Isolation of *Enterobacter spp.* and *Klebsiella pneumoniae* from ready-to-eat commercially sold frozen nuggets sold in Dhaka

By

Aditya Banik
ID: 17226011

Subah Basharat Mujtabah
ID: 19326009

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

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BRAC UNIVERSITY
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Declaration

It is hereby declared that

1. The thesis is our own original work while completing our undergraduate degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate cited though full and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help

Students full Name and Signature:

Aditya Banik
ID: 17226011

Subah Basharat
ID: 19326009

Approval:

The thesis Titled "Isolation of Enterobacter spp. and *Klebsiella pneumoniae* from ready-to-eat frozen nuggets from several parts of Dhaka city" submitted by

1. Aditya Banik; ID: 17226011
2. Subah Basharat Mujtabah; ID:19326009

Of Fall, 2017 and Summer 2019 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology in 12 October of Spring,2023.

Examine Committee:

Supervisor:
(Member)

Fahim Kabir Monjurul Haque, PhD
Associate Professor, Microbiology Program
Department of Mathematics and Natural Sciences

Program Director:
(Member)

Nadia Sultana Deen, PhD
Associate professor, Microbiology Program
Department of Mathematics and Natural Sciences
BRAC University

Departmental Head:
(Chair)

A F M Yusuf Haider, PhD
Professor and Chairperson,
Department of Mathematics and Natural Sciences
BRAC University

Abstract:

This study aimed to assess the microbiological quality of frozen foods that are currently very popular in Dhaka, Bangladesh. The demand for Ready-to-Cook (RTC) frozen foods have drawn public attention dramatically all over the world, but in recent years various bacterial contamination is being detected from the frozen foods which are the cause of food spoilage and food-borne diseases. Among those various bacteria that are pathogenic, the study aimed for detecting the presence of coliform bacteria that is more specifically *Enterobacter spp.* and *Klebsiella pneumoniae.* These two types of bacteria were isolated from two different branded frozen nuggets through proper preservation and transportation methods, which was followed by proper sample collection, processing, isolation and identification. For the purpose, both the culture-based method and Most Probable Number method (MPN) method was performed and then further continued by gram staining for morphological identification, biochemical tests, Polymerase Chain Reaction (PCR), Agarose based Gel Electrophoresis, Sequencing and then lastly the Antibiotic Susceptibility testing (AST). After performing all the methodologies mentioned above, from Sample 1, 13 isolates of *Enterobacter spp.* and from the Sample 2, 7 isolates of *Klebsiella pneumoniae* were obtained. However, more in depth research is needed on the factor of isolation of *Enterobacter spp.* from frozen nuggets for future aspects.

Acknowledgements:

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Lists of Acronyms:

1. Spp.: species
2. ESBL: Extended Spectrum β-Lactamase
3. MDR: Multidrug Resistance
4. MAR: Multiple Antibiotic Resistance.
5. RTC: Ready-to-Cook
6. AST: Antibiotic Susceptibility Test
7. *K. pneumoniae*: *Klebsiella pneumoniae*
8. TSI: Triple Sugar Iron
9. MIU: Motility Indole Urease test
10. MHA: Muller Hinton Agar
11. MAC: MacConkey
12. EMB: Eosin Methylene Blue
13. BGLB: Brilliant Green Lactose Bile Agar
14. CLSI: Clinical and Laboratory Standards Institute
15. CDC: Centers for Disease Control and Prevention
16. HACCP: Hazard Analysis & Critical Control Points
17. FDA: Food and Drug Administration
18. BAM: Bacteriological Analytical Manual
19. *S. typhi*: *Salmonella typhi*
20. WHO: World Health Organization
21. API: Analytical Profile Index
22. NICU: Neonatal Intensive Care Unit
23. PCR: Polymerase Chain Reaction
24. ECDP: European Central for Disease Prevention and Control

Chapter 1

1. Introduction

With the ongoing pace of urbanization, globalization and westernization, people's food habits are also changing rapidly from eating homemade cooked foods to prioritizing Ready-to-Cook (RTC) frozen foods (Sen et al., 2019).

