The Prevalence of Multi-Drug-Resistant *Staphylococcus aureus* and *Klebsiella pneumoniae* in Cooked Meat Samples Collected From Hospital Cafeterias in the Dhaka City Corporation Area

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A thesis submitted to the **Programme of Biotechnology** in the **Department of Mathematics and Natural Sciences** in partial fulfillment of the requirements for the degree of **Undergraduate Programme**

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Declaration

It is hereby declared that,

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4. We have acknowledged all primary sources of help.

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Abstract

Foodborne diseases are a common threat in Bangladesh since study showed that 30 million people suffer from food poisoning in our country annually. One of the most favorite and nutritious food items in Bangladesh is chicken curry. Foodborne pathogens present in cooked chicken curry have not been investigated in Bangladesh yet. On the other hand, there have been several food poisoning outbreaks in various nations like Italy and South Korea due to *Staphylococcus aureus* and *Klebsiella pneumoniae* contamination in food. The study aims to investigate the prevalence of multi-drug resistant *Staphylococcus aureus* and *Klebsiella pneumoniae* from cooked meat collected from government hospital cafeterias in Dhaka city. In the current experiment, samples were collected from 9 hospitals covering five prime locations in Dhaka city. The study was conducted from April 2023 to March 2024. Presumptive isolates of *Staphylococcus aureus* and *Klebsiella pneumoniae* through the usage of selective and differential media named Mannitol Salt Agar (MSA) and Eosin methylene blue agar (EMB) and biochemical tests- (i) Gram staining, (ii) Triple Sugar Iron test. (iii) Citrate Utilization test. (iv) Catalase test and (v) Oxidase Test.Out of 63 presumptive *Staphylococcus aureus* isolates based on MSA media, 49 were positive based on biochemical tests. Molecular detection of the presumptive isolates of *Staphylococcus aureus* and *Klebsiella pneumoniae* was carried out using polymerase chain reaction (PCR) using *nucA* and *ITS* sequence primers, respectively. Out of 49 presumptive *Staphylococcus aureus* isolates based on biochemical tests, 14.3% were confirmed positive isolates of *Staphylococcus aureus* through PCR and Gel Electrophoresis. All the *Staphylococcus aureus* isolates were multidrug-resistant (MDR), and among them, 57.1% are Methicillin-resistant *Staphylococcus aureus* (MRSA). Out of 14 isolates of presumptive *Klebsiella pneumoniae* isolates based on EMB media, all the isolates were positive for biochemical tests. Molecular detection also confirmed the presence of *Klebsiella pneumoniae.* Interestingly, this isolate was multi-drug resistant to ampicillin, Ceftazidime, and Colistin antibiotics.

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1. Introduction

1.1 Bacterial Contamination in Meat Products

Bacterial products in meat have gained significant attention in the food science industry. Various studies have been conducted worldwide, including multiple cities of Egypt- Damanhur, Benha, Greece, Indonesia, Poland, Iran, the United States of America, the United Kingdom, India, and Bangladesh on different types of meat products to understand their diversity of bacteria. These meat products encompass a wide range, including raw and poultry meat such as chicken and beef, Greek meat products, and processed and ready-to-eat (RTE) meats like minced meat, liver, luncheon, sausages, pork ham, chicken cold cuts, pork sausage, salami, nuggets, and pork luncheon meat, among others. (Ali et al., 2023; Theocharidi et al., 2022; Haghi et al., 2021; Farahmand et al., 2020; Sudarmadi et al., 2020; Uddin et al., 2019; Hassan et al., 2018; Schaeffer, 2017; Fijałkowski et al., 2016; Guo et al., 2016; Waters et al., 2011)

In all these studies, the main aim was to evaluate the prevalence of bacterial contamination, characterize the bacterial species present, and assess their impact on meat quality and public health. This focus concerns the association of several outbreaks linked to meat consumption, as animal-originated foods are more prone to microbial contamination.(Ali et al., 2023; Savini et al., 2023; Haghi et al., 2021; Farahmand et al., 2020; Sudarmadi et al., 2020; Theocharidi et al., 2022; Uddin et al., 2019; Hassan et al., 2018; Schaeffer, 2017; Fijałkowski et al., 2016; Guo et al., 2016; Waters et al., 2011)

Across these studies, several common findings have emerged regarding the bacterial species detected in meat products. The most frequently identified bacteria are *Staphylococcus aureus, Escherichia coli, Salmonella, Bacillus cereus, Clostridium perfringens,* and *Klebsiella pneumoniae*. (Ali et al., 2023; Farahmand et al., 2020; Fijałkowski et al., 2016; Guo et al., 2016; Haghi et al., 2021; Hassan et al., 2018; Savini et al., 2023; Schaeffer, 2017; Sudarmadi et al., 2020; Theocharidi et al., 2022; Uddin et al., 2019; Waters et al., 2011)

1.2 Meat Pathogens as Public Health Concerns and Worldwide Outbreaks of Drug-resistant Organisms from Meat

As discussed earlier, the common pathogens to be found are- *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, *Bacillus cereus*, *Clostridium perfringens,* and *Klebsiella pneumoniae* in a greater prevalence. (Ali et al., 2023; Farahmand et al., 2020; Fijałkowski et al., 2016; Guo et al., 2016; Haghi et al., 2021; Hassan et al., 2018; Savini et al., 2023; Schaeffer, 2017; Sudarmadi et al., 2020; Theocharidi et al., 2022; Uddin et al., 2019; Waters et al., 2011) Each of the bacterium mentioned is involved in the case of severe food poisoning and gastrointestinal diseases such as diarrhea, irritable bowel syndrome, hemorrhoids etc. (Ali et al., 2023) but among all bacteria, *Staphylococcus aureus* and *Klebsiella pneumoniae* were found in

all studies. They all showed significant disturbing results affecting public health. (Ali et al., 2023; Farahmand et al., 2020; Fijałkowski et al., 2016; Guo et al., 2016; Haghi et al., 2021; Hassan et al., 2018; Savini et al., 2023; Schaeffer, 2017; Sudarmadi et al., 2020; Theocharidi et al., 2022; Uddin et al., 2019; Waters et al., 2011)

1.2.1 *Staphylococcus aureus*

Staphylococcus aureus is known to be a major pathogen responsible for food poisoning, which causes up to 2,41,148 cases and six deaths annually (Haghi et al., 2021). It highlights the significant public health risk of meat contamination by *Staphylococcus aureus* (Haghi et al., 2021; Pal, 2022; Savini et al., 2023).

Numerous studies have shown high prevalence rates of *S. aureus* in meat samples, with a concerning number of isolates demonstrating resistance to multiple antibiotics, i.e., multiple drug-resistant *Staphylococcus aureus* isolates were obtained.(Haghi et al., 2021; Pal, 2022; Savini et al., 2023) The prevalence of *S. aureus* is exceptionally high in meat because the skin and mucous membranes of animals are known as the storage pools for *S. aureus*, and this bacteria has the immense ability to transmit and infect animal products such as meat, thus residing inside the meat. (Haghi et al., 2021) This high prevalence within meat presents a considerable challenge for public health, as it not only poses a direct risk of foodborne illness but also complicates treatment due to antibiotic resistance. In the studies, almost 52% of the meats were multiple drug-resistant *S. aureus* (MDR *S. aureus*). Moreover, these were highly pathogenic enough to cause an outbreak in Italy in 2019, where 72 patients died from food poisoning due to MDR *S. aureus* infection. (Haghi et al., 2021; Pal, 2022; Savini et al., 2023) The emergence of antibiotic-resistant strains further intensifies the situation, increasing the likelihood of spreading resistance through horizontal gene transfer (HGT) to a broader population.

1.2.2 *Escherichia coli*

Escherichia coli (E. coli) is extensively known as a fecal contamination indicator and has become a marker to quantify the hygienic quality of meat products. Several other studies have been carried out, showing the prevalence of *E. coli in* various types of meat, including poultry, seafood, swine, and processed meat products. For instance, the detection of *E. coli* was reported in some studies conducted in Egypt (Ali et al., 2023), Greece (Theocharidi et al., 2022), and Indonesia (Sudarmadi et al., 2020) in raw and processed meat samples.

There is plenty of evidence that *E. coli* is present in meat products, raising concerns. The foodborne illness risk associated with food products results from certain types of *E. coli*-infection, such as *E. coli* that produces Shiga toxin (STEC). In addition, this also raises concerns regarding the presence of meat products that are antibiotic-resistant. Researchers have determined the existence of *E. coli* participating in multi-drug resistance in meat items, only

revealing the need for monitoring the pattern of antibiotic resistance in foodborne pathogens (Haghi et al., 2021; Guo et al., 2016).

1.2.3 *Salmonella spp.*

Among the common bacterial contaminants in meat products worldwide is *Salmonella*. Research confirms *Salmonella's* dominance in poultry, beef, pork, and processed meat products. As indicated by close-knit research groups from countries such as Iran (Farahmand et al., 2020), the US (Waters et al., 2011), and Bangladesh (Uddin et al., 2019), numerous meat samples were found to have *salmonella*.

Salmonella bacteria pose a substantial danger in meat products, causing severe public health threats. The symptoms of salmonellosis include diarrhea, fever, and abdominal cramps, which are identifiable through it. Alongside a public health risk, the appearance of antibiotic-resistant strains raises the question of the effectiveness of antimicrobial treatment. Research has drawn findings about multidrug-resistant *Salmonella* isolates in meat samples, necessitating more comprehensive surveillance and control measures for overcoming antibiotic resistance (Haghi et al., 2021; Savini et al., 2023).

1.2.4 *Bacillus cereus*

Bacillus cereus is a spore-forming bacterium frequently found in soil but also invades meat products during the manufacturing process or by means of bad handling. Low-quality food is employed and home to all the microorganisms, and the unsystematic ways of handling it make it unfit for consumption. Researches show that in different countries, such as Poland (Fijałkowski et al., 2016), India (Hassan et al., 2018), and the United Kingdom (Schaeffer, 2017), *Bacillus cereus* was detected in various types of meat, such as poultry and processed meat products.

Bacillus cereus contamination in meat products can result in foodborne illnesses because some strains of the bacteria release toxins that induce symptoms like nausea, vomiting, and diarrhea. Furthermore, because *Bacillus cereus* spores can withstand cooking and other food processing methods, their capacity to produce heat-resistant spores presents a risk to food safety. Studies have also indicated that *Bacillus cereus* isolates from meat products may be antibiotic-resistant, highlighting the significance of taking preventative steps to reduce contamination (Fijałkowski et al., 2016; Sudarmadi et al., 2020).

1.2.5 *Clostridium perfringens*

The spore-forming bacteria *Clostridium perfringens* is frequently found in the environment and can infect meat products while they are processed and stored. Studies carried out in the United States (Waters et al., 2011), Iran (Haghi et al., 2021), and Egypt (Ali et al., 2023) have revealed the presence of *Clostridium perfringens* in a variety of meats, including beef and poultry.

The potential of *Clostridium perfringens* to create toxins that lead to foodborne illnesses, including food poisoning, makes the bacterium's contamination of meat products a matter of concern. Because *Clostridium perfringens* spores may withstand cooking and other food processing techniques, its spore-forming nature poses additional hurdles to food safety. Furthermore, research has shown that meat products include antibiotic-resistant strains of *Clostridium perfringens*, underscoring the significance of taking preventative steps to reduce contamination and the chance of contracting a foodborne illness (Haghi et al., 2021; Hassan et al., 2018).

1.2.6 *Klebsiella pneumoniae*

Klebsiella pneumoniae contamination in meat poses a significant threat to public health. It is an opportunistic bacteria that can cause various diseases, such as pneumonia, meningitis, blood and urinary tract infections, food poisoning, diarrhea, septicaemia, and liver abscesses, through meat consumption. The diseases caused by *Klebsiella pneumoniae* increase annually. (Hartantyo et al., 2020; Riwu et al., 2022; Zhang et al., 2018)

Meat, being a significant source of energy and essential nutrients, becomes a carrier of such bacterium that cannot only cause such a variety of diseases but also a carrier of antibiotic-resistant strains of *Klebsiella pneumoniae*. Multiple resistant *K. pneumoniae* strains and isolates have been found in various studies from various types of meat, and it was stated in the studies that dietary intake is one of the primary routes of introducing antibiotic-resistant bacteria to the human digestive tract. It would not only affect the treatment efficacy of patients but introducing antibiotic-resistant genes into the gut microbiota can disrupt the balance of beneficial bacteria, potentially leading to dysbiosis and associated health issues. (Hartantyo et al., 2020; Riwu et al., 2022; Zhang et al., 2018) The reason for the emergence of Multidrug-resistant Food *Klebsiella pneumoniae* is still not found. However, the concerning question is that *Klebsiella pneumoniae* is not limited to hospital environments but in other places as well, and as it is very contagious, the cross-contamination with bacteria from the raw yield, meat authorities, or food instructors contaminates the meat consumed by the public.(Riwu et al., 2022; Zhang et al., 2018) Therefore, controlling and preventing the spread of this pathogen through meat is crucial to safeguard public health and prevent foodborne illnesses. In addition to the concern of multi-drug resistance of *K. pneumoniae*, studies found an association between *K. pneumoniae* isolated from retail chicken meats and *K. pneumoniae* isolated from Urinary Tract Infections (UTI) patients, suggesting dietary intake of meat may cause UTI patients. (Schaeffer, 2017)

1.3 The Impact of Cooked Meat on Public Health is Uncharted Territory

Despite several studies on raw and processed meat, more research on bacterial identification from cooked meat and its effects on public health is needed. There have only been two studies on bacterial identification from cooked meat: one in Istanbul, Turkey, on "doner kebab" and another on "suya" (a roasted meat product) in Nigeria. In both studies, it is observed that studies have

tried to investigate the type of cooked meat that serves as a typical and staple cooked chicken dish in their nation. Both are traditional barbeque and roasted dishes where the meat is marinated with various spices such as clove, ginger, pepper, salt, vegetable oil, and other flavorings. The prolonged marination with spices and high-temperature roasting should eliminate bacterial contamination in the meat due to the antimicrobial properties of spices and high heat (Kaya et al., 2018; Orpin et al., 2019).

Nevertheless, surprisingly, in both studies, *Staphylococcus aureus* was found in a more significant percentage in cooked dishes (Kaya et al., 2018; Orpin et al., 2019) and in the paper of Orpin et al., 2019, *K.pneumonia* was also detected. (Orpin et al., 2019) In the paper (Kaya et al., 2018), a comparison of raw-doner kebab and cooked doner kebab was conducted. Interestingly, despite the earlier cooking conditions, the prevalence of *Staphylococcus aureus and K. pneumonia* was higher in cooked doner kebab than in raw doner kebab. The probable reasons described by the studies are as follows- **(i)** Poor hygiene practices during cooking, **(ii)** Usage of unsanitized utensils during cooking, **(iii)** Contamination from meat handlers may arise during the processing, and there is a likelihood that those contaminations did not die during the cooking processing indicating emergent strains of *S. aureus and K. pneumonia* who are unaffected by high heat and spices, **(iv)** The cooked chicken could be easily contaminated by the open air environment which consists of bacterial air spores or spores from insects such as flies, and lastly **(v)** Contamination may arise from spices used during cooking, either the spices become contaminated during the process of cooking, or the spices were contamination during the preparation of the spices. (Kaya et al., 2018; Orpin et al., 2019)

None of the previous studies have examined the effect of the microorganisms identified in cooked chicken on public health, including detecting antibiotic resistance patterns. This creates a significant need for further research and investigation into the potential impact of contamination of cooked chicken on the general public.

1.4 Hospitals- Homeground of Superbugs

Studies have shown a concerning prevalence of superbugs like *Acinetobacter spp, Escherichia coli*, *Klebsiella pneumoniae*, *and Staphylococcus aureus* in hospital settings, with high resistance rates to commonly used antibiotics. (Le Glass et al., 2022; Liu & Qin, 2022; Nath et al., 2020; Zhanel et al., 2010) The emergence of these superbugs is not a new phenomenon, as historical data from various countries such as Australia, Canada, and the USA dating back to the mid-20th century have already highlighted the challenges of antimicrobial resistance. (L Gilbert, 2007; Le Glass et al., 2022; Liu & Qin, 2022; Nath et al., 2020; Zhanel et al., 2010) Various studies have been conducted worldwide to determine the prevalence of multi-drug-resistant *Klebsiella pneumoniae* and *Staphylococcus aureus*. These studies collected clinical samples from patients attending clinics, emergency rooms, medical and surgical wards, and intensive care units (ICU).

Additionally, surfaces of hospital fomites, hands of hospital staff, and hospital wastages were examined. The studies were conducted in different countries, including Nigeria, USA, Canada, and Australia. The analysis revealed that both bacteria were present in all tested samples, with most exhibiting multidrug resistance. (L Gilbert, 2007; Le Glass et al., 2022; Liu & Qin, 2022; Nath et al., 2020; Zhanel et al., 2010) (Tula et al., 2022) in his paper compare the prevalence of each kind of bacteria isolated from the type of source it was extracted for, e.g., hospital environment samples such as sinks, beddings, door handles, staff tables, hands of hospital staff, and based on wards. Upon comparison, it was found that *Klebsiella pneumoniae* and *Staphylococcus aureus* were present in more significant quantities in the wards compared to other areas.

Furthermore, they were all multidrug-resistant, and all the *Staphylococcus aureus* were methicillin-resistant, stating they were all MRSA. The paper of (Liu & Qin, 2022) compares the prevalence and antibiotic-resistant pattern of the isolated bacteria from hospital environments from 2017 to 2019 for *Klebsiella pneumoniae* and *Staphylococcus aureus*. In the paper, it was evident that the prevalence of both bacteria has increased within these years. For *Staphylococcus aureus*, it was 20.3%, and for *Klebsiella pneumoniae*, it was 14%. Additionally, there were optimistic results for the drug resistance patterns of these two isolates; within three years, the drug resistance rates declined. For example, *Staphylococcus aureus*, penicillin, and clindamycin exhibited a reduction of 1.3% and 0.2%, respectively, but interestingly showed an increase for erythromycin with a rise of 0.9%. Ampicillin and aztreonam reduced 4.1% and 24.6% for *Klebsiella pneumoniae*, respectively. The results underscore the importance of hospital hygiene and how it positively impacts the reduction in the spread of antibiotic-resistant microorganisms inside the hospital environment.

However, this is only the case for some hospitals; most hospitals suffer from the spread of multidrug-resistant organisms. The emergence and spread of multidrug-resistant organisms among hospital patients is a significant concern as it can lead to transmission to non-infected patients. This highlights the need for strict hygiene practices in healthcare settings. Hospitals can become reservoirs for these superbugs, making it necessary to adopt comprehensive strategies for their containment and control. This is crucial to ensure patient's safety and well-being and prevent the escalation of antimicrobial resistance. (Córdova-Espinoza et al., 2023; Haghi et al., 2021; Herruzo et al., 2017; Le Glass et al., 2022; Liu & Qin, 2022; "Molecular Detection of Enterotoxins from *Staphylococcus Aureus* Isolated from Hospital and Environmental Samples in Osogbo," 2022; Nath et al., 2020; Odoya et al., 2015; Shrestha et al., 2017; University Ile Ife et al., 2017; Zhanel et al., 2010)

1.5 Rationale of Thesis

During the review of several studies, it became apparent that *Staphylococcus aureus* and *Klebsiella pneumoniae* are significant meat pathogens commonly found in hospitals and significantly impact human health. This led to a research gap regarding identifying these bacteria

and their impact on public health when isolated from cooked chicken, collected explicitly from hospitals, and finding an association between them. In South Asian regions, including Bangladesh, chicken curry is a staple and commonly consumed dish. It is not only common but a good, rich source of protein at an affordable price. The affordable price of this dish makes it an ideal source of protein to consume in a developing nation like Bangladesh. According to the Hindawi Journal of Food Quality, the mean Annual per capita consumption for the income group <'BDT 10,000' is 10.85, which signifies how the underprivileged community of Bangladesh is reliable in chicken dishes for their ultimate rich source of protein. Additionally, studies conducted in Bangladesh were mostly on different kinds of chicken but not on cooked chicken. Furthermore, studies conducted in Bangladesh were mostly on different kinds of chicken but not on cooked chicken. A study by (Akhi et al., 2019) aim was to detect methicillin-resistant *Staphylococcus aureus* from poultry samples, which included raw chicken as well, in this paper it was highlighted that the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) was highest from the raw chicken among all other kinds of samples they have studied on, i.e., 84%. Additionally, a study on frozen chicken meat in Bangladesh focused on the Antimicrobial Resistance Pattern of *Escherichia coli* and found out all the isolates were multi-drug resistant (MDR). (Parvin et al., 2020) Another similar study was conducted on the Antimicrobial Resistance Pattern of *Staphylococcus aureus* from frozen chicken meat, and the results were astonishing. Of 113 samples, 54.9% were positive for *Staphylococcus aureus*, 37.1% were MRSA, and all the isolates were multi-drug resistant. (Parvin et al., 2021). These studies have shown that many raw and processed chicken samples in Bangladesh contain MRSA and MDR bacteria, a cause for concern. However, whether this is also the case for cooked chicken is unclear.

However, studies have yet to be conducted on chicken curry. Therefore, a comprehensive investigation is necessary to understand the bacterial presence and its impact on public health. Although chicken curry is cooked at a high temperature and marinated with various spices, two studies on similar dishes have shown the prevalence of *Staphylococcus aureus* and *Klebsiella pneumoniae*. (Kaya et al., 2018; Orpin et al., 2019) This suggests the possibility of contamination in cooked chicken curry samples despite the precautions taken.

Moreover, the government hospitals in Bangladesh are often crowded, and many patients undergoing antibiotic treatment reside within the hospital. Due to the close contact of patients, staff, and visitors in hospital cafeterias, horizontal gene transfer (HGT) can occur, leading to the emergence of antibiotic-resistant bacteria. Since chicken curry is a popular dish in Bangladesh, it is a top priority for many visitors and staff. Therefore, it is crucial to examine the ongoing food safety of hospitals, specifically the cooked chicken curry served in the cafeteria.

Our study, of paramount importance, aims to isolate and identify *Staphylococcus aureus* and *Klebsiella pneumonia* from cooked chicken curry served in the cafeterias of government hospitals as these bacteria's emergence to resistance has a potential negative impact on public health, therefore assessing the antibiotic susceptibility of the characterized bacterial samples.

1.6 Objectives Of The Study

1. Sample preparation from cooked meat samples collected from government hospitals' cafeterias.
2. Isolation and Identification of *Staphylococcus aureus* and *Klebsiella pneumoniae* by morphological and biochemical tests, including selective and differential characterization
3. Molecular identification of *Staphylococcus aureus* and *Klebsiella pneumoniae* by PCR using the *nucA* and *ITS sequence,* gene-specific primers*,* respectively.
4. Detection of antibiotic susceptibility of the characterized bacterial samples using antibiotic susceptibility testing.

2. Materials and Methods

2.1 General Workflow

The study was designed to be conducted from April 2023 to March 2024. A general workflow was designed to understand the processing method of cooked chickens and screen for the prevalence of microbes, *such as Staphylococcus aureus* and *Klebsiella pneumoniae*. This study involved the identification of isolated *Staphylococcus aureus* and *Klebsiella pneumoniae* from high-heat-treated cooked chicken curry served in hospital cafeterias. Such pathogenic microbes may impose a potential risk of infection for patients, hospital staff, and visitors spanning all age groups. This research emphasizes the importance of continued monitoring and research to ensure food safety and public health in the region. The general workflow of the study is provided in **Figure-2.1**.

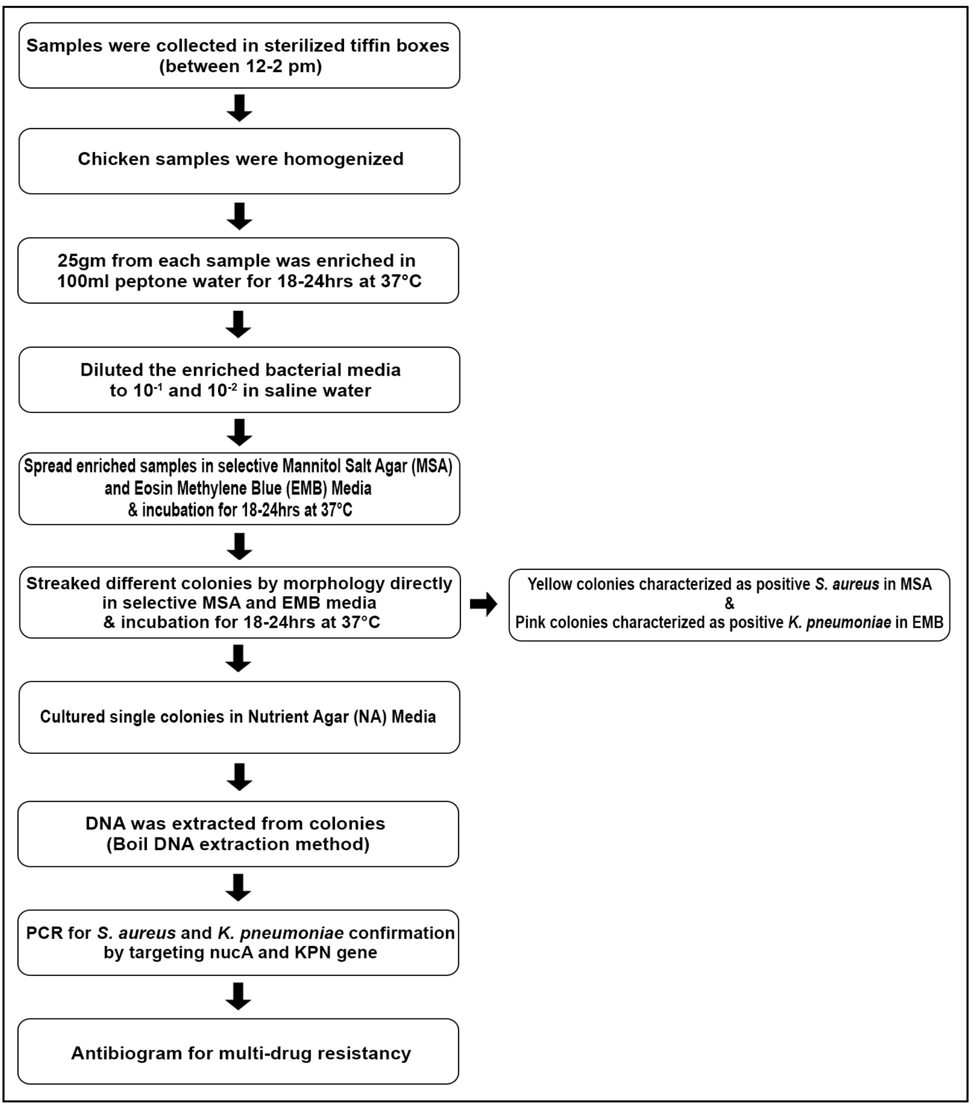


Figure 2.1: General Workflow

2.2 Sample Collection Site

The cooked chicken samples were collected from nine leading government hospital cafeterias of the five prime locations across both Dhaka South and North City Corporation between 12-2 pm. The locations and hospitals are accordingly:

- Ramna (Dhaka Medical College, DMC);
- Shahbagh (Bangabandhu Sheikh Mujib Medical University, PG);
- Old [Puran] Dhaka (Sir Salimullah Medical College Mitford Hospital, SMIT);
- Mohakhali (National Institute of Chest Disease and Hospital, TB; Sheikh Russel National Gastro Liver Institute & Hospital, SRG)
- Agargaon (Shaheed Suhrawardy Medical College and Hospital, SSH; National Institute of Cancer Research & Hospital, NICH; Dhaka Shishu Hospital, DSH; National Institute of Traumatology and Orthopaedic Rehabilitation, NITOR)

Isolation, Identification, and Characterization from the samples of these locations can represent a transparent scenario of what the general public, both patients and attendees, consumes daily from such vital places where they go for treatment to get a healthy life. The prominent government hospitals were chosen with the general public in mind.

