Isolation of Methicillin resistant *Staphylococcus aureus* from the environmental samples in Dhaka City

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DECLARATION

I hereby declare that the thesis work titled "**Isolation of Methicillin resistant *Staphylococcus aureus* from the environmental samples in Dhaka City**" has been written and submitted by me, Saria Farheen without the use of other sources than those mentioned. It is further asserted that this Bachelor's Thesis has never been submitted in the same or substantially similar version to any other examinations office. All explanations that have been adopted literally or analogously are marked as such. Any reference to work done by any other person or institution or any material obtained from other sources have been duly cited and referenced.

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Dedicated to my

Parents

Acknowledgement

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Abstract

Methicillin resistant *Staphylococcus aureus* have emerged over more than 50 years ago. The indiscriminate use of antibiotics is responsible for its prevalence. This comparative study has been carried out to show the antibiotic/Methicillin sensitivity of *Staphylococcus aureus* collected from the environmental samples of different locations of Dhaka city. The bacterial isolates were identified depending on their structural, morphological and biochemical characteristics. Antibiogram according to Clinical and Laboratory Standards Institute (CLSI) guidelines, was done in order to perceive the antibiotic susceptibility of the isolates. Antibiogram revealing the sizes of zone of inhibition of the isolates showed that the environmental samples are not exposed to the methicillin resistant *Staphylococcus aureus*. This result indicates that since environmental *Staphylococcus* species are not exposed to antibiotics so they have not achieved resistivity against methicillin. Commercial antibiotic disks of Erythromycin (E), Tetracycline (TE), Oxacillin (OX), Clindamycin (DA) and Vancomycin (VA) were used in disk diffusion process to observe the antibiotic sensitivity. Antibiotics have different mode of actions such as cell wall synthesis inhibitors, membrane permeability alternatives, protein synthesis inhibitors and DNA synthesis inhibitors. The presence of the methicillin sensitive *Staphylococcus aureus* was further ensured from the PCR result. DNA extraction from the samples was done to isolate the DNA template required to carry out PCR. Specific primers mec A P4 and mec A P7 were used in to amplify the methicillin resistant gene and then the amplicons were run on agarose gel electrophoresis to observe the DNA bands. The result showed no bands when viewed under the UV light and thus this indicated the absence of methicillin resistant *Staphylococcus aureus* in the environmental samples. The study will help to predict the future emergence and guide to develop strategies to maintain the sensitivity preventing the antibiotic resistance among the organisms in the environment.

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List of Abbreviations:

CA-MRSA	Community Acquired-Methicillin resistant *Staphylococcus aureus*
HCW	Healthcare workers
IMViC	Indole, Methyl red, Voges-Proskauer, Citrate
LB	Luria Bertani
MIU	Motility Indole Urease
MHA	Muller Hinton Agar
MR	Methyl Red
MSA	Mannitol Salt Agar
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
NA	Nutrient Agar
OD	Optical density
PCR	Polymerase Chain Reaction
TFTC	Too few to count
TNTC	Too numerous to count
TSI	Triple Sugar Iron
VP	Voges-Proskauer

Chapter 1: Introduction

1.1: Overview

Staphylococcus aureus is a gram positive coccal bacterium and a facultative anaerobe. *Staphylococcus* was first identified in 1880 in Scotland by the surgeon Sir Alexander Ogston in pus from a surgical abscess in a knee joint and was later named as *Staphylococcus aureus* by Friedrich Julius Rosenbach. [**31**]

S. aureus is found as part of the normal skin flora, healthy lower reproductive tract of women and in the nostrils. It can cause a range of illnesses such as pimples, impetigo, boils, folliculitis, scalded skin syndrome, abscesses, cellulitis, pneumonia, meningitis, osteomyelitis, toxic shock syndrome, bacteremia, sepsis etc. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections.

Pathogenic strains often promote infections by producing potent proteintoxins and expressing cell-surface proteins that bind and inactivate antibodies. The emergence of antibiotic-resistant strains of *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine. MRSA strains are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in community-acquired infections.

In the general community, MRSA most often causes skin infections. In some cases, it causes pneumonia (lung infection) and other issues. If left untreated, MRSA infections can become severe and cause sepsis - a life-threatening reaction to severe infection in the body. In a healthcare setting, such as a hospital or nursing home, MRSA can cause severe problems such as bloodstream infections, pneumonia and surgical site infections.

1.2: Character and Morphology

Classification of *Staphylococcus aureus* [15]:

Domain: Bacteria
Kingdom: Eubacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Staphylococcaceae
Genus: *Staphylococcus*
Species: *S. aureus*

S. aureus is a facultatively anaerobic, Gram-positive coccus. It appears as grape-like clusters when viewed through a microscope and has round usually golden-yellow colonies. *S. aureus* reproduces asexually by binary fission. It produces colonies having circular shape, convex elevation and entire margin. It usually gives beta-hemolytic colonies on sheep blood agar, when cultivated for 24 hours at aerobic atmosphere of 37°C. Carotenoid pigment staphyloxanthin is responsible for the characteristic golden colour of *S. aureus* colonies. This pigment acts as a virulence factor.
S. aureus is catalase-positive. Catalase converts hydrogen peroxide to water and oxygen. Catalase-activity tests are sometimes used to distinguish staphylococci from enterococci and streptococci. [**33**]

It is a normal inhabitant of the skin and mucous membranes in the nose of a healthy human. *S. aureus* is infectious to both animals and humans and may only survive on dry skin. It can be spread through contaminated surfaces, through the air and through people. Approximately 30% of the normal healthy population is affected by *S. aureus* as it asymptomatically colonizes on the skin of human hosts. Though some host colonization can be benign, a puncture or break in the skin can prompt this bacterium to enter a wound and cause infections. The best preventive measure is simply regular hand washing and daily bathing.

1.3: Emergence of MRSA:

Before antibiotics, a severe infection was fatal for many people. Penicillin was effective in treating infections from *S. aureus* until it became resistant. Throughout the second half of the 20th century, new antibiotics such as methicillin and vancomycin were developed which successfully treated *S. aureus* infections.

By 1950, 40% of hospital *S. aureus* isolates were penicillin-resistant and by 1960, this had risen to 80% [**11**]. MRSA is a dangerous strain of *S. aureus* which have become resistant to most β-lactam antibiotics. The use of different types of antibiotics over the years has led to the emergence of multi-resistant MRSA strains [**27**]. Besides, mutations in genes coding for target proteins and acquisition of antibiotic resistance-conferring genes are responsible for the increasing number of MRSA in the environment.

Vancomycin, a glycopeptide antibiotic, is commonly used to combat MRSA. Vancomycin inhibits the synthesis of peptidoglycan, but unlike β-lactam antibiotics, glycopeptide antibiotics target and bind to amino acids in the cell wall, preventing the formation of peptidoglycan cross-linkages.

A vancomycin-resistant strain of *S. aureus* emerged in Japan in 1996 **[19]**, and strains with partial resistance to vancomycin have been found in the USA, Australia and other countries.

Because of the high level of resistance to penicillins and because of the potential for MRSA to develop resistance to vancomycin, the U.S. Centers for Disease Control and Prevention has published guidelines for the appropriate use of vancomycin. In situations where the incidence of MRSA infections is known to be high, the attending physician may choose to use a glycopeptide antibiotic until the identity of the infecting organism is known. After the infection is confirmed to be due to a methicillin-susceptible strain of *S. aureus*, treatment can be changed to flucloxacillin or even penicillin, as deemed appropriate.

1.4: Mechanism of antibiotic resistance in *S. aureus:*

Staphylococcal resistance to penicillin is mediated by penicillinase production. Penicillinase is an enzyme that cleaves the β-lactam ring of the penicillin molecule and renders the antibiotic ineffective. Penicillinase-resistant β-lactam antibiotics such as methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, flucloxacillin etc. are able to resist degradation by staphylococcal penicillinase.

Resistance to methicillin is mediated via the *mec* operon, part of the staphylococcal cassette chromosome mec (SCC*mec*). The main mechanism of methicillin resistance in *S. aureus* is through the expression of a foreign and altered penicillin-binding protein (PBP) which is PBP2a. PBP2a has a lower affinity for binding β-lactams such as, penicillins, cephalosporins etc. [**17**]. It is resistant to the action of methicillin but which can take over the transpeptidation or the cross-linking reactions of the host PBPs. Synthesis of PBP2a is regulated and normally kept at low level but the level of synthesis can be enhanced if mutations occur in the regulatory genes. This allows for resistance to all β-lactam antibiotics and prevents their clinical use during MRSA infections. MRSA differ genetically from methicillin-sensitive *S. aureus* isolates by the presence of a large foreign DNA (40-60 Kb) which is known as the *mec* element and also by the presence of the *mecA* gene that encodes the 76 KDa penicillin-binding protein, PBP2a (also referred to as PBP2′). The *mecA* gene has been found to originate from *Staphylococcus sciuri* [**38**]. Two genes- *ccrA* and *ccrB*, present on the *mec* element are known to code for recombinase proteins that are capable of excising and integrating the *mec* element into the chromosome [**23**].

In common with other PBPs, PBP2a has the common structural motifs that are associated with penicillin binding yet its affinity for β-lactam antibiotics is greatly reduced. Consequently, at

therapeutic levels of methicillin that inhibits the transpeptidational activities of other PBPs, PBP2a remains active ensuring the cross-linking of the glycan chains in peptidoglycan. PBP2a is not able to completely recompense for the other PBPs since cells grown in the presence of methicillin exhibit a marked reduction in the degree of cross-linking. However, the limited degree of cross-linking is enough to ensure survival of the cell.

1.5: Transmission of MRSA:

The transmission of MRSA is an important source of nosocomial infection. Anyone can get MRSA on their body from contact with an infected wound or by sharing personal items, such as towels or razors that have touched infected skin.
There are two major ways people become infected with MRSA. The first is physical contact with someone who is either infected or is a carrier of MRSA and the second way is for people to physically contact MRSA from objects such as door handles, floors, sinks, or towels that have been touched by a MRSA-infected person or carrier. Normal skin tissue in people usually does not allow MRSA infection to develop; however, if there are cuts, abrasions, or other breaks in the skin such as psoriasis, MRSA may proliferate. Many otherwise healthy individuals, especially children and young adults, do not notice small skin imperfections or scrapes and may be lax in taking precautions about skin contacts. This is the likely reason MRSA outbreaks occur in diverse types of people. People including athletes, daycare and school students, military personnel in barracks and those who recently received inpatient medical care are at higher risk. MRSA infection risk can be increased when a person is in activities or places that involve crowding, skin-to-skin contact and shared equipment or supplies. Studies show that two in every 100 people carry MRSA [**26**].

The recognized risk factors for MRSA infection and colonization included recent hospitalization and other exposures to the health care system, residence in a long-term care facility acute-rehabilitation unit etc. [**8**]. The presence of an indwelling line or catheter, surgical wounds, chronic liver, lung, or vascular disease, malignancy, recent exposure to antibiotics, intravenous drug use ICU admission etc. are also the means of spread of MRSA [**10**].

1.6: Global epidemiology of MRSA

In the pre-antibiotic era, *S. aureus* bacteraemia was usually fatal. In a review of cases in the early 1940s, mortality amongst 122 consecutive patients was 82%, and was 98% in those aged >50 years. In the modern era, it is estimated that 25–35% of healthy human individuals carry *S. aureus* on the

skin or mucous membranes. This means that up to two billion individuals may currently carry *S. aureus* worldwide and conservative estimates based on Dutch and US prevalence data predict that about 2–53 million people carry MRSA.

