COMPARATIVE ANALYSIS OF PREVALENCE AND ANTIMICROBIAL RESISTANCE PATTERN OF *Staphylococcus aureus* ISOLATED FROM HOSPITAL EFFLUENTS WASTEWATER AND HOSPITAL ADJACENT COMMUNITIES IN DHAKA CITY.

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfilment of the requirements for the degree of

Bachelor of Science in Microbiology.

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Declaration

It is hereby declared that

1. The thesis submitted titled **"Comparative analysis of prevalence and antimicrobial resistance patten of *Staphylococcus aureus* isolated from hospital effluents wastewater and hospital adjacent communities in Dhaka city"** is my own original work while completing my degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted or submitted for any other degree or diploma at a university or other institutions.

4. I have acknowledged all main source of help.

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Ethics Statement:

For the completion of this research study, samples from selected places were collected following all the necessary precautions. All the experiments were done in BRAC University Life Sciences Laboratory. It should also be noted that no animal or human models were used in this study.

Abstract

Staphylococcus aureus is a gram-positive bacterium with spherical shape. They are member of Bacillota family. By nature, this organism is facultatively anaerobe, facultative anaerobe microorganisms don't require oxygen to grow. But they can also grow in presence of oxygen without any problem. So, basically *Staphylococcus aureus* have both the ability to grow by respiration and fermentation. This bacterium usually gives positive result in the nitrate reduction test as well as test of catalase. It is responsible for various infections including skin diseases, pyogenic infection, respiratory infection, food poisoning, toxic shock syndrome etc. In recent times, antibiotic resistant strains of microbes have increased at a terrible rate. Some of the strains of *Staphylococcus aureus* has already been resistant in methicillin that is also called MRSA strain. This clinic/hospital centred MRSA problem is now a worldwide problem. Among the clinical pathogens, mortality rate with antimicrobial resistance of *Staphylococcus aureus* is in one of the top positions. Hospital wastewater is a big source of the infectious diseases related with *Staphylococcus aureus* in hospital and community settings. Beside that, Hospital wastewater is the potential reservoir of ARGs and ARB.

Total 90 samples were collected from September 2022 to April 2023. Among them 43 were PCR confirmed *Staphylococcus aureus.* Cefixime, Amikacin, Erythromycin, Amoxycillin, Vancomycin, Gentamycin, Ceftriaxone all these antibiotics were 100% resistant to the Hospital isolate of *Staphylococcus aureus.* Cefixime was 100% resistant for both hospital and community isolates of *Staphylococcus aureus.*

From this research it is proven that ARGs in the Staphylococcus aureus strains has dangerously increased in both hospital and community settings which is really a alarming matter for us.

Dedication

I would like to dedicate this thesis paper to my beloved parents because they are the main source of my inspiration, strength and success. Words are not enough to express my gratitude towards you, thanks for everything that you have done for me in every step of my life. Without your support and inspiration, I might not be the person I am today. Every time when I was down, you both comforted me in every possible way and you have been there day after day to make sure my life turned out magically beautiful this way.

This thesis is also dedicated to my beloved younger sister Trisha, whom I love with all of my heart. I believe that one day you will be proud of me because of this little accomplishment of your sister.

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List of Acronyms:

MRSA- Methicillin resistant *Staphylococcus aureus*

MDR- Multi drug resistant

ET- Exfoliative toxin

SAg- Super antigen

PBP2a- Penicillin binding protein

PFT- Pore forming toxin

SCCmec: Staphylococcal cassette Chromosome mec

CDC- Centre for disease control and prevention

HA-MRSA- Health Associated Methicillin Resistant Staphylococcus aureus

ARB- Antibiotic resistant bacteria

NI- Nosocomial infection

HGT- Horizontal Gene Transfer

MHC- Major Histocompatibility Complex

WWTP- Waste water treatment plans

CNS- Central Nervous System

PCR- Polymerase Chain Reaction

CA-MRSA- Community-acquired Methicillin resistant Staphylococcus aureus

HWWs- Hospital Waste Waters

VRSA- Vancomycin Resistant Staphylococcus aureus

MIC- Minimum Inhibitory Concentration

TSB-Tryptone Soy Broth

AR- Antibiotic Resistance

ARGs- Antibiotic Resistance Gene

DNA- Deoxyribonucleic Acid

NA- Nutrient Agar

MSA-Mannitol Salt Agar

LB- Luria Bertani Broth

MHA-Mueller Hinton Agar

TE- Tris EDTA

EDTA- Ethylene di amine tetra acetic acid

RNA- Ribonucleic Acid

MCT-Micro Centrifuge Tube

TBE- Tris-Borate-EDTA

PBS- Phosphate-Buffered- Saline

BP- Base pair

CLSI- Clinical and Laboratory Standard Institute

HAI- Healthcare Associated Infection

ORSA- Oxacillin Resistant Staphylococcus aureus

AST- Antibiotic Susceptibility Test

UTI- Urinary Tract Infection

AMR- Anti-microbial Resistance

DNCC- Dhaka North City Corporation

NICRH- National Institute of Cancer Research & Hospital

NSH- National Shishu Hospital

Chapter 1

Introduction

1.1Background

Staphylococcus aureus produce extracellular cell clumping factor, they are coagulase and catalase positive. This organism cannot easily get into and cannot cross the barrier of intact or healthy skin. For crossing the barrier, at least they need a small cut or path in skin. However, most of the times, they enter into the body through any breakage or wounds (Brown et al.,2005). This organism is responsible for many pus-producing or in other word 'Pyogenic' infections, toxic shock syndrome, illness related with consumption of food, Endocarditis etc. some of the strains can produce capsules and are responsible for the production of different kinds of virulence factors. Pyogenic infection that *S. aureus* can produce are post-operative wound infections, breast abscesses, septic arthritis, folliculitis, lung abscesses etc. Denaturation of skin indicates different types of diseases that includes Ritter disease scalded skin syndrome by *staphylococcus*. This disease mainly caused by the production of exotoxins from *S. aureus* (Ross, A., & Shoff, H. W. 2023).

Most of the strains of *S. aureus* secrete exoproteins including enzymes as well as exotoxins. Some of the exotoxins and enzymes that are associated with this organism are lipases, proteases, collagenase, nucleases and hyaluronidase etc. There are different functions of these exotoxins and proteins. The first and foremost function of this protein is to convert host tissue (local) into the available nutrients for the requirement of bacterial growth (Justyna, Sokolova, September 14, 2011). By cytolytic activity, this organism can produce various exotoxins. The function of cytolytic toxin is to form β-barrel pores in the plasma membrane and because of this pore formation cell content's leakage will happen. As a result, cell lysis of target cell is so obvious. *S. aureus* is capable of producing different types of exotoxins that includes staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, and SEI), toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxins like ETA and ETB. TSST-1 and staphylococcal enterotoxins and TSST-1 both are counted as superantigens (PTSAg) of pyogenic toxin.

Many people have died from *Staphylococcus aureus* infection before the invention of beta-lactam antibiotic penicillin. Infections and diseases started to decrease by the invention of this antibiotic. However, some antibiotic resistant strains of *S. aureus* emerged. *Staphylococcus aureus* produce beta-lactamase enzyme that is plasmid encoded and destroys the ring of beta-lactam and because of the reason Beta-lactam antimicrobial is not anymore active against this organism.

1.2 Antibiotic-resistant pathogens transmission from hospital effluent to adjacent communities

Hospital is one the big reservoir of antibiotic resistance bacteria. Wastewater or hospital effluent serve as a pool of antibiotic resistance bacteria and genes resistant against antimicrobials. Resistance of antibiotic against bacteria has become a global issue by causing the transmission of different types of infection. Hospitals are recognized to have an excessive and long-term usage of antimicrobials/antibiotics. (Majlander et al.,2021). According to recent scientifical reports, most of the antibiotics that is approximately 30-90% are not working on human body anymore. The reason behind it is, those antibiotics are direct or indirectly released into different places and in wastewater. Further, from wastewater they can easily allowed to pass through the wastewater treatment systems. This circumstance generates a perfect environment that is very suitable for bacteria to get into touch with different types of antibiotics and increase rate of resistance to antibiotics, that is one of the major global concerns right now. Mostly, nosocomial infections are caused by multi-drug resistant pathogens, overuse and improper use of antimicrobials. Number of nosocomial infections are getting increased day by day. The greatest number of nosocomial infection cases occur in impoverished countries such as Bangladesh. Nosocomial infections referred as infections that are associated with hospital or healthcare system. This infection can occur for the improper managements of hospital effluents. Hospital waste referred as any kind of waste including fluid, the solid and liquid materials alongside with their containers and different types of items produced in long- or short-term clinical care like diagnostic, observational, therapeutic and services of rehabilitative for individuals (Sweta Pandey and Anil K Dwivedi et al.,2016). Hospital waste includes microorganisms, medicines, chemical, heavy metal, elements that can emit radiation are also present in waste of hospitals. As wastes of hospital carry many microbes, antibiotic resistance bacteria and antibiotic resistance genes, it can ultimately be a threat to public health.