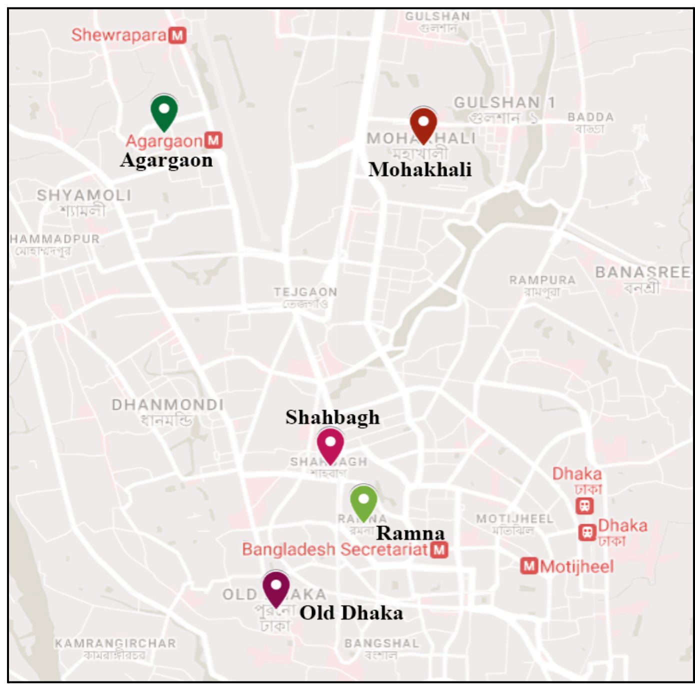


Figure 2.2: Map showcasing 5 prime locations of Dhaka City Corporation from where samples are collected.

2.3 Collection of Samples

18 cooked chicken samples were collected from 9 government hospitals covering five prime locations of Dhaka city- Ramna, Shabagh, Agargaon, Mohakhali, and Old Dhaka. The samples were taken in sterile food-grade plastic boxes during the lunch hour (between 12-2 pm), and sample collection was replicated two times in consecutive weeks on the same day and within the same timeframe. The samples were taken from serving dishes in hospital cafeterias using a spoon, the same way general people take their food inside the cafeteria.

2.4 Processing and Enrichment of Cooked Chicken Samples

In the laboratory, 25 gm of the cooked chicken curry samples (for each isolate) were cut with autoclaved scissors and forceps and homogenized using mortar and pestle. Then, the homogenized samples were inoculated into 100 ml of Peptone (HiMedia Laboratories) water in falcon tubes and then incubated at 37℃ for 18-24 hours for bacterial enrichment.

The details about the enrichment media used peptone are given below-

Peptone water/broth is a liquid medium not specific to any particular type of bacteria and can be used as the initial medium to promote the enrichment of bacteria. The microbial growth media consists of animal tissue peptate digestate and sodium chloride.

Peptone water serves as a diluting agent and nutrient-rich broth for reviving and cultivating various microorganisms in food, feed, and water samples. This product is well-suited explicitly for challenging strains, stringent growth conditions, and complex methods.

2.5 Microbiological Examination of *Staphylococcus aureus* and *Klebsiella pneumoniae* and Storage of the Isolates

After the incubation period, the bacterial-enriched peptone water was taken into other falcon tubes, and the meat pieces were discarded. Using the serial dilution technique, the enriched culture media was then diluted with saline water up to 10^{-1} and 10^{-2} dilution factor. After dilution, the original enriched media, the diluted 10^{-1} and 10^{-2} fold culture solution, were spread to Mannitol Salt Agar (MSA). The 10^{-2} fold solution was spread to Eosin Methylene Blue (EMB) Media. The MSA and EMB plates were incubated at 37°C for 24-36 hours.

The details about the types of media used and techniques used are described as follows.

2.5.1 Serial Dilution

Serial dilution is a technique deliberately used to decrease the concentration of a dense culture by a series of sequential dilutions to simplify the bacterial concentration. In this study, the ten-fold dilution was approached to reduce the bacterial concentration by a factor of ten, that is, to one-tenth the original concentration. To dilute 1 ml of bacterial broth of cooked chicken sample,

9 ml of general saline water was used for each fold. The samples were diluted to 10^{-1} and 10^{-2} for spread plating.

2.5.2 Spread Plate Method

A universal microbiological procedure known as the spread plate method is utilized for isolating and counting the total number of viable microorganisms in a liquid sample from evenly distributed discrete colonies all over the surface of the culture media.

2.5.3 Mannitol Salt Agar (MSA) Agar

Mannitol salt agar is a microbiological medium with selectivity and differentiation properties. Selective refers to the property of a medium that allows the growth of only specific microorganisms. Differential refers to the ability of the medium to exhibit certain strains of the microorganisms growing on it, allowing for the distinction between several species. This media is specific for isolating pathogenic *Staphylococcus spp.* from clinical samples, food, and others.

The selectivity of this medium is determined by its high salt concentration, which only supports the growth of halophilic (salt-loving) or halotolerant (salt-tolerant) organisms. For this particular study, the medium has a modified concentration of 12.5% NaCl (salt), while standard media usually has a concentration of around 7.5% NaCl.

The differentiability of this medium can detect the formation of acid resulting from the fermentation of mannitol, a sugar included in the medium. This medium can detect species that can ferment mannitol and create acid. The medium includes phenol red applied as a pH indicator, which appears red-orange when the pH is neutral, around pH 7. When a species undergoes mannitol fermentation and generates acid, the pH of the surrounding environment lowers, causing phenol red to change its color to yellow.

MSA agar distinguishes between species of *Staphylococcus* and *Micrococcus*. The fermentation of mannitol by pathogenic *Staphylococcus spp.*, such as *Staphylococcus aureus*, is evidenced by the medium turning yellow and producing yellow colonies. In contrast, *Staphylococcus epidermidis*, a non-pathogenic type of *Staphylococcus spp.*, does not produce a yellow hue, instead forming white raised colonies.

For this study, 27.75 grams of Mannitol Salt Agar (HiMedia Laboratories) and 12.5 (5% m/w) grams of sodium chloride (NaCl) were dissolved in 250 ml of distilled water. The medium was then entirely dissolved by boiling. The sterilization process involved autoclaving at a pressure of 15 psi and a temperature of 121℃ for 1.5-2 hours. The sterile media was poured into sterile petri dishes under aseptic conditions into the laminar flow.

2.5.4 Eosin Methylene Blue (EMB) Agar

Eosin methylene blue agar is also a selective and differential medium. Consequently, specific subset of bacteria will survive on this agar, and the visual characteristics of the growing bacteria will vary. EMB agar explicitly facilitates the proliferation of Gram-negative bacteria and assists in differentiating certain types of Gram-negative rods. The dyes 'eosin Y' and methylene blue are the selective and inhibitory agents of EMB. Methylene blue can hinder the growth of gram-positive bacteria. However, it also has a more negligible effect on eosin. On the other hand, eosin undergoes a color shift to a deep purple when the surrounding medium of the bacterial colony becomes acidic.

EMB medium consists of lactose and sucrose carbohydrates, with lactose playing a crucial role in the medium. Lactose-fermenting bacteria, such as *E. coli* and other coliforms, generate acid due to lactose metabolism. The presence of dyes in the medium, which act as pH indicators, leads to color changes in the colonies due to the acidic conditions. A high acidity results in the formation of a dark purple colony with a shiny green metallic appearance, whereas lower acidity levels may lead to the development of a colony with a brown-pink hue. Non-lactose fermenters exhibit a transparent or pink appearance.

For this study, 9.375 grams of Eosin Methylene Blue (EMB) (HiMedia Laboratories) was dissolved in 250 ml of distilled water. The medium was then entirely dissolved by boiling. The sterilization process involved autoclaving at a pressure of 15 psi and a temperature of 121℃ for 1.5-2 hours. The sterile media was poured into sterile petri dishes under aseptic conditions into the laminar flow.

After incubation, the colonies of the plates were examined according to morphology. Since the targeted organisms were *S. aureus* and *K. pneumonia*, yellow colonies with yellow zones from MSA and pink-mucoid and deep purple colonies from EMB were extracted. The extracted colonies from MSA were streaked in MSA media, and extracted colonies from EMB media were streaked in EMB media to achieve a single pure colony of that particular isolate, and they were incubated at 37°C for 24h.

A detailed description of the streak plate method is given below.

2.5.5 Streak Plate Method

The streak plate method is another widespread aseptic microbiological procedure for isolating and propagating pure colonies of bacteria from a mixed culture comprising various bacterial species. After the spread plate method for microbial growth, presumed colonies were collected using a sterile loop or needle and streaked onto selective MSA and EMB agar media surfaces. Due to the media's selective nature, the contamination probability is negligible.

While the single colonies of *Staphylococcus aureus* and *Klebsiella pneumoniae* were obtained, they were streaked on the surface of Nutrient Agar (NA) media to achieve the same single isolate without the interference of the composition of selective media, e.g., the dye. The details of nutrient agar are given below.

2.5.6 Nutrient Agar (NA)

Nutrient agar is a non-selective, multifaceted medium that facilitates the development of a diverse array of non-fibrous organisms. It is widely used due to its ability to promote the proliferation of diverse bacteria and fungi, quality control, and its rich composition of essential nutrients for bacterial growth and checking purity before biochemical tests, among other things.

In our study, 7 grams of the dehydrated powder (HiMedia Laboratories) was placed into a beaker containing 250 ml of distilled water. After that, the suspension was heated to its boiling point in order to fully dissolve the medium. The solution is subsequently sterilized by autoclaving at a pressure of 15 psi (121°C) for a duration of 1.5-2 hours. After the autoclaving procedure was finished, the beaker was removed and allowed to cool to a temperature of around 40-45°C. The sterile media was poured into sterile petri dishes under aseptic conditions into the laminar flow.

2.5.7 Storage of Isolates

From every streak result, a single colony and isolate were selected. This single colony was inoculated into 1 ml of nutrient broth, which was placed inside a 1.5 ml autoclave microcentrifuge tube. They were incubated for 24 hours at 37°C. Upon completion of incubation, 160-200µl of 60% glycerol was added to each NB stock, which was stored at -20°C in the fridge.

The details about the Nutrient broth are given below.

2.5.8 Nutrient Broth (NB)

Nutrient Broth (NB) is a frequently used medium for cultivating microorganisms with low nutritional requirements. It is advised in numerous standardized ways of analyzing food, dairy products, water, and other products. It is composed of a combination of Tryptone and meat extract that facilitates the proliferation of bacteria. Sodium chloride is used to regulate osmotic pressure. It can also be used for bacterial stocking purposes.

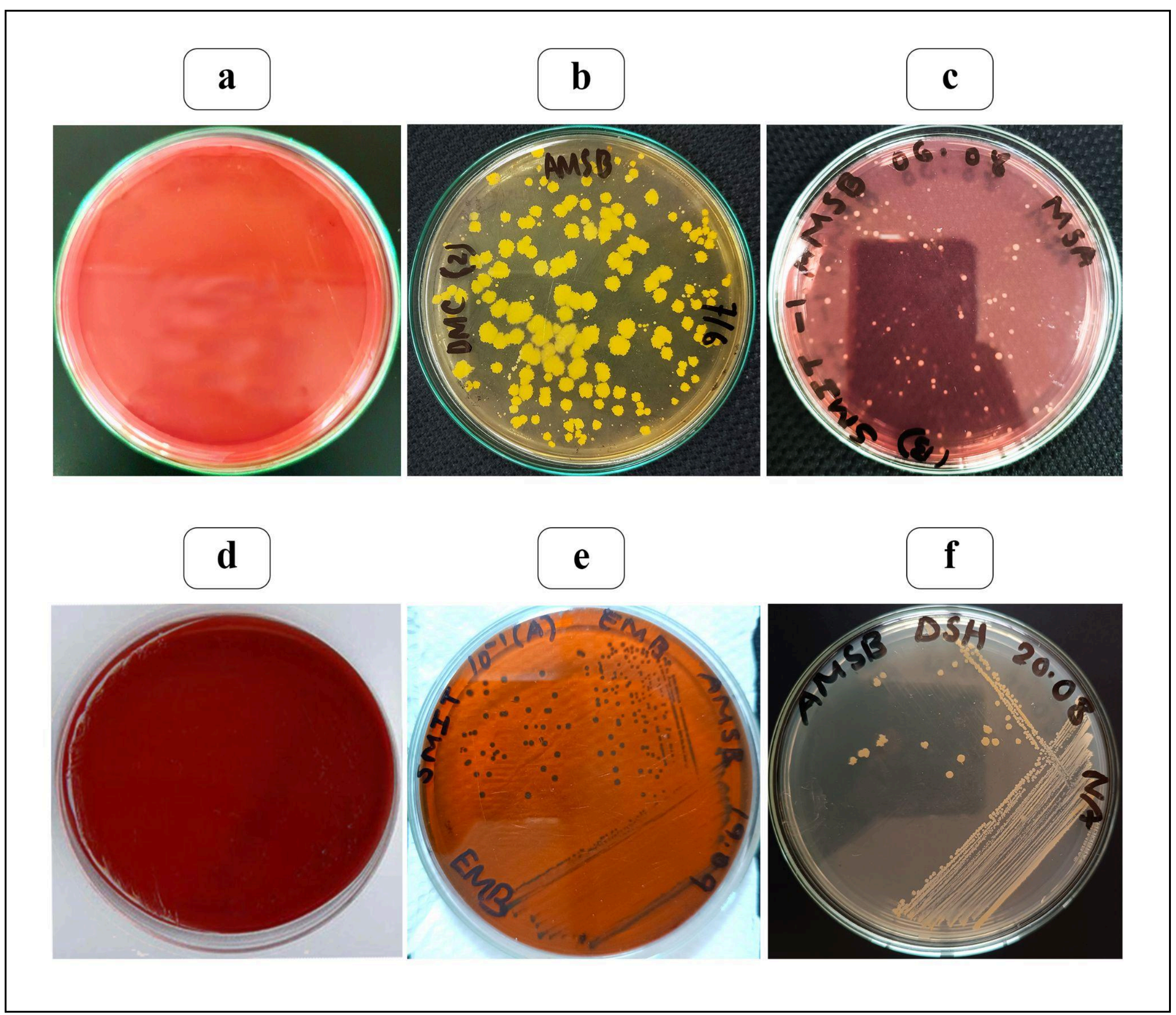


Figure 2.3: Bacterial Growth in MSA, EMB, and NA Agar Medium

(a) Uninoculated MSA Agar Medium; (b) Yellow colonies of *Staphylococcus aureus* by Mannitol fermentation (spread-plate method); (c) White colonies by *Staphylococcus epidermidis*, media color did not change as bacteria cannot ferment Mannitol; (d) Uninoculated EMB Agar Medium; (e) Deep purple/Pink colonies by *Klebsiella pneumoniae* (streak-plate method); (f) Bacterial growth by streaking in NA medium.

2.6 Identification of Presumptive Bacteria by Biochemical Tests

The colonies were revived from the stock for biochemical test purposes. Upon revival, freshly obtained single colonies were used throughout the biochemical testing process.

Different biochemical tests using standard protocols have been carried out to understand the characterization of the bacterial isolates. Conventional methods of culturing and biochemical profiling are still widely used to isolate and identify our targeted bacteria from various sources, including edible foods. Here, various types of biochemical tests— Gram Staining, Triple Sugar Iron (TSI) Test, Citrate Utilization Test, Catalase Test, and Oxidase Test—were used for both *S. aureus* and *K. pneumoniae*.

2.6.1 Gram Staining

The Gram staining technique is highly significant in the field of microbiology. The term "Gram" is derived from the Danish bacteriologist Hans Christian Gram, who initially presented it in 1882 as a means of identifying pneumonia-causing germs. The initial test commonly conducted, known as gram staining, utilizes crystal violet or methylene blue as the principal dye. When observed under a microscope, gram-positive organisms maintain the original hue and have a purple-brown appearance. Microscopic examination reveals that the microorganisms that do not absorb the main stain have a red coloration, indicating that they are Gram-negative organisms. (Aryal, 2022c)

The initial stage in gram staining involves the application of crystal violet dye to stain the slide. The subsequent stage, commonly referred to as dye fixation, entails using iodine to create a crystal violet-iodine complex, which impedes the facile removal of the dye. Afterward, a decolorizer, typically consisting of a mixture of ethanol and acetone, is employed to eliminate the dye. The fundamental idea of gram staining is around the bacterial cell wall's capacity to retain the crystal violet dye while exposed to a solvent. Gram-positive microbes contain a more significant amount of peptidoglycan, while gram-negative organisms contain a more substantial amount of lipids. (Aryal, 2022c)

At first, all bacteria absorb crystal violet dye. However, the lipid layer of gram-negative organisms is dissolved by a solvent. Gram-negative bacteria lose the primary stain when the lipid layer is dissolved. On the other hand, the solvent removes water from the gram-positive cell walls by closing the pores. This prevents the violet-iodine complex from diffusing, resulting in the bacteria retaining the stain. The duration of decolorization is a crucial stage in gram staining, as extended exposure to a decolorizing agent can eliminate all the stains from both types of bacteria (Aryal, 2022c).

The last stage of the gram-staining process involves applying a primary fuchsin stain to impart a pink hue to decolorized gram-negative bacteria, facilitating their identification. This stain is alternatively referred to as a counterstain. While safranin is used as a counterstain in some

laboratories, basic fuchsin is known to stain gram-negative organisms more intensely than safranin. Similarly, *Haemophilus spp., Legionella spp.,* and certain anaerobic bacteria exhibit little staining affinity with safranin (Aryal, 2022c).

2.6.2 Triple Sugar Iron (TSI) Test

The triple sugar iron (TSI) test identifies bacterial organisms capable of fermenting glucose, lactose, and sucrose and if they also produce hydrogen sulfide. Gram-negative bacteria from the Enterobacteriaceae family based on their physiological characteristics (glucose fermentation indicated by yellow butt, lactose fermentation indicated by yellow slant, and gas production marked by the presence of a crack or gas space or black precipitation, etc.) can be differentiated by this tube medium (Aryal, 2022e).

A specific agar slant medium was firstly made of the mixture of multiple sugars (0.1% glucose, 1% sucrose, 1% lactose) with pH-sensitive phenol red dye as well as sodium thiosulfate with ferrous sulfate (used 'Triple Sugar Iron' media by HiMedia Laboratories), then solidify at a slanted angle. This slanted form of the medium creates an array of surfaces that are either exposed to oxygen-containing air to variable degrees (an aerobic environment) or not exposed to air (an anaerobic environment), allowing for studying organisms' fermentation processes. Further, the microorganism is taken with the inoculum needle, stabbed into the agar medium's bottom, and streaked at the slant. Then, the cap-loose test tube was incubated at 37°C in ambient air for 18-24 hours. After the incubation period, different colors and conditions can be observed based on bacterial fermentation of the carbohydrates (Aryal, 2022e).

2.6.3 Citrate Utilization Test

The citrate utilization test assists in identifying the particular bacteria that are capable of utilizing the citrate as their sole energy source—gram-negative bacteria from the Enterobacteriaceae family commonly identified by this test. 'Simmons Citrate Agar' (HiMedia Laboratories) medium is primarily used in this test, which contains sodium citrate and a pH indicator bromothymol blue. The citrate utilization test is essential to check whether the bacteria can utilize the citrate as their carbon source and if the pH of the medium changes or not (Aryal, 2022e).

To conduct the experiment, citrate agar medium was taken into glass vials, and then the bacteria on the top of the inoculation needle were stabbed into the agar medium and streaked in the slant. Then, the vials were incubated at 37°C for 24 hours. After the incubation period, the color changes of the agar medium were observed. The media turned blue (positive result) from green when bacteria utilised citrate as their energy source, and the green color of media remained unchanged (negative result) when bacteria failed to utilize citrate (Aryal, 2022e).

2.6.4 Catalase Test

The catalase test detects the presence of the enzyme catalase, which protects organisms from oxidative damage from reactive oxygen species. (Aryal, 2022a; Sapkota, 2022)

An inoculum of the test organism was spread on a glass slide, and 2-3 drops of hydrogen peroxide (H_2O_2) were added; bubble formation was observed. Bubble formation indicated the presence of catalase to produce oxygen gas (bubbles), and no bubble formation stated the absence of catalase; thus, oxygen gas (bubbles) was not produced. (Aryal, 2022a; Sapkota, 2022)

2.6.5 Oxidase Test

This experiment aims to determine whether or not an organism possesses the cytochrome oxidase enzyme. The cytochrome-C-oxidase system, which catalyzes electron transfer between electron donors in bacteria and a redox dye called tetramethyl-p-phenylenediamine, can be identified by this test (Aryal, 2022d).

We took a single colony from a streaked colony onto filter paper with a needle, added 3-4 drops of freshly prepared 1% 'N,N,N′,N′-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD)' solution, and waited 5-10 seconds for the outcome. When the moist region took on a vivid purple-blue/blue hue, it suggested the existence of an oxidase product (positive result). On the other hand, no purple-blue/blue emerged in the negative results, indicating no oxidase was produced (Aryal, 2022d).

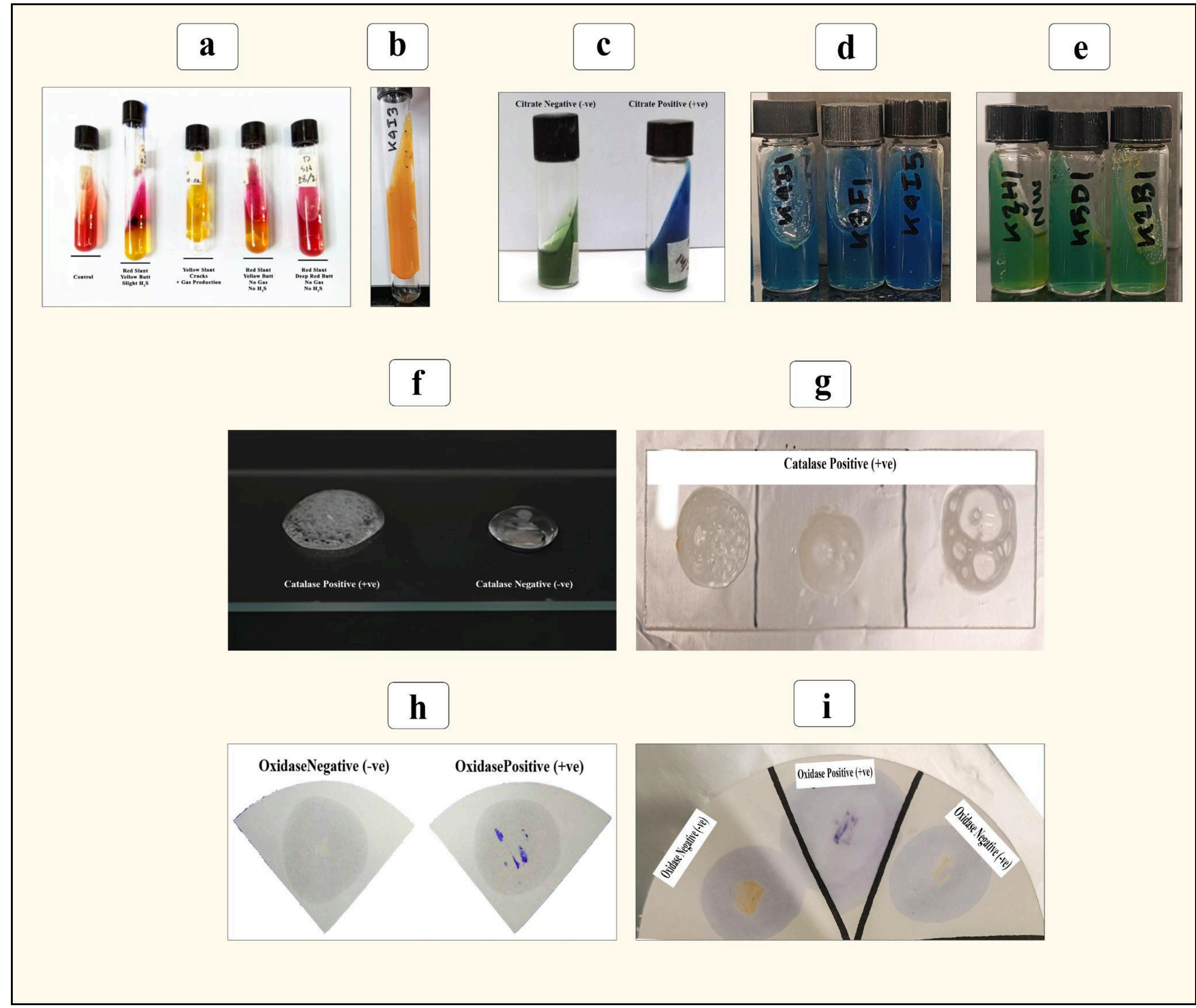


Figure 2.4: Biochemical Tests Control and Results

(a) Quality control and visual comparison of different microorganisms in TSI Tests; the microorganisms are differentiated by different physiological characters; (b) Bacteria fermented all the sugar strongly and produced gas; (c) Quality control and visual comparison of Citrate Utilization Test; (d) Citrate positive results, media turns blue from green; (e) Citrate negative results, media color unchanged; (f) Quality control and visual comparison of Catalase Test; (g) Catalase positive results, Bacteria produced the enzyme catalase and released gas bubbles; (h) Quality control and visual comparison of Oxidase Test (i) Oxidase Positive result in the middle indicates the related bacteria had cytochrome-C enzyme in their respiratory chain while the negative bacteria had not.

