	Amount g/L
Peptone	15.0
Chromogenic mixture	26.8
Agar	15.0
Final pH	6.8±0.2

Simmon's Citrate Agar:

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniundihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bactobromothymol blue	0.08

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

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar (TSI):

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Motility Indole Urease (MIU) Agar:

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± 0.2 at 25°C

Muller Hilton Agar:

Component	Amount (g/L)
Beef extract	2.0
Acid Hydrolysate of casein	17.5
Starch	1.5
Agar	17.0

Appendix – II Reagents and buffers:

1. Gram's iodine (300 ml)

To make this reagent, 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer and transferred to a reagent bottle. This solution was stored at room temperature and used for Gram staining.

2. Crystal Violet (100 ml)

To make this reagent, 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 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.

3. Safranin (100ml)

To make this solution, 2.5 g safranin was dissolved in 10 ml of 95% ethanol and distilled water was added to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature and used for Gram staining.

4. Kovac's Reagent (150 ml)

To make this solution, 150 ml of reagent grade isoamyl alcohol, 10 g of p- dimethylaminobenzaldehyde and 50 ml of concentrated HCL were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

5. 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.

6. Barrit's Reagent A (100 ml)

To make this reagent, 5% a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C and this reagent was used for MR VP test.

7. Barrit's Reagent B (100 ml)

To make this reagent, 40% of KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C and this reagent was used for MR VP test.

8. 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

9. 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

10. PBS buffer for DNA Extraction:

For 100 ml PBS buffer, the flowing components were added in duran bottle. The pH of PBS buffer was maintained (pH=7.6) and the buffer solution was autoclaved.

Components	**Amount (g)**
Sodium chloride	0.8
Potassium chloride	0.02
Disodium phosphate	0.14
Dipotassium phosphate	0.02

11. TAE buffer For Gel Electrophoresis:

Composition of 50X TAE buffer (100ml) (Stock Solution)	Tris base powder = 24.2g Acetic acid = 5.71ml 0.5M EDTA = 10ml Final pH = 8
Composition of 1X TAE buffer (1000ML) (Working solution)	50X TAE buffer = 20ml Distill water = 980ml

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