Chapter 2

Literature review

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive coccus. It grows in vine-like structures and produces golden or yellowish colonies on agar medium (Brenner's Encyclopaedia of Genetics, 2013). *S. aureus's* cell wall is a hard protective layer that has no define form of shape in appearance, approximate thickness of the wall is 20-40 nm. (Shockman and Barrett, 1983). This organism belongs to the group of bacilli and is immobile aureus or "Staph aureus" in the medical literature. It was discovered in 1880 by surgeon Sir Alexander Ogston. He basically found it into the purulent surgical abscesses. (Mandal, Ananya.2023, June 21). Because it can grow without oxygen, it is considered a facultative anaerobiotic. This organism usually lives on skin and in respiratory tract. It gives positive result for both test of catalase and nitrate reduction test. *Staphylococcus aureus* is responsible for pyogenic infections as well as superficial infections. It can also be associated with toxin-mediated disease. As an opportunistic pathogen, it can enter immunosuppressed patients through wounds, incisions, intravenous catheters, implanted prostheses, burns, etc. Pathogenic staphylococcus can haemolyze blood, coagulate plasma, produce various toxins and a special extracellular enzyme. Staphylococcus aureus are Gram-positive cocci. They are known as aureus or "Staph aureus" in medical literature. This organism is found in higher numbers mostly on surface of skin and in the upper respiratory tract. *S. aureus* almost always give positive result in catalase test and nitrate reduction test. This organism can cause pyogenic infections at the same time superficial infections also. As an opportunistic pathogen, it can enter immunocompromised patients through wounds, incisions, intravenous catheters, implanted prostheses, burns, etc. Pathogenic staphylococcus can haemolyze blood, coagulate plasma, produces various toxins (Döring M, R. 2023, September 21).

Staphylococcus aureus causes many infections and illness or diseases. This organism is responsible for community-acquired and hospital-acquired pathogenic infections after surgery. About 30% of people have *S. aureus* in the nose, throat or back of the throat and on the skin (D r. Annaya Mandal et al., 2021). *Staphylococcus aureus* causes different infections in different parts of the body. It causes skin infections in humans, including boils, papules, impetigo and other superficial skin infections. *Staphylococcus aureus* is responsible for surgical and traumatic wound infections. People who have chronic diseases, traumatic injury, diabetes, burns and immunosuppressive diseases are at higher risk to get infected by skin infection, deep abscesses and deeper tissue infection. (Ananya Mandal et al., 2021). This organism causes urinary infections, food poisoning or gastrointestinal infections, pneumonia, osteomyelitis, endocarditis, phlebitis, mastitis, septicaemia, toxic shock syndrome, bacteraemia and septicaemia, etc.

Staphylococcus aureus is Gram-positive and they are cocci in shape. Normally, this organism's size is 1μm in diameter. They form clumps. S. aureus is not encapsulated organism. As well as it has the lack of capability of sporulation. Besides that, this organism is non-motile. But they have one microcapsule and it can be observed by using high quality electrical microscope. Staphylococcus aureus growth and toxin production temperature is between 4-46 °C (39-115°F). S. aureus require the 7.5 pH and 37°C temperature respectively for optimum growth. At 37°C for 24 hours incubation, this organism normally produces smooth, round and convex, opaque colonies. The sizes of colonies in diameter are in between 1-3 mm in NA. Most of the strains of *S. aureus* produce golden yellowish strains.

2.2 Virulence factors

Virulence factor contribute to a pathogen's ability to cause disease. Some virulence factors of *S. aureus* give them the ability to adhere on to the surfaces of the cell wall and invade to immune system by breaking natural barrier. This organism can cause many types of diseases and infections.

First of all, several adhesins facilitate the attachment of *S. aureus* on to the cell surface of host, that mainly initiates the process of colonization. One of the most known adhesins of *S. aureus* consists of covalently anchored proteins to cell peptidoglycans. Besides that, these adhesins can specially attach to extracellular matrix (ECM) components or plasma. All these proteins are in group of MSCRAMMs or in other word- Microbial Surface Component Recognition Adhesive Matrix Molecules. Members of MSCRAMMs family are- Fibronectin-binding proteins A and B (FnbpA and FnbpB), Staphylococcal Protein A (SpA), Collagen-binding proteins etc (Justyna et al.,2011). Then, nearly all *S. aureus* secrete different kinds of enzymes and exotoxins. Some of them are proteases, nuclease, hyaluronidase, collagenase and lipase. For bacterial growth, these enzymes can help a lot by producing nutrients. These enzymes mainly convert local host tissue to available nutrients.

Exotoxins of *S. aureus* have cytolytic actions. Leakage of cell content is caused by cytolytic toxins. This is also responsible for the lysis of target cell through barrel pores in the plasma membrane. S. aureus secretes a number of cytolytic toxins, including -haemolysin, -haemolysin, leucocidin, and Panton-Valentine leucocidin (PVL). -haemolysin enters the eukaryotic membrane and oligomerizes to generate a hole. This hole further turned into bigger and cause osmotic cytolysis and human platelets and monocytes are more susceptible for cytolytic reactions (G. Menestrina et al.,2001).

Toxic Shock Syndrome is produced by an additional group of exotoxins that includes Toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEA, SEB, SEC n, SED, SEE, SEG, SEH, and SEI), and exfoliative toxins (ETA and ETB).

S. aureus's virulence factors are considered as multifactorial. Toxic shock syndrome toxin can cause toxic shock syndrome, food poisoning by staphylococcus species causes by different staphylococcal enterotoxins, SSSS by exfoliative toxins A and B and many others infections and illnesses are related with exotoxins. Based on mode of action, *S. aureus* can produce different toxins. Among them, cytotoxin can form pore and enhance pro-inflammatory changes. This change causes by alpha toxin and molecular weight of this toxin is 33kDA (Jensen, *et al.*, 1991).

2.3 Potential reservoir of ARBs and ARGs – Hospital Wastewater

A hospital is a public place. Various diseases and infections can spread from hospital premises to the environment and community. Hospital wastewater is a complex mixture of biological substances as well as chemical substances that is continuously discharged (Varricchi *et al.,* 2010). These mixtures consist of various chemicals, drugs alongside radioactive markers, metabolites of these drugs and chemicals, bacteria and antimicrobial resistance genes. It also contains residual amounts of disinfectants for the treatment of microbial skin infections and for the disinfection of hospital instruments, which are reservoirs of pathogenic microbes. Pathogenic microbes can also originate from medical devices, water and the environment. Another reservoir of pathogenic microbes is patient secretions. Therefore, HWW consists of various harmful microorganisms such as thermotolerant bacteria, bacteriophages, viruses, protozoa, algae, fungi, parasites, and parasites. In higher number of cases, this sewage remains untreated, untreated sewage can enter the food chain or remain in the soil for a long time and cause infection. (Wang et al., 2017).

By becoming resistant to different and wide range of drugs, ARBs and ARGs have almost loses their effectiveness of antibiotics. This happens for some reasons including misuse and overuse of antibiotics in human therapeutics and many other fields. Most of the antibiotics got resistant against pathogenic microbes. The presence of antibiotics in waters and microbes related to those antibiotics developed a serious antibiotic resistance problem worldwide in over the last two decades (Rodrguez, *et al.,* 2010). As it is mentioned earlier, hospital waster is potential reservoir of pathogenic microbes, chemicals, antibiotics and drugs. So, they are the major sources of antibiotic resistance also. In almost all locations, ARGs are found and are they are considered as an ecological problem and also a developing contaminant.

Even if the hospital effluent is treated, the resistance genes can still be there and can spread in community settings. Antibiotic resistance is a dangerous worldwide problem. So, it must be addressed sincerely to prevent the harmful impact on human health and as well as environment management. To addressed the augmentation of ARBs also ARGs, improved and developed

evaluations of risk assessment associated pathology and biology are needed as recent risk assessment is not sufficient anymore (Kaur et al.,2020).

2.4 Aim and objective of this study

Because of the above-mentioned issues, objective of my study was to identify the antibiotic-resistant *Staphylococcus aureus* from hospitals and their adjacent communities. Aim of this study was to make a comparison of the Anti-microbial resistance pattern of *Staphylococcus aureus* and their possibility to spread from hospital to environment.