2.7 Molecular Analysis of Isolated Bacteria

2.7.1 DNA Isolation from Isolated Bacteria

Two to three freshly cultured colonies were aseptically transferred and combined with 180µl of Tris-EDTA (TE) buffer within a sterile 1.5 ml microcentrifuge (Eppendorf) tube. The resulting suspension underwent heat incubation at 100℃ for 15 minutes using a heat block apparatus (ONiLAB Scientific Dry Bath Incubator). Subsequently, the sample was subjected to a heat shock treatment by immersion in an ice bath for 10 minutes to promote cell lysis.

Following the heat shock treatment, the suspension was centrifuged at 14,000 rpm for 5 minutes. This centrifugation step facilitated the separation of cellular debris, which formed a pellet, from the supernatant containing the DNA solution. The supernatant containing the DNA was carefully collected in a 1.5 ml sterilized microcentrifuge, while the resulting pellet, composed of cellular remnants, was discarded. The DNA samples were then stored at -20°C, wrapped with laboratory-grade parafilm. All the materials used were sterilized by autoclaving beforehand to avoid possible contamination.

2.7.2 PCR Preparation

Polymerase chain reactions were performed to identify the isolated bacteria. DNA extracted from presumptive *Staphylococcus aureus* cultures was amplified, targeting the *nucA* gene, while DNA from presumptive *Klebsiella pneumoniae* cultures was amplified, targeting the *ITS* sequence.

PCR amplification reactions were carried out in a total volume of 16µl, consisting of 7.5µL of Master Mix, 3µL of isolated DNA, and 4.5µL of nuclease-free water. Additionally, both forward and reverse primers were added at a volume of 0.5µL each.

Name of Gene	Primer Nucleotide Sequence	Target Organism	Band product size (bp)	Reference
nucA	**Forward Primer-** 5′-GCGATTGATGGTGATACGGTT-3′ **Reverse Primer-** 5′-AGCCAAGCCTTGACGAACTAAAGC-3′	*Staphylococcus aureus*	270	https://pubmed.ncbi.nlm.nih.gov/34434176/ https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9047149/#ref21

Klebsiella pneumoniae specific primer	**Forward Primer-** 5′-ATTTGAAGAGGTTGCAAACGAT-3′ **Reverse Primer-** 5′-TTCACTCTGAAGTTTTCTTGTGTTC-3′	*Klebsiella pneumoniae*	133	https://www.sciencedirect.com/science/article/pii/S1687157X17301191

Table 2.1: Primer List according to Organisms for PCR amplification

2.7.3 PCR Conditions and Amplification

For *Staphylococcus aureus*, conventional PCR was conducted targeting the *nucA* gene, utilizing the following PCR conditions: (Mashouf et al., 2015)

Steps	Temperature (℃)	Time	Cycle
Initial Denaturation	94°C	5 min	1x cycle
Denaturation	94°C	1 min (60 sec)	35x cycles
Annealing	50°C	1 min (60 sec)	
Extension	72°C	1 min (60 sec)	
Final Extension	72°C	10 min	1x cycle

Table 2.2: PCR Thermal Cycle Parameters for *nucA* gene

For *Klebsiella pneumoniae*, conventional PCR was performed targeting the *ITS* sequence, employing the following PCR conditions: (Mahmudunnabi et al., 2018)

Steps	Temperature (℃)	Time	Cycle
Initial Denaturation	94°C	10 min	1x cycle
Denaturation	94°C	30 sec	35x cycles
Annealing	60°C	45 sec	
Extension	72°C	45 sec	
Final Extension	72°C	10 min	1x cycle

Table 2.3: PCR Thermal Cycle Parameters for *Klebsiella pneumoniae* specific *ITS* sequence

2.7.4 Gel Electrophoresis

The PCR-amplified samples were subjected to agarose gel electrophoresis using a 1.5% agarose gel in 1x TBE buffer (pH 8.0-8.5), with 0.003% (wt/vol) ethidium bromide (Et-Br) added for DNA staining. A total of 5µL of the PCR-amplified samples and the ladder, named CLV-CSL-MDNA-1KB PLUS and Abclonal 100 bp ladder, were loaded onto separate occasions.

Gel electrophoresis was conducted at 110 volts for 40 minutes. Following electrophoresis, the resulting agarose gel was visualized and photographed on a transilluminator to detect DNA bands. Specifically, bands corresponding to a size of 270 bp were sought for *Staphylococcus aureus*, while bands of 133 bp were targeted for *Klebsiella pneumoniae*.

2.8 Antibiotic Susceptibility Test (AST)

The positive isolates obtained from gel electrophoresis results were subjected to antibiotic susceptibility testing using the Kirby-Bauer Disk Diffusion Susceptibility method. The fresh cultures of the positive samples were inoculated in 9 ml saline water and were adjusted to 0.5 McFarland turbidity standard to ensure standardized concentrations of bacterial suspensions. The suspensions were evenly spread (lawned) on Mueller-Hinton Agar (MHA) media using sterile cotton swabs. Upon lawning, antibiotic discs were placed on top of the media using autoclaved tweezers. Then, the petri dishes were incubated for 24 hours at 37°C.

The details about the Mueller-Hinton (MHA) Agar are given below.

2.8.1 Mueller-Hinton (MHA) Agar

Mueller-Hinton (MHA) Agar was developed specifically to isolate pathogenic *Neisseria* species. By this time, it was a widely employed microbiological growth medium used for antibiotic susceptibility testing using the Kirby-Bauer disc diffusion method. MHA is the preferred medium for the dispersion of antimicrobial agents infused into a paper disc, which is done by placing the disc over this agar gel. This method is detailed in the Clinical and Laboratory Standards Institute (CLSI) approved standard. It exhibits satisfactory batch-to-batch reproducibility in the context of susceptible testing.

2.8.2 Conducting AST of *Staphylococcus aureus*

The positive isolates of *Staphylococcus aureus* were tested against 12 antibiotics named Penicillin (10µg/disk- HiMedia), Methicillin (5µg/disk- HiMedia), Levofloxacin (5µg/disk- HiMedia), Ampicillin (25µg/disk- HiMedia), Novobiocin (30µg/disk- HiMedia), Azithromycin (30µg/disk- HiMedia), Erythromycin (15µg/disk- Oxoid), Amoxicillin (30µg/disk- Oxoid), Kanamycin (30µg/disk- Oxoid), Doxycycline hydrochloride (25µg/disk- Oxoid), Vancomycin (30µg/disk- Oxoid), Clindamycin (2µg/disk- BioAnalyse). The zone sizes of bacteria were compared with standard guidelines for resistance and sensitivity stated by the Clinical and Laboratory Standards Institute (CLSI). The following table represents CLSI guidelines for *Staphylococcus aureus* and their respective antibiotics. (Mayrhofer et al., 2014)

Antibiotic Name	**Disk Concentration (µg)**	**Symbol**	**Interpretive Categories & Zone Diameter (mm)**		
			Sensitive (S)	**Intermediate (I)**	**Resistant (R)**
Penicillin	10	P	≥29	-	≤28
Methicillin	5	MET	≥22	-	≤17
Amoxicillin	30	AMC	≥22	-	≤15
Levofloxacin	5	LE	≥19	16-18	≤15
Ampicillin	25	AMP	≥29	-	≤28
Novobiocin	30	NV	≥22	18-21	≤17

Kanamycin	30	K	≥18	14-17	≤13
Vancomycin	30	VA	≥17	15-16	≤14
Erythromycin	15	E	≥21-23	14-20	≤13-18
Azithromycin	30	AZM	≥18	14-17	≤13
Doxycycline	25	DO	≥21-22	15-20	≤14-19
Clindamycin	2	DA	≥21	15-20	≤14

Table 2.4: Different Antibiotics Zone Diameter for *Staphylococcus aureus*

2.8.3 Conducting AST of *Klebsiella pneumoniae*

The positive isolates of *Klebsiella pneumoniae* were tested against 11 antibiotics named Amikacin (30µg/disk- HiMedia), Ceftazidime 30µg/disk- HiMedia), Levofloxacin (5µg/disk- HiMedia), Cefepime (30µg/disk- HiMedia), Colistin (10µg/disk- HiMedia), Chloramphenicol (30µg/disk- HiMedia), Ampicillin (25µg/disk- HiMedia), Meropenem (10µg/disk- HiMedia), Amoxicillin-clavulanate (30µg/disk- Oxoid), Tetracycline (30µg/disk- Oxoid), Aztreonam (30µg/disk- BioAnalyse). The zone sizes of bacteria were compared with standard guidelines for resistance and sensitivity stated by the Clinical and Laboratory Standards Institute (CLSI). The following table represents CLSI guidelines for *Klebsiella pneumoniae* and their respective antibiotics. (Mayrhofer et al., 2014)

Antibiotic Name	**Disk Concentration (µg)**	**Symbol**	**Interpretive Categories & Zone Diameter (mm)**		
			Sensitive (S)	**Intermediate (I)**	**Resistant (R)**
Amikacin	30	AK	≥17	15-16	≤14
Ceftazidime	30	CAZ	≥21	18-20	≤17
Levofloxacin	5	LE	≥21	17-20	≤16

Cefepime	30	CPM	≥25	19-24	≤18
Colistin	10	CL	≥14	12-13	≤11
Chloramphenicol	30	C	≥18	13-17	≤12
Ampicillin	25	AMP	≥17	14-16	≤13
Meropenem	10	MRP	≥23	20-22	≤19
Amoxicillin-clavulanate	30	AMC	≥18	14-17	≤13
Tetracycline	30	TE	≥15	12-14	≤11
Aztreonam	30	ATM	≥21	18-20	≤17

Table 2.5: Different Antibiotics Zone Diameter for *Klebsiella pneumoniae*

2.9 Calculation of Multiple Antibiotic Resistance Index (MAR Index)

Multi-antimicrobial resistance (AMR) is a global threat, and hence, it is highly crucial to determine Multiple Antibiotic Resistance Index (MARI) of identified positive isolates of *Staphylococcus aureus* for several reasons- **(i)** It provides insight into the prevalence and distribution of resistant strains within a specific population or geographical area, aiding in the formulation of targeted intervention strategies; **(ii)** Understanding the extent of antimicrobial resistance helps healthcare providers make informed decisions regarding antibiotic prescribing practices, infection control measures, and public health policies; **(iii)** Monitoring multi-antimicrobial resistance patterns over time allows for the early detection of emerging resistance trends and the identification of high-risk areas or populations, this would prevent the further spread of resistant bacteria and mitigate the potential consequences of widespread antimicrobial resistance and finally; **(iv)** Helps assess the effectiveness of existing antimicrobial stewardship programs and infection control measures.

Multiple Antibiotic Resistance Index (MARI) is estimated by dividing the number of antibiotics that displayed resistance by the number of total antibiotics tested for each microorganism. A

MAR index >0.2 demonstrates that the particular isolates have originated from high-risk contamination where antibiotics are extensively used.

3. Observation and Result

3.1 Isolation of *Staphylococcus aureus*

Isolation of *Staphylococcus aureus* was carried out using selective and differential media named Mannitol Salt Agar- MSA. The bacteria that showed distinct yellow colonies with yellow zones are considered *Staphylococcus aureu*s, and pre-assumptive *Staphylococcus epidermidis* showed white colonies in MSA are considered *Staphylococcus epidermidis*. 56 and 7 pre-assumptive *Staphylococcus aureus* and *Staphylococcus epidermidis* using selective media-MSA, respectively. Detailed descriptions of the distinct morphologies observed on each media type are provided in the subsequent **Table-3.1.**

	Number of isolates	**Observation**	**Picture**	**Hypothesised Organism**	**Percentage of Positive Presumptive** *Staphylococcus aureus* and *Staphylococcus epidermidis* using MSA media
Mannitol Salt Agar (MSA)	56	Yellow colonies with yellow zones		*Staphylococcus aureus*	88.9%
	7	White raised colonies		*Staphylococcus epidermidis*	11.1%

Table 3.1: Observational Result for MSA Agar Medium.

Distinct colonies from spread plates were streaked in Mannitol Salt Agar (MSA) media, where the single distinct colonies were obtained. To achieve single colonies, those single colonies were streaked again in Nutrient Media (NA). The picture of single colonies obtained in Nutrient Agar (NA) media is shown in **Figure-3.1.**

These single colonies were stocked for further usage in Nutrient broth (NB). They were used for other confirmatory identification purposes, such as biochemical tests and bacterial genomic Deoxyribonucleic acid (DNA) extraction for PCR assay.

The following biochemical tests were performed-

- Gram Staining
- Triple Sugar Iron (TSI) test
- Citrate Utilization test
- Catalase Test
- Oxidase Test

Figure 3.1: Single Colonies obtained in Nutrient Agar (NA) media

3.2 Identification of *Staphylococcus aureus* Using Biochemical Tests

Some fundamental characteristics of biochemical tests indicate the possible presence of specific kinds of bacterial species. **Table-3.2** mentions a chart for these characteristics for *Staphylococcus aureus***.**

Basic Characteristics	**Result for *Staphylococcus aureus***
Gram staining	Gram-positive (+ve)
Fermentation of Mannitol	Positive (+ve)
Fermentation of Glucose	Positive (+ve)
Fermentation of Sucrose	Positive (+ve)
Fermentation of Lactose	Positive (+ve)
Production of Gas	Negative (-ve)
Production of H_2S	Negative (-ve)
Citrate	Positive (+ve)
Catalase	Positive (+ve)
Oxidase	Negative (-ve)

Table 3.2: Observational Characteristics of *Staphylococcus aureus*

3.2.1 Gram Staining

All 63 presumptive isolates of ***Staphylococcus aureus*** underwent the Gram staining procedure. The staining characteristics were consistent with gram-positive ***Staphylococcus aureus*** bacteria. All of the 63 isolates retained the crystal violet stain, resulting in a purple coloration under the microscope, indicating the presence of a peptidoglycan cell wall. Therefore, these findings confirm the Gram-positive nature of the isolates, which provides a solid foundation for subsequent characterization and identification processes in the investigation of *Staphylococcus aureus.* **Figure-3.2** shows one of the results of the sample after the gram-staining process.

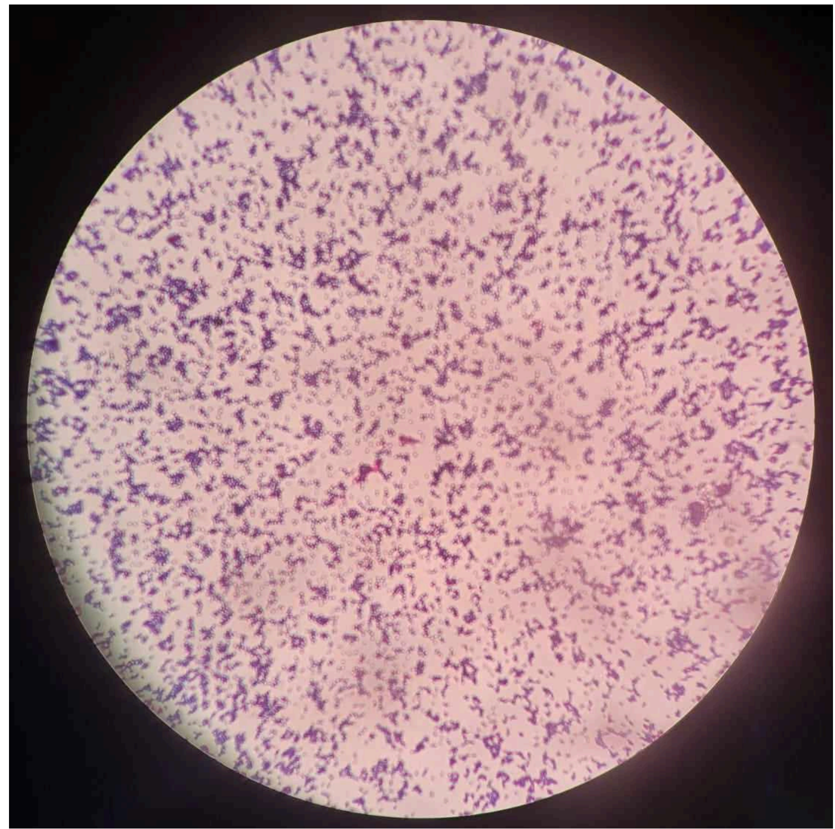


Figure 3.2: Gram-positive Result of the Sample.

3.2.2 Biochemical Identification Using Triple Sugar Iron Test (TSI)

The Triple Sugar Iron (TSI) test determines an organism's ability to ferment glucose, lactose, and sucrose and produce hydrogen sulfide.

All 63 isolates were inoculated in TSI media and were incubated for 24 hours. After incubation, none of the isolates showed the ability to produce hydrogen sulfide (black precipitate) and production gas such as carbon dioxide and hydrogen, thus indicating none of the isolates could be *Staphylococcus epidermidis*. *Staphylococcus aureus* can ferment all three sugars- glucose, lactose, and sucrose. So, as per it, if fermentation occurs to all three sugars, the medium color of the butt and slant will change from red to yellow due to the production of acids. These acids will change the pH from alkaline to acidic, and due to the presence of the indicator phenol red in the media, the color changes from red to yellow. If the organism was able to ferment only dextrose only, the slant color changes to yellow, and the butt color remains the same, which means it remains alkaline. If the organism was not able to ferment none of the sugars, the slant and the butt both remain alkaline, and the color does not change. **Figure-3.3** represents a positive control of *Staphylococcus aureu*s and a negative control in TSI agar media after incubation, compared with the positive result obtained from the sample.

The chart in **Figure-3.4** represents the results of the pre-assumptive *Staphylococcus aureus* in TSI where A/A denotes Acid slant/Acid butt, K/A denotes Alkaline slant and acidic butt, and K/K denotes Alkaline slant and butt. As per the observational result in TSI, 77.78% of the isolates (49 isolates) showed A/A results, and these organisms can be hypothesised as *Staphylococcus aureus*. In contrast, the remaining 22.22% can not be hypothesised as *Staphylococcus aureus*.

The distribution of the 77.78% isolates presumptively identified as *Staphylococcus aureus* among the nine hospitals collected is illustrated in **Figure-3.5**. The bar chart shows that the number of positive isolates from each hospital varies distinctly. Based on TSI results, the highest number of positive *Staphylococcus aureus* isolates was found in Sir Salimullah Medical College Mitford Hospital (SMIT), with a value of 11. In contrast, the lowest number was observed at the Dhaka Medical College (DMC), with only two isolates. Interestingly, Bangabandhu Sheikh Mujib Medical University, commonly referred to as PG Hospital, and the National Institute of Traumatology and Orthopaedic Rehabilitation (NITOR) have the same number of positive isolates with a value of 3.

Figure 3.3: Different TSI Test Results

(a) Positive control for *Staphylococcus aureus*; **(b)** Positive result of A/A for sample to be hypothesized *Staphylococcus aureus*; **(c)** Negative control for *Staphylococcus aureus*

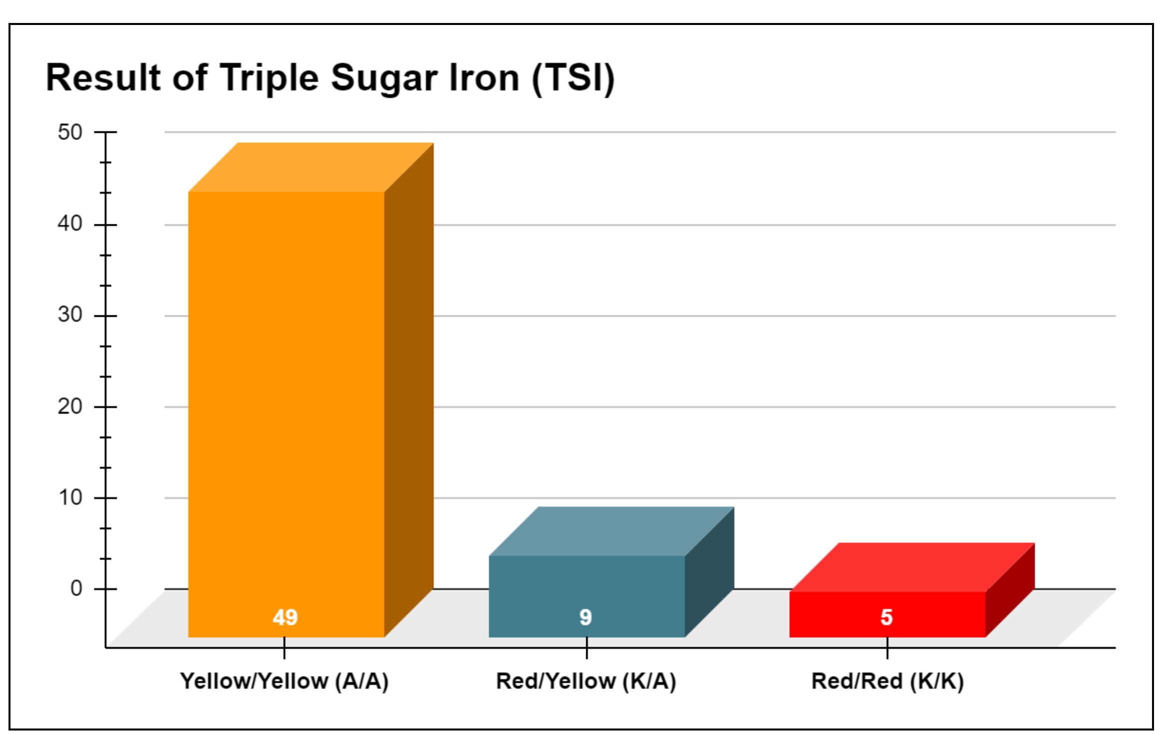


Figure 3.4: Observational Result of Triple Sugar Iron Test.

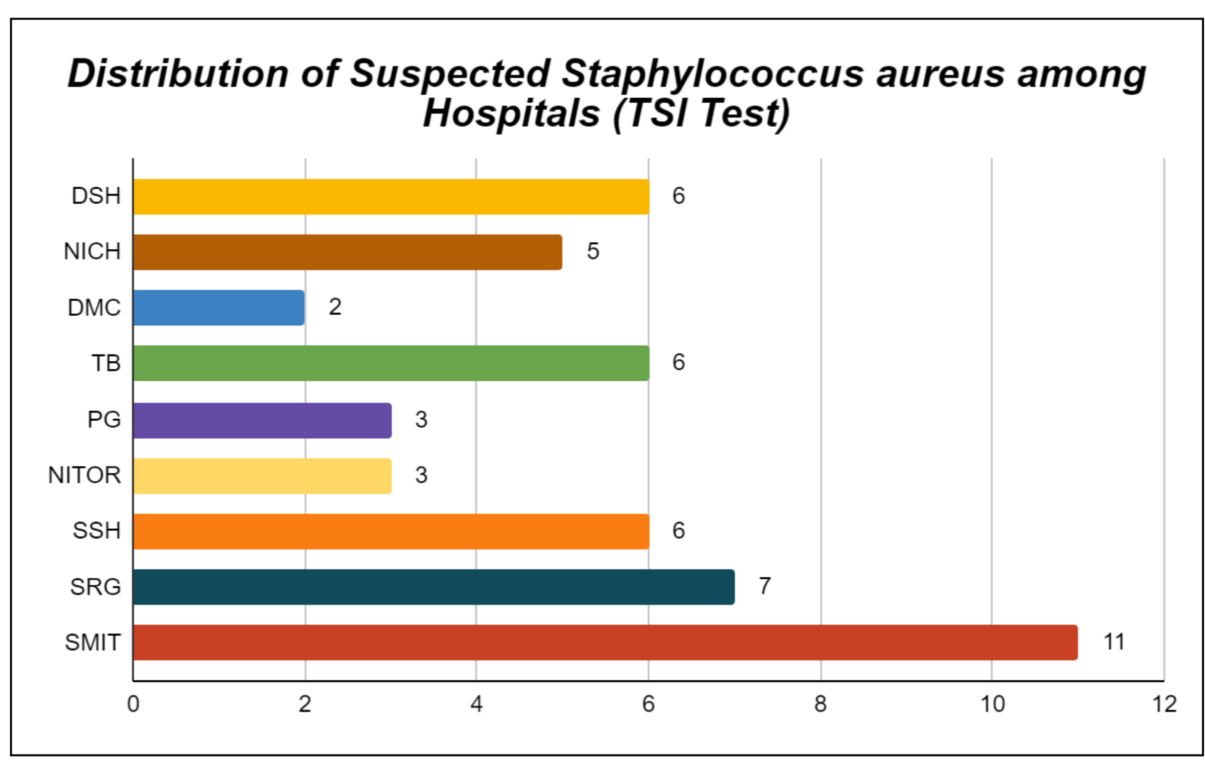


Figure 3.5: Distribution of Pre-assumptive *Staphylococcus aureus* based on TSI results among the collected hospitals.

3.2.3 Biochemical Identification Using Citrate Utilization Test

The Citrate Utilization Test distinguishes microorganisms using citrate as the carbon source. Citritase breaks down citrate into oxaloacetate and acetate. Sodium citrate is used as the carbon source. The pH change helps detect organisms using the indicator bromothymol blue. The test sample is inoculated into Simmon's citrate agar media and incubated for 24 hours. The test is positive if the tube changes color or if bacterial growth occurs. The test is negative if the color remains green and no bacterial growth is observed.

Staphylococcus spp. can use citrate as its carbon source; thus, fermenting citrate produces oxaloacetate and acetate acids, which would change the pH. Because of the indicator bromothymol blue, the color changes to blue from green.

Figure-3.6 represents a positive control of *Staphylococcus aureus* in Simmon's citrate agar media after incubation, compared with the positive result obtained from the sample.

All 63 isolates were inoculated in Simmon's citrate agar media and were incubated for 24h at 37°C. The following pie chart in **Figure-3.7** depicts the observational results of the citrate utilization test, where blue indicates that the color had shifted to blue from green (citrate positive results). Green suggests the color remains unchanged (citrate negative results). 77.8% of the isolates (49 isolates) were citrate-positive, indicating these organisms could be hypothesised as *Staphylococcus aureus*. The isolates showed positive results in TSI, and the same isolates showed positive results in the Citrate Utilization Test.

The distribution of the 77.8% of isolates presumptively identified as *Staphylococcus aureus* among the nine hospitals collected is illustrated in **Figure-3.8**. The bar chart shows that the number of positive isolates from each hospital varies distinctly. Based on citrate utilization test results, the highest number of positive *Staphylococcus aureus* isolates was found in Sir Salimullah Medical College Mitford Hospital (SMIT), with a value of 11. In contrast, the lowest number was observed at the Dhaka Medical College (DMC), with only two isolates. Interestingly, Bangabandhu Sheikh Mujib Medical University, commonly referred to as PG Hospital, and the National Institute of Traumatology and Orthopaedic Rehabilitation (NITOR) have the same number of positive isolates with a value of 2.