Staphylococcus aureus is the number one cause of hospital-associated infections and a high proportion of these are caused by MRSA. The mortality rate associated with invasive MRSA infections is estimated to be 20% but varies considerably between studies in different settings. In a recent large European prospective cohort study, mortality and length of stay attributable to *S. aureus* bloodstream infections were evaluated. Results demonstrated the clinical importance of *S. aureus* invasive infections and unequivocally underlined the additional burden imposed by resistance for 30-day mortality.

The evolution of MRSA has paralleled that of penicillin-resistant *S. aureus* from the 1940s. MRSA is now pandemic. MRSA is highly prevalent in hospitals worldwide. Although epidemiological data from separate studies are often not comparable owing to differences in study design and populations sampled, the highest rates (>50%) are reported in North and South America, Asia and Malta. Intermediate rates (25–50%) are reported in China, Australia, Africa and some European countries [e.g. Portugal (49%), Greece (40%), Italy (37%) and Romania (34%)]. Other European countries have generally low prevalence rates (e.g. The Netherlands and Scandinavia).

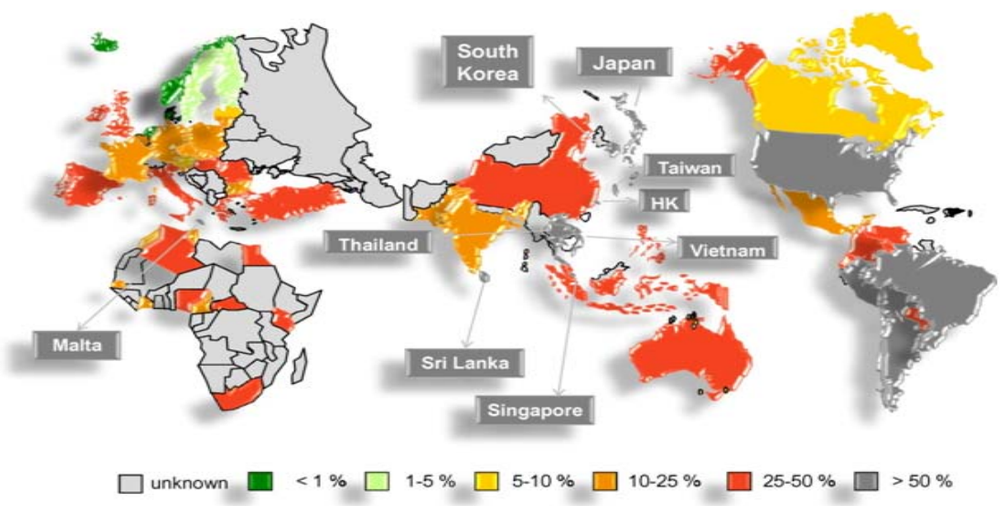

Fig 1: Worldwide prevalence of MRSA (35)

The prevalence of HA-MRSA (hospital-acquired MRSA) has declined in recent years in some European countries, e.g. Austria, France, Ireland, the UK and Greece. In other European countries the prevalence has remained fairly stable. However, very high rates of MRSA are reported in East Asia, especially in Sri Lanka (86.5%), South Korea (77.6%), Vietnam (74.1%), Taiwan (65.0%), Thailand

(57.0%) and Hong Kong (56.8%). In contrast, the values are much lower in India (22.6%) and The Philippines (38.1%).

1.7: Prevention of MRSA spread

The standard precautions as mentioned in the following can help to control the spread of MRSA.

- **Hand Hygiene**
  Hand hygiene needs to be performed after touching blood, body fluids, secretions, excretions and contaminated items, whether or not gloves are worn. Immediately after removing the gloves the hands should be washed properly. When hands are visibly soiled with blood or other body fluids then hands must be washed with soap and water. It may be necessary to perform hand hygiene between tasks and procedures on the same patient to prevent cross-contamination of different body sites.
- **Mouth, nose and eye protection**
  The mucous membranes of the eyes, nose and mouth must be protected during procedures and patient-care activities that are likely to generate splashes or sprays of blood, body fluids, secretions and excretions. Proper masks, goggles and face shields must be used according to the need anticipated by the task performed.
- **Gowning**
  Gown as deemed appropriate to the task must be worn to protect skin and prevent soiling or contamination of clothing during procedures and patient-care activities when contact with blood, body fluids, secretions or excretions is anticipated.
- **Appropriate device handling of patient care equipment**
  Handle used patient-care equipment are usually soiled with blood, body fluids, secretions and excretions in a manner that prevents skin and mucous membrane exposures, contamination of clothing and transfer of microorganisms to other patients and environments. It must be ensured that reusable equipment is not used for the care of another patient until it has been appropriately cleaned and reprocessed and that single-use items are properly discarded. Surfaces that are likely to be contaminated with pathogens, including those that are in close proximity to the patient e.g., bed rails, over bed tables etc. and also those that are frequently-touched surfaces in the patient care environment e.g., door knobs, surfaces etc. must be cleaned and disinfected on a more frequent schedule compared to that for other surfaces.

1.8: Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a technique for the amplification of a single copy or a few copies of DNA to generate thousands to millions of copies of a particular DNA sequence. The method includes the steps of thermal cycling causing alternate heating and cooling of PCR sample through a defined series of temperature steps, primers containing sequences complementary to the target region along with a DNA polymerase that enables selective and repeated amplification. PCR employs a heat-stable DNA polymerase- Taq polymerase which enzymatically assembles a new DNA strand from the nucleotides by using single-stranded DNA as a template and thus initiates DNA synthesis. PCR can be extensively modified to perform a wide array of genetic manipulations.

In this study, PCR has been conducted to identify presence of the methicillin resistance gene in the *S. aureus* using the suitable primers- mecA P4 and mecA P7.

1.9: Agarose Gel Electrophoresis

Agarose gel electrophoresis is the standard lab procedure for separating DNA by size that is, length in base pairs, for visualization and purification. Agarose is a polysaccharide, generally extracted from seaweed. It is frequently used in molecular biology for the separation of DNA by size using electrophoresis. Agarose gels are typically used to visualise fragments of DNA. The concentration of agarose used to make the gel depends on the size of the DNA fragments. Smaller fragments of DNA are separated on higher concentrations of agarose whilst larger molecules require a lower concentration of agarose

Electrophoresis uses an electrical field to move the negatively charged DNA through an agarose gel matrix toward a positive electrode. Shorter DNA fragments migrate through the gel more quickly than longer ones and this process is called sieving. In this way it helps to determine the approximate length of a DNA fragment by running it on an agarose gel alongside a DNA ladder. The DNA bands are visualized in the gel by the addition of ethidium bromide and exposing it under the UV light. The required DNA band can be obtained by comparing the size of the respective band with that of the DNA ladder.

1.10: General Objectives of the study

The objectives of the project include the isolation of specific organism *Staphylococcus aureus* from the environmental samples in Dhaka city, followed by the isolation of the specific gene called methicillin resistant mecA gene by PCR and finally comparative analysis of the clinical and environmental samples through PCR result.

Chapter 2: Materials and Methods

2.1: Working laboratory

All project tasks were executed in the Microbiology and Biotechnology Laboratory, Department of Mathematics & Natural Sciences, BRAC University.

2.2: Sample collection

In this study, 10 environmental samples of *Staphylococcus aureus* have been used. All these species were obtained from different environmental locations of Dhaka city.

Table 1: Sample Collection: Sample Number, Source, Location and number of *S.aureus* isolates

Sample No.	Source	Location	Presence of *Staphylococcus* species
A	Lake Water	Dhanmondi Lake	2/3
B	Jheel water	Hatirjheel	3/5
C	Tea water	Local tea stall at Khilgaon	_
D	Raw fish water	Farmgate fish market	2/6
E	Raw fish water	Adabor fish market	2/5
F	Salad Sauce	Local restaurant at Mohakhali	1/3
G	Vegetable water	Malibagh grocery market	_
H	Dish washing scrub	My home at Malibagh	1/2
I	Door knob	Female washroom of 10th floor of BRAC university	2/4
J	Door knob	Public toilet at Gulshan	2/2
Total			15 0f 30 Isolates

2.3: Method of sample collection:

Autoclaved sterile Duran flasks were used for collecting all the liquid samples. A piece of dish washing scrub was cut using a sterile scissors and placed on 0.85% saline taken on a sterile test tube. Autoclaved sterile cotton swabs were used to collect the species from door knob and then immersed on saline held in a sterile test tube. All samples were inoculated within 30 minutes of collection.

2.4: Preparation of the bacterial plating

- Luria-Bertani (LB) medium was used for enrichment.
- 5 ml of LB broth was prepared into a screw capped test tube.
- 1 ml of each liquid sample was inoculated into 10ml of LB broth
- The inoculated LB medium was then incubated overnight at 37°C
- 1µl of enriched broth was spread plated onto nutrient agar
- The plates were then incubated at 37°C for 24 hour
- Following the incubation, heterotrophic plate count were taken from the nutrient agar
- The count was considered between 30-300 colonies per plate. A count of more than 300 colonies is said to be 'too numerous to count' (TNTC) and that less than 30 colonies is said to be 'too few to count' (TFTC). All the plates for all ten sources showed TNTC count.
- For isolation of *S.aureus*, cultural characteristics of the colonies on the selective media were observed.

2.5: Isolation and identification of the reference strains

For the isolation and identification of *S.aureus* from the collected samples, two selective media were used- Mannitol salt agar (MSA) and Blood agar. The colony morphology and the cultural characteristics of the inoculated samples on these two media ensured the confirmation of *S.aureus*.

2.5.1: Mannitol Salt Agar (MSA):

Mannitol Salt Agar is a selective and differential medium. *Staphylococcus aureus* produce yellow colonies with yellow zones as a result of mannitol fermentation. Whereas, other Staphylococci do not ferment mannitol and produce small pink or red colonies with no color change to the medium. [**9**]

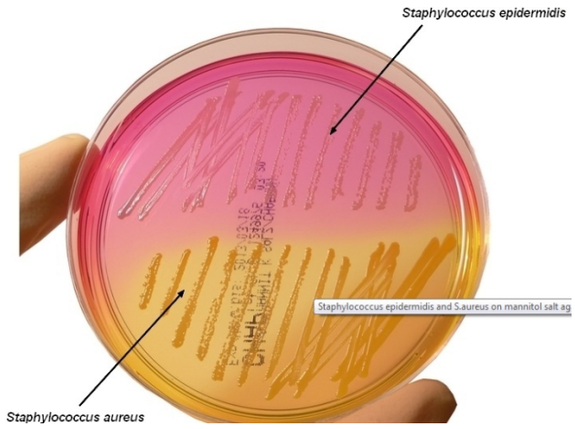

Fig 2: *Staphylococcus aureus* on Mannitol Salt Agar [**39**]

- Sample to be tested was inoculated into 20ml of MSA taken on a medium sized petri plate and incubated at 37^{o}C for 24 hours.
- After the 24 hours incubation, the morphology and cultural characteristics of the colonies on the media was observed to identify and confirm the presence of *S.aureus*
- Appearance of yellow color on the media was taken a positive result for the presence of *S.aureus*
- Further confirmation for the presence of *S.aureus* was done by observing the growth on Blood agar.