Chapter 3

Methods and Materials:

3.1 Site for the Sample Collection

The sample collection site for this research study was in Dhaka Metropolitan areas of Dhaka North City Corporation. This study "Comparative analysis of prevalence and antimicrobial resistance pattern of *Staphylococcus aureus* isolated from hospital effluents wastewater and hospital adjacent communities in Dhaka city" was conducted from September 2022 to April 2023. Sample were collected from mainly three hospitals and those hospital adjacent communities in DNCC. Hospitals that were main focus of this study are- National Institute of Cancer Research and Hospital (NICH), Mohakhali-1212, National Shishu hospital (NSH), Shyamoli -1207 and DNCC Dedicated Covid-19 Hospital, Mohakhali-1212. Samples have been collected in each week of every month from the selected hospitals and their adjacent community households. Samples were mainly hospital effluents or wastewater and hospital adjacent community's tap water. Community sample point within a 300-meter range has been selected, with the hospital area as the centre. If clinical hazardous effluents from hospital release into environment without proper treatment, it can spread in soil, water, air etc and can cause different type of diseases in nearby communities. Sometimes those disease can turn out as outbreak. As those hospitals were government hospitals, so there was mass gathering of all type of people including patients that's why these sites were chosen for my study.

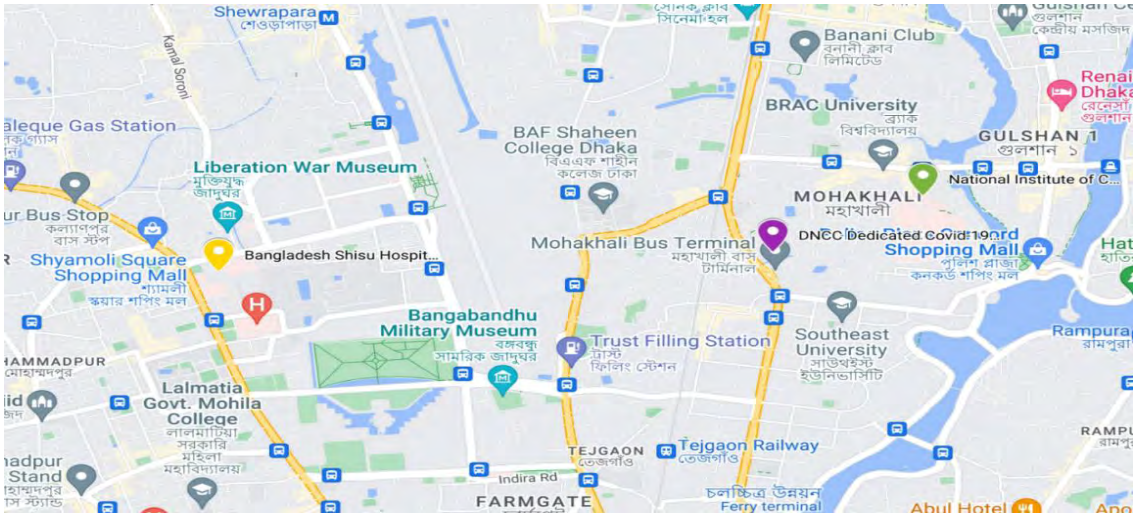

Figure 1: Sample collection sites in GIS map

3.2 Collection of Sample

Samples for this research work were collected from selected hospitals wastewater and their adjacent communities within 300m. samples were used to be collected in the morning on Saturday in every week. Every time, four samples have been collected from one selected hospital and its communities. Among them there were one effluent wastewater and three community tap water. Before sample collection, all the equipment and utilities associated with sample collection were properly autoclaved. For sample collection, the items needed were- sample collection bottle which must be sterile(500ml), falcon tubes of 50ml size that also must be sterile or disinfected and one pair of gloves. Icebox and icepacks were used to collect sample and to carry them from sampling site to laboratory. Without icebox, samples and organism can be deteriorate their condition.

Hospital wastewater was taken in sterile falcon tube of 50ml size. Hospital adjacent community pipe/tap water were collected in separated autoclaved sample collection bottle (500ml) and those bottles were marked by different identification like sample number, name etc. After pouring falcon tubes and bottles by water, tubes were sealed with caps very tightly to avoid contamination risk of running anywhere. After that, the samples were putted into the box of ice to carry them in laboratory. In next step, when the samples were already collected, the gloves that were used for collection purposes were putted in different bag specially in Ziplock so that they cannot contaminate the other things and later gloves were discarded in discard bin. As, wastewater and community water can carry harmful microbes so to avoiding risk of contamination and contracting any infection 70% ethanol was used to sanitize the hands. For further processing, collected samples were sent to the lab immediately.

3.3 Sample processing

For processing of the sample, the materials and utilities were needed are- filter apparatus, falcon tubes (50ml), test tube (10ml), sterile filter paper (Whatman filter paper), modified TSB (with 15% NaCl), normal physiological saline containing NaCl 0.9%, MSA +NaCl (7.5%) and gloves etc. Approximately 50 ml water was poured into the filter apparatus from all the water collected from hospital adjacent communities. After that, samples were filtered by using Whatman filter paper. Modified TSB that was made with 15% NaCl in TSB was poured into the sterile falcon tube. After the filtration, the filter papers were transferred into the modified TSB containing falcon tube with sterile tweezer. In next step, modified falcon tubes those were containing samples for enrichment were placed in a beaker and by using foil paper they were sealed. Next, in shaker incubator they were incubated for 48 hours at 37°C. Growth was observed after 48 hours by observing the turbidity of TSB broth. Every turbid sample was gone through 6-fold dilution in normal saline containing 0.9% NaCl. Then, from each sample 0.1 ml

(Raw, 10^{-2}, 10^{-4} ,10^{-6}) were poured into MSA media plate and was spread in the plate evenly. Then media containing plates were labelled properly and together they were stacked by using masking tape and media plates were placed in the incubator at 37°C for 24 hours.

For hospital wastewater, wastewater from falcon tubes were diluted serially in saline till 8 folds dilution. From diluted samples, (Raw, 10^{-2}, 10^{-4}, 10^{-6}) each sample 0.1 ml was poured into modified MSA and spread evenly until they got sticky. Then the media plates were labelled and stacked together by masking tape and placed on incubator for 24 hours to observe the growth of microorganism.

Bacteriology count was also done from hospital wastewater. For bacteriology count, the wastewater was diluted serially in saline (normal) till 7-fold. From diluted samples, each of samples from tube (10^{-1}-10^{-7}) 0.1ml was poured into Nutrient agar (NA) media and spread evenly. Then the plates were labelled properly and stacked together by masking tape. After that the plates were putted in incubator at 37°C for 24 hours.

3.4 Growth on selective media and media enrichment

Mainly this research study emphasized on the characterization and isolation of *Staphylococcus aureus* from hospital effluents and community tap water. For this, the media were used are- Nutrient Agar (HiMedia), Mannitol Salt Agar (HiMedia), Tryptic Soy Broth (TSB), Luria Bertani broth etc. For enrichment, samples collected from hospital adjacent communities, TSB modified containing 15% NaCl was used. Because loads of bacteria are comparatively lower in water of hospital adjacent communities.15% NaCl was added as a growth requirements of *Staphylococci spp.* into the samples. MSA (Mannitol Salt Agar) for *Staphylococci spp.* Worked as selective growth media. This media can differentiate between *staphylococcal spp.* Species that is mainly used for the identification of *Staphylococcus aureus*. Besides that, NaCl added into the MSA media that helps to isolate and identify *Staphylococcus aureus.* (Chapman, n.d.). Luria-Bertani Broth and NA media were used for regular microbiological purposes.

3.5 Selection of Colony morphology and analysis

For further process, all MSA media plates were out of the incubator after the selected incubation period. Then morphology of bacterial colony and growth were observed. If *Staphylococcus aureus* is present, it will ferment the mannitol sugar and because of this fermentation MSA containing media would change its colour from pink to yellow or golden yellow. By standard plate count methods, total colony forming unit/ml was calculated. Next, 5-8 white (circled by yellow zone) / yellow, yellow and pink colonies were possibly selected from each of the sample for streaking on plates containing MSA media. After streaking, MSA plates containing sample were incubated for 24 hours at 37°C to observe growth of bacteria. These possible *S. aureus* colonies were then stored carefully and properly for further processing.