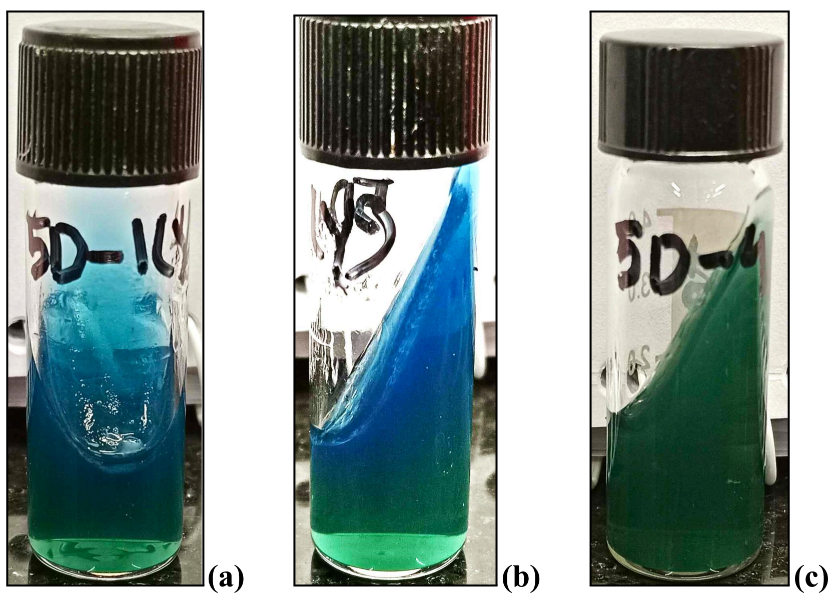


Figure 3.6: Results for Citrate Utilization Test
(a) Positive Citrate Utilization Test result for sample; (b) Positive control for Citrate Utilization Test; (c) Negative control for Citrate Utilization Test

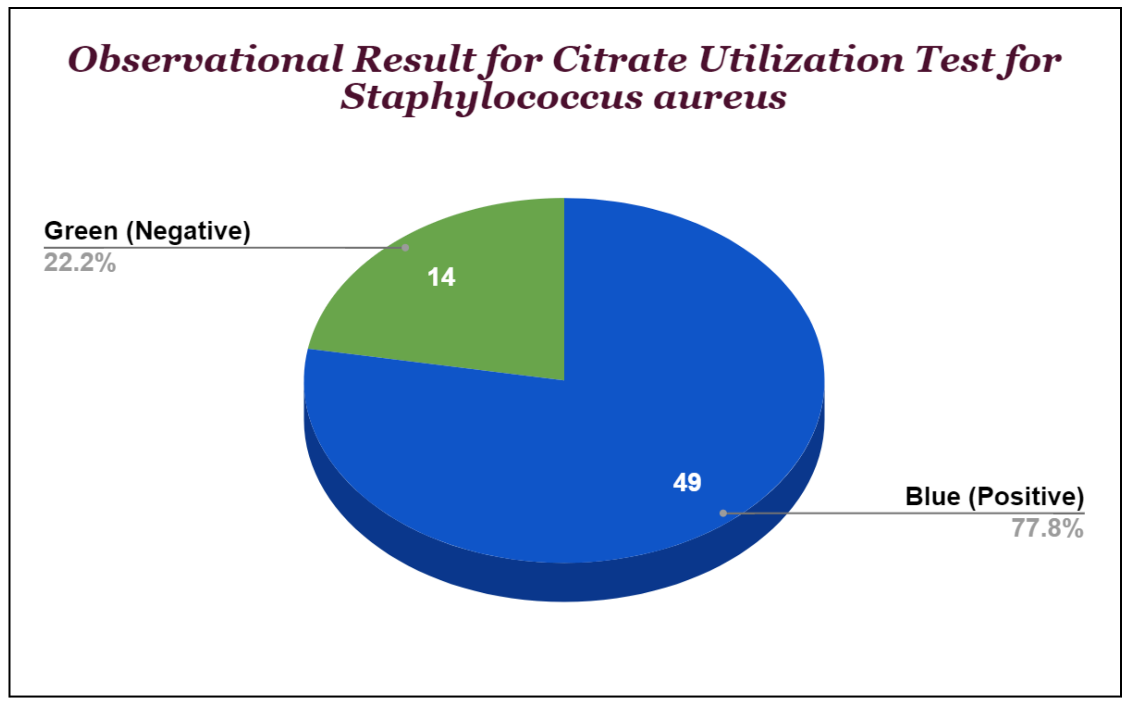


Figure 3.7: Observational Result of Citrate Utilization Test.

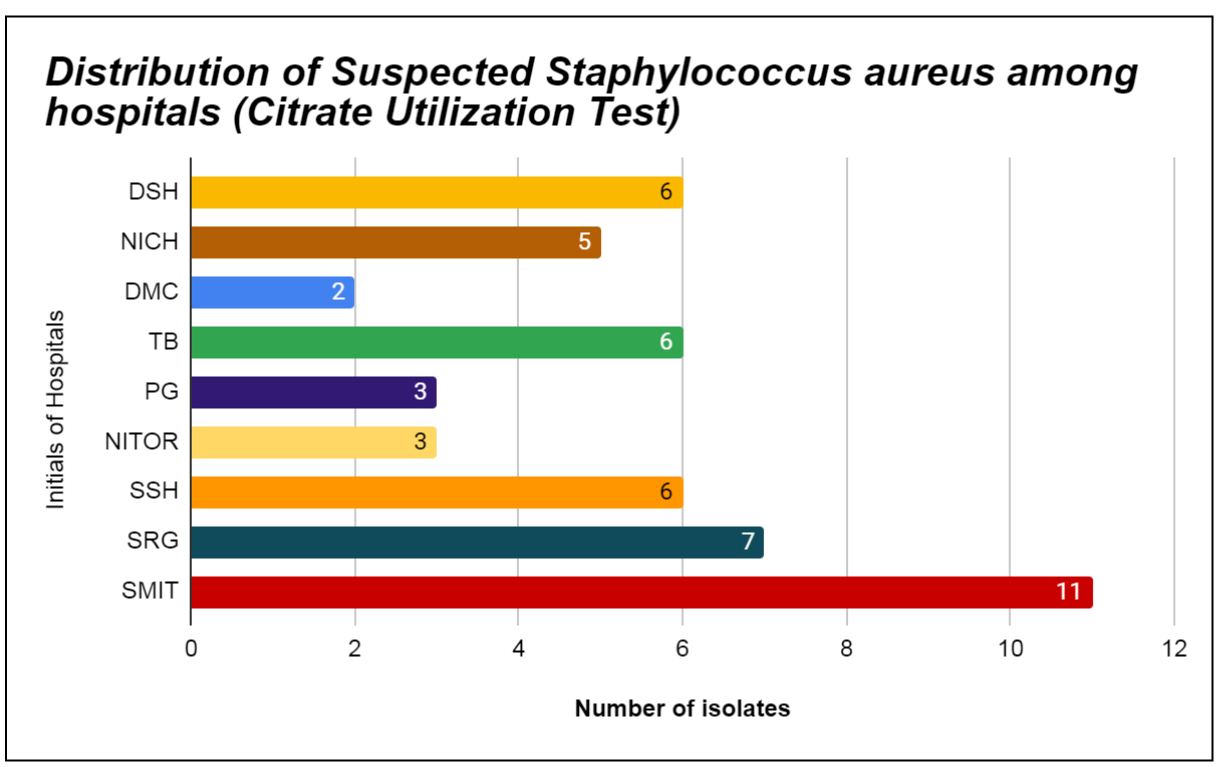


Figure 3.8: Distribution of Pre-assumptive *Staphylococcus aureus* based on citrate utilization results among the collected hospitals.

3.2.4 Biochemical Identification Using Catalase Test

The catalase test detects the enzyme that protects organisms from oxidative damage. Organisms produce hazardous by-products such as hydrogen peroxide and superoxide radicals. Pathogenic organisms break down these compounds into non-toxic chemicals. Bacteria use catalase to break down hydrogen peroxide into water and oxygen. The test distinguishes catalase-positive organisms from catalase-negative organisms. Oxygen bubbles indicate the presence of catalase. The absence of bubbles indicates the lack of the enzyme.

Staphylococcus spp. has the enzyme 'catalase'. All hypothesised *Staphylococcus spp.* Isolates were tested for the precedence of catalase enzymes by adding 2-3 drops of hydrogen peroxide (H_2O_2). Within a few seconds, all of them produced bubbles, as per the picture shown in **Figure-3.9**.

The bar chart in **Figure-3.10** depicts the observational results of the catalase test, where oxygen bubble formation indicates that the test species have the catalase enzyme. Here, 100% of the isolates were catalase-positive, indicating these organisms could be hypothesised as *Staphylococcus aureus.*

The graph in **Figure-3.11** shows that the number of positive isolates from each hospital varies distinctly. Based on catalase test results, the highest number of positive *Staphylococcus aureus*

isolates was found in SRG, with a value of 14. The nearest positive result, with 13 isolates, is from SMIT. DSH had eight positive isolates. NICH and TB both shared seven positive isolates. Along with this, PG and NITOR both shared three positive isolates. The lowest positive isolates were found from DMC, with only two isolates.

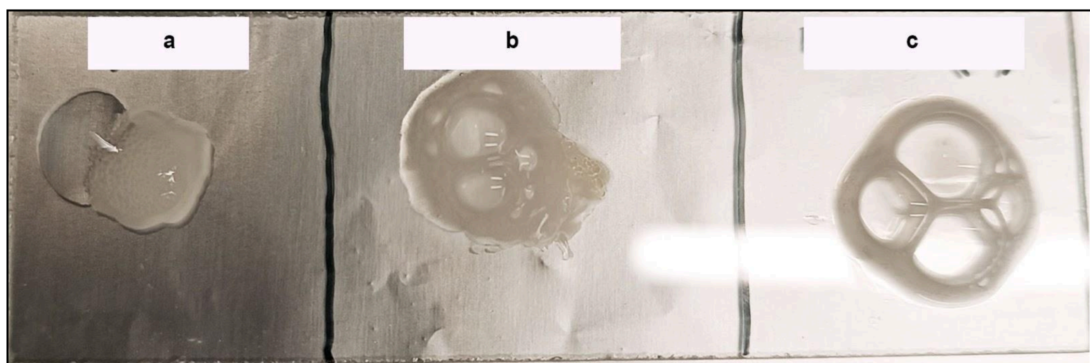


Figure 3.9: Bubble formation after adding H_2O_2 to the test species, (a) & (b) Positive Result; (c) Positive control

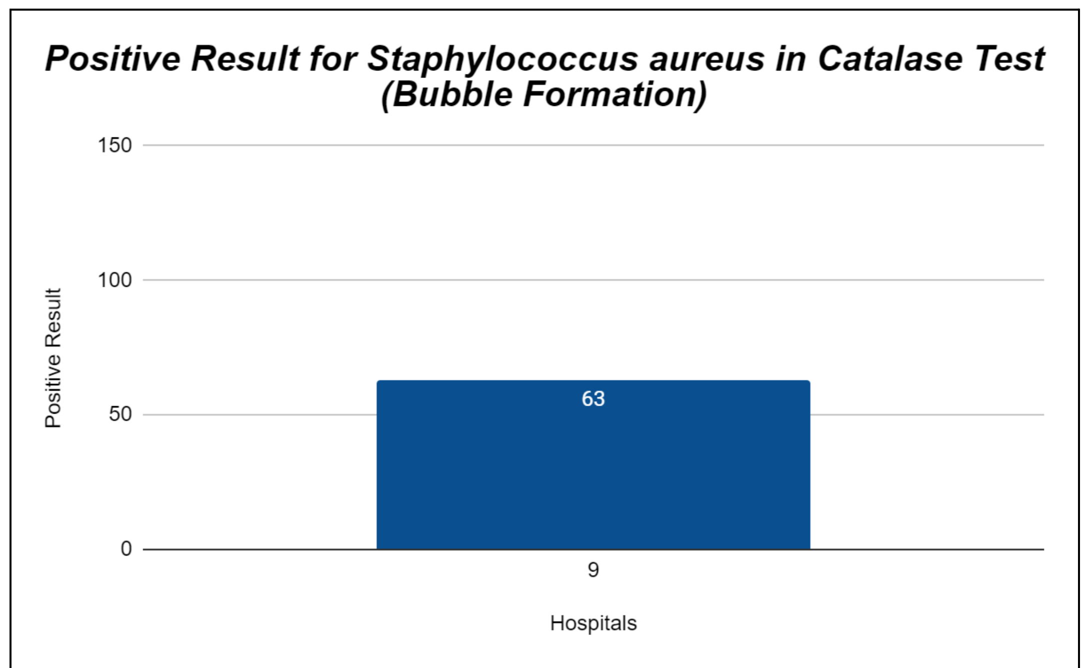


Figure 3.10: Observational Result of Catalase Test.

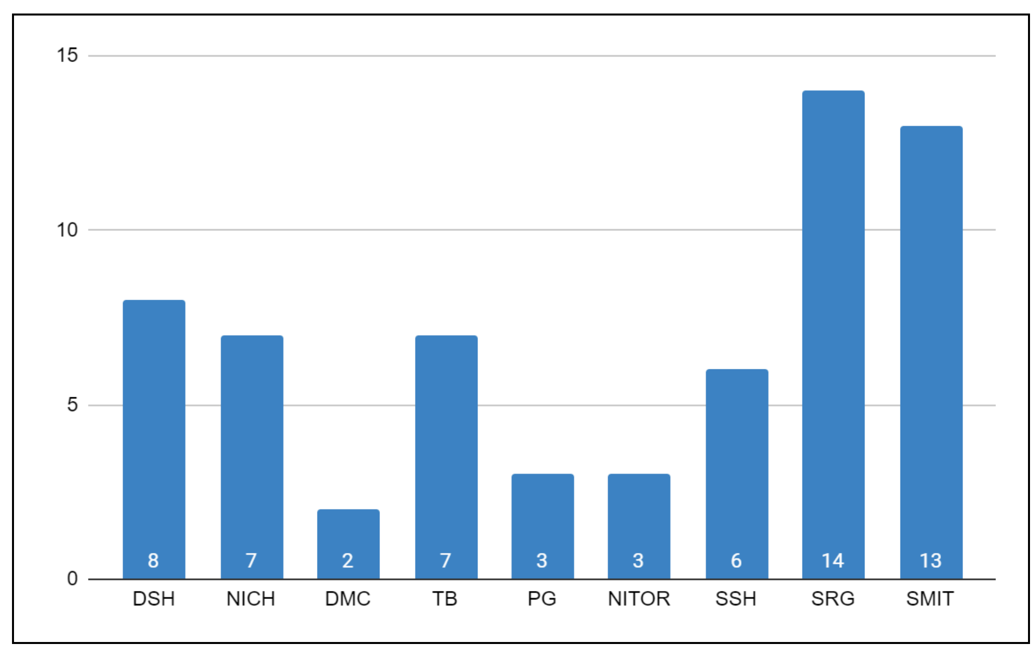


Figure 3.11: Distribution of Pre-assumptive *Staphylococcus aureus* based on Catalase results among the collected hospitals.

3.2.5 Biochemical Identification Using Oxidase Test

Oxidase tests detect if the organism has the cytochrome oxidase enzyme present in Gram-negative bacteria such as *Pseudomonas*. An absorbent paper disc is taken, and the disc is impregnated with N`-Tetramethyl-p-phenylenediamine dihydrochloride. Then, the test species are rubbed on the paper. If the color changes to blue, it is oxidase-positive. Otherwise, no color indicates a negative result. **Figure-3.12** depicts the results of some of the samples for the Oxidase test.

The pie chart in **Figure-3.13** shows the observational results of the oxidase test. Here, 59 isolates (93.7%) among 63 isolates were oxidase-negative, indicating these organisms could be hypothesised as *Staphylococcus aureus.* On the contrary, only four isolates were oxidase-positive.

The hospital-based bar chart **Figure-3.14** shows that the number of positive isolates from each hospital varies distinctly. Based on oxidase test results, the highest number of positive *Staphylococcus aureus* isolates were found in Sheikh Russel GastroLiver Hospital, with a value of 13. In contrast, the lowest number was observed at Dhaka Medical College (DMC), with only two isolates. The second highest positive isolates were from Sir Salimullah Medical College Mitford Hospital (SMIT), with a value 11. Interestingly, Bangabandhu Sheikh Mujib Medical University is commonly called PG Hospital. The National Institute of Traumatology and Orthopaedic Rehabilitation (NITOR) has the same number of positive isolates with a value of 3, and the National Institute of Cancer Research & Hospital and National Institute of Chest Disease

and Hospital, commonly called TB Hospital, have the same number of positive isolated with a value of 7.

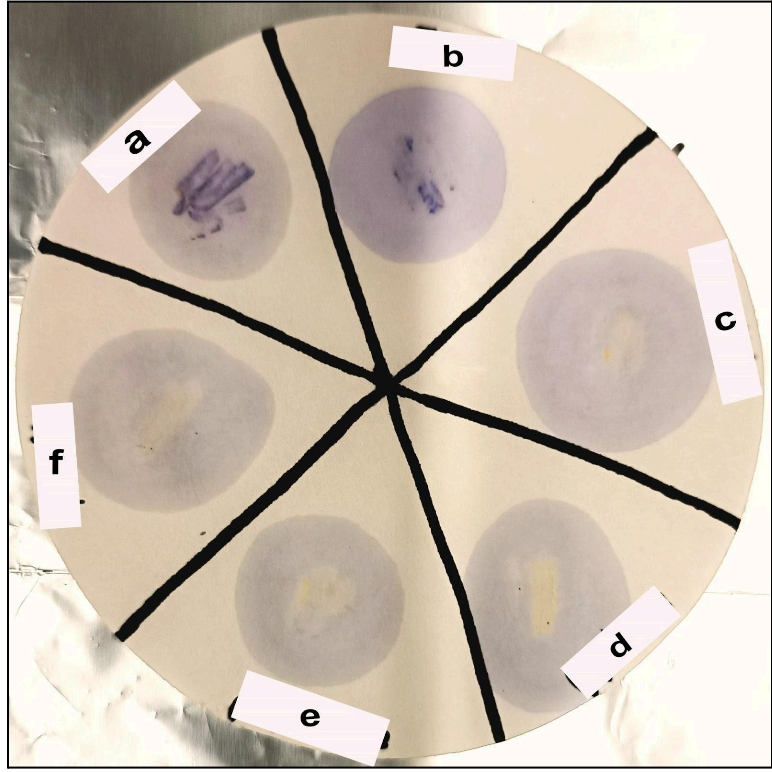


Figure 3.12: Result of Oxidase Test
(a) and (b) shows a blue hue, indicating the oxidase-positive result
(c), (d), (e), and (f) have no color change indicating the oxidase-negative result.

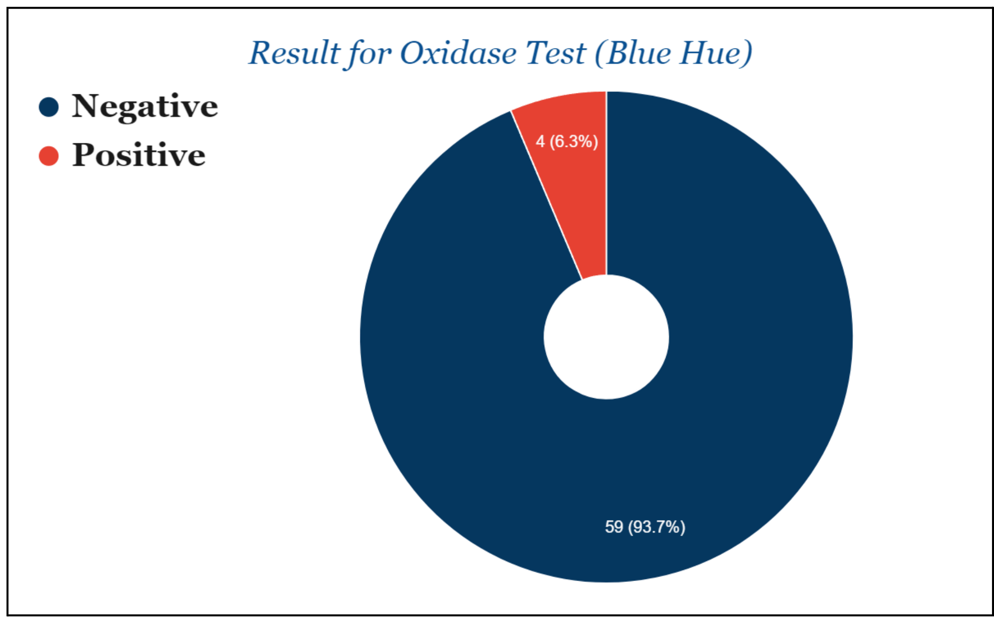


Figure 3.13: Observational Pie Chart of Oxidase Test.

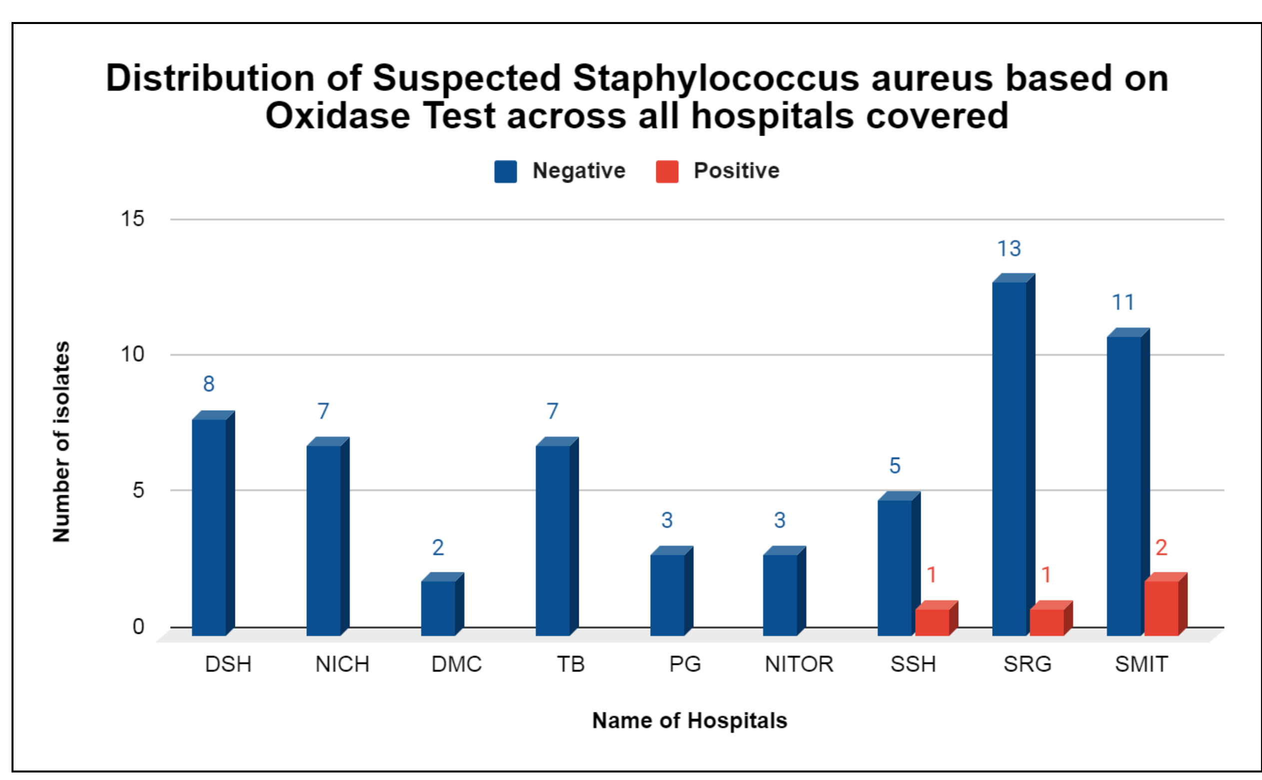


Figure 3.14: Distribution of Suspected *Staphylococcus aureus* based on Oxidase test across hospitals covered.

Eventually, **Table-3.3** summarizes the biochemical results of all the presumptive *Staphylococcus aureus* isolates and showcases the results location-wise. In this result, we can observe that isolates from Ramna and Shahbagh demonstrate the highest percentage of 100%. All the presumptive isolates of *Staphylococcus aureus* obtained were positive by all the biochemicals used. The next highest are from Puran Dhaka and Agargaon, with 84.62% and 83.33%, respectively. Mohakhali area had the lowest percentage of presumptive positive isolates of *Staphylococcus aureus* by all biochemical tests.

Area	Total No. of Isolates	Commonly Positive by All the Biochemical Tests	Overall Percentage (%)
Ramna	**2**	**2**	100
Shahbagh	**3**	**3**	100
Puran Dhaka	**13**	**11**	84.62
Agargaon	**24**	**20**	83.33
Mohakhali	**21**	**13**	61.9

Table 3.3: Overall result of Biochemical tests of presumptive *Staphylococcus aureus* isolates

3.3 Identification of *Staphylococcus aureus* isolates by PCR Assay and Gel Electrophoresis

The identified 49 pre-assumptive *Staphylococcus aureus* isolates from biochemical tests underwent conventional PCR using a *nucA* primer, as described in section 2.7.4. The gel electrophoresis method was used to examine the results, as mentioned in section 2.7.4. The study found that out of the 49 isolates, only 7 showed positive results for the *nucA* gene, which produced a band of 270 bp in the gel electrophoresis observed in **Figure-3.15**. Therefore, only 14.3% of the isolates were confirmed as *Staphylococcus aureus* isolates.

The distribution of the 14.3% identified isolates of *Staphylococcus aureus* among the nine hospitals collected is illustrated in **Figure-3.16**. Interestingly, there were no positive isolates of *Staphylococcus aureus* found from Dhaka Shishu Hospital (DSH), Dhaka Medical College (DMC), National Institute of Chest Disease and Hospital, commonly referred to as TB Hospital (TB), National Institute of Traumatology and Orthopaedic Rehabilitation (NITOR) and Shaheed Suhrawardy Medical College and Hospital (SSH). The highest positive isolates were obtained from Bangabandhu Sheikh Mujib Medical University, commonly known as PG Hospital, with a 42.9%. This higher prevalence may be due to differences in food handling practices, hygiene standards, or variations in the chicken sources supplied to each hospital. Additionally, the patient population and the number of attendees and staff served by PG Hospital may increase the chance of cross-contamination of *Staphylococcus aureus* in the cafeteria, resulting in the highest number of positive isolates. Furthermore, Sir Salimullah Medical College Mitford Hospital (SMIT) and the National Institute of Cancer Research & Hospital (NICH) have the same amount of positive *Staphylococcus aureus* isolates. The remaining 28.6% of Sheikh Russel National Gastro Liver Institute & Hospital (SRG) is the second-highest abundance of positive *Staphylococcus aureus* isolates.