1.1 Background:

Food is considered as one of the most important necessities of each and every living being (Shamimuzzaman et al., 2022) and when it comes to the matter of foods that are related to animal origin, then it is the first and foremost priority for every human being (Ema et al., 2022). However, Ready-to-Eat (RTE) or Ready-to-Cook (RTC) foods are also becoming a popular phenomenon in recent days, as they do not require any additional cooking preparation or arrangement (Ema et al., 2022). Especially the frozen foods or frozen RTC foods have gathered more public attention due to globalization and most significantly due to the COVID-19 outbreak. Because, during this period of time, people were bound to stay at their home, cook, eat, work and everything else from home. So, they developed the idea of using the frozen foods more than anything as it doesn't need any additional requirements. The term frozen is commonly used in food sectors all over the world. It means that a particular food item is obtained through freezing and it is kept frozen so that it can be readily cooked (Speck & Ray, 1977). There are some prior reasons behind keeping food frozen. Most of the time, food is kept in a freezing environment in order to inhibit microbial growth so that it can be stored for a particular period of time and also to maintain its nutritional values through preservation (Shamimuzzaman et al., 2022). Although, both the raw and cooked foods can be kept frozen along with the processed foods, raw food is mainly kept frozen for preservation for a long time, on the other hand, cooked food is kept frozen for the same purpose but only for a small period of time. Also, the processed food is kept frozen for two separate purposes, first one is that it can be cooked at any time and the other purpose is also to store it for a particular time period so that it can be cooked whenever it is needed to. Additionally, it is also important to maintain the quality and safety of those frozen snacks along with its rapid increase in demand at least for the sake of consumers well-being.

1.2 Food spoilage and food safety:

Food is widely considered as one of the basic needs of people because it provides proper nutrition and energy for the human body. But unfortunately, due to lack of proper supply chain and monitoring it is not still equally distributed all over the world. For instance, during the time span of 2020-2022 more than 800 million people were deprived of the severe necessities of food due to a sudden outbreak of Covid19 (Tomaszewska et al., 2022). Although foods are generally made up of several biological elements, such elements can also lead to food spoilage and food wastage (Steele, 2004, p.3). Food spoilage and food security are one of the heaviest concerns all over the world, where a major part of food is being wasted due to improper food supply and manufacture process (Odeyemi et al., 2019). Here, food spoilage refers to alteration in food either to degrade or to upscale its quality for the consumers which actually change the whole property of that particular food. This spoilage eventually ends up in several food borne diseases through several microbial or chemical changes (Odeyemi et al., 2019). In fact, several pathogenic and non-pathogenic microbes play the integral part in food spoilage as they are really hard to notice even after their minimal or maximum exposure to food and this exposure may occur in several ways such as handling, transportation, mixing and packaging of food (Odeyemi et al., 2019). Some of the widespread pathogens may include *Bacillus cereus, E. coli, Klebsiella, Enterobacter, Salmonella* etc. However, both Food spoilage and food safety are two opposite phenomena with different parameters. One refers to the importance of safe and healthy food, while the other refers to the damage in the quality of foods. Furthermore, food safety is widely preferred as an important part for the existence of mankind. But in some countries, they fail to reach the minimal standard of food safety parameters even after facing different challenges related to different foodborne outbreaks. That is why efficient and strict guidelines must be enforced and monitored properly in order to confirm the best practices of food safety (Lee et al., 2023). Additionally, the United Nations prepared an Agenda for food safety within 2030, where they will be reflecting about the importance of food waste management along with a mindset to achieve food security, better nutrition and also elimination of hunger (Tomaszewska et al., 2022)

1.3 *Enterobacteriaceae*

Enterobacteriaceae are usually known as the gram-negative bacteria with facultative anaerobic characteristics, as in recent times they have been recognized as the major cause of various nosocomial infections in association with some other urinary tract infections (UTI) or respiratory tract infections, which has also become a great concern widely all over the world (Teklu et al., 2019). However, like other gram-negative bacteria species, this *Enterobacteriaceae* also have both pathogenic and nonpathogenic strains, where among the pathogenic strains some includes- *S. typhi* and *S. paratyphi* are associated with typhoidal salmonella serovars, which are known as the most life-threatening strains of bacteria, whereas strains like *S. typhimurium & S. enteritidis* are among the non-typhoidal *S. enterica* serovars (Cassotta et al., 2020). Classification *Enterobacteriaceae* is displayed below-

Domain: Bacteria

 Phylum: *Pseudomonadota*

  Class: *Gammaproteobacteria*

   Order: *Enterobacterales*

    Family: *Enterobacteriaceae*

1.3.1 Transmission of *Enterobacteriaceae:*

The gram negative facultative anaerobic bacteria *Enterobacteriaceae* are mostly found in the human gastrointestinal tract which leads to many community-acquired-infectious diseases like- UTI, respiratory tract infections etc. (Amare et al., 2022). However, the emergence of ESBL (Extended Spectrum Beta-Lactamase) producing *Enterobacteriaceae* was reported in the early 80's, which were then only confined to healthcare related facilities. And the mortality rates of such resistant isolates are much higher than the ones with non ESBL producing strains, which possess a greater health risk to worldwide mankind (Subramanya et al., 2021). Furthermore, in recent times using such resistant strains of bacteria at a higher rate may cause an alarming situation for the healthcare facilities in the near future. Moreover, along with the infections related to clinical samples, there might be a good possibility that the infections might also occur from any sort of food samples, as in some reports it was mentioned that such antimicrobial resistant strains might also transfer from mishandling of food or contaminated food (Amare et al., 2022). So, more critical study on the fact of foodborne transmission is also needed for the betterment of public health.