3.6 Molecular Detection

3.6.1 DNA Extraction

DNA extraction is very important for further molecular analysis of any microorganisms/bacteria. Because of the extraction of DNA, micro-centrifuge tubes were taken. In each micro-centrifuge tubes, 150ml 10X TE (Tris-EDTA) buffer was added. Then from the subculture NA plates, colony was picked and mixed well in the TE buffer of those micro-centrifuge tubes. Vortex machine was used to mix properly. Then pre-heat (100°C for 14 minutes) was used to heat the machine to come into appropriate state. After that, the microcentrifuge tubes were placed on the heating machine at 95°C for 15 minutes. This heating is necessary to denature the cell membrane and proteins. High heat helps to disrupt the cell and nuclear envelope that release the DNA into the lysate form. After 15 minutes heating, tubes that carrying isolate were set for centrifugation for 5 minutes at 13000 rpm. After that, pellet and supernatant were clearly visible to differentiate. Then, the supernatants were transferred by a micropipette to a new set of microcentrifuge tubes and the pellets were discarded. Then the DNA carrying supernatant containing MCT were kept in an airlocked bag and at -20°C they were kept in refrigerator.

3.6.2 Primer's preparation from stock for PCR

In this study, for molecular analysis of *Staphylococcus aureus*, three types of primers were used for PCR. For the detection of genus level that is staphylococcus, TStaG422 primer was used. Sa442 also NUC primers were used to detect species level of Staphylococcus aureus. Stock solutions for three of them were available at lab.

For the preparation of 100 µl solutions (10µM) of TStaG442 primer from 100 µM, 10 µl for forward and 10 µl for reverse primers were taken in different Micro centrifuge tubes. By using Molecular-grade nuclease-free water, rest of the 70 µl was then fill up in each tube. Short spins and re-pipetting were done for approximately 20 seconds to mix them gently. The same process was followed for the rest of the two-primer solution of Sa442 and NUC primer.

Gene	Primer Sequence	Target organism	Product size	Reference
TStaG422	5′- GGCCGTGTTGAACGTGGTCAAA TCA-3′ 5′-TIACCATTTCAGTACCTTCTGGTAA-3′	*Staphylocous spp.*	370bp	(**Martineau** et al.,1998)
Sa442	5′- AATCTTTGTCGGTACACGATATTCTTCACG-3′ 5′- CGTAATGAGATTTCAGTAGATAATACAACA-3′	*Staphylococcus aureus*	108bp	(**Martineau** *et al.*,1998)
NUC	5′-GCGATTGATGGTGATACGGTT-3′ 5′-AGCCAAGCCTTGACGAACTAAAGC	*Staphylococcus aureus*	279bp	(**Martineau** *et al.*,1998)

Table 1: Primer's sequence used for the PCR amplification.

3.6.3 Control's Preparation for PCR

To do molecular analysis of this bacteria, **Polymerase chain reaction** or (PCR) is necessary, every time a positive control was used that is mainly serves as quality control of all the methods of molecular detection of *Staphylococcus aureus*. In the laboratory, laboratory standard true positive isolate of *Staphylococcus aureus* was available. That positive isolate was used as positive control for the molecular detection of this study. As negative control, master mix with nuclease free water was used.

3.6.4 PCR assay

PCR is very important to determine the genetic identification for bacteria like *Staphylococcus aureus*. At the molecular level, to detect bacterial isolates PCR allows rapid amplification of

certain genes. Again and again throughout the study in every week, by amplifying TStaG422, Sa442 and NUC PCR-based detection of this organism was done regularly.

To perform PCR, PCR tubes were taken and PCR mixtures were taken in a 13µl volume in which water (nuclease free) 2.3µl, 2X PCR master mix emerald (Takara Bio) 7.5 µl, forward and reverse primers 0.6 µl and DNA template 2µl. After taking all these, proper mixing is necessary. For proper mixing, short spinning or gentle re-pipetting were done in a careful way so that bubble cannot form into tubes. In an Applied Thermo-Fischer Bio-system thermal cycle the PCR was performed and the programme was run according to this serial: 5 minutes at 94°C set for initial denaturation, 94°C for 1 minute and 30 cycles were set for denaturation, 55°C set for annelation of primers (for TStaG422 and Sa442), NUC primer annelation for 1 minute at 56°C, at 72°C for extension about 1 minute and for 10 minutes final extension. For positive control, positive isolates were used and nuclease free water was used for negative control. This PCR methods were used for TStaG422, Sa442 and NUC primers.

3.6.5 Gel Electrophoresis

To see the PCR confirmation of the amplified target genes, the conventional agarose gel electrophoresis was performed in this study. From the mixture, 10µl products of PCR was selected for electrophoresis for 60 minutes at 110 V in TBE buffer with 2% agarose gel (that contains Tris 40mM, EDTA 1mM, 20mM Boric acid and pH approximately 8.). With Ethidium bromide 0.5 µg/ml, the prepared gel was stained. When gel electrophoresis was done, by using UV trans-illuminator, prepared gel was visualized and with appropriate labelling pictures taken of the gel. From the visualization, when the band was matched and by using TStaG422 expected size 370 bp was detected they were considered as positive isolates, expected size for NUC and Sa442 were 279 bp and 108 bp accordingly. When band length properly marched with the associated length of those primers, that means the *Staphylococcus aureus* is present in the sample. Besides that, ladder with various sizes were used to make comparison between amplicons with the band size of expectation.

3.7 Antimicrobial Susceptibility test

Antimicrobial susceptibility is a term in which microbes like bacteria cannot grow when one or multiple drugs of antimicrobials are present. In this research, all the positive isolates that were confirmed by the test of PCR were further chosen for antimicrobial susceptibility test. Because to see how the antibiotics react with the microbes and identify the pattern of resistance by antibiotics. By following Kirby-Bauer disk diffusion method and CLSI guidelines, antibiotic susceptibility was done in lab. Nine antibiotics were selected for the test. Those antibiotics were: Vancomycin, Cefixime, Amikacin, Gentamycin, Ceftriaxone, Erythromycin, Amoxicillin, Imipenem and Cefepime.

Isolates that were got positive by PCR confirmation, they are further processed for sub-culturing in NA agar media plates and at 37°C they were grown overnight in incubator to make bacterial suspension. For the test, firstly, from bacterial culture, a loop full of fresh colonies

were taken for dipping in normal saline 0.9 % and properly mixing. With 0.5 McFarland turbidity standard, it was further matched and compared. By using a sterile cotton swab bacterial isolates from suspension were taken and on Mueller Hinton Agar (MHA) plate lawn of bacteria was prepared. Antibiotic-impregnated disks were carefully picked by using sterile forceps and pressing the disks to set and to make it sure that the antibiotic diffused properly on agar surface. Then the plates were stacked and labelled and place for 24 hours at 37°C in incubator. MHA plates were coming out from the incubator to identify inhibition zone after the incubation period. Further, it was ready for the comparison with CLSI guidelines to see the match. By using millimetre (mm) scale, inhibition zone was measured properly.

Antibiotic name	Antibiotic Class	Zone interpretation
Vancomycin	Glycopeptide	S>=12, I=10-12, R<=13
Cefixime	Cephalosporin	S>=19, I=16-18, R<=15
Amikacin	Aminoglycosides	S>=21, I=18-20, R<=17
Gentamycin	Aminoglycosides	S>=15, I=13-14, R<=12
Ceftriaxone	Cephems	S>=21, I=14-20, R<=13
Erythromycin	Macrolides	S>=23, I= 14-22, R<=13
Cefepime	Cephems	S>=18, I=15-17, R<=14
Amoxycillin	Penicillin	S>=18, I=14-17, R<=13
Imipenem	Carbapenem	S>=16, I= 14-15, R<=13

Table 2: Antibiotics and their class and zone interpretation.

Chapter 4

Result and Observation:

4.1 Isolation of *Staphylococcus aureus*

43 samples were confirmed by PCR for *Staphylococcus aureus* selected from the hospital effluents and hospital adjacent community water during September 2022-April2023. Among them, 40 were from community water and 3 were hospital wastewater. For PCR processing, on MSA agar plates, the colony structure was observed carefully and the isolates were picked based on the colony morphology on the agar plates. And further those isolates that were selected from MSA plates were run for the confirmation of PCR.

Figure 2: The yellow colonies of *Staphylococcus aureus*

4.2 PCR-based identification of *Staphylococcus aureus*

In next step that is gel electrophoresis, amplified products in the gel were observed and viewed by using an UV illuminator. After that, the size of the amplified products was matched with the expected band size. When the PCR product give the expected size value under the illuminator, it will be considering as positive isolates. For matching the sizes, DNA ladder was used for comparison. Figures in the below shows the finding and demonstration of PCR amplified product under UV illuminator.