2.5.2: Blood Agar:

Blood agar is an enriched and differential medium. Some bacteria produce exoenzymes breaking red blood cells and degrade hemoglobin known as called hemolysins. 3 different types of hemolysis can occur- alpha hemolysis, beta hemolysis and gamma hemolysis. *Alpha-hemolysis*, turns the medium under the bacterial growth brown-green. *Beta-hemolysis* produces clear zone of the medium under the bacterial colonies. Some bacteria leave red blood cells essentially untouched and the medium is not cleared by growth, known as *gamma-hemolysis*. *S.aureus* usually produces beta hemolysis which leaves a clear zone around the bacterial colony. [**9**]

Fig 3: Types of hemolysis on Blood agar

- Samples were inoculated into 20ml of Blood agar taken on a medium sized Petri plate and incubated at 37^{o}C for 24 hours.
- After the 24 hours incubation, the morphology and cultural characteristics of the colonies on the media was observed to identify and confirm the presence of *S.aureus*
- Presence of a clear zone around the colonies that is, beta hemolysis was taken to be positive result for the presence of *S.aureus*
- Biochemical tests were performed next for further confirmation of *S.aureus*

2.6: Biochemical Confirmation of the *S. aureus* isolates

Biochemical tests were performed for the further confirmation of the selected *S.aureus* isolates according to the methods described in the Microbiology Laboratory Manual [**9**]. The biochemical tests performed were indole production test, methyl-red test, Voges-Proskauer test, citrate utilization test, triple sugar iron (TSI) agar test, catalase test, oxidase test, motility indole urease (MIU) test and gram staining. Selected *S.aureus* isolates were grown on the nutrient agar plates with an incubation period of 24 hours at 37^{o} C.

2.6.1: Indole Test

This test determines the ability of the organism to convert tryptophan into the indole by the activity of the tryptophanase enzyme through deamination. Final products of the reaction are indole, pyruvic acid and ammonium.

The selected isolates were incolulated in 6ml of peptone water broth and incubated overnight at 37^{o} C. Following overnight incubation, five drops of Kovac's reagent were added. Then the colors of the cultures were examined and the results were recorded. Formation of a rose red ring at the top of the liquid surface indicates a positive result. A negative result can have a yellow or brown layer. Usually *S.aureus* gives negative result for indole production test.

2.6.2: Methyl Red (MR) test

The MR test detects the production of sufficient acid during the glucose fermentation. The glucose is metabolized to pyruvic acid**,** which is further metabolized through the 'mixed acid pathway' to produce the stable acid. The acid decreases the pH to 4.5 or below indicated by a change in the color of the indicator, methyl red (p-dimethylaminoaeobenzene-O-carboxylic acid) from yellow to red, showing a positive result. Appearance of orange color of the broth indicates a pH of 6 and is considered as a negative result due to insufficient accumulation of acids. *S.aureus* gives positive result for MR test. [**29**]

Bacterial isolates were inoculated into 5 ml of dextrose phosphate broth (MR-VP broth) and incubated at 37^{o}C for 48 hours. Following the 48 hours of incubation, the pH of the medium was tested by the addition of five drops of MR reagent.

2.6.3: Voges-Proskauer (VP) Test

Voges-Proskauer test determines an organism's ability to produce acetoin from glucose fermentation. Pyruvic acid, the end product of glucose metabolism, is further metabolized by using the Butylene glucol pathway to produce end products such as acetoin and 2,3-butanediol. Addition of Barrit's reagent A (40% KOH) and Barrit's reagent B (5% solution of alpha naphthol) can detect the presence of acetoin. Development of a deep rose colour within 15 minutes of the Barritt's reagent addition indicates presence of acetoin and represents a positive result. The absence of a rose colour is a negative result. *S.aureus* gives positive result for VP test. [**29**]

Bacterial isolates were inoculated into 5 ml of dextrose phosphate broth and incubated at 37^{o}C for 48 hours. Following the incubation, 10 drops of Barritt's reagent A was added to the aliquots of each broth cultures and was shaken. Immediately, 10 drops of Barritt's reagent B was added and the cultures were shaken again. The cultures were then kept aside for 15 minutes for the reaction to occur. After 15 minutes, the colors of the cultures were examined and the results were recorded.

2.6.4: Citrate Utilization Test

This test identifies the ability of an organism to use citrate as the sole source of carbon. Organisms that metabolize citrate utilize the ammonium salts releasing ammonia which creates an alkaline environment in the medium and increase the pH of the medium. At pH 7.5 or above, bromthymol blue turns royal blue or Prussian blue indicating a positive result and at a neutral pH, bromthymol blue is green which indicates a negative result. *S.aureus* is usually a citrate positive organism. [**16**]

A single colony collected from the bacterial isolates were stabbed into the slant of 3 ml of Simmon's citrate agar and incubated at 37^{o}C for 24 hours. Following the 24 hours of incubation, the color change of the media was observed and the results were recorded.

2.6.5: Triple Sugar Iron (TSI) test

Triple sugar iron (TSI) agar is a differential medium which differentiates bacteria on the basis of their ability of hydrogen sulphide (H_2S) production and the type of carbohydrate (lactose, glucose, sucrose) fermentation. TSI contains three carbohydrates: glucose (0.1%), sucrose (1%), and lactose (1%). If the organism is able to ferment all the three sugars, then the butt would turn into yellow color indicating the production of acid and the subsequent decrease in the pH of the media. Whereas, a red color in the slant and butt indicates that the organism being tested is a non-fermenter and the media remains alkaline. Sodium thiosulfate in the medium is reduced by some bacteria to hydrogen sulphide which reacts with ferric ions in the medium and produces iron sulphide, a black insoluble precipitate. *S.aureus* usually undergoes acid fermentation on the TSI agar. [**37**]

A single bacterial colony of each of the isolates was picked up from each nutrient agar plates by a needle and stabbed into 6ml of TSI agar. Caps of the tubes were loosened and incubated at 35°C overnight and were observed after 18-24 hours for carbohydrate fermentation, CO_2 and H_2S production.

2.6.6: Motility Indole Urease (MIU) test

This test simultaneously checks indole production, urease hydrolysis and bacterial motility. Motile bacteria move with structures called flagella. In MIU media, motile bacteria 'swarm' and give a diffuse spreading growth that is easily recognized by the naked eye. Indole production in MIU is detected by the presence of rose red ring after the addition of 5 drops of Kovac's reagent to the medium followed by the incubation. The media will turn to deep pink from its original deep orange color if the organism is positive for urease test. [**18**]

5ml MIU media was prepared in a screw capped test tube. A single colony of the bacterial isolate was picked from a nutrient agar plate by a needle and stabbed straight into the test tube. It was then incubated at 37^{0}C for 24 hours to observe the results.

2.6.7: Catalase test

This test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate bacteria that produce catalase, such

as *Staphylococci*, from non-catalase producing bacteria such as *Streptococci*. Catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of this enzyme in a bacterial isolate can be detected when a small amount of the inoculum is introduced into hydrogen peroxide and the rapid formation of oxygen bubbles occurs. [**12**]

$$2H_2O_2 \xrightarrow[\text{catalase}]{} 2H_2O + O_2 \text{ (gas bubbles)}$$

A drop of the catalase reagent (hydrogen peroxide) was taken on autoclaved glass slides. The glass slides were labeled according to the sample being tested. A colony for each of the bacterial isolates was taken from a nutrient agar plate and placed on the reagent drops on the glass slides. An immediate bubble formation indicated a positive result.

2.6.8: Oxidase test

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. When cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) it produces indophenols, which is a purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless. *S.aureus* usually gives negative result for oxidase test. [**22**]

Two drops of oxidase reagent (p- Aminodimethylaniline oxalate) were added on the filter papers.The filter papers were labeled according to the sample being tested. A loopful of each bacterial isolate to be tested was streaked onto the filter paper. A positive reaction would turn the paper from violet to purple within a few seconds.

2.6.9: Gram staining

Gram staining is a used to differentiate two large groups of bacteria on the basis of their cell wall constituents into -Gram positive and Gram negative groups violet. Gram positive bacteria are stained violet as they contain a thick layer of peptidoglycan in their cell walls. This thick layer of peptidoglycan layer can retain the crystal violet which is used for staining purpose. Alternatively, Gram negative bacteria are stained red due to their thinner peptidoglycan wall, which does not retain the crystal violet during the decoloring process. *S.aureus* is a gram positive organism and thus appears violet on staining.

A colony of the bacterial isolate was touched with a burnt loop and placed on a sterile glass slide. A drop of saline was taken by the loop and then the bacterial colony was smeared on the glass slide. The glass slide was heat fixed and the smear was allowed to dry. A drop of crystal violet, the primary stain was added to the smear and after one minute, the crystal violet was carefully washed off the glass

slide. A drop of the mordant, iodine was next added and then after one minute the grams iodine was carefully washed off the slide. A drop of 70% ethanol was added as the decolorizing agent and washed off after 15 seconds. Safranin, the counter stain was added and after 60 seconds it was washed off the glass slide. The slide was allowed to dry off completely, after which it was observed under the microscope.

2.7: Antibiotic disk diffusion

The antibiotic disk diffusion test is done to detect antimicrobial susceptibility in the bacterial isolates and to assure susceptibility to drugs of choice for infections caused by these bacteria. Kirby-Bauer antibiotic testing (also called KB testing or disk diffusion antibiotic sensitivity testing) was used to test whether particular bacteria are susceptible to specific antibiotics.

In this study, the potency of five different commercially available antibiotics was determined. The list of antibiotics used is as follows:

Table 2: Antibiotic disks, their amount, identification number and Zone of Inhibition size [13]

Provided Antibiotic disks	Identification Number and Amount	Disk diffusion diameter (in millimeter, mm)		
		Resistant	Intermediate	Sensitive
Erythromycin	E(15µg)	≤ 13	14-22	≥23
Clindamycin	DA(2 µg)	≤14	15-20	≥21
Tetracycline	TE(3 µg)	≤14	15-18	≥19
Oxacillin	OX(1 µg)	≤10	11-12	≥13
Vancomycin	VA(30 µg)	≤9	-	≥12

2.7.1: Preparation of McFarland Solution

McFarland Turbidity Standard is a precursor for carrying out the antimicrobial susceptibility. McFarland standards are used to adjust the turbidity of both the test and control microorganisms. It allows the tests to be carried out with a defined concentration of the test and control microorganisms and avoids the possibilities of working with a high amount of the bacterial culture. Thus, it ensures that the number of bacteria stays in a given range. This also avoids the possibility of any sort of errors in the results since too much concentrated or diluted bacterial suspension can lead to inappropriate results about the potency of the antimicrobial agents against the test organisms.

2.7.2: Preparation of inoculums of the bacterial isolates:

- The test tubes were labeled carefully with the number of the samples to be prepared
- .85% of physiological saline was prepared and taken on the test tubes
- Using a burnt inoculating loop, one or two colonies of the bacterial isolates were picked up from sub cultured nutrient agar plates and suspended on the saline
- All the test tubes were vortexed properly to make the suspension homogenous

2.7.3: Comparison with the McFarland Solution

- The optical density (OD) of the McFarland solution was measured using the colorimeter
- The OD of all the prepared inoculums was taken using the colorimeter
- This helped to balance the OD of the inoculums in the same range as that of the OD of the McFarland solution
- The inoculums with the same OD to that of the McFarland solution was taken
- The inoculums with a higher OD were diluted to bring the OD back into the reference range
- Finally, all the suspensions were in the same range as the McFarland solution and the inoculums were ready to be inoculated as pure lawn on Muller-Hinton Agar (MHA) plates.