1.4 *Enterobacter spp.*

Being a part of the *Enterobacteriaceae* family, *Enterobacter* is also a motile, gram-negative bacilli (Davin-Regli et al., 2019), which was first observed in early 1960 by Hormaeche and Edwards (Iversen C, 2014). This gram-negative bacterium is mostly found in nature in association with prevalence in commensal microflora of humans (Wu et al., 2020). However, along with the continuous revolution of different bacteria, scientists have also found more than 20 types of *Enterobacter* species including- *E. aerogenes, E. asburiae, E. cloacae, E. sakazakii* etc. (Davin-Regli et al., 2019), where both the *Enterobacter cloacae* and *E. asburiae* are known to be causing major types of human nosocomial infections (Harada et al., 2017) which are basically made available on the basis of their phenotypic and genotypic characteristics (Davin-Regli et al., 2019). Additionally, some genus of *Enterobacter* are found as environmental inhabitants, while some other among them are used in bioprocessing mechanisms and metabolic engineering (Davin-Regli et al., 2019). In recent times, *Enterobacter spp.* has become a great matter of concern due to its rapid increase in antimicrobial resistance worldwide which causes many clinical problems like treatment failure, health complexes etc., which is why it is important to understand the microbial resistance pattern of the *Enterobacter* isolates in every part of the health sector (Harada et al., 2017).

1.4.1 Transmission of *Enterobacter spp.*:

The gram-negative bacterium *Enterobacter spp.* is known for the occurrence of nosocomial infections where they mostly target neonates, infants, and other immuno-compromised patients. However, in case of ICU (Intensive Care Unit) patients, they mostly spread through catheterization and intubation. Additionally, some clinical features like High Charlson Sore, diabetes mellitus, and digestive carriage represent higher probability of bacterial transmission (Davin-Régli et al., 2019). Moreover, the *Enterobacter* strains associated with ESBL possess less chance of Zoonotic transmission even though some isolates of *Enterobacter spp.* are mostly found in animals that are full of ESBLs (Harada et al., 2017).

1.4.2 *Enterobacter spp.* from food:

Although such *Enterobacter spp.* isolates are known to be one of the most lethal clinical pathogens, some of its strains are also found to be isolated from different food samples and other environmental samples, for example- *Cronobacter* spp. also known as *Enterobacter sakazakiii* is a part of other several *Enterobacter* strains (Jaradat et al., 2009). These strains have been found in several food samples like dry food, dairy products, cheese, meat, milk, vegetables etc. (Shaker et al., 2007. Similarly, several studies in Namibia proved that *Enterobacter cloacae* are also one of the most contaminating bacteria at high levels on RTE vegetables, processed meat, chicken snacks etc., which caused a serious havoc through an extensive outbreak by its multidrug resistant mechanism in susceptible patients in France's Neonatal intensive care units (2012-2018) along with serious diseases like Gastrointestinal infections and meningitis in Nepal (2012-2013) (Budiarso et al., 2023). However, more extensive research is required in order to find out whether there are more *Enterobacter* strains prevalent in frozen foods.