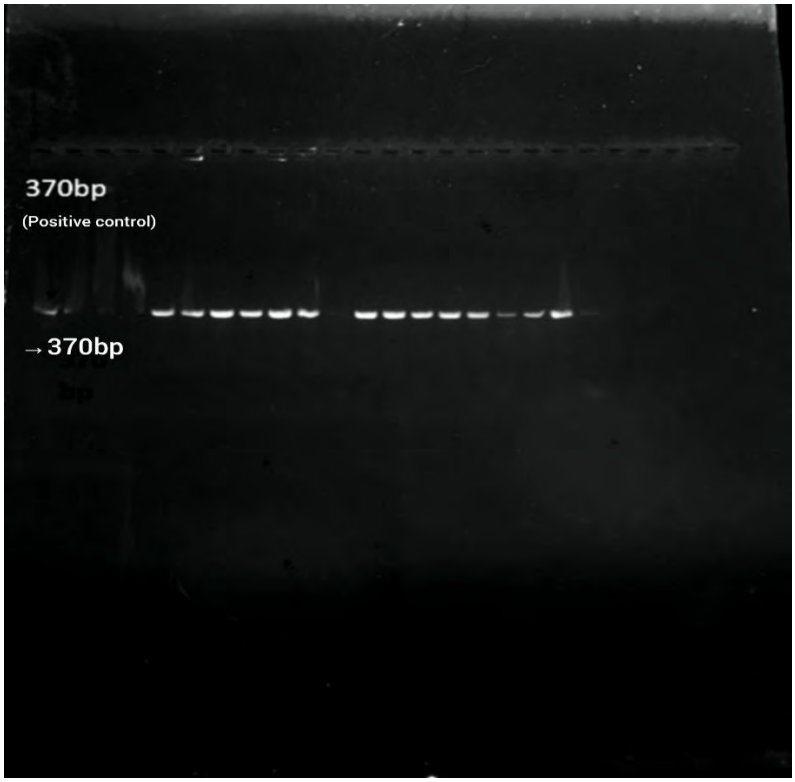

Figure 3: TStaG422 PCR for the detection of the genus *Staphylococcus* that is showing 370bp band.

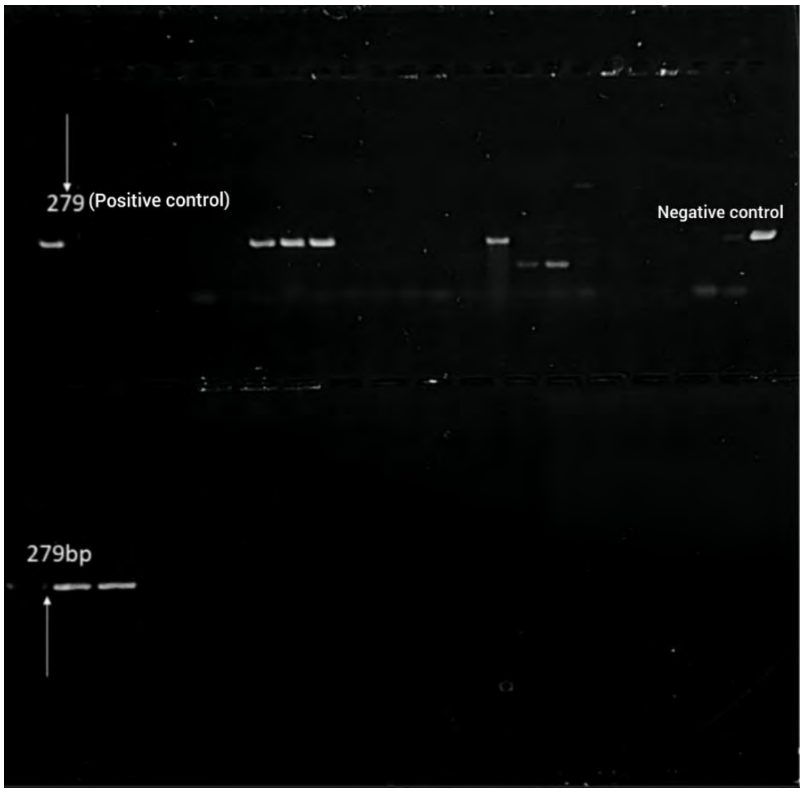

Figure 4: NUC primer used for PCR and that showed 279 bp band.

4.3 Month-wise Distribution of Positive isolates

This study figured out positive isolates pattern from September 2022 to April 2023. Total 43 PCR confirm *Staphylococcus aureus* were found in this time frame. Among them, 40 isolates were from Hospital adjacent community water and 3 were from hospital wastewater. In September 2022 from the sampling sites and the isolates numbers were 16 which was about 37% of total positive isolated *Staphylococcus aureus* and this was the highest number of isolates among all. In December, 10 positive isolates were found that is 23% of total PCR-confirmed Staphylococcus aureus. In February 2023, 9 positive isolates were confirmed by PCR which is about 21% of total isolates. In October 2022, the positives isolates were total 2 that is 5%. In November, 1 isolate was found and that was 2 % of total positive isolates. In March 2023, 2 positive isolates were found that is 5%. In April, total 3 positive isolates were found that is 7% of total positive isolates but in January 2023, no positive isolates were found.

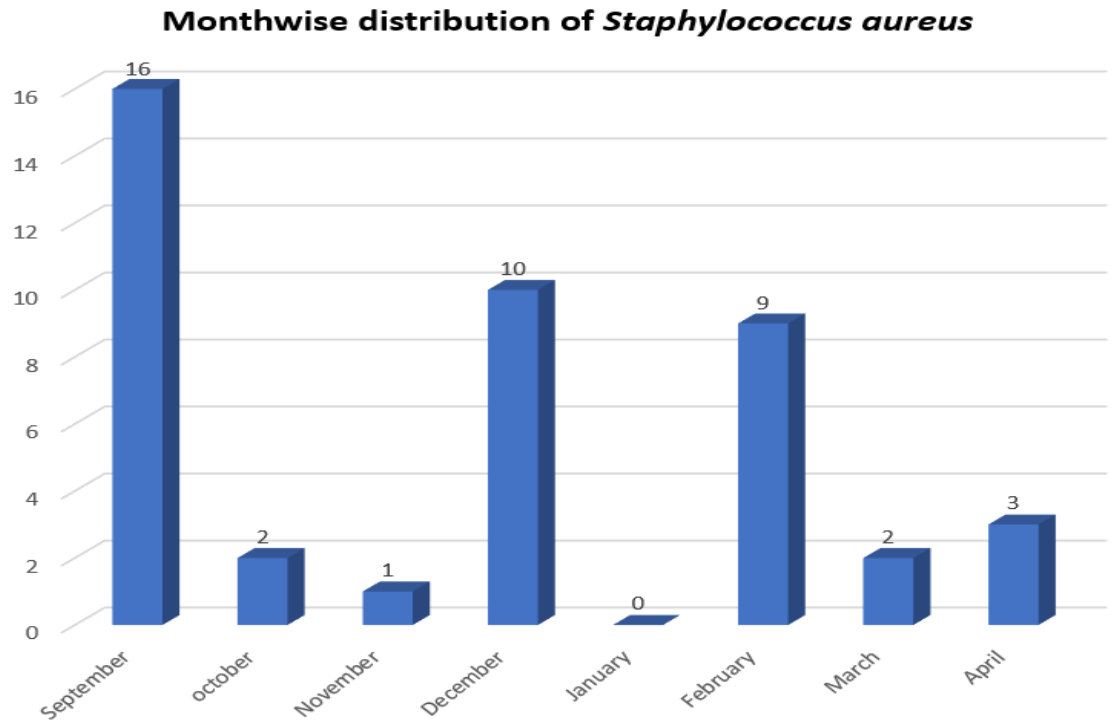

Figure 5: Distribution of positive isolates of *Staphylococcus aureus* month-wise.

4.4 *Staphylococcus aureus* distribution from Hospital wastewater and Community water

In this study, from September 2022 to April 2023 total 43 positive isolates of *Staphylococcus aureus* were found by PCR confirmation. Among them 40 was from community water and 3 were from hospital water. So, the percentage of community water was 93% in positive isolates and the percentage of hospital waste water *S. aureus* was 7%.

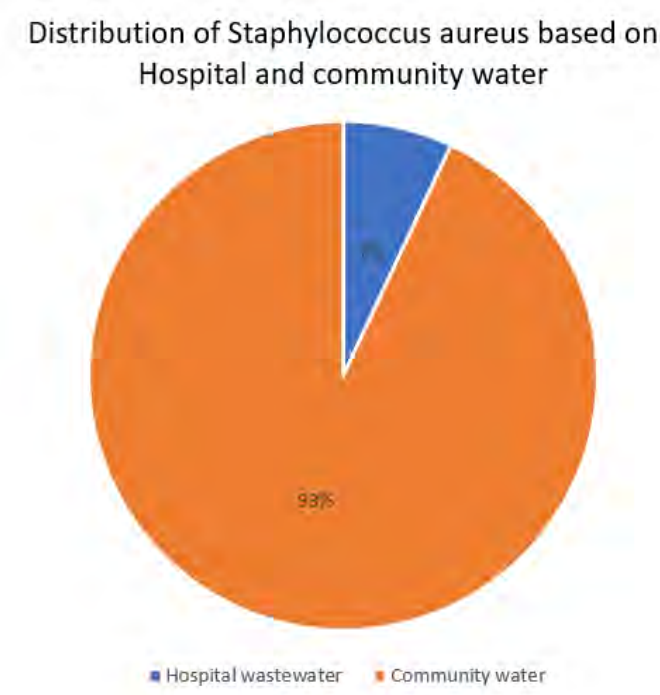

Figure 6: Positive isolates of *Staphylococcus aureus* based on hospital and community water.