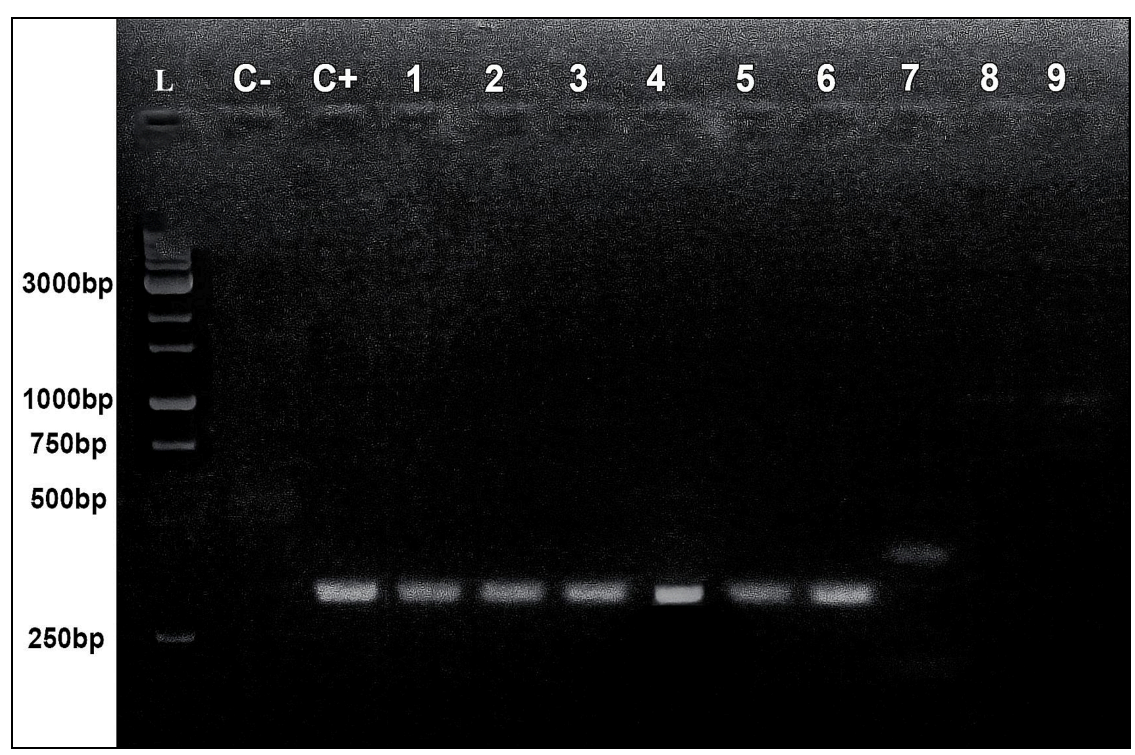


Figure 3.15: PCR amplified products of 270 bp of *nuc* gene of *Staphylococcus aureus* in 1.5% agarose gel electrophoresis and ethidium bromide staining.
Legends: L= DNA Ladder; C- = Negative control; C+ = Positive control of *S. aureus*; Lane 1-9 = Tested *S. aureus* isolates; Lane 1-6 = Positive isolates of *S. aureus*

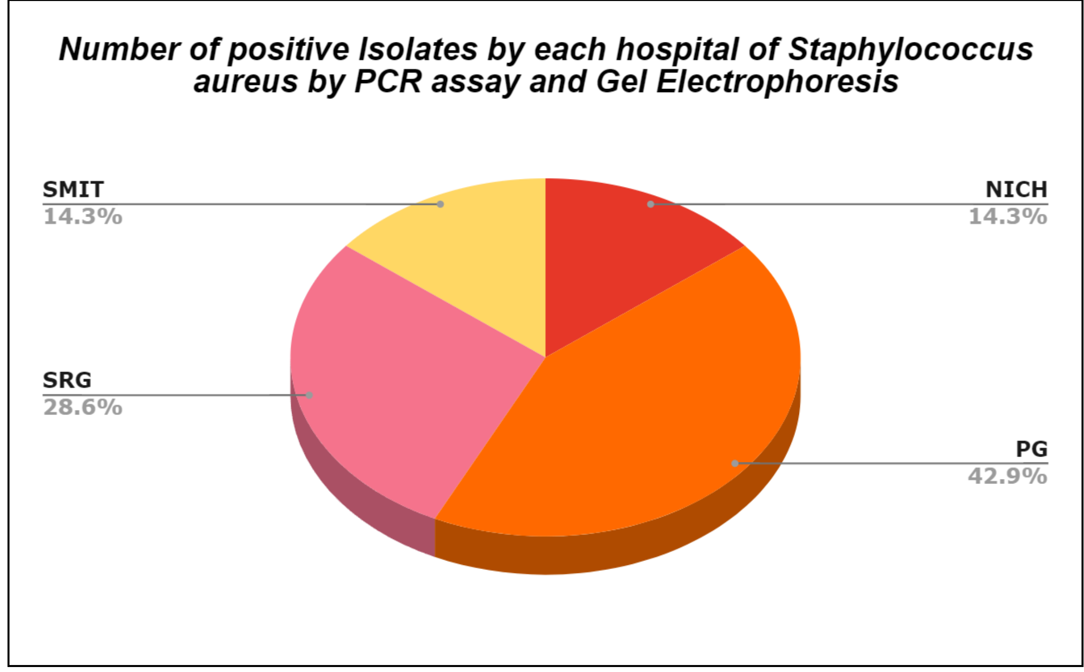


Figure 3.16: Distribution of Positive *Staphylococcus aureus* isolates among the collected hospitals.

3.4 Antibiotic Susceptibility Testing of *S. aureus*

Seven positive isolates of *S. aureus* underwent antibiotic susceptibility testing using disk diffusion methods against a panel of 12 antibiotics.

As demonstrated in **Figure-3.18**, ampicillin-25 and penicillin-10 have exhibited the highest resistance rates- 100%, followed by clindamycin-2 and methicillin-5 with 42.9%, erythromycin-15, doxycycline-30, and kanamycin-30 with 28.6%, azithromycin-30, vancomycin-30, and novobiocin-30 with 14.3%, and the lowest observed was a resistance of 0%, which is amoxicillin-30 and levofloxacin-5.

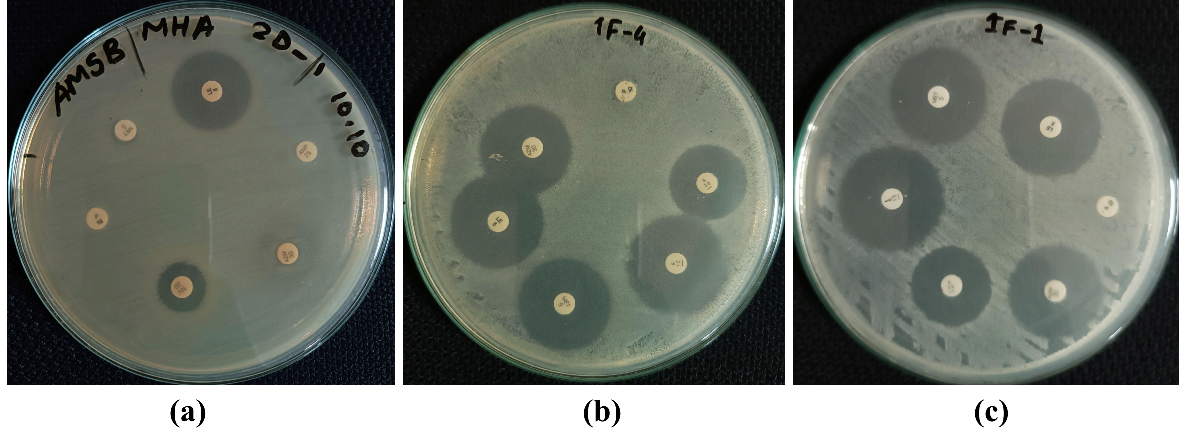
Conversely, Levofloxacin (100%) and Amoxicillin (100%) exhibited the highest sensitivity rates, followed by Erythromycin (83.33%) and Kanamycin (83.33%). Azithromycin (75%), Vancomycin (75%), and Novobiocin (75%) displayed similar sensitivity patterns.

Mult-antimicrobial resistance (AMR) is a global threat, and hence, it is highly crucial to determine the Multiple Antibiotic Resistance Index (MARI) of identified positive isolates of *Staphylococcus aureus*. **Figure-3.19** depicts the MARI- Multiple Antibiotic Resistance Index of seven isolates of *Staphylococcus aureus*. The figure signifies out of seven positive isolates of *Staphylococcus aureus*, four isolates have a MAR index greater than; these are S-4 to S-7 consecutively, where S-4 has the highest MAR index of 0.58, and the next subsequent one is S-6 with a value of 0.417. Out of these 4, the S-4 is from the National Institute of Cancer Research & Hospital (NICH), which is situated in Agargaon. S-5 and S-6 are from Sheikh Russel National Gastro Liver Institute & Hospital, located at Mohakhali, and S-7 is from Sir Salimullah Medical College Mitford Hospital, in Old Dhaka. A MAR index value greater than 0.2 indicates that the particular organism likely originated from sources with a high risk of resistance. Therefore, according to the result derived, the organism obtained from the National Institute of Cancer Research & Hospital (NICH) has the highest likelihood of originating from a source with a high risk of resistance. The next consecutive one with the highest resistance is from Sheikh Russel National Gastro Liver Institute & Hospital. Thus, it is impeccable to monitor the resistance patterns among the patients of these hospitals and try to find an association with their food habits, if consumption of cooked chicken from the cafeteria has an effect on them, or the multi-drug resistance pattern of the admitted patients are getting cross-contaminated to the cooked chicken food items in the cafeteria via the staff such as nurses and doctors.

In **Figure-3.20**, the hospital-wise distribution of *Staphylococcus aureus* resistance to different antibiotics is observed. From the figure, it can be depicted isolates obtained from SRG are resistant to 10 antibiotics named Penicillin-10, Ampicillin-25, Methicillin-5, Azithromycin-30, Erythromycin-15, Vancomycin-30, Doxycycline-30, Kanamycin-30, Novobiocin-30 and Clindamycin-2. The isolate from NICH is resistant to 8 antibiotics named— Penicillin-10, Ampicillin-25, Methicillin-5, Erythromycin-15, Doxycycline-30, Kanamycin-30, Novobiocin-30, and Clindamycin-2. The isolate from SMIT is resistant to Penicillin-10,

Ampicillin-25, and Methicillin-5. The isolates from PG are resistant to Penicillin-10 and Ampicillin-25. Therefore, all 7 isolates from the four locations are multidrug-resistant (MDR) as they all are resistant to more than one antibiotic. Explicitly, the isolate from SRG situated in Mohakhali showcases the highest MDR, and the isolate from SMIT situated in Old Dhaka showcases the lowest MDR rate among all. The highest MDR rate in SRG (Mohakhali) symbolizes in this particular hospital, there is an extensive use of antibiotics that leads to resistance development. Furthermore, the lack of proper antimicrobial stewardship and the emergence of resistance genes contribute to the emergence of resistant bacteria. It is important to note that 1 isolate from NICH, 2 isolates from SRG, and 1 isolate from SMIT were resistant to methicillin, making them MRSA, that means in total 4 isolates of *Staphylococcus aureus* are resistant to methicillin and can be classified as MRSA. Therefore, 57.1% of the S*taphylococcus aureus* are MRSA.

In **Figure-3.21,** the hospital-wise distribution of *Staphylococcus aureus* sensitivity to different antibiotics is observed. Only three positive isolates are sensitive to methicillin (100%), and they belong to PG; these isolates can be defined as MSSA (Methicillin Sensitive *Staphylococcus aureus*). The three isolates of PG are sensitive to the highest number of antibiotics— 10 antibiotics—and only the isolate from NICH is sensitive to the least number of antibiotics— 3. Therefore, 42.9% of isolates are MSSA.



(a) **(b)** **(c)**

Figure 3.17: Results for Antibiotic Susceptibility Testing for *Staphylococcus aureus*.

(a) AST result of a sample-1 against methicillin, levofloxacin, ampicillin, azithromycin, novobiocin, and penicillin; (b) AST result of a sample-2 against penicillin, kanamycin, erythromycin, methicillin, levofloxacin, and azithromycin; (c) AST result of a sample-3 against methicillin, levofloxacin, ampicillin, azithromycin, novobiocin, and penicillin.

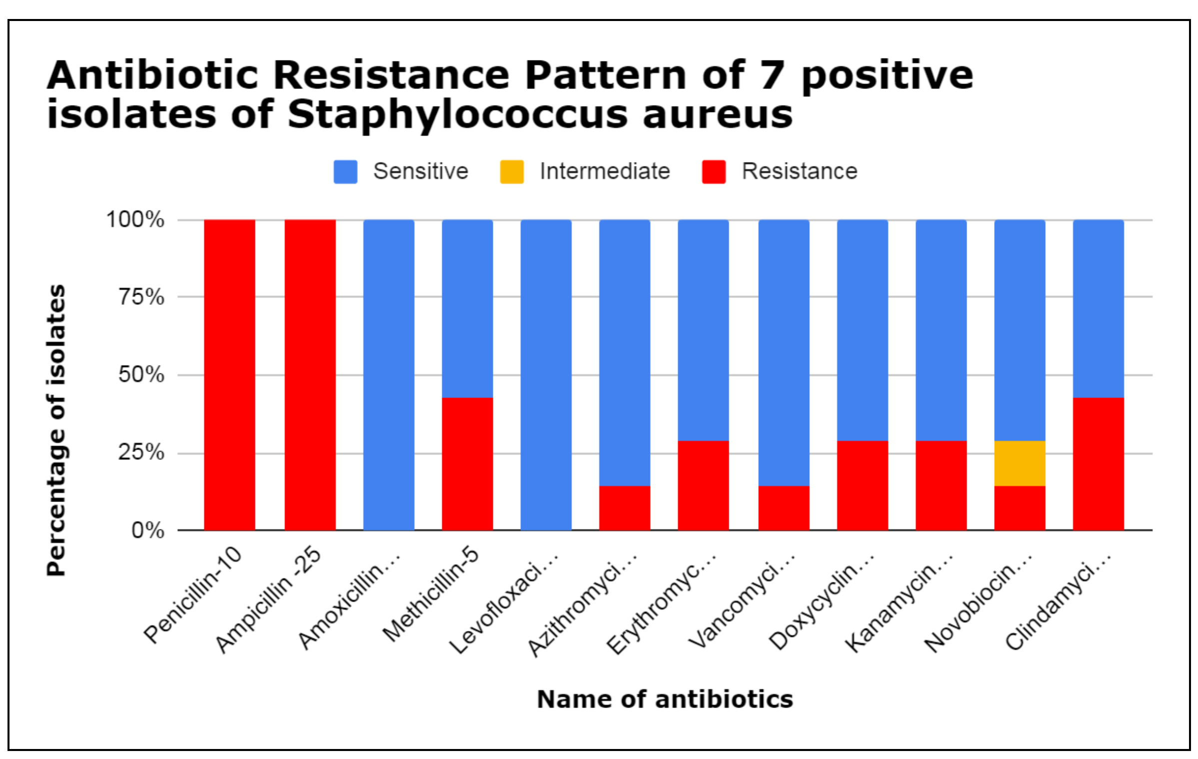


Figure 3.18: Bar Graph showing the Antibiotic susceptibility pattern of 12 different antibiotics in *S. aureus* isolates

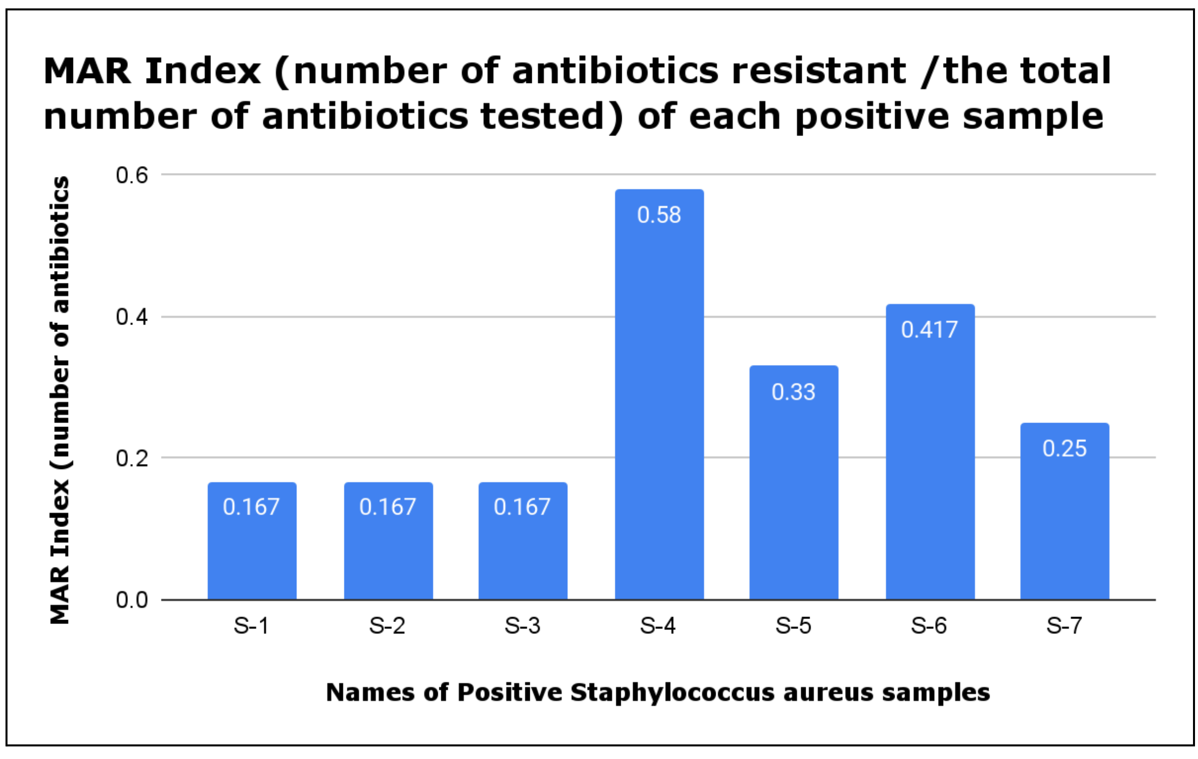


Figure 3.19: MAR Index of positive isolates of *Staphylococcus aureus* samples.

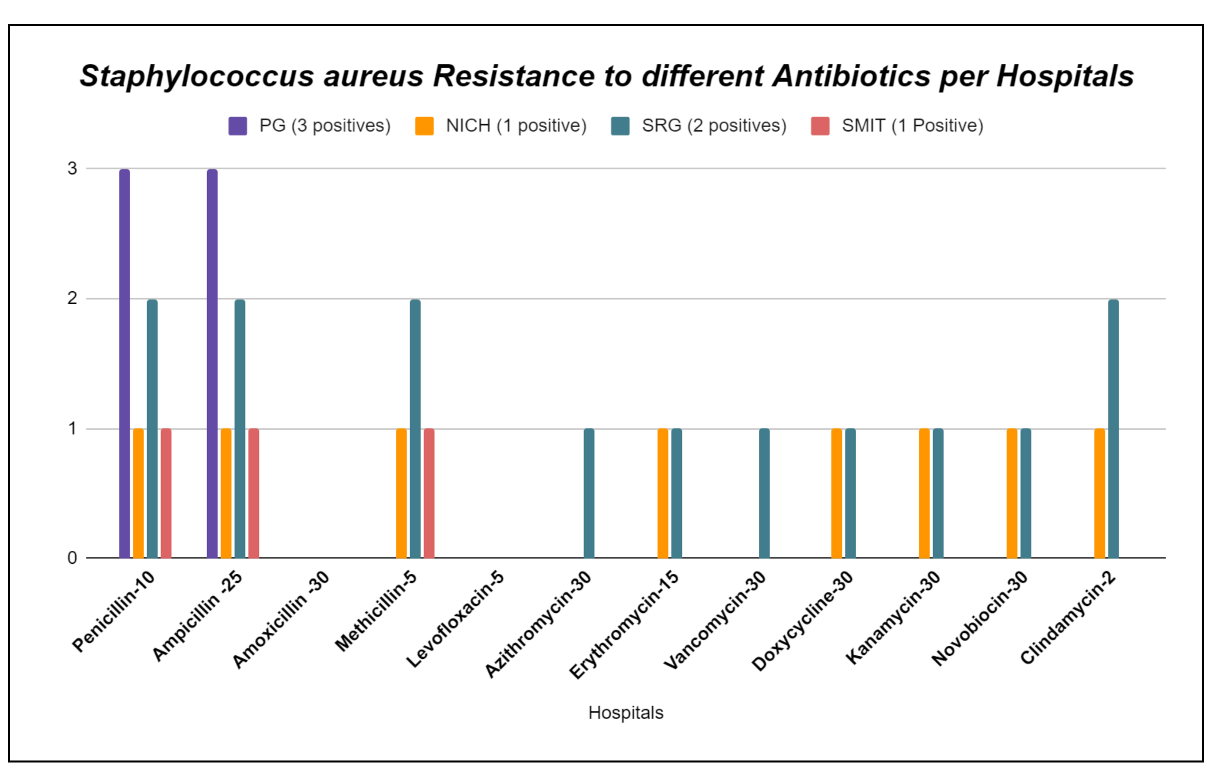


Figure 3.20: Hospital-wise Comparison Graph for *Staphylococcus aureus* resistance to different antibiotics.

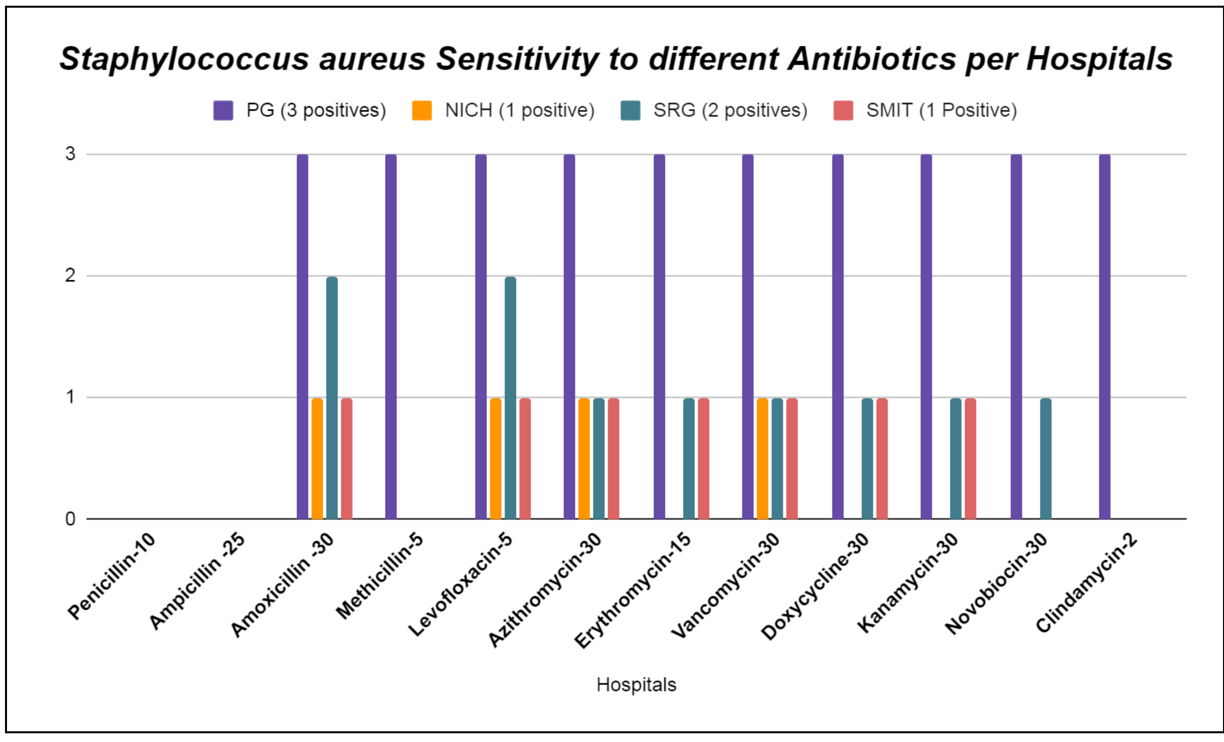


Figure 3.21: Hospital-wise Comparison Graph for *Staphylococcus aureus* sensitivity to different antibiotics.

3.5 Isolation of *Klebsiella pneumoniae*

Isolation of ***Klebsiella pneumoniae*** was carried out using selective and differential media named Eosin methylene blue agar (EMB). The bacteria that showed distinct mucoid pink colonies and dark purple colonies were considered *Klebsiella pneumoniae*. Using selective media, 14 isolates were determined to be presumptive ***Klebsiella pneumoniae***.

Detailed descriptions of the distinct morphologies observed on EMB media type are provided in the subsequent table.

<table>
<tr><th></th><th>Number of isolates</th><th>Observation</th><th>Picture</th><th>Hypothesised Organism</th><th>Percentage of Positive Presumptive Klebsiella pneumoniae</th></tr>
<tr><td rowspan="2">Eosin Methylene Blue (EMB)</td><td>8</td><td>Pink mucoid colonies</td><td></td><td rowspan="2">Klebsiella pneumoniae</td><td rowspan="2">100%</td></tr>
<tr><td>6</td><td>Dark purple colonies</td><td></td></tr>
</table>

Table 3.4: Observational Result for EMB Agar Media.

Distinct colonies from spread plates were streaked in EMB media, where the single distinct colonies were obtained. To achieve single colonies, those single colonies were streaked again in

Nutrient Media (NA). These single colonies were stocked for further usage in Nutrient broth (NB). They were used for other confirmatory identification purposes, such as biochemical tests and bacterial genomic Deoxyribonucleic acid (DNA) extraction for PCR assay.