1.4.3 *Enterobacter cloacae*

In the United States of America, *Enterobacter spp.* is considered as the second most dangerous carbapenem-resistant bacterium among the *Enterobacteriaceae* group for causing a rapid increase in causing carbapenem-resistant infections. However, among several *Enterobacter spp.* strains, *Enterobacter cloacae* is also known to be causing nosocomial infections including pneumonia, UTI etc. (Annavajhala et al., 2019). Although, more clear and relevant information regarding *E. cloacae* is still unavailable, its biofilm forming and toxin production ability are well known among its other several pathogenicity features. In fact, in recent times, this *E. cloacae* has also become very popular for its resistance to extended spectrum beta-lactamases (ESBL), carbapenems and many other groups of antibiotics including Ampicillin, Amoxicillin etc. Moreover, this *Enterobacter* species have the ability to make its one habitat in unique environments like aquatic, terrestrial etc. for which they can easily come in contact with human and animal's normal microflora through intestines and cause serious diseases like- bacteremia, endocarditis septic arthritis etc. (Davin-Régli & Pagès, 2015). Furthermore, in recent investigations it has also been bound that, *E. cloacae* also have the potential to trigger its formation early in neonatal microbiota causing several diseases like- early and late-onset sepsis, and sometimes even death also (Ferry et al., 2020). Most importantly, *E. cloacae* complex which includes the species *E. cloacae, i*s also known to cause a large number of serious outbreaks worldwide, as in an article, it was mentioned that, this *E. cloacae* complex caused at least 26 neonatal outbreaks along with 16 more bloodstream infections related outbreaks (Girlich et al., 2021).

1.4.4 *Enterobacter asburiae*

E. asburiae also known as enteric group 17, also a part of *Enterobacteriaceae* family, is basically a gram-negative motile anaerobic bacterium, which was founded by Brenner and coworkers in 1986. This particular bacterium was primarily obtained from soil, water surface and also human properties, for which it is known as an opportunistic pathogen that causes several types of infections. In fact, it mostly targets the hosts that are mostly immunocompromised humans such as neonates. For which, several types of infectious diseases occur in infants (Mardaneh, 2016). However, *E. asburiae* also have the ability to interact through the signaling of autoinducers, which is carried on through its gene expression with the help of its excessive population and thus triggers the certain activities like- enzyme secretion, symbiosis etc. (Lau et al., 2014). Additionally, according to Brenner et al. *E. asburiae was found to be* susceptible to antibiotics like Gentamicin, Kanamycin, Chloramphenicol's etc. whereas they were resistant to Imipenem (Davin-Régli et al., 2019).

1.4.5 *Enterobacter mori*:

Among 23 known species of *Enterobacter, Enterobacter mori* has been widely known for its isolation from the Rhizosphere part of the plants (Fadiji et al., 2023). This particular *Enterobacter* species was primarily identified as a phytopathogenic bacterium, which was isolated from the roots of a plant (mulberry) (Davin-Régli & Pagès, 2015). However, some strains of the *Enterobacter mori* were isolated from a certain type of crude oil, which also had the ability to degrade such chemical substances like petroleum (Zhang et al., 2015). Similarly, in China, *Enterobacter mori* was found in peach fruits, which caused potential rot immediately after infection (Abedinzadeh et al., 2023). Moreover, even though *Enterobacter mori* is widely known as a plant pathogen, there have been some reports where it was found to be a causative agent of human infection in some parts of Austria (Davin-Régli & Pagès, 2015). Additionally, certain studies carried out in Vietnam and Congo, reported a minimal prevalence of *E mori* in some particular types of foods such as fermented peppers or fermented sauce etc. (La Anh, 2015) (Boumba, 2022)

1.5 *Klebsiella pneumoniae*:

In the year 1882, Carl Friedlander is the one who first described *Klebsiella pneumoniae* as a gram-negative, non-motile bacteria that belongs to the Family *Enterobacteriaceae* (Dai & Hu, 2022). Being an encapsulated bacteria *Klebsiella pneumoniae* are universally found in soil, surface water, medical devices and most importantly in human mucosal surfaces such as, Gastrointestinal tract (GI tract), oropharynx etc. (Paczosa & Mecsas, 2016). According to European Central for Disease Prevention and Control (ECDP), *Klebsiella pneumoniae* is currently gaining a worldwide attention due to its rapid increase in infection number and resistance to antibiotics, as they have found one third of their tested strains resistant to certain groups of antibiotics like- Fluoroquinolones, Third generation Cephalosporins etc. In addition to that, a particular amount of *Klebsiella* species is found to be an acknowledged reservoir for antibiotic-resistant genes that has the ability to grow alongside with other gram-negative bacteria (Bengoechea & Sá-Pessoa, 2018). In USA, right after some other gram-negative bacterium, *Klebsiella pneumoniae* is considered as the third leading cause for hospital acquired infections including pneumoniae, UTI and bloodstream infections, ventilator associated pneumonia (VAP;83%) etc. where mortality rates are shown above 50% (Martin & Bachman, 2018). Even though *Klebsiella pneumoniae* is known to cause some clinical infections like Nosocomial infections they are also found in several food products (Crippa et al. 2023) such as-meat, fresh vegetables, milk, fish, frozen food etc. (Junaid et al., 2022). In recent years, common and unexpected food contaminants like *Klebsiella pneumoniae* are also being found in Asian and non-Asian cohorts where it has been identified as a risk factor for gastro-intestinal transport (Riwu et al., 2022). Moreover, during ongoing research in Australia it was found that 48% of *Klebsiella pneumoniae* infected patients showed a noticeable intestinal colonization before the occurrence of infection (Riwu et al., 2022). Additionally, some specific strains of *Klebsiella pneumoniae* that are isolated from vegetables, shrimp and chicken samples have also been found resistant to certain antibiotics (Guo et al., 2016). In fact, in certain reports it is also feared that *Klebsiella pneumoniae* might be subjected to severe Nosocomial Outbreaks (Guo et al., 2016). Unfortunately, being a clinical pathogen more than the foodborne one, there's not been any extensive research on isolation of *klebsiella pneumoniae* from frozen foods (Crippa et al., 2023). As a result, numerous studies are being carried out by experts to identify the presence of rare food-borne pathogens like *Klebsiella pneumoniae,* where in most of the preliminary studies