4.5 *Staphylococcus aureus* positive isolates distribution based on the sampling sites

This research was done by emphasizing three locations of Dhaka Metropolitan areas. These three locations were including hospitals and their adjacent community. Sites selected for sample collection of this study were Dhaka Shishu Hospital (DSH), Shaymoli-1207, DNCC Dedicated Covid-19 Hospital (DNCC, DCH), Mohakhali, Dhaka-1212 and National Institute of Cancer Research and Hospital (NICRH). After analysing collection and sample test report, it was observed 64% positive isolates were achieved from Covid Hospital and its associated

communities, 26% positive isolates of Staphylococcus aureus were found from Dhaka Shishu Hospital and 10% were found from National Institute of Cancer Research Hospital.

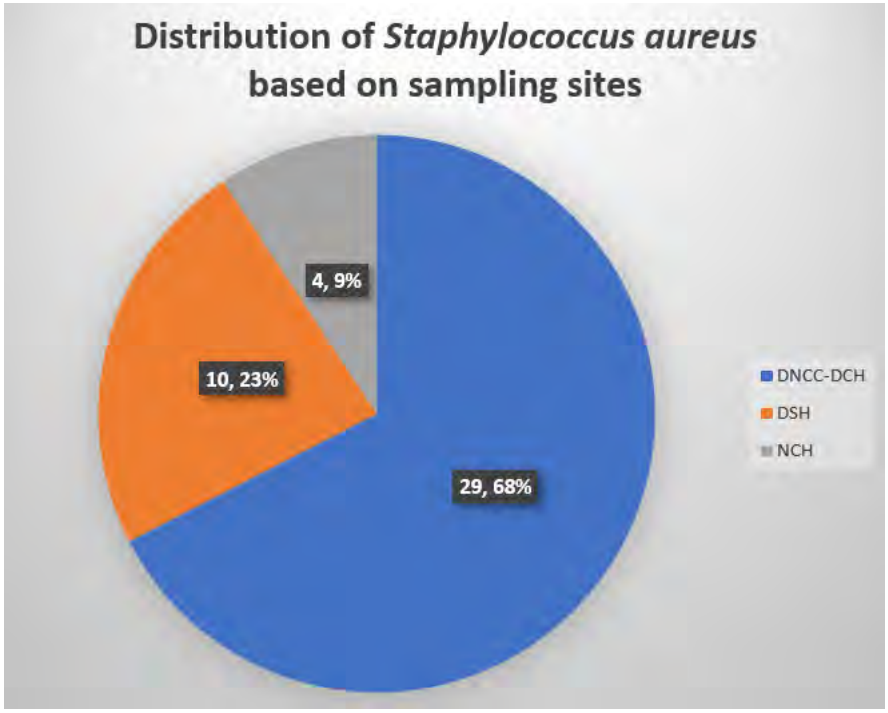

Figure 7: On the basis of sampling sites *Staphylococcus aureus* positive isolates distribution.

4.5 Antimicrobial Susceptibility test

Kirby-Bauer disk diffusion method was used for the susceptibility testing of antibiotics. After the incubation period (24hours) at 37°C, MHA plates were observed to examine whether the isolates were sensitive, intermediate or resistance to antibiotic-impregnated discs. According to CSCI guidelines results were interpreted.

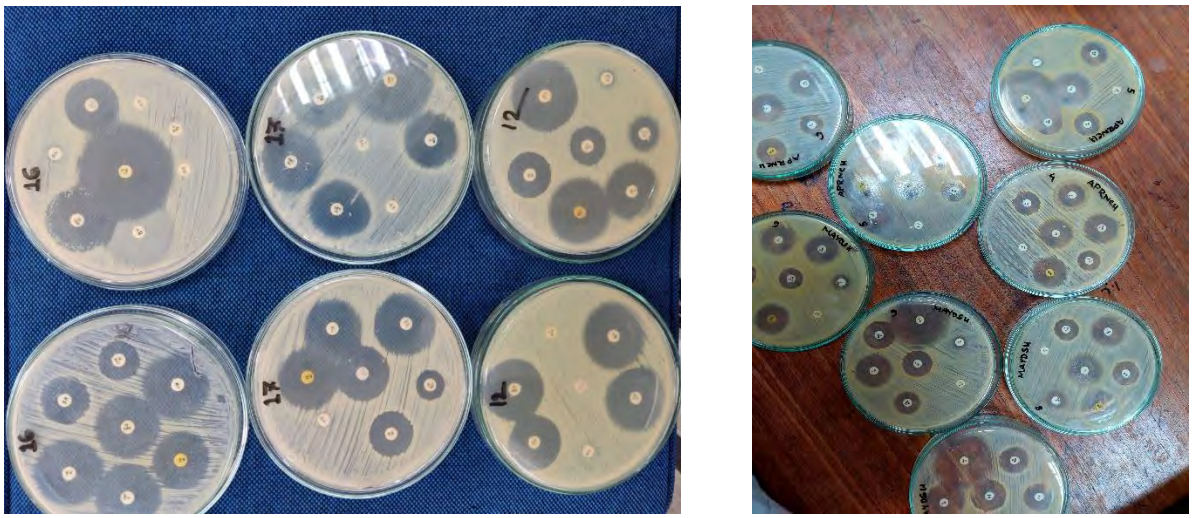

Figure 8: *Staphylococcus aureus*- Susceptibility testing for Antibiotics.

4.5.1 Pattern of Antimicrobial Resistance for total isolates

From the analysation on total isolates for the susceptibility testing, it was found that vancomycin was 38% resistant, Cefixime (100%), Amikacin (28%), Gentamycin (12%), Ceftriaxone (43%), Erythromycin (92%), Cefepime (35%), Amoxycillin (42%) and Imipenem 8%. From this analysis, it was showed that Imipenem has the highest susceptibility against *Staphylococcus aureus.*

Antibiotics	Resistant	Intermediate	Sensitive
Vancomycin	38%	0%	62%
Cefixime	100%	0%	0%
Amikacin	28%	4%	68%
Gentamycin	12%	0%	88%
Ceftriaxone	43%	37%	20%
Erythromycin	92%	0%	8%
Cefepime	35%	20%	45%
Amoxycillin	42%	0%	58%
Imipenem	8%	0%	92%

Table 3: Resistance pattern of total isolates for different antibiotics.

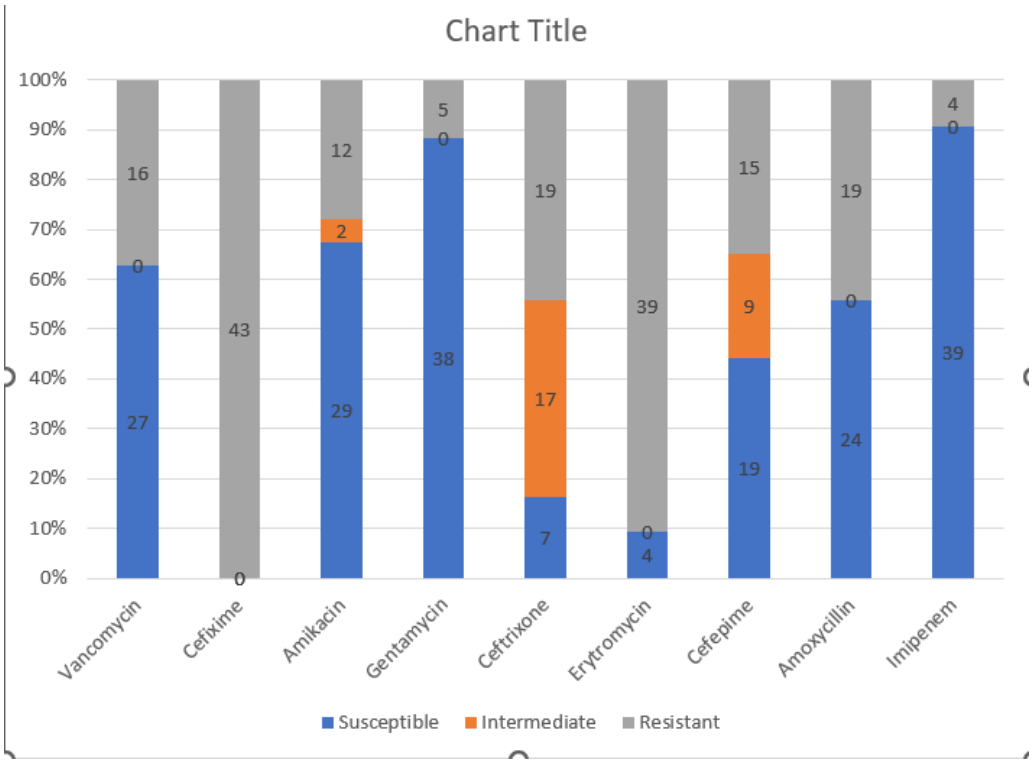

Figure 9: Anti-microbial resistance pattern of all isolates.