2.7.4: Inoculation on the MHA plates

- For eight test samples, eight different MHA (Muller-Hinton Agar) plates with proper labeling of the samples were prepared
- Autoclaved swab was dipped into the bacterial suspensions and rotated so that it is completely wet with the suspension
- The test tubes having the bacterial suspension were vortexed before dipping the cotton swab
- The swab was then streaked several times on the dried surface of the MHA plate to make a pure lawn ensuring the contact of the cotton of the swab with all the edges of the plate.
- The agar plate was being rotated 90 degrees each time it was being streaked, to ensure the even distribution of the inoculums
- The plates were next allowed to dry out

2.7.5: Placement of the antibiotic disks:

- A burnt sterile forceps was used to insert the antibiotic discs on the MHA plate
- Five different sterile antibiotic discs were placed on the surface of the inoculated MHA plates

- Each of the discs was slightly pressed with the forceps on the MHA plate so that it sticks properly to the agar surface
- The discs were not placed close to the edge of the plates was as the zones will not be fully round and lead to inaccuracy of the test as the measurement cannot be taken properly
- The MHA plates were next inverted and incubated at 37^0C for 24 hours

2.7.6: Measurement of the zone of inhibition

- Following the incubation, the zone of inhibition for each of the antibiotics was observed on the MHA plate
- The size of zones for each antibiotic were measured carefully in millimeters (mm) using a ruler
- All the measurements were taken viewing the back of the Petri dish
- The zone size was recorded on the recording sheet in a chart

2.8: Preparation of stock sample

When good strains of microorganisms are developed, it must be preserved properly for use in future. If the cultures are not properly preserved, their characteristic features may decline after certain period. Consequently production of product also gets decreased.

In this study, two methods have been used for maintaining the organisms in viable conditions over a long period of time.

2.8.1: Long term preservation

The media Trypticase Soy broth was prepared in a sterile cryovial. For long-term preservation, 500µl of bacterial culture was grown in Trypticase Soy Broth at 37^0C for 6 hours. After the incubation period, 500µl of sterile glycerol was added to the broth culture and the cryovial was stored at -20^0C.

2.8.2: Short term preservation

- **Stock:** 3ml of T1N1 media was prepared in sterile vials. Colonies from the bacterial samples to be preserved were touched by a needle from nutrient agar plates and stabbed onto the butt of the vials. Then the vials were incubated at 37^0C for 6 hours. Following the incubation period, 200µl of paraffin oil was added into the surface of the medium contained in each of the vials. All the vials were carefully labeled and stored at room temperature.

- **Subculture:** The selected colonies from nutrient agar were re-cultured onto fresh NA plates for further stocking and biochemical tests.

2.9: Amplification of the template DNA

Polymerase chain reaction (PCR) technique is used to amplify trace amounts of DNA. In this study, PCR is used to enhance the amounts of the unique DNA sequences that is, methicillin resistant gene (mecA), which can then ensure with a very high probability, the presence of MRSA in the environment. In other words, the aim was to determine the presence of mecA genes which will aid us to know whether or not MRSA are abundant in environmental samples.

However, DNA was extracted from the confirmed selected bacterial isolates and then the template was stored until the PCR was being carried out.

2.9.1: Template preparation

- The colony of each of the isolates were taken from the stock and sub-cultured on 5ml of Luria Bertani (LB) medium and incubated at 37°C overnight
- 2ml of each of the overnight *S. auerus* isolates was transferred to Eppendorf tube and centrifuged at 13,500 rpm for 3 minutes to pellet the cells
- Following the centrifugation, the supernatant was discarded to as much as possible without disturbing the cell pellets
- The cell pellets were resuspended in 600µl lysis buffer and vortexed completely to resuspend the cell pellets and incubated at 37^{o}C for 1 hour
- An equal volume (600µl) of phenol: chloroform: isoamyl alcohol in the ratio 24:25:1, was added and mixed well by inverting the tube until the phases are completely mixed
- The tube should not be vortexed as it can shear the DNA
- It was next spun at 13,500 rpm for 5 minutes at room temperature
- A white protein layer appeared in the aqueous interface
- The upper aqueous part was transferred to a new 1.5ml eppendorf tube by using a 1ml pipette
- An equal volume as that of the separated aqueous layer, of chloroform was added and mixed well by inverting the tube
- Next, it was spun at 13,500rpm for 5 minutes
- The aqueous layer was removed to a new eppendorf tube
- 2.5 ml of cold 70% ethanol was next added and mixed gently in order to precipitate the DNA
- The tube was next incubated at -20^{o}C for 30 minutes
- After the incubation, it was spun at 13,500rpm at for 15 minutes

- The supernatant was next discarded and the DNA pellet was rinsed with 1ml of 70% ethanol, kept at room temperature
- It was next spun at 13,500rpm for 3 minutes
- The supernatant was next discarded carefully and the DNA pellet was air dried
- The air dried DNA was resuspended (Tris-ethylenediaminetetraacetic acid) in 25µl of TE buffer
- It was next stored at -20^0 C to be used for PCR run later

2.9.2: Preparation of the Reaction Mixture for PCR

PCR is being carried out in this study to amplify copies of methicillin resistant gene containing DNA. MRSA gene if present, then will b amplified on PCR run and will be visible as bands on electrophoresis gel run.

The Reaction mixture was prepared with the following constituents:

Table 3: Reaction Mixture preparation

Component	Volume
Commercial Mastermix	125µl
Nuclease free water	50µl
Forward primer	25µl
Reverse primer	25µl
Template DNA	2µl
Total	227µl

2.9.3: Primer Sequences used for PCR

Table 4: PCR Primers

Primer name	Primer # (EURLAR)	Sequences	Amplicon size (base pairs, bp)
mecA P4	2821	5'- TCCAGATTACAACTTCACCAGG-3'	162
mecA P7	2822	5'- CCACTTCATATCTTGTAACG-3'	162

2.9.4: Dilution of PCR primers

To get a 100µL 5µM solution, 5µL of 100µM oligo stocks was mixed with 95µL of TE buffer.

2.9.5: Conditions for PCR

The Polymerase Chain Reaction (PCR) method has 3 main steps:

- **Denaturing:** Separation of the double stranded DNA template at a high temperature to give single stranded DNA
- **Annealing:** Primers anneal to the single stranded target DNA sequence
- **Elongation:** DNA polymerase extends the primers by adding deoxyribonucleotide triphosphate (dNTPs) to the phosphate backbone

For 30 thermal cycles, the following conditions were followed:

Table 5: PCR Thermal Cycling parameters

Temperature		Time	Purpose
Step 1:	94^{o} C	5minutes	Initial Denaturation
Step 2:	94^{o}C	30 seconds	Denaturation
	69^{o}C	1 minute	Primer anneaing
	72^{o}C	1 minute	Elongation
Step 3:	72^{o}C	10 minutes	Final Extension Step
Step 4:	4 ^{o}C	Until use in future	Storage

2.9.6: Agarose Gel Electrophoretic Analysis of Amplified DNA Product

- To prepare 100ml of a 1.5% agarose solution, 1.5g agarose was measured on an electric balance and 100ml of 1X buffer was added to it
- This solution was heated in a microwave oven until the agarose dissolved and the solution became transparent
- When the temperature went down a little, 2µl of ethidium bromide dye (EtBr) was added. EtBr dissolves on the major groove of the DNA and makes the DNA band visible when viewed under the ultra violet (UV) light
- The solution was poured into the gel tray and the comb set having 12 wells capacity, was placed close to one end of the gel
- The gel was left at room temperature for 10 minutes to allow uniform solidification
- Afterwards the comb was gently removed and the gel tray with the gel was placed in the electrophoresis chamber
- The electrophoresis chamber was filled with TBE buffer keeping the wells submerged under the buffer
- 3µl of the 100bp DNA ladder along with 2µl of loading dye, was taken on the first well
- 2µl of gel loading dye was added for every 6µl of DNA solution and inserted on different wells placed side by side
- The sequence followed to load the samples in the wells were as follows- DNA ladder, Positive Reference Strain, Negative control- water, A2, B1, D3, E3, F3, H2, I2 and J1.
- The gel was run at 60 volts for about 1 hour until the run was complete
- Following the gel run, the gel was viewed under the short wave UV light for desired DNA band detection.

CHAPTER 3: Results

3.1: Confirmation of *Staphylococcus aureus*:

10 different environmental samples of *S. aureus* were collected from different locations of Dhaka city. The samples were enriched in LB broth and after enrichment, the culture broth was subjected to a two folds dilution. The samples from the appropriate dilutions were next spread plated on the Nutrient agar to identify the heterotrophic count. For the isolation of staphylococcal species, two selective media- Blood agar and Mannitol Salt agar were used. From 10 samples, 30 isolates have been obtained and culture on the selective media ensured the presence of 15 staphylococcal isolates. Colonies showing typical morphological characteristics of *Staphylococcus* were further taken for biochemical tests. A series of biochemical tests- IMVIC, MIU test, TSI test, Gram staining etc. investigated the presence of *S.aureus*. Through the biochemical tests, 8 *S.aureus* isolates were confirmed from the 15 staphylococcal isolates.

Table 6: Heterotrophic count of the 10 samples collected

Sample number	**Nutrient Agar**			
	Raw	**10^{-1}**	**10^{-2}**	**CFU**
A – Dhanmondi lake water	TNTC	TNTC	52	$5.2*10^5$
B – Hatirjheel	TNTC	TNTC	72	$7.2*10^5$
C – Tea water	TNTC	TNTC	54	$5.4*10^5$
D – Raw fishwater	TNTC	TNTC	65	$6.5*10^5$
E – Raw fishwater	TNTC	TNTC	56	$5.6*10^5$
F – Salad sauce	TNTC	TNTC	59	$5.9*10^5$
G – Raw vegetable	TNTC	TNTC	75	$7.5*10^5$
H – Dish washing scrub	TNTC	TNTC	68	$6.8*10^5$
I – Doorknob	TNTC	TNTC	62	$6.2*10^5$
J – Doorknob	TNTC	TNTC	68	$6.8*10^5$

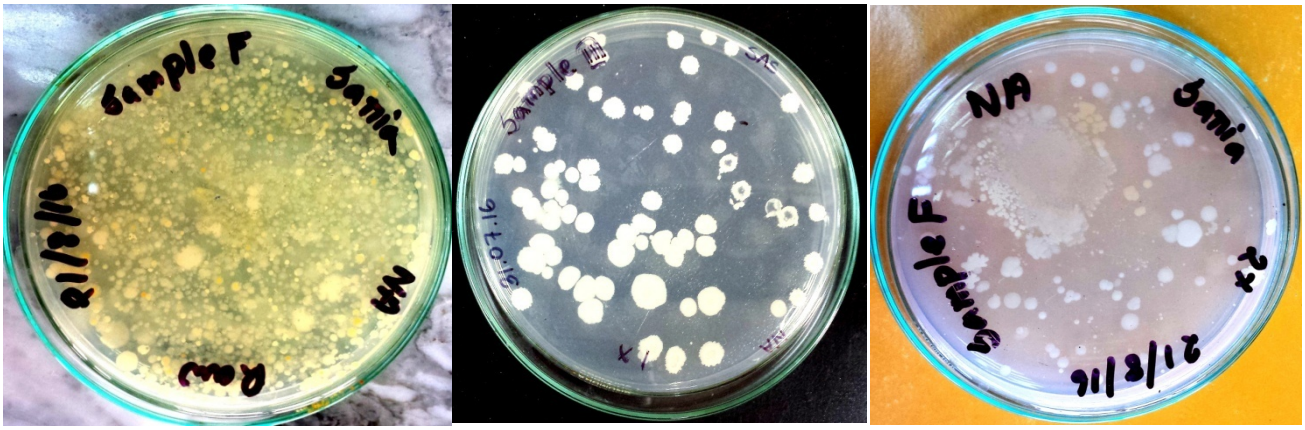

Fig 4: Heterotrophic bacterial count on nutrient agar on different dilution folds

The cultural and morphological features of the isolates as seen on the Mannitol Salt Agar and Blood agar are stated on the table below:

Table 7: Cultural characteristics of 15 Staphylococcus isolates on selective media