The following biochemical tests were performed-

- Gram staining
- Triple Sugar Iron (TSI) test
- Citrate Utilization test
- Catalase Test
- Oxidase Test

3.6 Identification Of *Klebsiella pneumoniae* Using Biochemical Test

There are some fundamental characteristics of biochemical tests in which results indicate the possible presence of specific kinds of bacterial species. A chart for these characteristics for *Klebsiella pneumoniae* is given below:

Basic Characteristics	**Result for *Klebsiella pneumoniae***
Gram staining	Gram-negative (-ve)
Fermentation of Glucose	Positive (+ve)
Fermentation of Sucrose	Positive (+ve)
Fermentation of Lactose	Positive (+ve)
Production of Gas	Positive(+ve)
Production of H_2S	Negative (-ve)
Citrate	Positive (+ve)
Catalase	Positive (+ve)
Oxidase	Negative (-ve)

Table 3.5: Observational Characteristics of *Klebsiella pneumoniae*

3.6.1 Identification using Gram staining

All 14 presumptive isolates of *Klebsiella pneumoniae* underwent the Gram staining procedure. The staining characteristics were consistent with gram-negative *Klebsiella pneumoniae* bacteria. All of the 14 isolates retained the dye- safranin, resulting in a pink coloration under the microscope, indicating the presence of lipopolysaccharide cell wall. Therefore, these findings confirm the Gram-positive nature of the isolates, which provides a solid foundation for subsequent characterization and identification processes in the investigation of *Klebsiella pneumonia*. **Figure-3.22** shows one of the results of the sample being gram-negative after the gram-staining process.

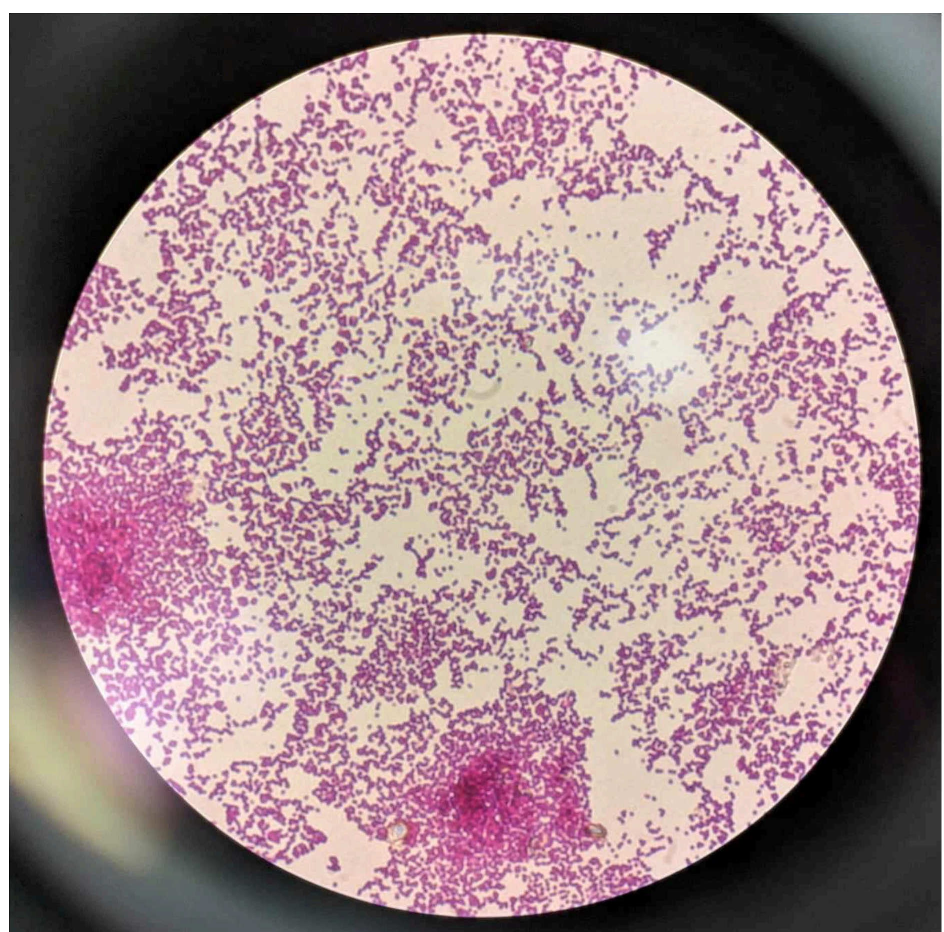


Figure 3.22: Gram staining of the sample showing gram-negative bacteria.

3.6.2 Biochemical Identification Using Triple Sugar Iron (TSI)

The Triple Sugar Iron (TSI) test determines an organism's ability to ferment glucose, lactose, and sucrose and produce hydrogen sulfide.

All 14 isolates were inoculated in TSI media and were incubated for 24 hours. After incubation, all of the 14 isolates showed yellow slant/yellow butt indicating acidic/acidic condition, which is

denoted by A/A, and cracks in the media were observed, indicating the formation of bubbles. *Klebsiella pneumoniae* can ferment all three sugars- glucose, lactose, and sucrose and can produce gas, so, upon fermentation of all three sugars, the slant and butt both would turn yellow due to pH change. Thus, 100% of all fourteen (14) isolates depicted a positive result for suspected *Klebsiella pneumoniae*. **Figure-3.23(a)** demonstrates one of the positive results of the sample, and **Figure-3.23(b)** represents positive control for *Klebsiella pneumoniae***;** the results are confirmed by comparison with positive control.

Figure-3.24 shows that the number of positive isolates from each hospital varies distinctly. Based on TSI results, the highest number of positive ***Klebsiella pneumoniae*** isolates was found in Sir Salimullah Medical College Mitford Hospital (SMIT), with a value of 5. In contrast, no isolates were observed at Dhaka Medical College (DMC). The second highest number of positive isolates was from Dhaka Shishu Hospital (DSH), with a value of 3. Interestingly, PG Hospital, NITOR, SRG, SSH, TB, and NICH have the same number of positive isolates with a value of 1.

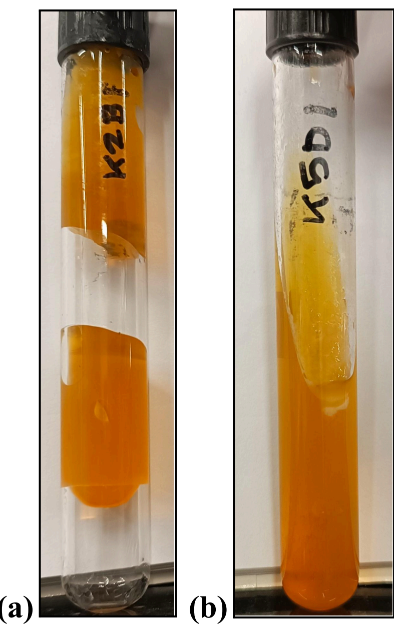


Figure 3.23: TSI Test Result for *Klebsiella pneumoniae*
(a) Positive result for sample demonstrating Y/Y with bubbles; (b) Positive control

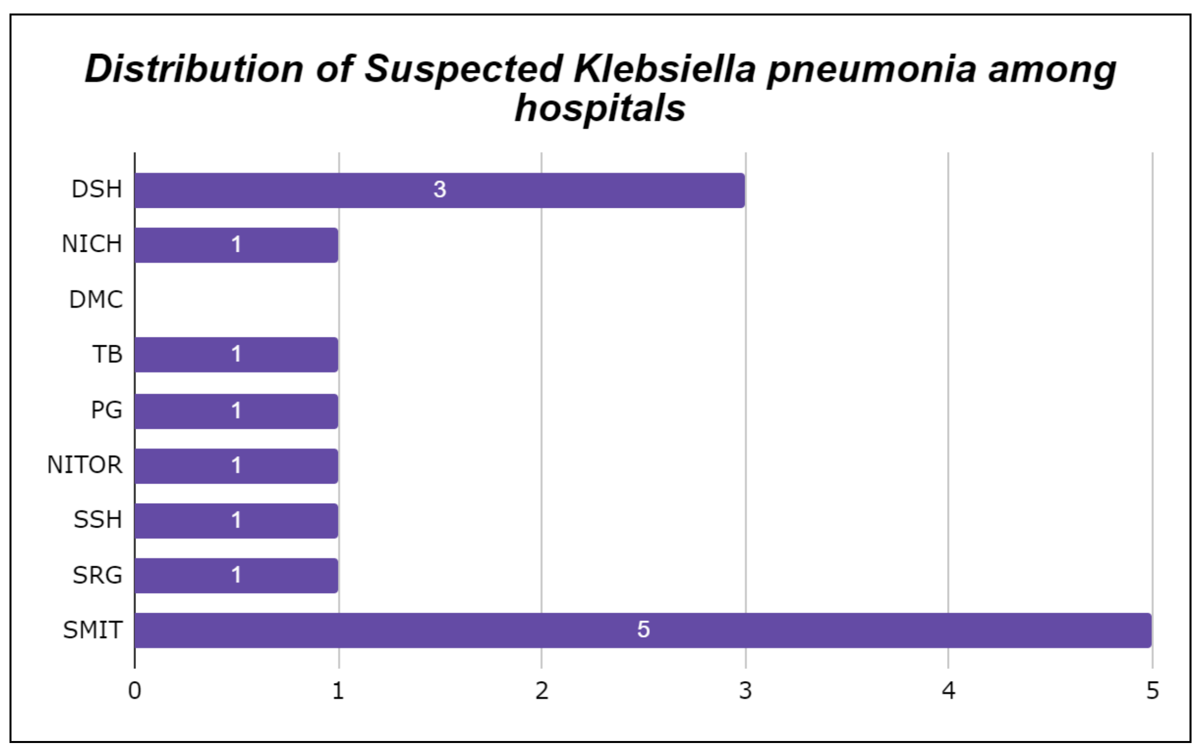


Figure 3.24: Distribution of Suspected *Klebsiella pneumoniae* isolates based on TSI results among the collected hospitals.

3.6.3 Biochemical Identification Using Citrate Utilization Test

The Citrate Utilization Test distinguishes microorganisms using citrate as the carbon source. Citritase breaks down citrate into oxaloacetate and acetate. Sodium citrate is used as the carbon source. The pH change helps detect organisms using the indicator bromothymol blue. The test sample is inoculated into Simmon's citrate agar media and incubated for 24 hours. The test is positive if the tube changes color or if bacterial growth occurs. The test is negative if the color remains green and no bacterial growth is observed.

Klebsiella spp. can use citrate as its carbon source; thus, fermenting citrate produces oxaloacetate and acetate acids, which would change the pH. Because of the indicator bromothymol blue, the color changes to blue from green.

Figure-3.25 represents a positive control and negative control of *Klebsiella pneumoniae* in Simmon's citrate agar media after incubation, compared with the positive result obtained from the sample.

Figure-3.26 shows all 14 isolates were inoculated in Simmon's citrate agar media and were incubated for 24h at 37°C. The following pie chart depicts the observational results of the citrate utilization test, where blue indicates that the color had shifted to blue from green (citrate positive results). Green suggests the color did not move (citrate negative results) 71.4% of the isolates

were citrate-positive, indicating these organisms could be hypothesized as *Klebsiella pneumoniae*. The remaining 28.6% could be other organisms other than *Klebsiella pneumoniae*. Upon analysis of the rest of the biochemical tests, identification of the remaining 28.6% can be predicted.

Figure-3.27 illustrates the distribution of the 57.1% of isolates presumptively identified as *Klebsiella pneumoniae* among the nine hospitals collected. The bar chart shows that the number of positive isolates from each hospital varies distinctly. Based on citrate utilization test results, the highest number of positive isolates was found in Sir Salimullah Medical College Mitford Hospital (SMIT), with a value of 4. In contrast, no isolates were found from NICH, DMC, SSH, and SRG, indicating the absence of *Klebsiella isolates* in these hospitals. Surprisingly, DSH, TB, PG, and NITOR all have one distinct suspected *Klebsiella* isolate according to the citrate utilization test.

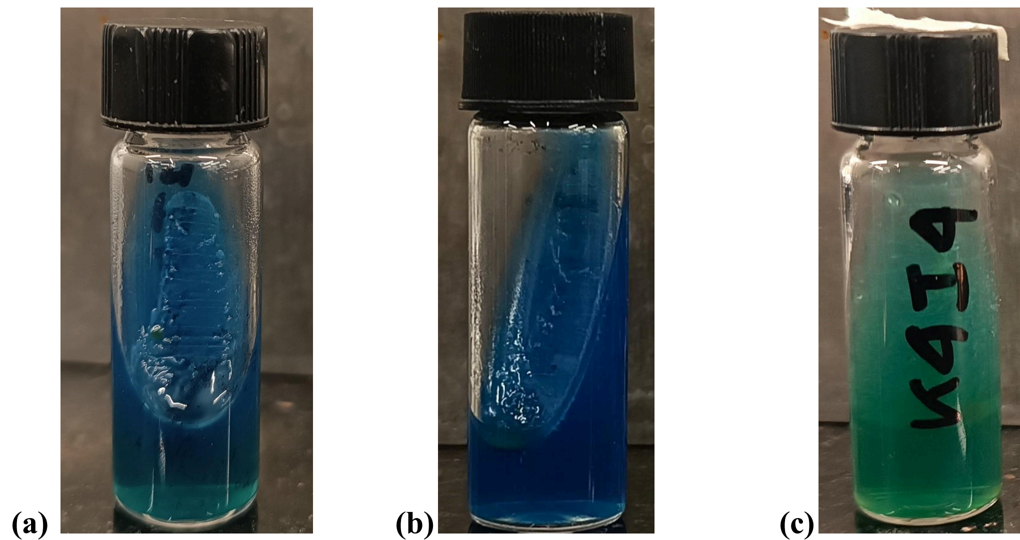


Figure 3.25: Citrate Utilization Test Result for *Klebsiella pneumoniae*

(a) Positive control for Citrate Utilization Test; (b)Positive Citrate Utilization Test result for sample; (c) Negative control for Citrate Utilization Test

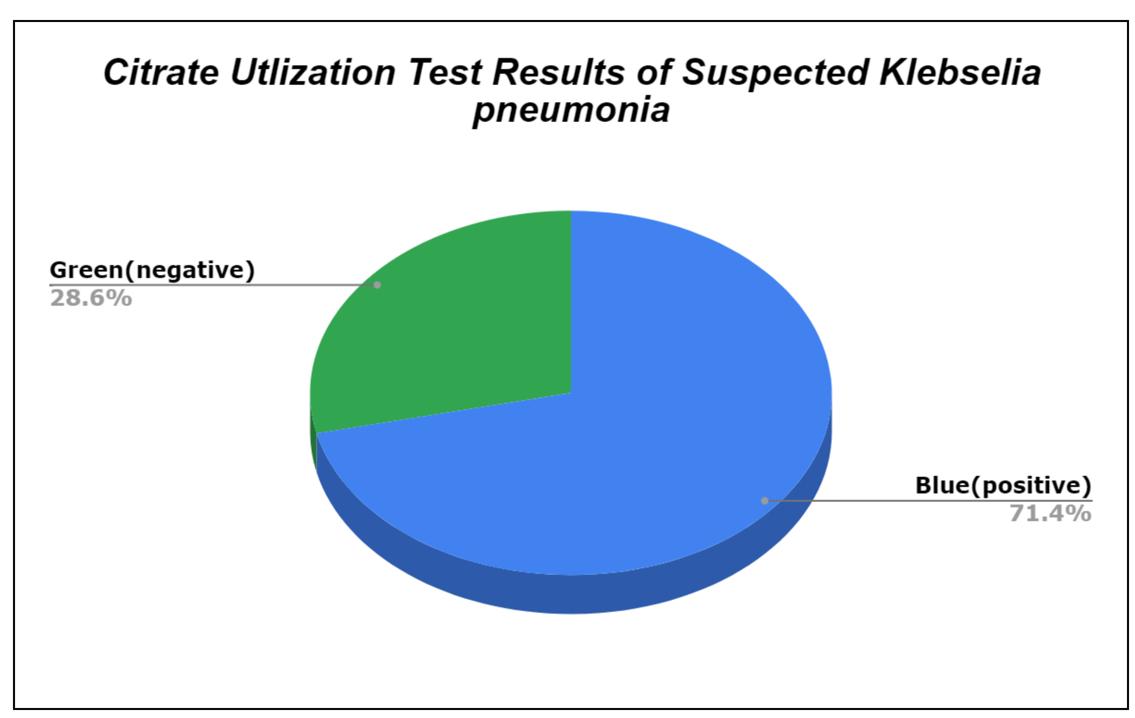


Figure 3.26: Observational Result of Citrate Utilization Test in Pie Chart.

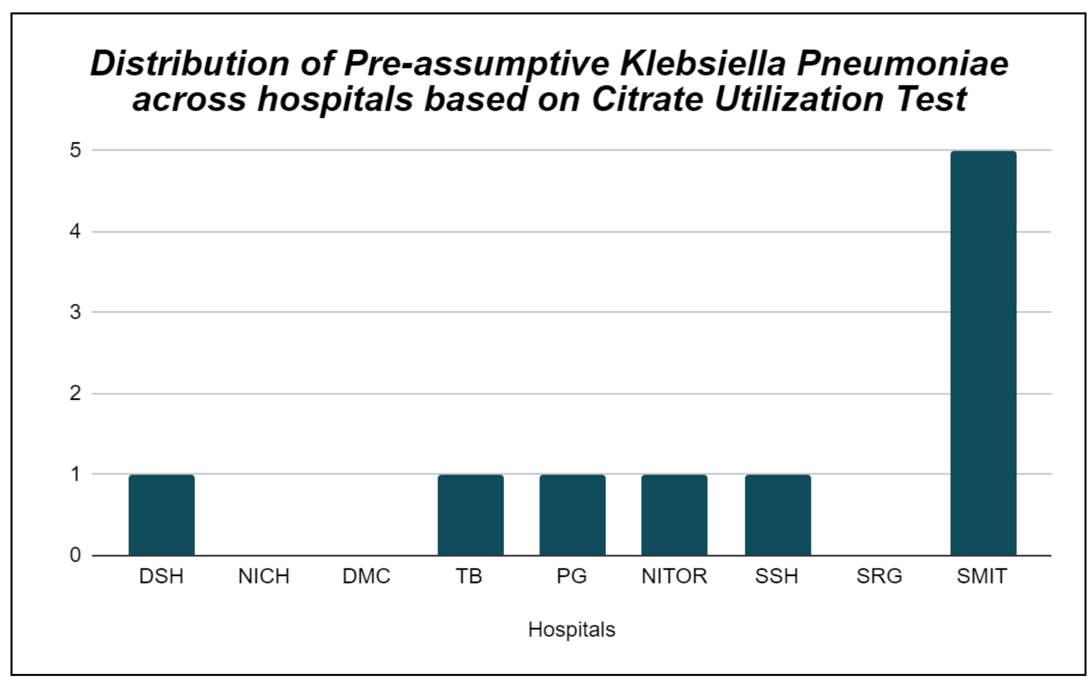


Figure 3.27: Distribution of Presumptive *Klebsiella pneumoniae* based on citrate utilization results among the collected hospitals.

3.6.4 Biochemical Identification Using Catalase Test

The catalase test detects the enzyme that protects organisms from oxidative damage. Organisms produce hazardous by-products such as hydrogen peroxide and superoxide radicals. Pathogenic organisms break down these compounds into non-toxic chemicals. Bacteria use catalase to break down hydrogen peroxide into water and oxygen. The test distinguishes catalase-positive organisms from catalase-negative organisms. Oxygen bubbles indicate the presence of catalase. The absence of bubbles indicates the lack of the enzyme.

Klebsiella spp. has the enzyme 'catalase'. All presumptive *Klebsiella spp* isolates were tested for the precedence of catalase enzymes by adding 2-3 drops of hydrogen peroxide (H_2O_2). Within a few seconds, they all produced bubbles, demonstrating that 100% of the isolates were catalase-positive.

Figure-3.28 demonstrates the results of the test samples to be positive in (a) and (b), whereas the test samples are compared with the positive controls of *Klebsiella pneumoniae* in (c).

Figure-3.29 illustrates the distribution of the 100% of isolates presumptively identified as *Klebsiella pneumoniae* among the nine hospitals collected. The bar chart shows that the number of positive isolates from each hospital varies distinctly. Based on catalase test results, the highest number of positive *Klebsiella pneumoniae* isolates was found in SMIT, with a value of 5. The second highest is from DSH. NICH, TB, PG, and SSH all shared one positive isolate. Interestingly, there were no isolates from DMC.

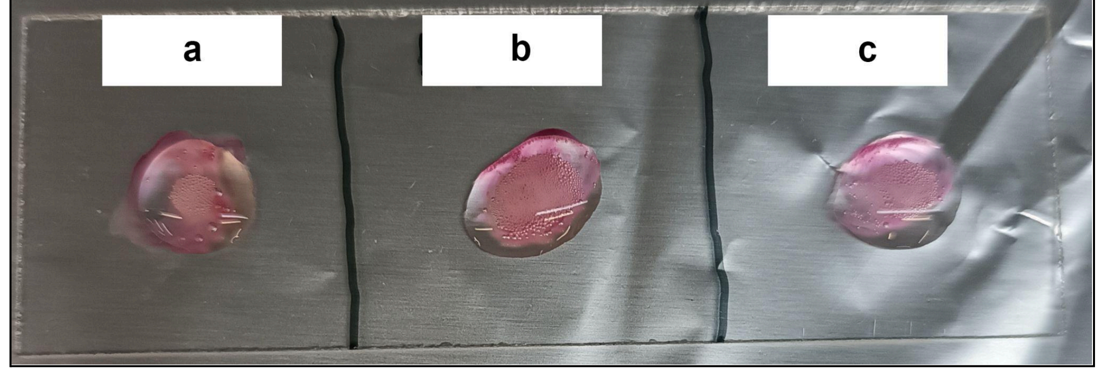


Figure 3.28: Bubble formation after adding H_2O_2 to the test species (a,b); (c) Positive control

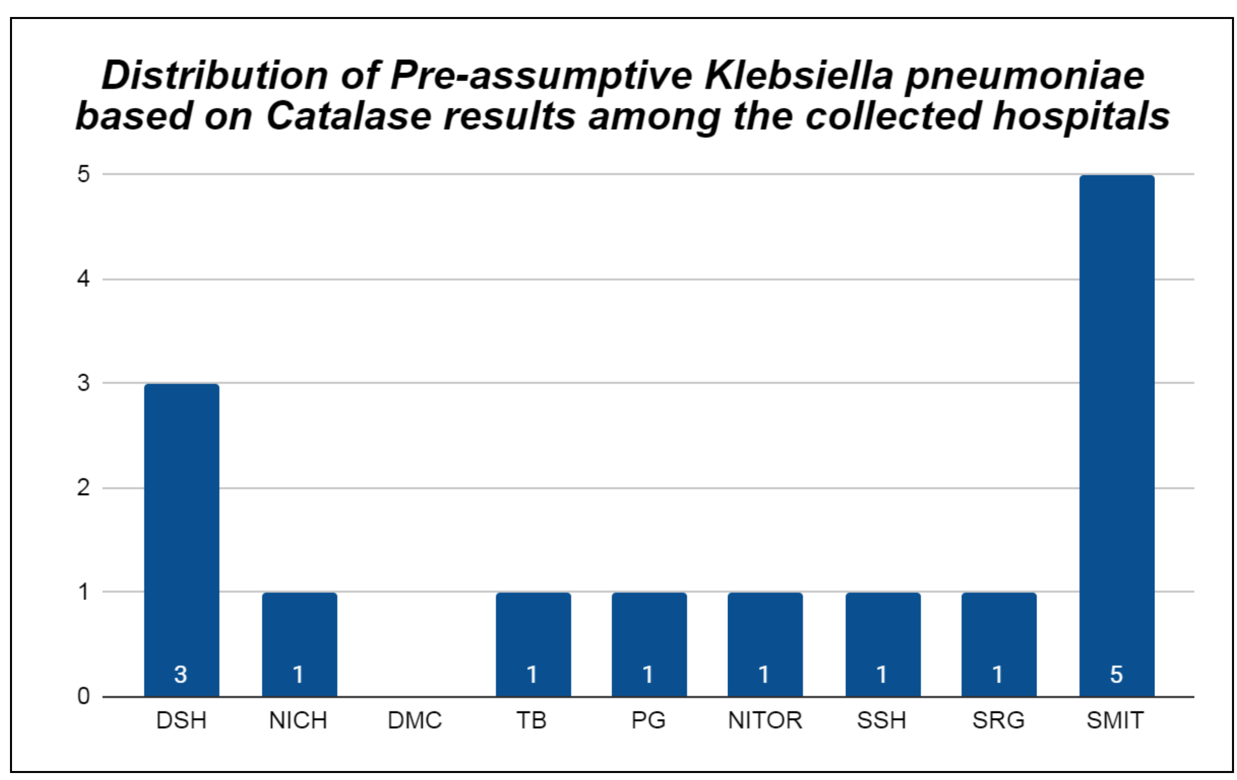


Figure 3.29: Distribution of Presumptive *Klebsiella pneumoniae* based on catalase test results among the collected hospitals.

3.6.5 Biochemical Identification Using Oxidase Test

Oxidase tests detect if the organism has the cytochrome oxidase enzyme present in Gram-negative bacteria such as *Pseudomonas*. An absorbent paper disc is taken, and the disc is impregnated with N`-Tetramethyl-p-phenylenediamine dihydrochloride. Then, the test species are rubbed on the paper. If the color changes to blue, it is oxidase-positive. Otherwise, no color indicates a negative result.

Figure-3.30 depicts the results of some of the samples for the Oxidase test. No color change is observed, demonstrating that all are oxidase-negative.

Klebsiella pneumoniae does not possess the enzyme cytochrome oxidase. While all 14 isolates were tested for oxidase, 100% were negative.

Figure-3.31 is an illustration of the distribution of the 100% of isolates presumptively identified as *Klebsiella pneumoniae* among the nine hospitals collected. The results show the same tendency as obtained in the catalase test. The bar chart shows that the number of oxidase-negative (positive for *Klebsiella pneumoniae*) isolates from each hospital varies distinctly. Based on catalase test results, the highest number of positive *Klebsiella pneumoniae*

isolates was found in SMIT, with a value of 5. The second highest is from DSH. NICH, TB, PG, and SSH all shared one positive isolate. Interestingly, there were no isolates from DMC.