4.5.2 Hospital waste water isolates and their resistance pattern for antibiotics

From the study it was found that hospital isolates were mostly resistant to almost all antibiotics. From hospital wastewater, all isolates were resistant against Amoxycillin, Ceftriaxone, Erythromycin, cefixime, vancomycin etc. Imipenem showed resistance against 33% isolates, 34% susceptibility and 33% intermediate zone. Cefepime showed resistance in 67% isolates and susceptibility in 33% isolates for this antibiotic.

Antibiotics	Resistant	Intermediate	Sensitive
Cefixime	100%	0%	0%
Amoxicillin	100%	0%	0%
Amikacin	100%	0%	0%
Erythromycin	100%	0%	0%
Imipenem	33%	33%	34%
Vancomycin	100%	0%	0%
Gentamycin	100%	0%	0%
Ceftriaxone	100%	0%	0%
Cefepime	67%	0%	33%

Table 4: Hospital effluents isolates and their resistance pattern for antibiotics.

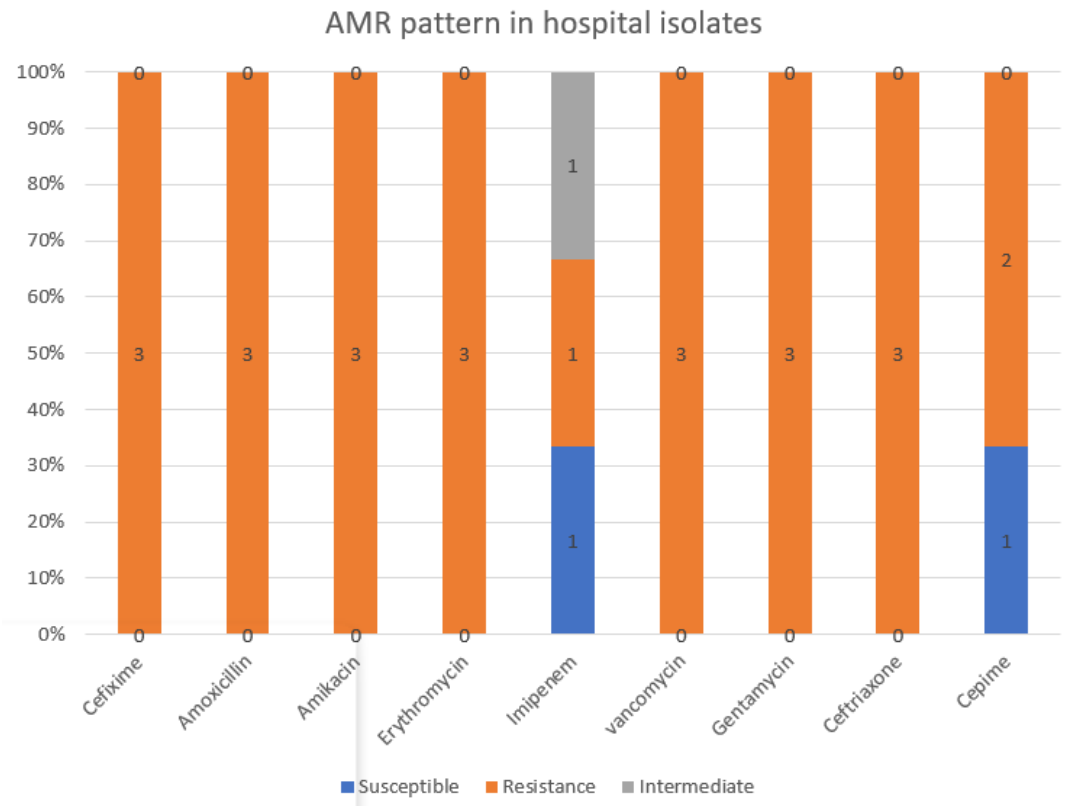

Figure 10: Hospital wastewater positive isolates resistance pattern for antibiotics.

4.5.3 Isolates of hospital adjacent communities and their antibiotic resistance pattern

By observing the data, it shows that Cefixime and Amoxycillin is resistant for all isolates. Amikacin was 68% susceptible,27% resistant and shows 5% intermediate zone over all isolates of hospital adjacent communities. Erythromycin shows 95% resistance and 5% susceptibility. Imipenem shows 73% susceptibility, 13% resistance and 14% intermediate pattern. Gentamycin shows 90% susceptibility that is the highest percentage among all and shows 10% resistance. Ceftriaxone shows 10% susceptibility, 38% resistance and 52% intermediate pattern. Lastly, Cefepime shows 42% susceptibility, 30% resistance and 28% intermediate zone

Antibiotics	Resistant	Intermediate	Sensitive
Cefixime	100%	0%	0%
Amoxicillin	100%	0%	0%
Amikacin	27%	5%	68%
Erythromycin	95%	5%	0%
Imipenem	13%	14%	73%
Vancomycin	35%	0%	65%
Gentamycin	10%	0%	90%
Ceftriaxone	38%	52%	10%
Cefepime	30%	28%	42%

Table 5: Antimicrobial resistance pattern of community water isolates.

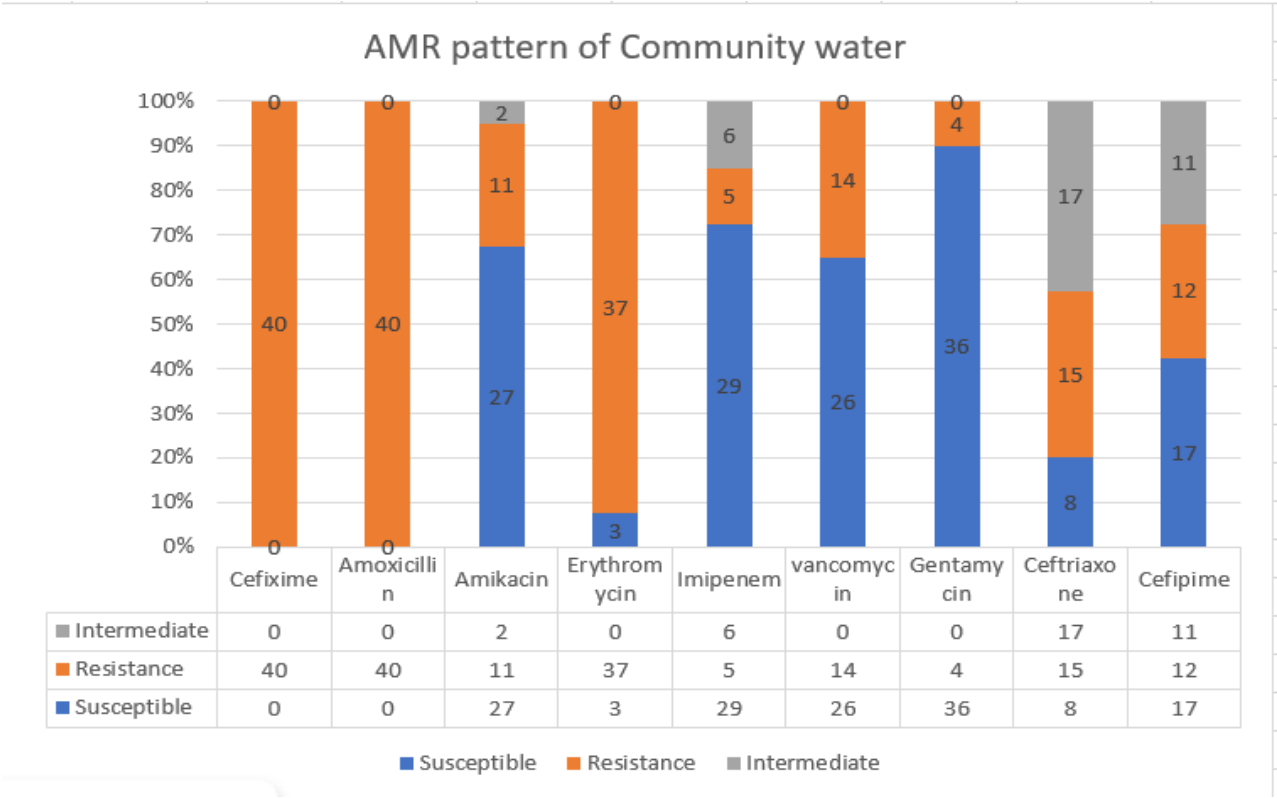

Figure 11: Hospital adjacent communities isolates and their antimicrobial resistance pattern.