Isolates no.	**Shape**	**Elevation**	**Margin**	**Consistency**	**Media color change (MSA)**	**Hemolysis On Blood Agar**
A2	Circular	convex	Entire	Smooth	Pink to yellow	β hemolysis
A3	Circular	convex	Entire	Creamy	Pink to yellow	β hemolysis
B1	Circular	convex	Entire	Smooth	Slight yellow	Slight β hemolysis
B3	Circular	convex	Entire	Smooth	Pink to yellowish	β hemolysis
B5	circular	convex	Entire	Creamy	Pink to yellow	Scarce β hemolysis
D1	Circular	convex	Entire	Smooth	Pink to yellow	β hemolysis
D3	Circular	convex	Entire	smooth	Pink to slight yellow	β hemolysis

E1	Circular	slight convex	Entire	creamy	Pink to yellowish	Abundant β hemolysis
E3	Circular	convex	Entire	smooth	Slight yellow	β hemolysis
F3	Circular	convex	entire	smooth	Slight yellow	β hemolysis
H1	Circular	slight convex	entire	smooth	Pink to yellow	Very scarce β hemolysis
I1	Circular	convex	entire	creamy	Pink to yellow	Abundant β hemolysis
I2	Circular	convex	entire	smooth	Pink to yellow	β hemolysis
J1	Circular	convex	entire	smooth	Slight yellow	β hemolysis
J2	Circular	convex	entire	creamy	Slight yellow	β hemolysis

*******A2, A3= Dhanmondi lake water

B1, B3, B5= Hatirjheel water

D1, D3= Fish water from Farmgate

E1, E3= Fish water from Adabor Market

F3= Salad water from Mohakhali local restaurant

H1= Dish washing scrub

I1, I2= Female toilet of 10^{th} floor of BRAC University

J1, J2= Gulshan public toilet

The confirmed Staphylococcal isolates were chosen to undergo the biochemical tests in order to get *S. aureus* isolates. The isolates showed patterns of biochemical reactions that are typical for each strain of bacteria according to Microbiology Laboratory Manual [**9**].

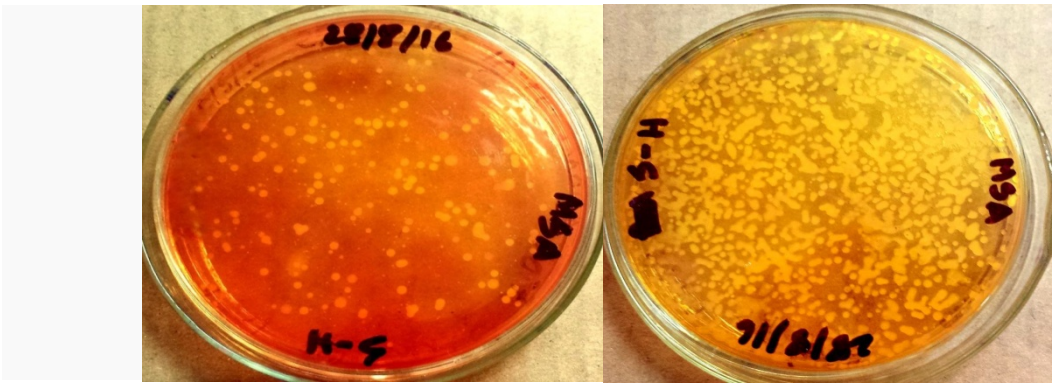

Fig 5: Bacterial growth on the Mannitol Salt Agar media

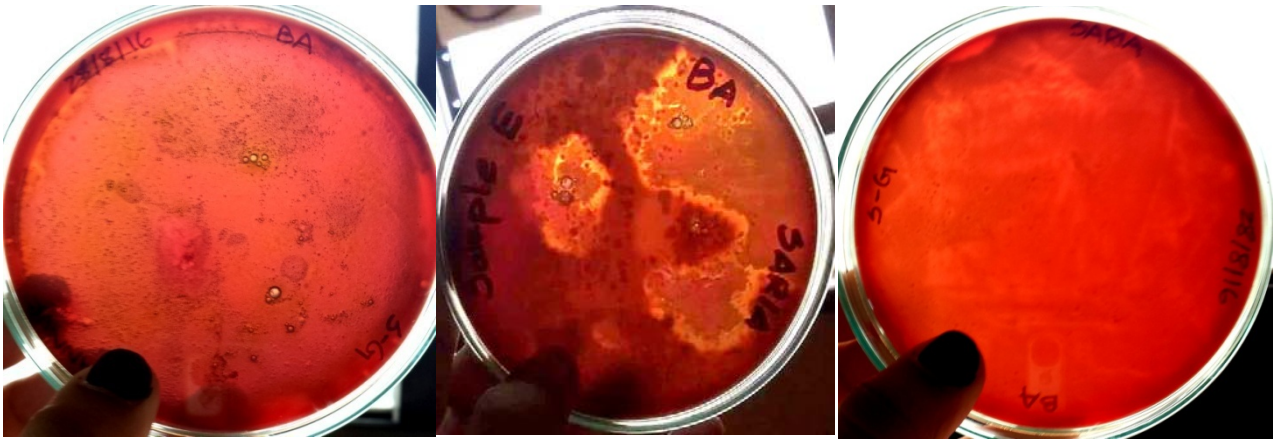

Fig 6: Alpha, Beta and Gamma hemolysis on the Blood Agar media

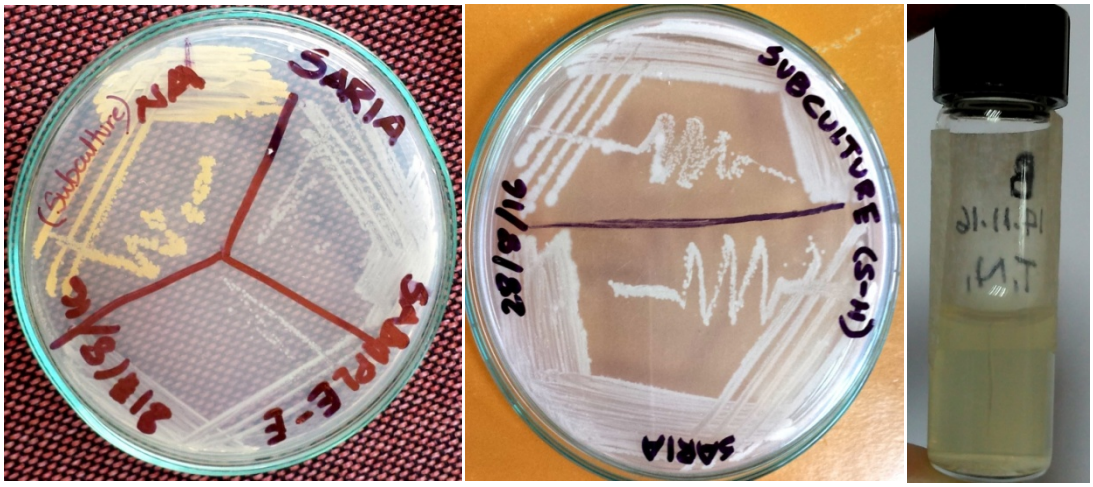

Fig 7: Subculture and stock used for preservation purpose

3.2: Biochemical Identification

Biochemical tests were conducted for the 15 selected staphylococcal isolates. Among these, 10 of the isolates showed standard results for the *S.aureus*. Standard results of the biochemical tests of the isolates are as follows:

Table 8: Biochemical Identification Results

Isolate no.	IMViC				Catalase test	Oxidase test	MIU Test		Triple Sugar Iron (TSI) test			
	Indole Test	Methyl Red Test	Voges Proskaur Test	Citrate Utilization Test			Motility	Urease	Slant	Butt	H_2S	Gas
A2	_	+	+	+	+	_	_	+	A	A	_	_
A3	_	+	+	_	+	_	+	_	A	K	_	_
B1	_	+	+	+	+	_	_	+	A	A	_	+
D3	_	+	+	+	+	_	_	+	A	A	_	_
E1	_	+	+	_	+	_	_	_	A	A	_	_
E3	_	+	+	+	+	_	_	+	A	K	_	_
F3	_	+	+	+	+	_	_	+	A	K	_	_
H1	_	+	+	+	+	_	_	_	A	A	_	+
I2	_	+	+	+	+	_	_	+	A	A	_	_
J1	_	+	+	+	+	_	_	+	A	A	_	_

KEY: + = positive, - = negative, A= acidic condition (Yellow), K= alkaline (Red) condition