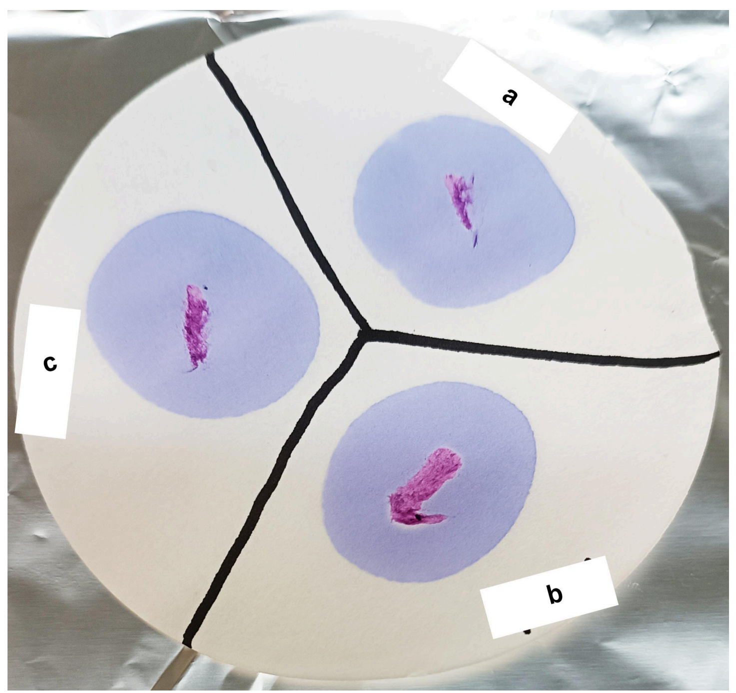


Figure 3.30: Result of Oxidase Test for *Klebsiella pneumoniae*
(a), (b) and (c) test samples are purple in color, there is no color change to blue. Test organisms are oxidase-negative

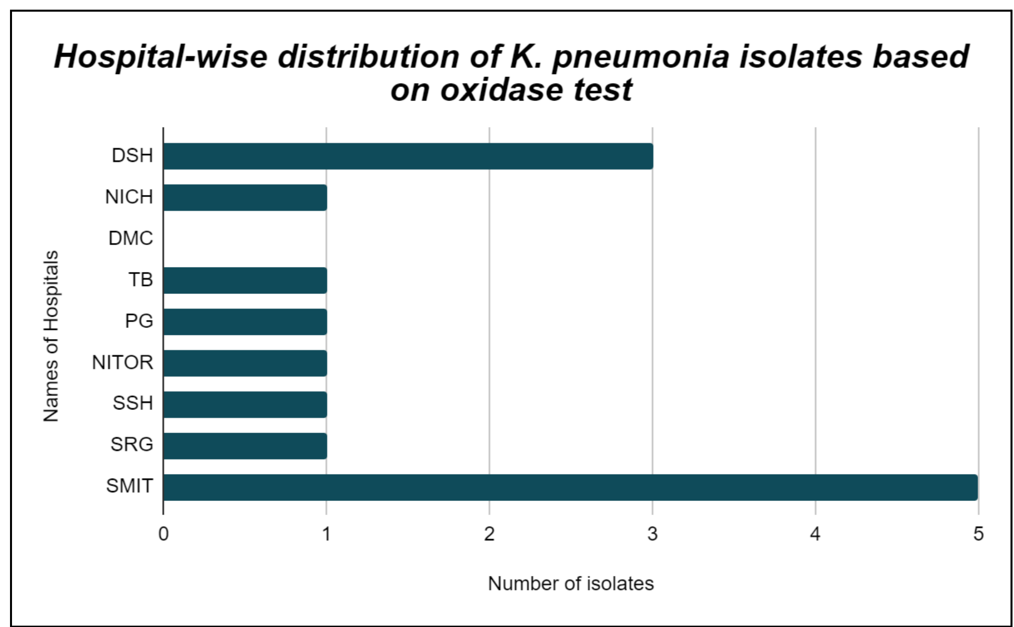


Figure 3.31: Distribution of Presumptive *Klebsiella pneumoniae* based on oxidase test results among the collected hospitals.

Finally, **Table-3.6** summarizes the biochemical results of all the presumptive ***Klebsiella pneumoniae*** isolates and showcases the results location-wise. In this result, we can observe that isolates from Old Dhaka and Shahbagh demonstrate the highest percentage of 100%. All the presumptive isolates of *Klebsiella pneumoniae* obtained were positive by all the biochemicals used. The next highest are from Mohakhali and Agargaon, with 50% each. Ramna area did not have any isolate of *Klebsiella pneumoniae* by all biochemical tests. It is evident that out of 14 samples. 4 samples were citrate negative, but they all showed other characteristics of *Klebsiella pneumoniae; from* this observation, it could be predicted, and there is a probability that those 4 isolates were *Escherichia coli* because it possesses the same characteristics as *Klebsiella pneumoniae***,** but the only difference is it is Citrate Utilization negative.

Area	Total No. of Isolates	Commonly Positive by All the Biochemical Tests	Overall Percentage (%)
Ramna	**0**	**0**	0
Shahbagh	**1**	**1**	100
Old Dhaka	**5**	**5**	100
Mohakhali	**2**	**1**	50
Agargaon	**6**	**3**	50

Table 3.6: Overall result of Biochemical tests of presumptive *Klebsiella pneumoniae*.

3.7 Identification of *Klebsiella pneumoniae* Isolates by PCR Assay and Gel Electrophoresis

The identification of 14 presumptive *Klebsiella pneumoniae* isolates was conducted by performing conventional PCR using a primer targeting the *ITS* sequence*,* and its sequence is mentioned in 2.6.1. Gel electrophoresis was carried out as mentioned in 2.6.3, and the results showed that out of 14 *Klebsiella pneumoniae* isolates, one isolate was found positive for the *ITS* sequence, showing a band in 133 bp, as observed in **Figure-3.32**. Hence, out of 14 pre-assumptive *Klebsiella pneumoniae* isolates, only 7.14% of isolates were positive for the *ITS* sequence, demonstrating these isolates are *Klebsiella pneumoniae* isolates

The positive isolate was only from PG Hospital, which is located in Shahbagh. No positive isolates were from the other eight hospitals and four rest locations, which suggests that *Klebsiella pneumoniae* does not stay in a higher number in cooked chicken because of the cooking condition. Also, maybe good hygiene practices are maintained to prevent the contamination of *Klebsiella pneumoniae* in cooked chicken.

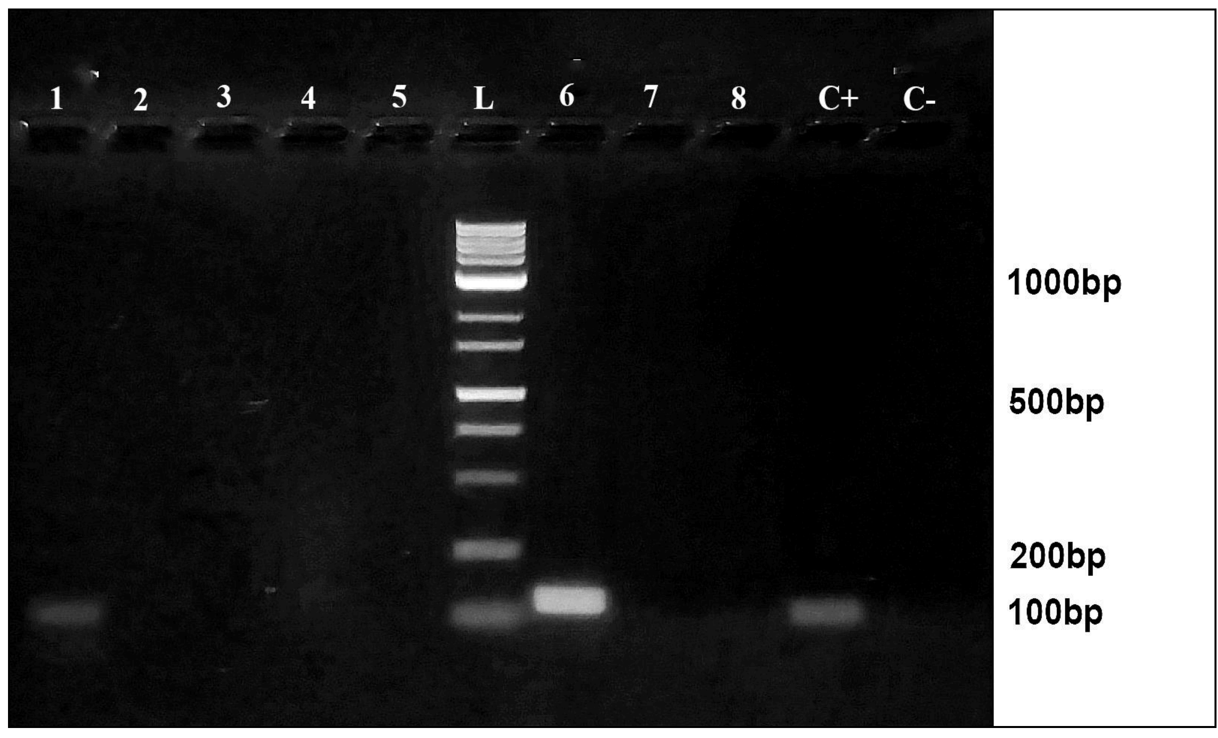


Figure 3.32: PCR amplified products of 133 bp of *ITS* sequence of *Klebsiella pneumoniae* in 1.5% agarose gel electrophoresis and ethidium bromide staining.
Legends: L= DNA Ladder; C- = Negative control; C+ = Positive control of *K. pneumoniae*; Lane 1-8 = Tested *S. aureus* isolates; Lane 6 = Positive isolate of *K. pneumoniae.*

3.8 Antibiotic Susceptibility Testing of *Klebsiella pneumoniae*

One positive isolate of *Klebsiella pneumoniae* underwent antibiotic susceptibility testing using disk diffusion methods against a panel of 11 antibiotics.

As demonstrated in **Figure-3.34**, Ampicillin (100%), Ceftazidime (100%), and Colistin (100%) have the highest resistance rates. Otherwise, the rest of the eight antibiotics named- Amikacin (AK-30), Amoxicillin-clavulanate (AMC-30), Cefepime (CPM-30), Tetracycline (TE-30), Levofloxacin (LE-5), Chloramphenicol (C-30), Meropenem (MRP-10) and Aztreonam (ATM-30) are sensitive towards it.

In **Figure-3.35**, the hospital-wise distribution of *Klebsiella pneumoniae* isolated from PG hospital situated in Shabagh is resistant to three antibiotics: Ampicillin, Ceftazidime, and Colistin. Since this positive isolate is resistant to more than one antibiotic, it could be classified as multi-drug-resistant (MDR) *Klebsiella pneumoniae*. The MDR *Klebsiella pneumoniae* indicates that there is an extensive use of antibiotics in PG hospitals that leads to resistance development. Furthermore, the lack of proper antimicrobial stewardship and the emergence of resistance genes contribute to the emergence of resistant bacteria.

Mult-antimicrobial resistance (AMR) is a global threat, and hence, it is highly crucial to determine the Multiple Antibiotic Resistance Index (MARI) of identified positive isolates of *Klebsiella pneumoniae.* **Figure-3.36** depicts the MARI- Multiple Antibiotic Resistance Index of one isolate of *Klebsiella pneumoniae*. As we know, a MAR index value greater than 0.2 indicates that the particular organism likely originated from sources with a high risk of resistance. The only positive sample of *Klebsiella pneumonia* has a MAR index of 0.273, which is greater than 0.2, and this particular isolate is from PG Hospital situated in Shahbagh. It indicates that the organism from this hospital has the likelihood of originating from a source with a high risk of resistance. Thus, it is impeccable to monitor the resistance patterns among the patients of these hospitals and try to find an association with their food habits, if consumption of cooked chicken from the cafeteria has an effect on them, or the multi-drug resistance pattern of the admitted patients are getting cross-contaminated to the cooked chicken food items in the cafeteria via the staff such as nurses and doctors.

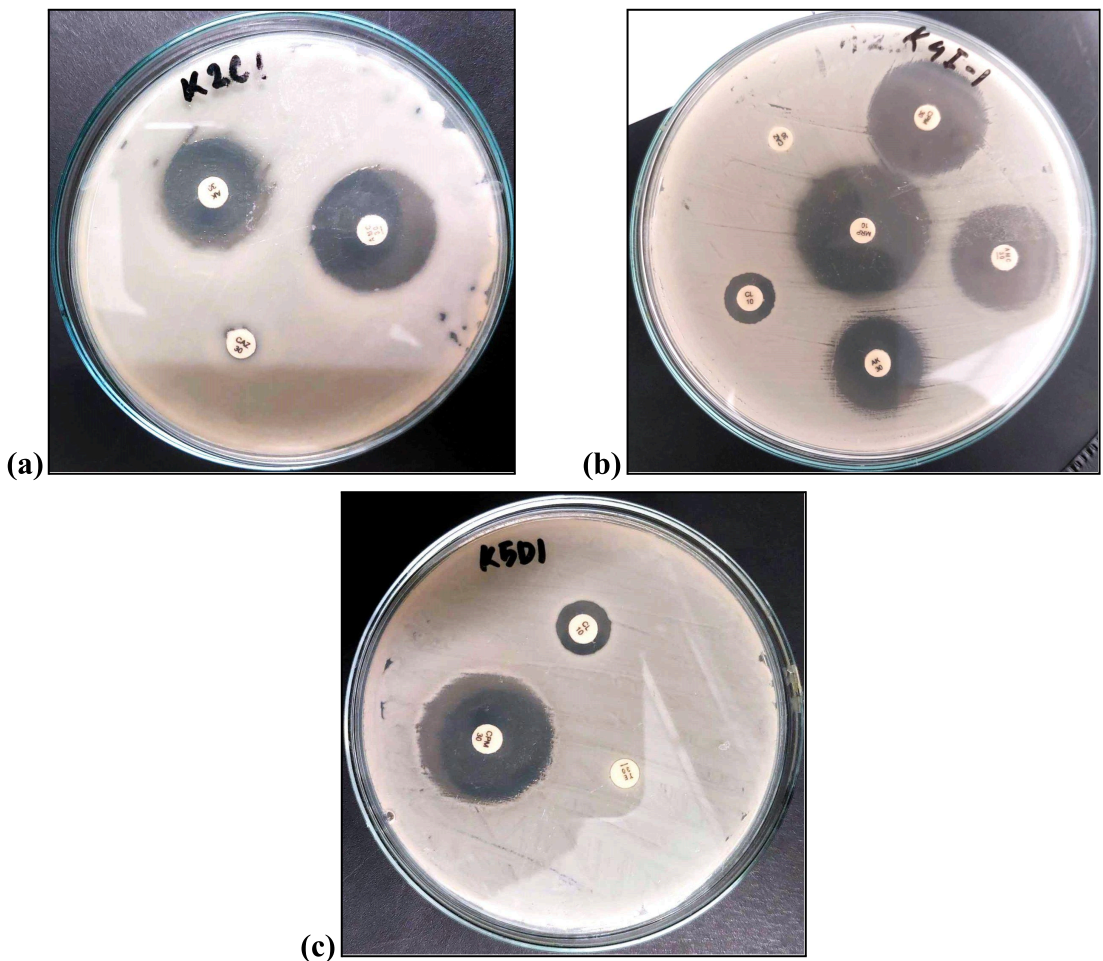


Figure 3.33: Results for Antibiotic Susceptibility Testing for *Klebsiella pneumoniae*
(a) AST result of sample-1 against AK-30, AMC-30 and CAZ-30; (b) AST result of sample-1 against CPM-30, MRP-10,AK-30 and CL-10; (c) AST result of sample-1 against TE-30.

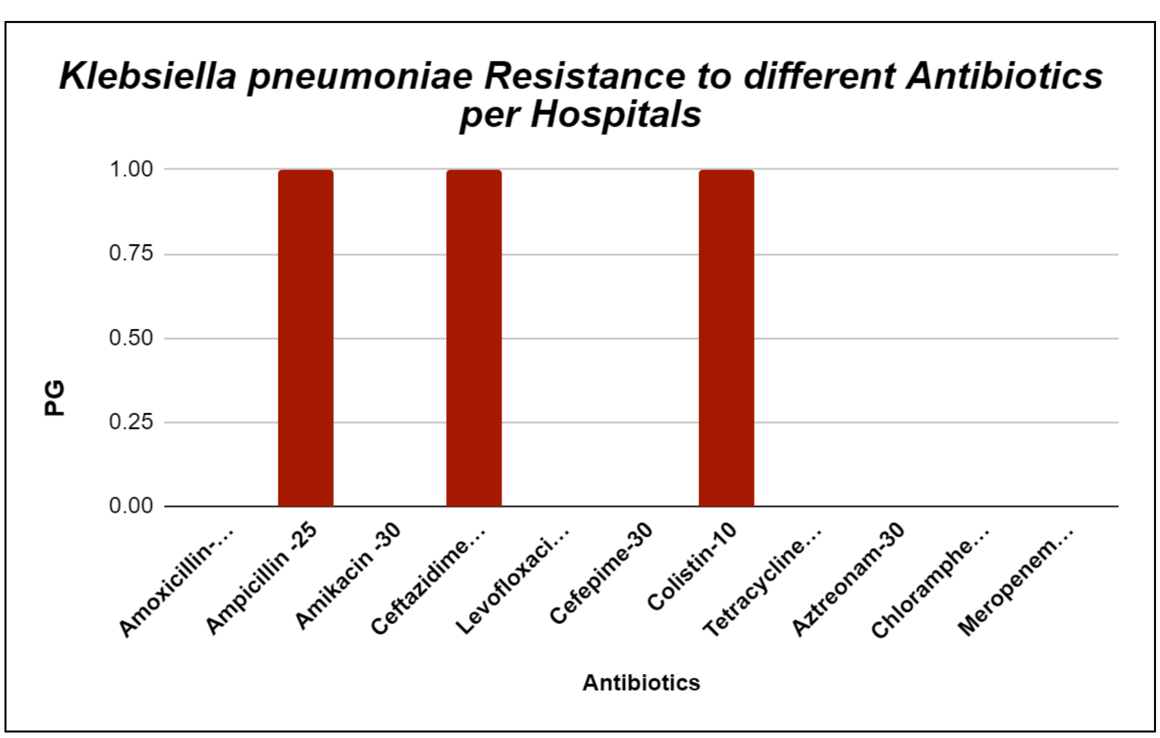


Figure 3.34: Hospital-wise comparison graph of *Klebsiella pneumoniae* and their resistance to different antibiotics.

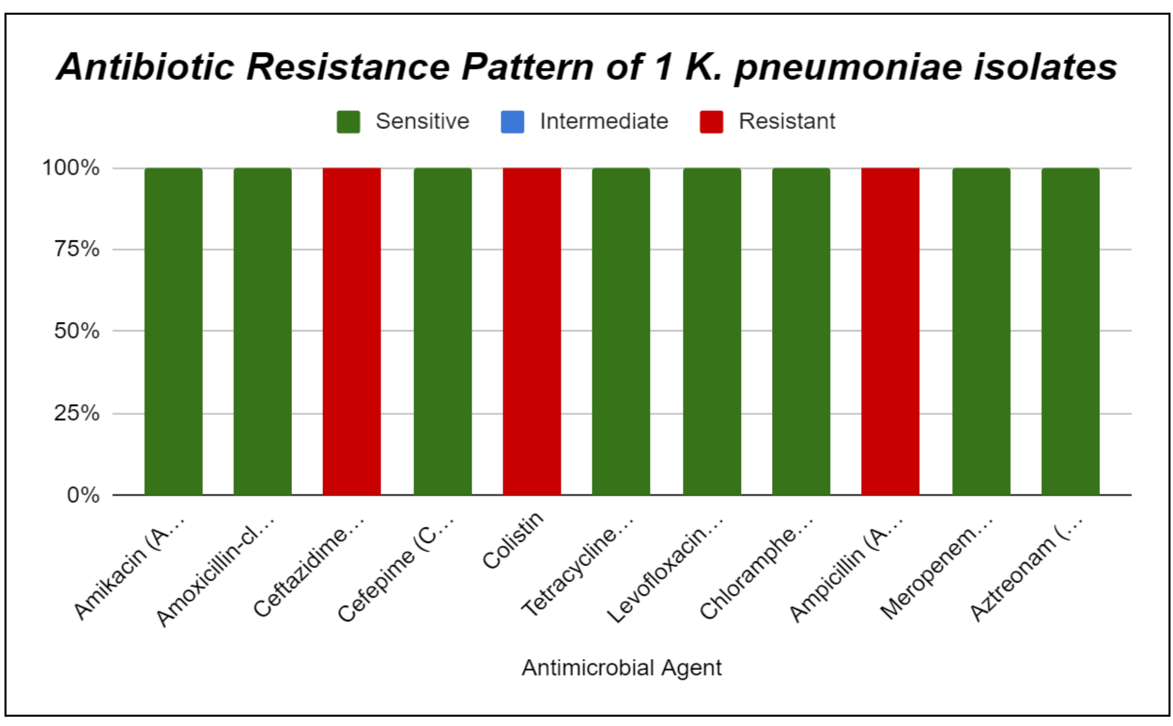


Figure 3.35: Bar Graph showing the Antibiotic susceptibility pattern of 12 different antibiotics in *K. pneumoniae* isolates.

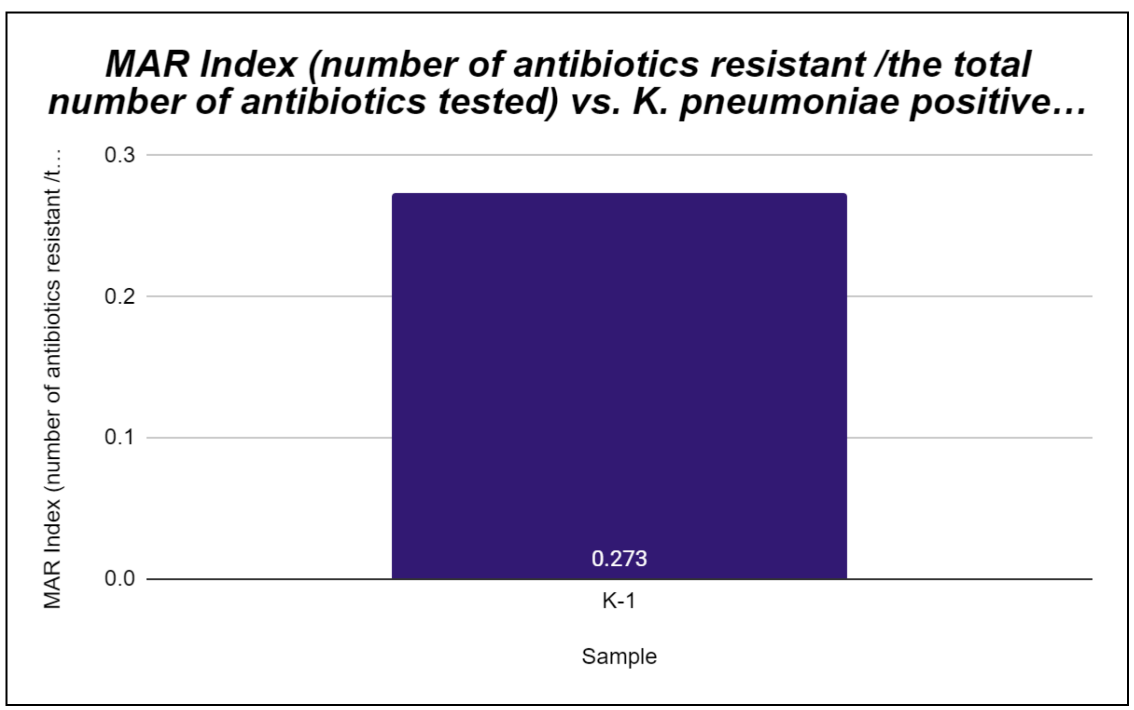


Figure 3.36: MAR Index of positive isolates of *Klebsiella pneumoniae* sample.

3.9 Comparative Analysis of Results between *Staphylococcus aureus* and *Klebsiella pneumoniae*

Prevalence between *Staphylococcus aureus* and *Klebsiella pneumoniae*

The following table compares the prevalence of *Staphylococcus aureus* and *Klebsiella pneumoni*ae in each location and hospital and their overall percentage. It is highly observed that the prevalence of *Klebsiella pneumoniae* is hugely less compared to *Staphylococcus aureus*. This may indicate that the probability of *Klebsiella pneumoniae* surviving in cooked chicken due to the cooking style is less than *Staphylococcus aureus*. It is observed that *Klebsiella pneumoniae* was found only from one location- Shahbagh, PG hospital- and that it was also multi-drug resistant. In contrast, positive isolates of *Staphylococcus aureus* are from Shahbagh, Old Dhaka (Puran Dhaka), Mohakhali, and Agargaon, covering 4 locations out of 5 covered in this study. All the isolates of *Staphylococcus aureus* are MDR. **Table-3.7** provides an insightful summary based on differences in the prevalence of *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Sl. No.	Area	Hospital Name	Total Isolates	Identified Bacteria as positive for *Staphylococcus aureus*	Identified Bacteria as positive for *Klebsiella pneumoniae*	Overall Percentage (%)
1	**Ramna**	Dhaka Medical College (DMC)	2			0
2	**Shahbagh**	Bangabandhu Sheikh Mujib Medical University (PG)	4	3	1	100
3	**Puran Dhaka**	Sir Salimullah Medical College Mitford Hospital (SMIT)	18	1		5.56
4	**Mohakhali**	National Institute of Chest Disease and Hospital (TB)	8			0
		Sheikh Russel National Gastro Liver Institute & Hospital (SRG)	15	2		13.33
5	**Agargaon**	Shaheed Suhrawardy Medical College and Hospital (SSH)	7			0
		Dhaka Shishu Hospital (DSH)	11			0
		National Institute of Traumatology and Orthopaedic Rehabilitation (NITOR)	4			0
		National Institute of Cancer Research & Hospital (NICH)	8	1		12.5
	Total		**77**	**7**	**1**	**10.4**

Table 3.7: Summarized comparative analysis result of the prevalence of *Staphylococcus aureus* and *Klebsiella pneumoniae*

4. Discussion

The cafeteria of a government hospital within Dhaka City Corporation was selected as the sampling site, keeping in mind that hospitals in Dhaka are the busiest on a daily basis. People from every corner of the country came here to get better treatment. Not only patients but also patient attendants, medical staff, office staff, and many other attendants visit the hospital for various purposes. At various times of the day, especially at lunch hour (12 pm to 2 pm) they take food from the cafe located inside the hospital. So, there is a high chance of bacterial contamination in the food inside the hospital area; as stated in this study previously, hospitals are places of superbugs with virulent and resistant bacteria. However, the meat samples collected at lunch hour between 12-2 pm were decided as sample collection time because, at this time, most of the people stay present together at the cafeteria for their lunch. This would give us an insight into what may happen to a larger population of the public.