Chapter 5

Discussion

5.1 Result-analysis based discussion

Healthcare-associated infections (HAIs) are contracted by patients while they receive medical care and intervention for different medical condition. Any institution for the treatment or medical care that includes Surgery centres, hospitals, clinic, pathology, renal-illness treatment centre, long-term care home are in high risk of HAIs. Bacteria, viruses, fungi or other pathogens can be the cause of HAIs. Hospital Associated Illnesses can be the significant reason of illness and death. *Staphylococcus aureus* is one of the most known pathogens for hospital related illnesses. Anyone can be infected by staph infection but some people are at high risk to get infected by staph related infections than any other people. These some people include immune suppressed people with chronic diseases like cancer, diabetes, people who are into drugs, people with vascular diseases or lung or heart diseases. As these people are already immune-compromised, so any harmful pathogens can easily get into their bodies and can create infections. Besides that, people who have already gone under surgery, they are at high risk to get infected by staph infection. Patients admitted in intensive care units or (ICUs) can easily get infected by staph. Most importantly, sometimes medical interventions or medical devices that attach with patient body even a needle can lead serious staph infection. So, overall hospital or medical care centre is a big source of diseases that caused by staph organisms.

Staphylococcus aureus can cause nosocomial infections, especially blood-steam infection (SA-BSI). Additionally, *Staphylococcus aureus* is a commensal bacterium as it is human bacteria. *S. aureus* is a colonizer and 30% of human population can be affected by it. This organism is also responsible for Skin and tissue infections, osteoarticular, pleuropulmonary and many other infections related with medical devices. Infective endocarditis and bacteraemia can also can be caused by it (Tong & Fowler (2015). *Staphylococcus aureus* is a gram-positive opportunistic pathogen. Toxic Shock Syndrome (TSS) can also occur by Staphylococcus aureus that is life threatening. Despite of inventing new and improved antibiotics, severe morbidity and death rate have been shown worldwide because of *Staphylococcus aureus*. *Staphylococcus aureus* shown resistance against various antibiotics like Vancomycin, Cefixime, daptomycin etc. there might be several reason of this antibiotic resistance pattens including misuse and overuse of antimicrobials.

These antibiotic resistant *Staphylococcus* is a great threat to our environment as well as public health. Untread hospital effluent is an important source of these resistant *Staphylococcus aureus*. The misuse or inappropriate contribution of antimicrobials to treat or prevent diseases in humans and animals is one of the main contributors to the rise of antibiotic resistance. Some of the examples of misuses are- administering antibiotics to both humans and animals when they are not necessary, sharing of antibiotics or self-medication, using antibiotics in ways other than those recommended by a doctor, using antibiotics to treat a viral infection that is not bacterial in origin etc. This misuse of antibiotics raised the resistance pattern of *Staphylococcus*

aureus by horizontal gene transfer. Most of the time, hospital effluents are not treated in our country and without treating them they are often released into the environment that has a great role in acquisition of ARGs. Besides that, most of the nosocomial or hospital pathogens are resistant to almost all of the antibiotics because of their heavy use of antibiotics in hospital. These nosocomial pathogens can easily spread in hospital adjacent communities through the untreated hospital effluents.

This study actually targeted on areas of hospitals and its adjacent communities in Dhaka North City corporation. For this research, three hospitals and their adjacent areas were selected. Those hospitals were- DNCC Dedicated Covid-19 Hospital, Mohakhali, Dhaka-1212, Dhaka Shishu (Children) Hospital, Shyamoli-1207 and National Institute of Cancer Research and Hospital, Mohakhali, Dhaka-1212. From those areas, *Staphylococcus aureus* isolates were found and categorized as "Hospital wastewater isolates" and "Community water isolates". Among the isolates 7% were from waste water of hospital and 93% were from water of nearby communities. Based molecular detection that PCR was used for the confirmation of the isolates.

It was observed that hospitals isolates were more prone to resistance of antibiotics. Most of the hospital isolates were 100% resistant against Cefixime, Amikacin, Gentamycin, Vancomycin, Amoxycillin, Erythromycin and Ceftriaxone. It was also found that Imipenem had also 33% resistant pattern for Staphylococcus aureus. But Imipenem was 34% susceptible against Staphylococcus aureus. Cefepime was resistant in 67% isolates and 33% isolates were susceptible to it.

Isolates from community water were founded to be resistant to Amoxycillin and Cefixime in this research. Amikacin was 27% resistant, Erythromycin shows 95% resistance, Imipenem (13%), Vancomycin (35%), Gentamycin (10%), Ceftriaxone (38%), Erythromycin (95%), Cefepime (30%). From the AST results, it was viewed that Cefixime was resistant for 100% organisms.

From this study, it was found that from hospital untreated wastewater ARGs transmitted to environment. That's the reason why, many of the community water isolates were became resistant to various antibiotics. As different types of antibiotics are used in hospital, when they released from the hospital settings without any treatment to environment, they speeded resistant bacteria and as a result ARGs got speeded more easily in environment. Because of the increasing rate of ARGs, treating any infection is getting difficult day by day. Besides that, because of it, diseases are increasing that is great threat to Public Health and mortality rate is also increasing.

5.2 Limitation of this study

The sampling sites were just limited by hospital sites, there was no chemical sites or other source that can be potential for harmful pathogen reservoir. Besides that, sampling sites were limited to only three hospitals in Dhaka North City Corporation area. But, if the samples were collected from two city corporation's hospitals, then the real scenario might have revealed. This study only worked on microbiological assay but chemical and physical evaluation is also necessary. Physical and chemical evaluation might have revealed different types of harmful substances including chemical, heavy metals etc. because of the limitation of the lab facilities further different kinds of molecular analysis were not done, if there all primers related to ABRs and ARBs were present, then detection might be further progressed that could have added a little more information in this study.

Chapter 6

6.1 Conclusion

Treating hospital waste or effluents is really very important for public health. In recent past few years, this issue has dragged attention to the world. Different types of analysis and studies were done worldwide on the management of hazardous effluents of Hospital. But the scenario is different here in Bangladesh, these types of studies were not conducted to monitor the hospital effluents and diseases related with them. Because of these hospital's waste and effluents, human health has been very harmfully impacted for a long time. And the cases are increasing day by day because of the lack of management and monetization. Because of this carelessness in terms of treating hospital waste water increasing the antibiotic resistance bacteria day by day. Resistance pattern of antibiotics has developed in the whole world and pointed to be serious problem for mankind. In hospital, not only the use of different types of antibiotics but also release and spreading of resistant bacteria to nearby area and environment that made ARGs more common in environment. This is really a matter of worry. As a result, treating any infection and fighting against any disease is getting difficult day by day.

In this study, it was observed that, ARGs of *Staphylococcus aureus* has been increased at a high rate. From the hospital settings ARBs and ARGs transmitted to untreated hospital effluents. Most isolates were resistant to Cefixime, amoxycillin, Ceftriaxone, Amikacin, vancomycin etc. In community water, by observing the data, it shows that Cefixime and Amoxycillin is resistant for all isolates. Amikacin was 68% susceptible,27% resistant and shows 5% intermediate zone over all isolates of hospital adjacent communities. Erythromycin shows 95% resistance and 5% susceptibility. Imipenem shows 73% susceptibility, 13% resistance and 14% intermediate pattern. Gentamycin shows 90% susceptibility that is the highest susceptibility rate among all and shows 10% resistance. Ceftriaxone shows 10% susceptibility, 38% resistance and 52% intermediate pattern. Lastly, Cefepime shows 42%

susceptibility, 30% resistance and 28% intermediate zone. Almost all the antibiotics are getting resistant against *Staphylococcus aureus*.

To avoid this high rate of antibiotic resistance bacteria, management and treatment of hospital effluent is really necessary. Because of extensive impact, resistance of antibiotic has on human health, more surveillance and control of its spread also prevalence is necessary. Again, planning and implementation on antibiotic use, limit the antibiotic use, proper use of antibiotics, detect microbial communities from hospital wastewater must be carried out by public health authorities and scientific communities. Overall, these steps might improve the scenario of the disease and infection related to ARBs and ARGs.

Chapter 7

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