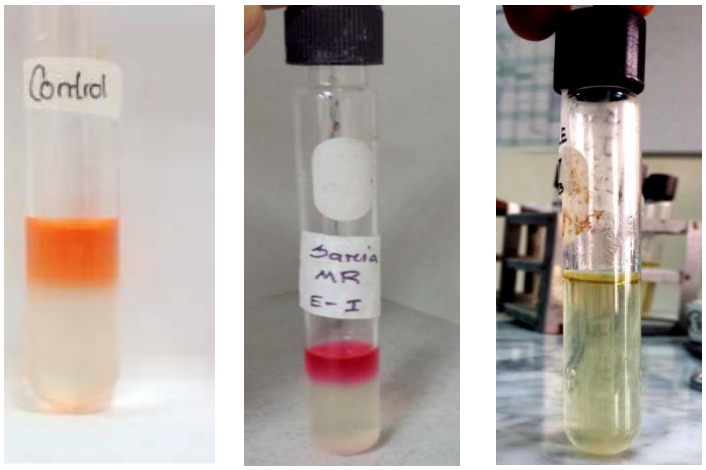

Fig 8: Control, Positive and Negative results in Methyl Red test

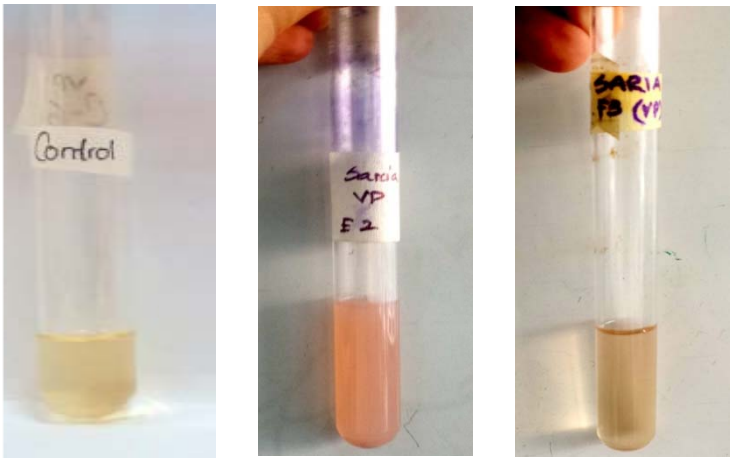

Fig 9: Control, Positive and Ne gative results in Voges Proskauer test

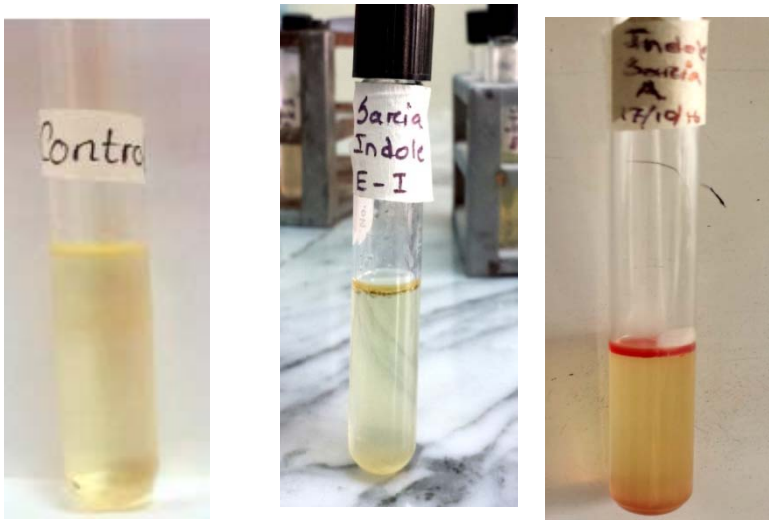

Fig 10: Control, Negative and Positive results in Indole test

Fig 11: Bacterial motility and urease production in MIU media

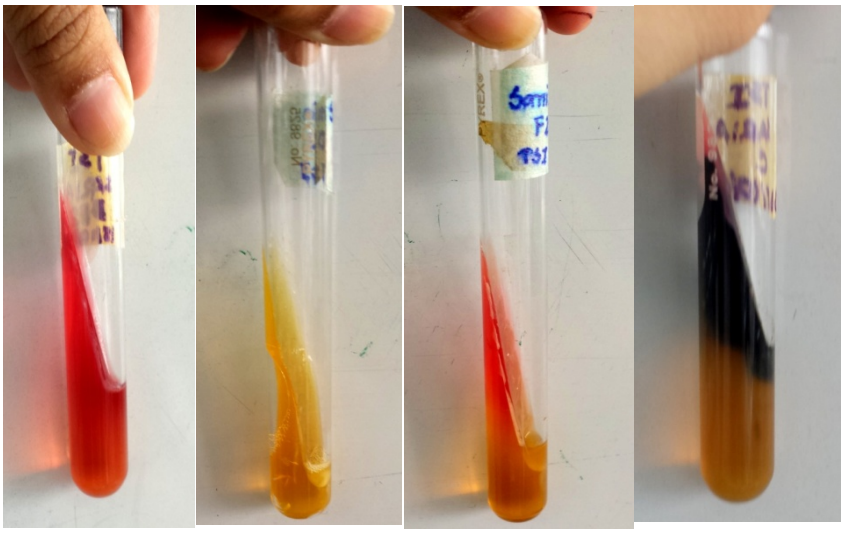

Fig 12: TSI test showing control, positive result with yellow butt and slant and gas production and negative result with H_2S production

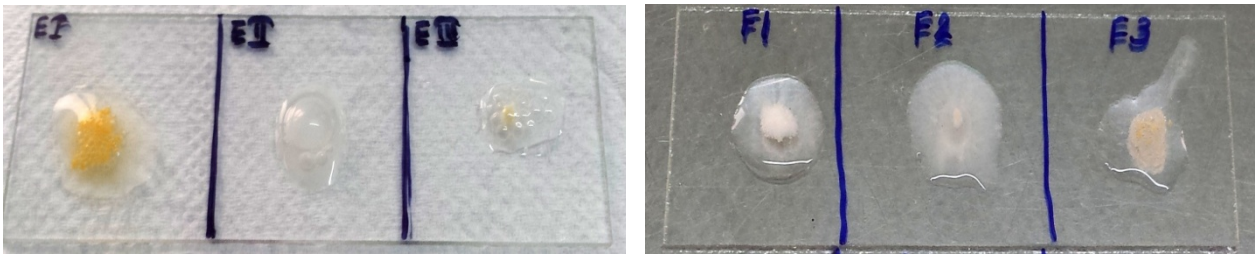

Fig 13: Catalase test showing positive result with bubble formation and negative result having no bubbles

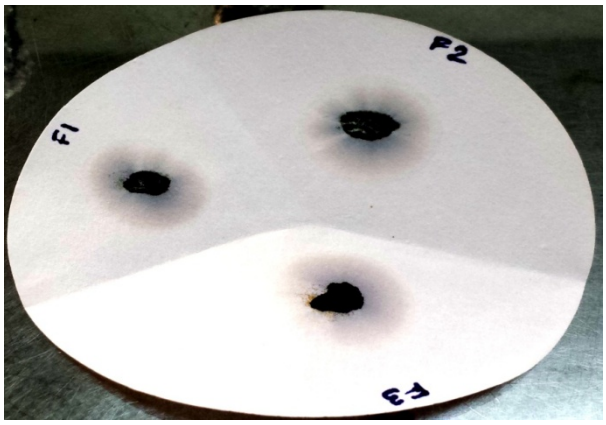

Fig 14: Oxidase test showing negative result

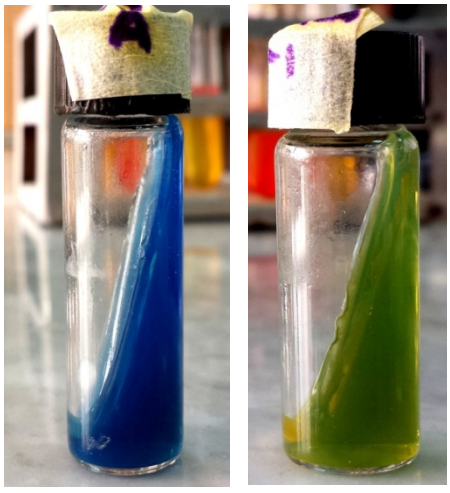

Fig 15: Citrate utilization test showing positive result with blue media and negative result with original green media

3.3: Morphology confirmation of the isolates through gram staining

The morphology of the confirmed isolates from the biochemical test was checked through the gram staining. *S. aureus* are usually gram positive organisms and would give purple color on staining as seen under the microscope.

Table 9: Morphological properties of the isolates from gram staining

Isolates	Shape under microscope	Color after staining	Arrangement	Inference
A2	Cocci	Purple	Cluster	Gram positive
A3	Rod	Purple	Free	Gram positive
B1	Cocci	Purple	Free and cluster	Gram positive
D3	Cocci	Purple	Free and cluster	Gram positive
E1	Rod	Purple	Free	Gram positive
E3	Cocci	Purple	Cluster	Gram positive
F3	Cocci	Purple	Cluster	Gram positive
H1	Cocci	Purple	Free and cluster	Gram positive
I2	Cocci	Purple	Cluster	Gram positive
J1	Cocci	Purple	Cluster	Gram positive

After conducting all the biochemical and morphological tests, 8 isolates were chosen- A2, B1, D3, E3, F3, H1, I2 and J1, which gave very identical results when compared with the standard results. The research work from here was next conducted with these 8 isolates and their antibacterial resistance pattern were tested, observed and compared.

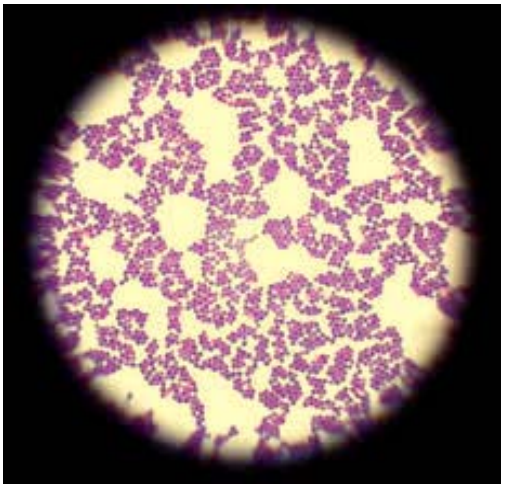

Fig 16: Gram staining showing positive result for *Staphylococcus aureus*

3.4: Selective antimicrobial activity test by means of antibiogram method

The confirmed 8 isolates were chosen to carry out the standard disc diffusion test. The test was carried out with five commercial antibiotics- Erythromycin (E 15), Tetracycline (TE 3), Oxacillin (OX 1), Clindamycin (DA 2) and Vancomycin (VA 30). The size of zone of inhibition indicates the resistance and sensitivity of the organisms to the respective antibiotics. The antibiotic disk diffusion test carried out for each of the 8 isolates showed the following results.

Table 10: Antibiotic susceptibility test of A1 isolate (Dhanmondi Lake)

Antibiotic Disks	Diameter of zone of inhibition(mm)
E 15	26 (Sensitive)
TE 3	30 (Sensitive)
OX 1	15 (Sensitive)
DA 2	24 (Sensitive)
VA 30	22 (Sensitive)

Table 11: Antibiotic susceptibility test of B1 isolate (Hatirjheel)

Antibiotic Disks	Diameter of zone of inhibition(mm)
E 15	21 (Intermediate)
TE 3	22 (Sensitive)
OX 1	20 (Sensitive)
DA 2	12 (Resistant)
VA 30	7 (Resistant)

Table 12: Antibiotic susceptibility test of D3 isolate (Raw Fish water from Farmgate)

Antibiotic Disks	Diameter of zone of inhibition(mm)
E 15	0 (Resistant)
TE 3	20 (Sensitive)
OX 1	16 (Intermediate)
DA 2	22 (Sensitive)
VA 30	8 (Resistant)

Table 13: Antibiotic susceptibility test of E3 isolate (Raw fish water from Adabor)

Antibiotic Disks	Diameter of zone of inhibition(mm)
E 15	8 (Resistant)
TE 3	18 (Intermediate)
OX 1	14 (Sensitive)
DA 2	14 (Intermediate)
VA 30	12 (Sensitive)

Table 14: Antibiotic susceptibility test of F3 isolate (Salad Sauce from Mohakhali)

Antibiotic Disks	Diameter of zone of inhibition(mm)
E 15	10 (Resistant)
TE 3	20 (Resistant)
OX 1	26 (Sensitive)
DA 2	11 (Intermediate)
VA 30	9 (Resistant)

Table 15: Antibiotic susceptibility test of H1 isolate (Dish washing scrub)

Antibiotic Disks	Diameter of zone of inhibition(mm)
E 15	8 (Resistant)
TE 3	18 (Intermediate)
OX 1	0 (Resistant)
DA 2	0 (Resistant)
VA 30	2 (Resistant)

Table 16: Antibiotic susceptibility test of I2 isolate (Door knob of BRAC University washroom)

Antibiotic Disks	Diameter of zone of inhibition(mm)
E 15	10 (Resistant)
TE 3	20 (Intermediate)
OX 1	0 (Resistant)
DA 2	0 (Resistant)
VA 30	4 (Resistant)

Table 17: Antibiotic susceptibility test of J1 isolate (Door knob of Gulshan public toilet)

Antibiotic Disks	Diameter of zone of inhibition(mm)
E 15	20 (Intermediate)
TE 3	4 (Resistant)
OX 1	0 (Resistant)
DA 2	0 (Resistant)
VA 30	0 (Resistant)