The study identified 77 isolates in total, out of which only 10.4% were positive for the targeted bacteria, namely *Staphylococcus aureus* and *Klebsiella pneumoniae* (**Table-3.7**). This indicates a very low prevalence of these bacteria in general. However, the rate of 10.4% is still quite concerning since the isolation was conducted from cooked chicken, where the cooking style and procedure should have destroyed these bacteria. Each part of the obtained results will be discussed in detail below.

4.1 Prevalence of *Staphylococcus aureu*s

This study has yielded significant findings, revealing 49 positive *Staphylococcus aureus* isolates from specific biochemical tests. The molecular identification of *S. aureus* from these isolates was conducted using the PCR procedure, targeting the *nucA* gene. Notably, seven (7) isolates from the initial 49 showed a band (at 270bp) in gel electrophoresis after PCR (Mashouf et al., 2015). This indicates a prevalence rate of 14.3% for *Staphylococcus aureus* in cooked meat samples collected from hospital cafeterias, as specified in **Figure-3.16**.

The 14.3% prevalence rate of *Staphylococcus aureus* is significantly high. This high prevalence was observed in several studies which have worked on raw and poultry meat such as chicken and beef, greek meat products, and processed and ready-to-eat (RTE) meats like minced meat, liver, luncheon, sausages, pork ham, chicken cold cuts, pork sausage, salami, nuggets and pork luncheon meat and many more (Ali et al., 2023; Farahmand et al., 2020; Fijałkowski et al., 2016; Guo et al., 2016; Haghi et al., 2021; Hassan et al., 2018; Schaeffer, 2017; Sudarmadi et al., 2020; Theocharidi et al., 2022; Uddin et al., 2019; Waters et al., 2011). These researches showed sufficient prevalence of *S. aureus* in raw meat samples. According to Igbinosa et al., 2016 & Farahmand et al., 2020, the prevalence of *S. aureus* was about 25 to 40% in raw meat samples. Furthermore, Shields & Tsang, 2006 the characterization of *S. aureus*, specifically from different food items in hospitals, within the findings that raw chicken samples and cooked meat barbeque have *S. aureus* prevalence of 27.02% and 26.31%, respectively. Chicken samples interestingly

showed the highest prevalence than other meat samples there. Different studies stated that raw chicken/meat samples exhibited enough prevalence of *S. aureus* and their possible reasons.

The reasons could be: **(i)** Failure to maintain hygienic procedures for processing, handling, or storing meat; **(ii)** Insufficient cleaning and post-microbial contamination; **(iii)** Skins or other parts of broiler meat could be the reservoir of antimicrobial-resistant strains; **(iv)** Virulence factors, and genetic clustering, indicating contamination sources and so on (Thakur et al., 2023; Igbinosa et al., 2023).

At the same time, several causes can be speculated for the prevalence of cooked chicken as well, such as **(i)** Inadequate cooking temperatures, **(ii)** Cross-contamination during serving handling, **(iii)** Antimicrobial resistance with specific resistance genes (Thakur et al., 2023; Doiphode et al., 2022). In addition, the collected cooked chicken from hospital cafeterias and the hospital environment also impacted the samples.

However, cooked chicken samples were less prevalent than raw chicken/meat samples. In this study, a prevalence of 14.3% for *S. aureus* was achieved. Some previous studies also showed a close prevalence of 9.3% (Mashouf et al., 2015), 8.4% (Orpin et al., 2019), and 6.66% (Kaya et al., 2018) *S. aureus* in cooked meat samples. The prevalence of *S. aureus* is relatively higher than the previous studies conducted. The rate of prevalence is lower in cooked chicken and this may be due to adequate cooking temperature, use of medicinal spices, and maintaining proper cleanliness during cooking and serving. Thus, the prevalence of *S. aureus* is reduced in cooked chicken than in the raw/retail chicken sample.

As observed in **Figures-3.15 and 3.16,** seven positive isolates in molecular detection were obtained from four (4) hospital samples out of the nine (9). The highest prevalence of *S. aureus* was observed from PG Hospital (Shahbagh, three isolates), with 42.9%. This increase in frequency may be due to the variety of chicken sources each hospital receives, the hygienic requirements, or the methods for processing food differently. In addition, *S. aureus* will cross-contaminate the cafeteria due to the patient population, number of attendees, and personnel served in the PG Hospital cafeteria, which could lead to the highest number of positive isolates. Besides, SMIT (Old Dhaka, one isolate) and NICH (Agargaon, one isolate) have the same amount of positive *Staphylococcus aureus* isolates with a value of 14.3%. The remaining 28.6% of SRG (Mohakhali, two isolates) is the second-highest abundance of positive *Staphylococcus aureus* isolates. The positive isolates of *S. aureus* from these hospitals may demonstrate the kitchen's poor hygiene policy where the meat was cooked. Cross-contamination from the hospital environment is possible because all kinds of people, like- attendees, staff, and even patients, also take their food from a typical cafeteria. This culture indeed increases the risk of food poisoning and other diseases while people intake meat in search of proteins and other nutrients.

Nevertheless, the prevalence of *S. aureus* in cooked chicken is alarming due to the threat of foodborne illness, which negatively impacts public health. This pathogen, like *S. aureus,* causes

an outbreak of foodborne intoxication that may lead to morbidity and mortality (*Savini et al., 2023*). Moreover, enterotoxicity and antimicrobial resistance of the bacteria may cause severe harm and permanent damage to the immune system (Pal, 2022).

In summary, kitchen culture and the hospital environment are significant causes of public health complications because of the prevalence of *Staphylococcus aureus* in meat samples from the hospital courtyard cafeteria. A better and hygienic way of processing and handling food, adequate cooking temperature, and cleanliness in serving could help reduce the chances of bacterial contamination in food.

4.2 Prevalence of *Klebsiella pneumoniae*

Klebsiella pneumoniae is also a crucial concern of our study. However, the sample size of *K. pneumoniae* is less than that of *S. aureus*. 14 biochemical positive isolates were taken for molecular identification targeting *Klebsiella-specific ITS* sequence (*Liu et al., 2008*) to confirm whether the isolate is *K. pneumoniae*. Only one (1) displayed the band (at 133 bp) in gel electrophoresis from those distinct isolates. Consequently, the prevalence rate found for *Klebsiella pneumoniae* is about 7.14% from cooked meat samples collected from the hospital cafeterias.

Usually, *Klebsiella pneumoniae* is not regarded as a foodborne pathogen. Still, recent studies have raised a frightening concern that antimicrobial-resistant strains of *K. pneumoniae* have been found in retail meat products, farm-raised chicken, and other food samples (Guo et al., 2016). *K. pneumoniae* has different virulence factors, genetic diversity, and pathogenic strains [*ecpA*, *fimH*, *wcaG*, *ampC*, ***β****-lactamases*, *OXA-48,* etc.] that are closely related to antimicrobial-resistance (Theocharidi et al., 2022; HARTANTYO et al., 2020). However, most studies reported that the prevalence of *K. pneumoniae* is higher in raw meat and chicken, just in cases ranging from an average of 3.6 to 60% in different countries. (Deepan et al., 2023). Unlike *Staphylococcus aureus*, specific causes are responsible for this maximum prevalence in raw meat. For instance: (i) in various environmental reservoirs, including water, soil, and animal gut, *K. pneumoniae* has been found; if chickens are raised or processed in *K. pneumoniae* area, then the risk will be increase; (ii) the poultry/broiler farm's chicken is reservoir of pathogenic *K. pneumoniae* itself; (iii) in case of hospitals, *K. pneumoniae* can be found any patient ward from infections, it can be transmitted through staff and people around hospitals and came into hospital's cafeteria as well.

In contrast, the prevalence of *K. pneumoniae* from cooked chicken/meat samples is comparatively lower than the raw samples. According to the report of Guo et al., 2016, the prevalence of *K. pneumoniae* in cooked chicken was about 7-8%. This prevalence is relatively matched with our study, which has a prevalence of 7.14%. The decreased prevalence of cooked chicken could be due to inadequate cooking time and temperature, using spices, maintaining cleanliness, etc. But, even though there is some prevalence, it can be caused by **(i)** A thermotolerant strain of *K. pneumoniae* (Jørgensen et al., 2016), **(ii)** Cooking procedure and

inadequate temperature, **(iii)** Failed hygiene procedure in retailing as well as in the kitchen. Even so, the chances of cross-contamination in the hospital environment sustain the prevalence of pathogenic *K. pneumoniae*.

The positive isolate belongs to PG Hospital (Shahbagh area). This scenario may suggest a hazardous situation in the respected hospital/area. It also raises questions about the cafeteria's environment, kitchen cleanliness, alertness during food processing, general people/staff awareness, and more.

Ultimately, the outbreak of *K. pneumoniae* will be an alarming issue because it will not only cause intoxication but also induce other diseases like- pneumoniae, septicaemia, liver abscesses, and diarrhea in humans (Zhang et al., 2018). The potential health risks posed by such virulent strains and antimicrobial-resistant genes of *K. pneumoniae* should not be underestimated. Maintaining proper personal hygiene and diet are essential for reducing antibiotic resistance. Therefore, improved monitoring policies and prevention strategies are urgently needed to control the emergence and transmission of such foodborne pathogens.

4.3 Prevalence of Multi-Drug Resistant Bacteria in the Present Study

As stated previously, seven positive isolates of *Staphylococcus aureus* and one positive isolate of *Klebsiella pneumoniae* underwent antibiotic susceptibility testing. The results of the antibiotic susceptibility testing are discussed-

4.3.1 *Staphylococcus aureus*

The results of the antibiotic susceptibility testing reveal that the seven positive isolates of *Staphylococcus aureus* are all multi-drug resistant. As observed in 3.4 (**Figure-3.18)**, all seven isolates are 100% resistant to ampicillin-25 and penicillin-10. Additionally, the resistance rates are followed by clindamycin-2 and methicillin-5 with 42.9%, followed by erythromycin-15, doxycycline-30, and kanamycin-30 with 28.6% followed by azithromycin-30 and vancomycin-30 and novobiocin-30 with 14.3%. Since the samples were obtained from the hospital cafeteria, the prevalence of having all multi-drug resistant *Staphylococcus aureus* aligns with the results obtained in earlier studies conducted in hospital settings. (L Gilbert, 2007; Liu & Qin, 2022; "Molecular Detection of Enterotoxins from *Staphylococcus aureus* Isolated from Hospital and Environmental Samples in Osogbo," 2022; Tula et al., 2022; University Ile Ife et al., 2017; Zhanel et al., 2010)

The highest resistance towards penicillin and ampicillin of *Staphylococcus aureus* was discussed earlier (L Gilbert, 2007). In 1946, when penicillin became the most available treatment against *Staphylococcus aureus,* it shortly emerged to penicillin-resistant *Staphylococcus aureus* that has caused at least 50% of *staphylococcal* surgical wound infections at the Royal Prince Alfred Hospital (RPAH), in Sydney. (L Gilbert, 2007) Additionally, the findings of (Tula et al., 2022), where they detected multi-drug resistant (MDR) bacterial isolates from hospital fomites and

hands of healthcare workers in Mubi General Hospital, also found all the isolates they have found, including *Staphylococcus aureus,* were resistant to penicillin and ampicillin. A study by (Liu & Qin, 2022) also highlights a 91.3% resistance rate of penicillin for *Staphylococcus aureus*. In addition to the hospital environment, previous studies for poultry and processed meats from the local market region have shown the highest resistance rates for penicillin and ampicillin. (Hassan et al., 2018; Rahman et al., 2018; Waters et al., 2011) *Staphylococcus aureus* develops resistance to ampicillin and penicillin through the production of a penicillin-binding protein 2a (PBP2a) encoded by the *mecA* gene, resulting in poor affinity towards β-lactam antibiotics such as ampicillin and penicillin. (El Shazely et al., 2020) This mechanism happens to missense mutations in PBP2a, such as E239K, E239R, G246E, and E447K, with the E239R mutation inducing conformational changes that hinder interactions with cefoxitin, leading to high-level resistance (Asyraf et al., 2022)

The study found that clindamycin and methicillin had the second-highest resistance rates. Additionally, 42.9% of the isolates were identified as methicillin-resistant *S. aureus*. Methicillin-resistant *S. aureus* is a significant finding due to its heavy impact on human and animal health, which causes hospital and community-acquired infections. (Kasela et al., 2023; Patel & Rawat, 2023; Shoaib et al., 2023) MRSA can form robust biofilms and secrete virulence factors contributing to its pathogenicity. (Alkharsah et al., 2018) It has been observed that MRSA strains are highly prevalent in hospital environments. (L Gilbert, 2007) reported that over 95% of isolates were MRSA. Additionally, (Zhanel et al., 2010) found that the rate of MRSA in Canadian hospitals was 27%, the second-highest in their study. This finding was based on a sample size of 5,282 bacterial isolates. A study of (Waters et al., 2011)from US poultry meat also revealed that 75% of *S. aureus* isolates were MRSA. The study by (Safarpoor Dehkordi et al., 2017) that took food samples from hospitals found that the highest number of *S. aureus* was from chicken. Additionally, all the isolates from cooked chicken products had complete resistance against methicillin, and all were classified as MRSA. The reasons for having MRSA in cooked chicken samples from the hospital cafeteria could be – (i) due to contamination during processing or handling. (Bernier-Lachance et al., 2020) (ii) the presence of resistance genes like mecA, VanA, ermA, and tet L, facilitating antibiotic resistance transmission from animals to humans through food production processes. (Zaher et al., 2023), (iii) may originate from human sources, two distinct MRSA lineages were found that are in association with human contamination - (ST398-MRSA-V and ST8-MRSA-IVa), (Bernier-Lachance et al., 2020) (iv) overuse of antibiotics in livestock and finally (v) The up-and down-regulation of adhesion genes for biofilm formation and genes for virulence factor synthesis during infection stages act as a genetic regulatory see-saw in MRSA pathogenesis. (Patel & Rawat, 2023)

The resistance rates for erythromycin-15, doxycycline-30, and kanamycin-30 with 28.6% followed by azithromycin-30 and vancomycin-30 and novobiocin-30 with 14.3% are relevant by the studies conducted by (Liu & Qin, 2022; Tula et al., 2022; Zhanel et al., 2010) demonstrates that the resistance rates of these antibiotics keep on mainly varying depending on the usage of

antibiotics on each hospital and geographical location. The reasons for having resistant isolates for these antibiotics could be similar to having MRSA, as mentioned above.

Figure-3.18 also illustrates an optimistic result, a very high percentage of sensitivity of the found *S.aureus* isolates for Levofloxacin (100%) and Amoxicillin (100%), Azithromycin (75%), Vancomycin (75%), and Novobiocin (75%). Similar results have been found in various studies, including (Kasela et al., 2023; Patel & Rawat, 2023; Shoaib et al., 2023) where all the isolates were MRSA but showed higher sensitivity against Levofloxacin and Amoxicillin. The reasons for this could be because– **(a)** Both show significant efficacy against planktonic and biofilm-associated forms of the bacteria- one of these is *S.aureus*, **(b)** Levofloxacin, fluoroquinolone has shown enhanced antimicrobial activity against *S.aureus* that can form biofilm.(Sultan et al., 2022) Moreover, **(c)** Amoxicillin, a beta-lactam antibiotic, effectively reduces the counts of *S.aureus* in the intracellular environment when the concentration of this antibiotic reaches the serum concentration level in humans. (Ledger et al., 2023) In contrast, their varying antibiotic resistance profiles and mechanisms explain the higher sensitivity towards Azithromycin, Vancomycin, and Novobiocin. The higher sensitivity of *Staphylococcus aureus* to erythromycin, kanamycin, azithromycin, vancomycin, and novobiocin is due to varying antibiotic resistance profiles and mechanisms observed in the study. Erythromycin and azithromycin mechanisms work by binding to the 50S subunit of the bacterial ribosome, thereby inhibiting protein synthesis; therefore, it is effective against *S. aureus* strains that do not possess resistance mechanisms such as efflux pumps or ribosomal modification enzymes. Kanamycin operates by binding to the 30s subunit of the bacterial ribosome, thus inhibiting protein synthesis. Vancomycin inhibits cell wall synthesis in bacteria by binding to the D-alanyl-D-alanine terminus of cell wall precursors. It is effective against S. aureus due to its unique mechanism of action, which targets cell wall components. Novobiocin inhibits DNA gyrase, an essential bacterial enzyme involved in DNA replication. The sensitivity of *S. aureus* to novobiocin is linked to its interference with DNA replication, leading to bacterial cell death. (Selim et al., 2022)

Most importantly, in this study, the optimistic result was that 42.9% of the isolates of *S. aureus* were sensitive towards methicillin (MSSA). (L Gilbert, 2007) states that the ratios of MRSA to MRSA reflect the quality of hospital care, infection control, and antibiotic prescribing surveillance measures. There were only three positive isolates from PG, and out of these three, all were MSSA, indicating a high quality of control of the hospital PG. (L Gilbert, 2007) also finds in his study that MSSA tends to be less resistant towards a vast number of antibiotics, which is evident by the result found from PG; the three MSSA isolates from PG are only resistant to two antibiotics- penicillin and ampicillin and sensitive to the rest of other ten antibiotics tested. Despite the reasons for MSSA, other reasons, except for the environment and usage control, cannot be classified as being affected by various factors. Studies by (Abdulgader et al., 2020; Villegas et al., 2022) have shown that these isolates can be the natural variant

lacking the specific DNA regions associated with resistance. Thus, they cannot emerge into MRSA, or the hospital and particular location must strictly maintain antibiotic surveillance.

Figure-3.19 demonstrates the MAR index of the seven positive isolates of *S.aureus,* where it is revealed that out of seven isolates, four isolates have a MAR index>0.2, meaning 57.1% of positive isolates of *S.aureus* have a MAR index greater than 0.2. A MAR index>0.2 demonstrates that the particular isolates have originated from high-risk contamination where antibiotics are extensively used.(Jaja et al., 2020; Saber et al., 2022) This signifies that 57.1% of *S. aureus* have originated from a high-risk contamination source where antibiotics are extensively used. Sample numbers named from S-4 to S-7 consecutively all have a MAR index>0.2. Amongst them, S-4 has the highest MAR index- 0.58, which is from NICH, situated in Agargaon; the next subsequent is S-6, with a value of 0.417, which is from Sheikh Russel National Gastro Liver Institute & Hospital, located at Mohakhali and the next subsequent ones are S-5 also from SRG and S-7 from SMIT (Old Dhaka) which has a value of 0.25. Therefore, according to the result, the hospitals named NICH, SRG, and SMIT from Agargaon, Mohakhali, and Old Dhaka, respectively, have the likelihood of originating from a source with a high risk of resistance. To prevent drug-resistant infections in hospitals, it is essential to monitor patients' resistance patterns and Investigate if there is an association between food habits and resistance. It is essential to monitor patient resistance patterns to prevent hospital drug-resistant infections. Investigate if there is an association between food habits and resistance. Check if cooked chicken in the cafeteria affects resistance. Also, explore whether patients with multi-drug resistance patterns transfer to cafeteria food through staff. Address cross-contamination in hospital cafeterias.

Figures-3.20 & 3.21 highlight the antibiotic resistance and susceptibility pattern hospital-wise; two isolates obtained from SRG (Mohakhali) are resistant to 10 antibiotics out of 12 and are sensitive to only Levofloxacin and Amoxicillin. It shows a growing concern about the antibiotic surveillance system of Sheikh Russel National Gastro Liver Institute & Hospital in Mohakhali; the next one with extreme concern is NiCH in Agargaon, which is resistant to eight antibiotics. This brings questions about their antibiotic surveillance system, which needs to be lit over here immediately to ensure the safety of patients, staff, and doctors in their hospital.

4.3.2 *Klebsiella pneumoniae*

There was only a positive isolate of *Klebsiella pneumoniae,* and it has undergone antibiotic susceptibility testing against eleven antibiotics. As shown in **Figure-3.35**, *Klebsiella pneumoniae* isolate is resistant to three antibiotics named- Ampicillin, Ceftazidime, and Colistin and sensitive to rest eight antibiotics named– Amikacin, Amoxicillin-clavulanate, Cefepime, Tetracycline, Levofloxacin, Chloramphenicol, Meropenem and Aztreonam indicating that this isolate is multi-drug-resistant (MDR). Since the samples were obtained from the hospital cafeteria and meat, the prevalence of all multidrug-resistant isolates of *Klebsiella pneumoniae* aligns with the

previous works where the MDR of *Klebsiella pneumoniae* is found. (*Córdova-Espinoza* et al., 2023; Guo et al., 2016; Herruzo et al., 2017; Schaeffer, 2017) As per the paper of (*Córdova-Espinoza* et al., 2023), 46% of the isolates of *Klebsiella pneumoniae* were MDR. In a prior survey in the United States, 53 (16.1%) MDR *K. pneumoniae* strains were found in 330 farm-raised frozen shrimp imported from Thailand. (Zhang et al., 2018)

Complete resistance was revealed against Ampicillin, Ceftazidime, and Colistin. As per (*Córdova-Espinoza* et al., 2023), they have found *K. pneumoniae* isolates that were 96% resistant to Ampicillin. *Klebsiella pneumoniae* exhibits resistance against ampicillin due to the production of various beta-lactamase enzymes, including extended-spectrum beta-lactamases (ESBLs), AmpC-type beta-lactamases, and carbapenemases. These enzymes confer resistance by inactivating the antibiotic, rendering it ineffective against the bacteria. (Sobia et al., 2011) Additionally, studies have shown that *K. pneumoniae* biofilms impede the penetration of ampicillin, contributing to its reduced efficacy in eradicating the bacteria. (Sheff, 2000) The resistance pattern of *K. pneumoniae* to ampicillin is part of a broader multidrug resistance profile, making the bacterium challenging to treat with conventional antibiotics. (Anderl et al., 2000; Sheff, 2000; Sobia et al., 2011) Therefore, understanding the mechanisms behind this resistance is crucial for developing effective treatment strategies against *K. pneumoniae* infections.

K. pneumoniae isolates have shown resistance towards ceftazidime and colistin in various mainly clinical studies (Liu & Qin, 2022; Nath et al., 2020; Zhanel et al., 2010), where all exhibit resistance towards clindamycin. The reasons for this resistance could be (i) the presence of carbapenemase-encoding genes like blaKPC, blaOXA-48, and blaNDM-1 and mutations in porin genes named ompK35 and ompK36 (Bulman et al., 2022; El-Kady et al., 2022) (ii) emergence on a novel variant of KPC genes such as KPC-70, with specific amino acids substitutions like D179Y and T263A and finally (iii) the loss of function in genes named ompK36 and ramR with the carriage of specific carbapenemases. (Resistance to Ceftazidime_Avibactam Plus Meropenem_Vaborbactam When Both Are Used Together Achieved in Four Steps From Metallo-β-Lactamase Negative *Klebsiella pneumoniae*..Pdf, n.d.) The reasons for resistance against colistin could be- a) mutations in the chromosomal gene named mgrB affects the membrane permeability resulting in resistance against colistin. (Yap et al., 2022) b) the activation of the arn operon, regulated by PhoPQ and PmrAB systems, plays a crucial role in colistin resistance; c) emergence of extensively drug-resistant strains, like ST656, further complicating treatment options, with factors such as mcr genes and efflux pumps contributing to high-level colistin resistance. (Phoebe Cheung, C. H., Dulyayangkul, P., Heesom, K. J., & Avison, M. B., (2020); Proteomic Investigation of the Signal Transduction Pathways Controlling Colistin Resistance in Klebsiella pneumoniae. Antimicrobial Agents and Chemotherapy. University of Bristol - Explore Bristol Research, 2020; Wang et al., 2023)

K. pneumoniae isolates have shown sensitivity against Amikacin, Amoxicillin-clavulanate, Cefepime, Tetracycline, Levofloxacin, Chloramphenicol, Meropenem, and Aztreonam. The isolates are from a hospital environment, so it is rare for them to be sensitive to many antibiotics. The isolate was from PG, demonstrating that the antibiotic supervenience system of PG hospital could be strict, ensuring that the isolate does not emerge and become resistant to these antibiotics.

Figure-3.36 demonstrates the MAR index of only one positive isolate of *K. pneumoniae.* The MAR index is 0.273, which is greater than 0.2, implying it originated from high-risk contamination where antibiotics are extensively used. The isolate is from PG Hospital, Shahbagh, so isolates of *K. pneumoniae* have great MAR indexes.

5. Limitations

There are various limitations in this study. Firstly, the sample size of the study is small; this gives a higher risk of error rates and less precise information. Additionally, it may overestimate the magnitude of the associations the study is trying to make, thus making the reason less reliable and valid. Secondly, the prevalence present in food in hospital cafeterias may vary daily to daily basis as it is more based on the contamination present in the hands of the chef and staff, in utensils, spices, and plates served, and they can alter every day. Lastly and most importantly, this study is one of a kind; no other works related to this have ever been done to compare and contrast with. There have been only two studies on cooked chicken samples where further characterization was not carried out, and neither such work- i.e. cooked food items from hospital cafeterias was carried out. This makes it hard to make an association whether the multi-drug resistant *Staphylococcus aureus* and *Klebsiella pneumoniae* obtained reside in all cooked chicken items or is mostly due to the hospital environment.

6. Conclusion

This research aims to determine the occurrence of drug-resistant *Staphylococcus aureus* and *Klebsiella pneumoniae* in cooked meat samples collected from hospital cafeterias in Dhaka city. Over the course of nine months, 18 samples were meticulously collected from nine hospitals located in five prominent locations in Dhaka city.

The noteworthy findings from this research are the prevalence of *S. aureus* of 14.3% through molecular analysis with a concerning exhibition of 57.1% Methicillin-resistant *S. aureus* (MRSA), and all isolates were found to be multi-drug resistant (MDR), causing consequential challenges in treatment and control. Concurrently, the prevalence of *K. pneumoniae* is 7.14% within the demonstration of multi-drug resistance to ampicillin, colistin, and ceftazidime-type antibiotics.

The referred findings highlight the vital requirement for strict regulations to address the matter of foodborne pathogenicity through antimicrobial resistance in cooked meat/chicken supplied in hospitals. The findings of multi-drug-resistant isolates of *S. aureus* and *K. pneumoniae* in cafeteria food raise major public health concerns, especially when considering the possibility of spreading the diseases to vulnerable populations.

In conclusion, the data reported in this study will help in our understanding of the potential risk of *S. aureus and K. pneumoniae* in retail food hygiene, food safety and public health in Bangladesh. It also emphasizes the importance of continued research and observation in monitoring antimicrobial resistance trends in foodborne pathogens. Therefore, more extensive research is needed for a longer time frame in order to further characterize the prevalence and

resistance profile of foodborne *S. aureus and K. pneumoniae* strains and to take the relevant safety measurements related to the contamination. Additionally, an extensive study on the identified isolates for their virulence factors and genotypic analysis of their resistance patterns would give us further insight.

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