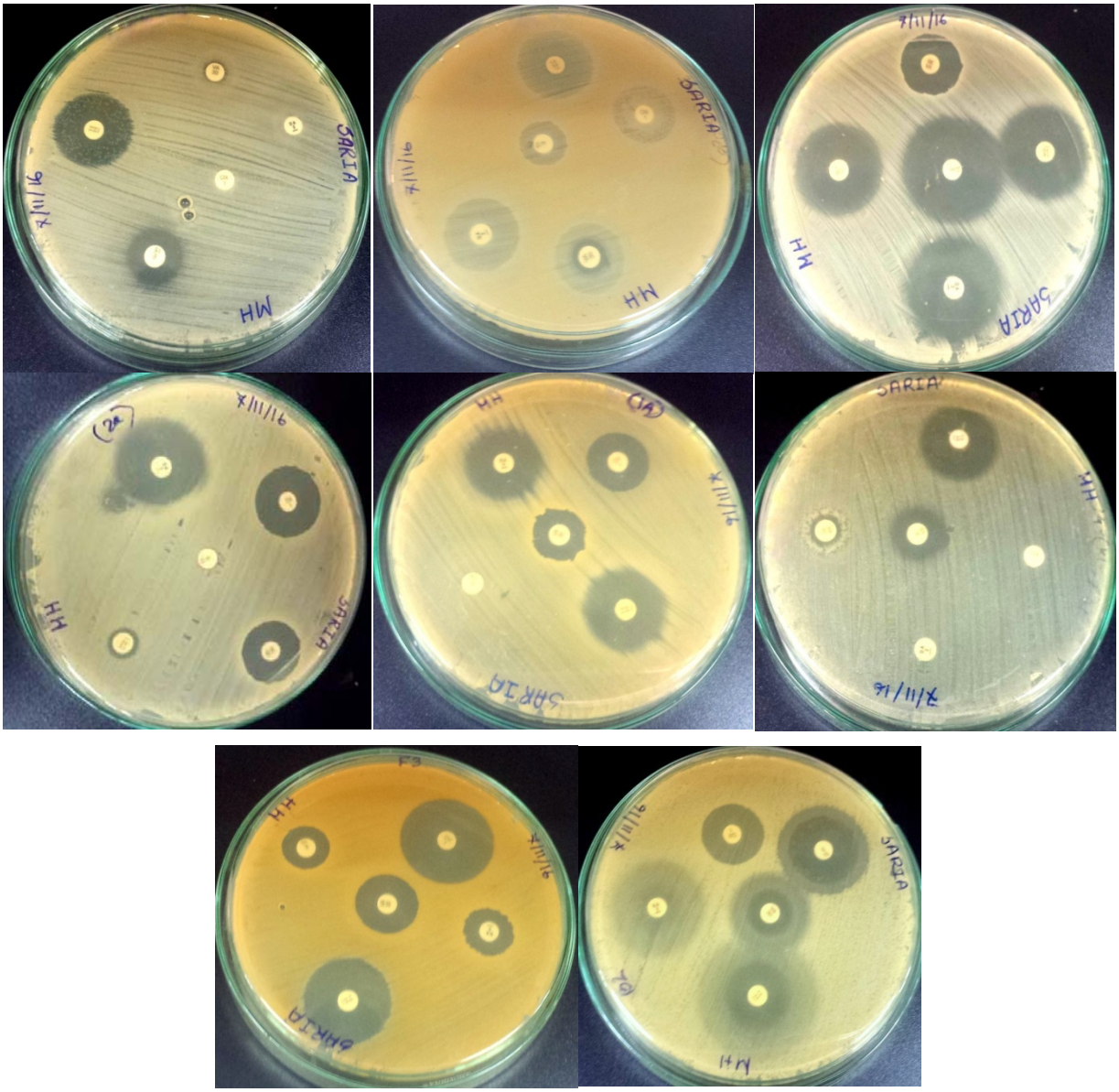

Fig 17: Antibiotic susceptibility test result of the confirmed 8 *S. aureus* isolates

3.5: Analysis of antibiotic susceptibility profiles of the isolates

The profile for the susceptibility of the environmental strains of *S.aureus spp* showed a very significant pattern. It can be observed that a particular antibiotic did not have the same effect on all the environmental strains of *S. aureus*. There are various factors that have influenced these variations which can be only concluded after carrying out PCR with these 8 *S. aureus* isolates.

A graph showing the pattern of antibiotic susceptibility of the different environmental isolates is given as follows:

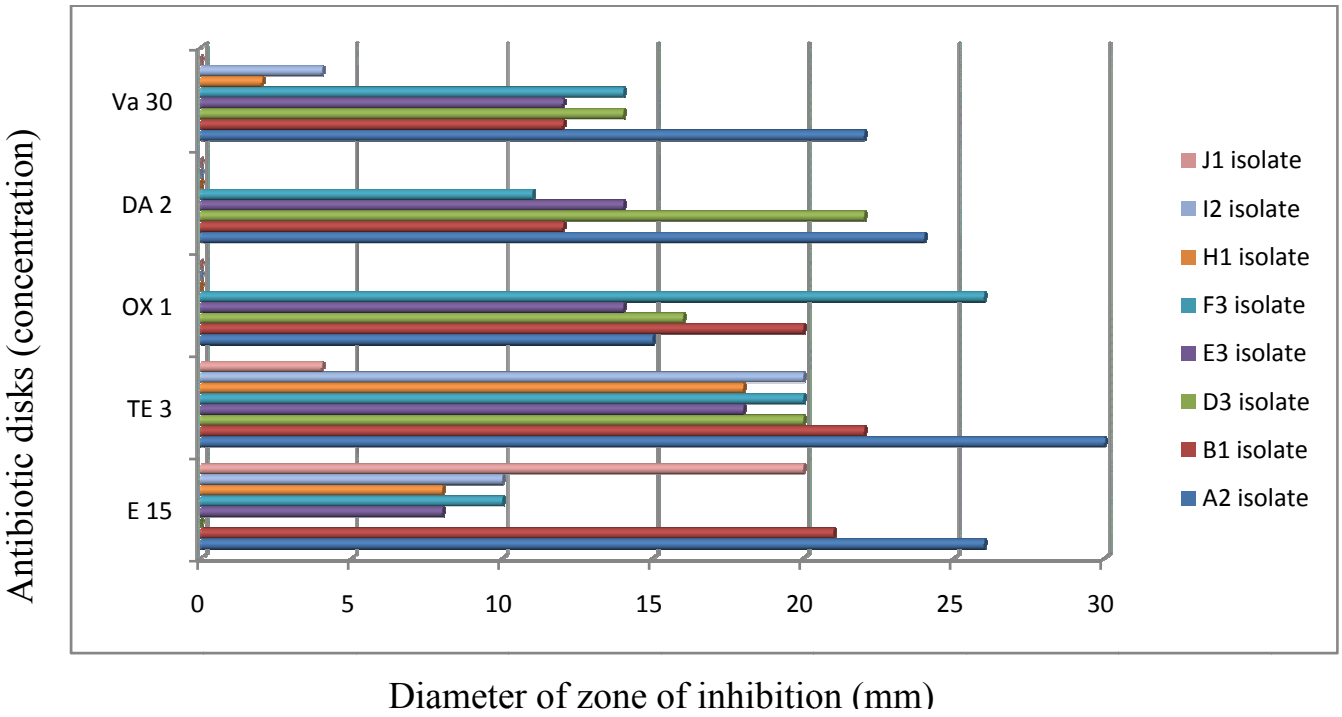

Fig 18: Efficiency of different antibiotics on the environmental strains of *S. aureus*

3.6: Genotypic Characterizations of the Isolates by PCR

DNA from the isolates was extracted in order to amplify the desired MRSA gene through the PCR. Template DNA was prepared from biochemically identified isolates by the Phenol-Chloroform method. 2µl of template DNA was subjected to PCR to increase the chances of detecting MRSA gene of *S.aureus* by using specific primers- mecA P4 and mecA P7.

The PCR result is illustrated in the figure as follows:

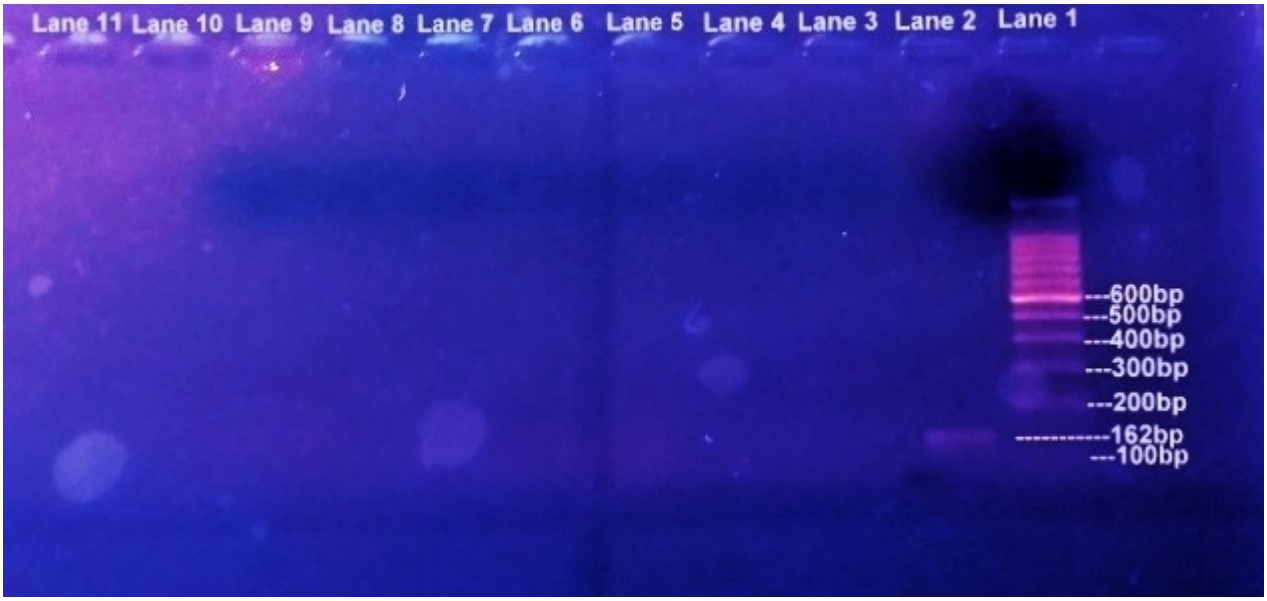

Figure 19: Gel Electrophoresis of *S.aureus* samples

Lane 1: **100bp DNA ladder**

Lane 2: **Positive Control-Reference strain**

Lane 3: **Negative Control- Water**

Lane 4: A2 isolate

Lane 5: B1 isolate

Lane 6: D3 isolate

Lane 7: E3 isolate

Lane 8: F3 isolate

Lane 9: H2 isolate

Lane 10: I2 isolate

Lane 11: J1 isolate

The PCR results were found negative for the presence of MRSA gene in any of the isolates. The absence of MRSA gene was confirmed by comparing the PCR result with that of MRSA positive PCR result of another study which is given as follows:

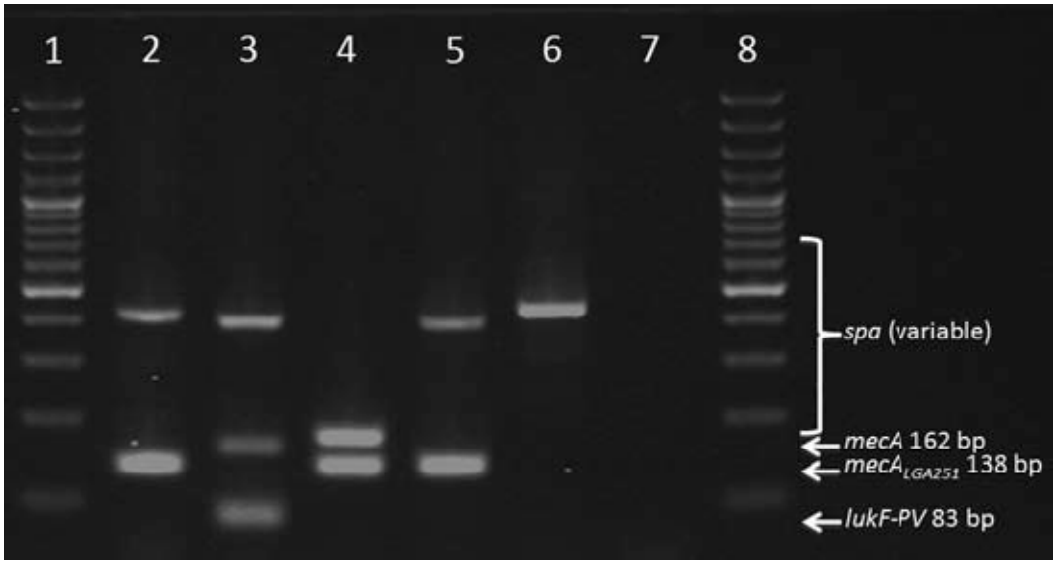

Figure 20: Multiplex PCR for detection of *mecA*, *mecC (mecALGA251*), *lukF-PV (PVL)* and *spa* [**36**]

Chapter 4: Discussion and Conclusion

From observing the result, it can be said that none of the samples collected from the environmental locations contained MRSA. The morphological and cultural characteristics as seen from the biochemical tests, confirmed the presence of *S. aureus*. The presence of *S. aureus* was further confirmed from the gram staining mechanism and the confirmed isolates were next taken for antibiotic susceptibility test. The confirmed 8 isolates exhibited the following pattern of antibiotic susceptibility:

Table 18: Antibiotic sensitivity pattern of the confirmed *S. aureus* isolates

Antibiotics	% of Susceptibility
Erythromycin	12.5
Tetracycline	37.5
Oxacillin	50
Clindamycin	25
Vancomycin	37.5

This result is compared with that of the susceptibility pattern of MRSA found in another study as follows:

Table 19: Antibiotic susceptibility pattern

Types of Antibiotics	% MRSA
Oxacillin	0
Cefoxitin	0
Azithromycin	37.2
Chloramphenicol	86.7
Clindamycin	54.9
Erytromycin	23
Gentamicin	47
Levofloxacin	42.5
Linezolid	96.5
Nitrofurantin*	90.3
Penicillin G	5.3
Rifampin	85.8
Tetracycline	58.4
Trimeth/Sulpha	58.4
Teicoplanin	97.3
Vancomycin	100

The comparison of the two results can help to reach to the conclusion that there is no MRSA present in the 8 isolates since the sensitivity pattern does not match. [**4**]

However, from the PCR result as mentioned earlier, it is very clear that the collected environmental samples lack the presence of MRSA. This may be since the environmental samples do not come in contact with the antibiotics so they are found to be sensitive to methicillin and other antibiotics.

It has been mentioned earlier that the transmission of MRSA is an important source of nosocomial infection. The prevalence of methicillin resistance among *S. aureus* isolates in intensive care units in the United States is 60 percent [**30**] and more than 90,000 invasive infections due to MRSA occurred in the United States in 2005 [**25**]. Healthcare workers (HCWs) are most likely to be important in the transmission of MRSA, but more frequently act as vectors, rather than being the main sources of MRSA transmission [**3**].The most important mode of MRSA transmission is through contamination of the hand. An alternative mechanism of transmission is airborne dispersal of staphylococci in association with an upper respiratory tract infection. Colonised HCW are most

often transiently colonised, but they may become persistent carriers if they have chronic dermatitis or sinusitis and this may lead to prolonged MRSA transmission [**14**].

Studies show that antimicrobial resistance is wider for nosocomial-acquired MRSA [**28**]. Methicillin-resistant *Staphylococcus aureus* may be nosocomial or community-acquired. MRSA is defined as community-acquired if the positive culture was obtained outside hospital settings or within two days of hospital admission, and if the subject had no prior history of hospitalization within the preceding two years [**34**]. Boyce reviewed the incidence of community-acquired MRSA and he pointed out that it is difficult to prove that the organism was not acquired as the result of a health care facility visit [**6**]. Other authors suggested that MRSA incidence is low in the community [**1**]. Because MRSA colonization acquired in a hospital may persist for months to years and given that most studies of community-acquired MRSA (CA- MRSA) conducted in the 1990s did not exclude patients who had been hospitalized during the year preceding their MRSA infection, the frequency of true CA-MRSA has been a matter of considerable debate.

In Bangladesh, the occurrence of MRSA was alarming due to indiscriminate use of antibiotics [**32**]. In 1991, the prevalence of MRSA was reported as 62.61% in clinical environment when even methicillin was not yet introduced in Bangladesh market [**24**]. However, in 2002, 47.2% MRSA was investigated on clinical *S. aureus* isolates [**32**]. In 2011, the prevalence of MRSA on clinical samples has been found to be 43·48% [**20**]. From these findings, it can be said that the prevalence of MRSA has been high on clinical environment due to the indiscriminate use of antibiotics among the patients and also due to the health care environment being exposed to patients carrying this organism. The environmental samples as collected in this study did not show the presence of MRSA may be as a result of not being exposed to the antibiotics and the conditions that will cause mutation in the *S. aureus* gene to make it methicillin resistant.

In an investigation, among the 23 clinical isolates of *S.aureus*, 43.48% isolates were classified as methicillin resistant and 56.52% isolates were found to be methicillin-sensitive (MSSA) by disk diffusion method using 1 µg oxacillin disk [**20**]. So taking this finding into consideration, it can be said that even in clinical set up, the prevalence of MRSA has been found to be less than that of the methicillin sensitive *S.aureus* (MSSA) and so the absence of MRSA in environmental samples as found in my dissertation can be considered a justified hypothesis.

From Table 18 [**4**], it is seen that the MRSA gives 100 percent susceptibility for vancomycin, whereas, in this study a susceptibility percentage of 37.5 is found for vancomycin. This

comparison can conclude that the collected *S.aureus* isolates did not have MRSA gene in the nuclear material and the hypothesis stands correct.

Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria are more and more prevalent in the hospital environment and represent a challenge to infection control practices in most countries [**2**]. Incidence and prevalence of MRSA varies widely between countries, geographical regions, hospitals and even wards in the same hospital [**5**]. The main mechanism of transmission of infections within hospital is by direct contact, in particular with the hands of health professionals, which may both contaminate or be contaminated by hospital surfaces [**7**].

In conclusion it can be said that unlike the environmental condition, hospital atmosphere is a complicated ecosystem and many interventions are needed for optimal infection control. Hospitals provide reservoirs of multi resistant microorganisms borne by patients and staff. Lack of a universal procedure for surveillance of nosocomial infection, poor hand hygiene and high level of bacterial contamination on hospital environmental surfaces are the reasons for the high prevalence of MRSA. Preventing the spread of relevant bacteria depends on the quality of hospital routine cleaning services. Monitoring of bacterial susceptibility to antimicrobials and disinfectants is necessary to manage nosocomial infections and become aware of the MRSA in hospitals and community arenas in Bangladesh.

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APPENDIX I

Media composition

The composition of the media used in the present study has been given below. Unless otherwise mentioned, all the media were autoclaved at 121°C for 15 min.

1. Nutrient Agar (Himedia,India)

Ingredients	Amount (g/L)
Peptic digest of animal tissue	5.0
Beef extract	1.50
Sodium chloride	5.0
Yeast extract	1.50
Agar	15.0

2. Nutrient Broth (Oxoid, England)

Ingredients	Amount(g/L)
Lab-lemcopowder	1.0
Yeastextract	2.0
Peptone	5.0
Sodiumchloride	5.0

3. T1N1 soft agar

Ingredients	Amount(g/L)
Tryptone	0.6 g
Sodiumchloride	0.3g
Agar	0.42

4. Simmon's citrate agar (Oxoid, England)

Ingredients	Amount(g/L)
Magnesiumsulfate	0.2
Ammonium dihydrogenphosphate	0.2

Ammoniumphosphate	0.8
Sodiumcitrate	2.0
Sodiumchloride	5.0
Agar	15.0
Bactobromthymolblue	0.08

5. MR-VP broth

Ingredients	Amount(g/L)
Peptone	7 g
Dextrose	5 g
Potassiumphosphate	5 g

6. Tryptone soy broth, (Oxoid, England)

Ingredients	Amount(g/L)
Pancreatic digest of Casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Di-basic potassium phosphate	2.5
Glucose	2.5

7. Triple sugar iron agar (Himedia, India)

Ingredients	Amount(g/L)
Peptic digest of animal tissue	10.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulfate	0.20
Sodium thiosulfate	0.30
Casein enzymatic hydrolysate	10.0

Yeast extract	3.0
Beef extract	3.0

8. Peptone Water

Ingredients	Amount(g/L)
Peptone	10
Sodium chloride	5

9. Luria Bertani broth

Ingredients	Amount(g/L)
Tryptone	1
Yeast extract	0.5
Sodium chloride	1
Bacto agar	1.5

10. Mannitol Salt agar (Oxoid, England)

Ingredients	Amount(g/L)
Peptone	10.0
Manitol	10.0
Lab-lemco powder	1.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0

11. Blood Agar

Ingredients	Amount(g/L)
Peptone	0.5
Beef extract	0.3
Sodium chloride	0.5
Sheep blood	5
Agar	15

12. Motility Indole Urease Agar

Ingredients	Amount(g/L)
Protease Peptone Difco	10
Beef extract	5
Sodium Chloride	5
KH_2PO_4	2
Phenol Red	6
Agar	15

APPENDIX-II

Buffers and reagents

1. Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4 and 2.0 g of KH2PO4 in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl. The final volume was adjusted to 1 liter by distilled water. The solution was sterilized by autoclaving and was stored at room temperature.

2. Kovac's reagent

5 g of para-dimethylaminobenzaldehyde was dissolved in 75 ml of amyl alcohol. Then concentrated HCl was added to make the final volume 25 ml. This reagent was covered with aluminum foil and stored at 4^{o}C.

3. Methyl red reagent

0.1 g of methyl red was dissolved in 300 ml of 95% ethyl alcohol. Then distilled water was added to make the final volume 500 ml. This reagent was covered with aluminum foil and stored at 4^{o}C.

4. Barritt's reagent

Solution A

5 g of alpha-naphthol was dissolved in 95% ethanol. This solution was covered with aluminum foil and stored at 4^{o}C.

Solution B

40 g of KOH was dissolved in distilled water. The solution became warm. After cooling to room temperature, creatine was dissolved by stirring. Distilled water was added. This solution was covered with aluminum foil and stored at 4°C.

5. 10 x TBE (pH 8.3)

54.0gm of Tris-base, 27.5gm of boric acid and 20ml of 0.5 M EDTA (pH 8.0) were taken and distilled water was added to the mixture to make 500 ml. The buffer was stored at room temperature.

6. Gel loading buffer

10x concentrated loading buffer consisted of 800µl of 20% Ficoll 400, 400µl of 0.1 M EDTA (pH 8.0), 10 µl of 0.25% bromophenol blue and 200µl of 1% SDS in 590µl of distilled water. It was stored at 4°C in 1ml aliquot.

7. Ethidium bromide solution

2.5mg of ethidium bromide (Sigma, USA) was dissolved in 5 ml of distilled water at a concentration of 0.5mg/ml. This solution was covered with aluminum foil and stored at room temperature.

8. Oxidase reagent

100 mg of N,N,N1,N1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 ml of distilled water and covered with aluminum foil. Then the solution was stored at 4^{o}C.

APPENDIX-III

Instruments

The important equipments used through the study are listed below:

Autoclave, Model No: WAC-47	Korea
Balance(Core series): Adam	UK
Centrifuge, Model No: Code: 5433000.011	Eppendorf, Germany
Digital Homogenizer (Wise Tis)	Korea
Freezer (-20°C)	Siemens Germany
Gel Documentation System: Major Science	Taiwan
Horizontal Gel Electrophoresis Unit	Wealtec Corporation, USA
Incubator	UK
Laminar Airflow Cabinet	UK
Micropipettes	Eppendorf, Germany
Oven (Universal drying oven) Model: LDO-060E	Labtech, Singapore
pH meter, Model: E-201-C	Shanghai Ruosuaa Technology Company, China
Refrigerator, Model: 0636	Samsung
Sterilizer, Model No: NDS-600D	Japan
Thermal Cycler, Model No: 2720	Applied Biosystems, USA
Vortex Mixture	VWR International