they have been found to be resistant to more than four classes of antibiotics which is actually alarming for the future (Riwu et al., 2022).

1.5.1 Transmission of *Klebsiella Pneumoniae*:

Researchers have found several potential sources that are related to the transmission of *K. pneumoniae,* which includes- patient-healthcare worker contact, link of surface contamination and clinical instruments, where the contact of worker and patients are considered as the highest probable way of transmission, as the hands or the wearables of the workers may often remain dirty or may come in touch with some other infectious agents in the hospitals (Martin & Bachman, 2018). However, even if this *Klebsiella pneumoniae* is known to be transmitted through fecal-oral route in case of hospital spread, the actual reason and clarification for the transmission mechanism is still unclear (Zhu et al., 2021).

1.6 Aim and Objective:

Following the ongoing worldwide popularity of frozen foods, there also have been several reports of presence of pathogenic bacteria along with the multi-drug resistant ones. That is why the aim of this study was to isolate *Enterobacter spp.* and *Klebsiella pneumoniae* from frozen RTC food (frozen nuggets) from the market of Dhaka city and also to identify and analyze their antibiotic resistance. The concluded objective of this study will provide insight into the potential risks associated with the contamination in the RTC frozen nuggets by multi-drug resistant *Enterobacter spp.* and *Klebsiella pneumoniae*.

Chapter 2

Materials and methods

Sample collection, preparation, processing and enrichment was done following the procedures of Sultana et al. (2014) & Borty et al. (2018)

2.1 Sample collection:

To conduct the research on frozen food, frozen nuggets were chosen. So, 2 separate brands of frozen nuggets were bought from among 8 other available brands from 2 different locations of Dhaka City. Once they were bought, they were safely carried in a compact icebox and carefully stored at -20°C fridge temperature before further use.

2.2 Sample preparation and processing:

Once all the preparations were complete, the samples were taken out of the fridge and kept in a cool-dry place to bring it to a room temperature. Then one piece of nugget was taken randomly from each separate package of frozen nuggets, they were cut and sliced very well, measured 10 gm of each sample and then transferred into a sterile glass beaker containing sterile distilled water. After that, all two processed samples were grinded properly using a homogenizer machine.

2.3 Media preparations:

For the research purpose, several culturing techniques were applied for which a variety of culture media were used, such as- Peptone water for enrichment; Sodium Chloride or saline for dilution & Antibiotic Susceptibility Testing; Hicrome^{TM} KPC Agar Base, Hicrome^{TM} UTI Agar (Modified), Mannitol Salt Agar (MSA), MacConkey Agar (MAC), Eosin Methylene Blue (EMB) Agar (Levine's) for spread plate method; Lactose broth and Brilliant Green Lactose Bile broth(BGLB) for MPN method; Nutrient Agar (NA) for fresh culture & DNA extraction; Muller Hinton Agar (MHA) for AST; Gram's Iodine, Safranin, Crystal Violet and Ethanol for Gram staining and finally TSI agar (Triple Sugar Iron Agar), Simmons Citrate Agar and MIU Medium Base (Motility Indole Urease) for Biochemical testing.

2.4 Sample Enrichment:

For both the samples, 5ml from its grinded solution was mixed into 2 separate 45 ml peptone water containing flask which was then incubated into a shaker incubator for 18-24 hours at 37°C

2.5 Most Probable Number (MPN) method:

The Most probable number method (MPN) is a statistical, multi-step assay, is mainly used for enumeration and identification of coliform bacteria from food samples. Here, in this research, the Most Probable Number method was performed for both the samples specifically for isolation, following the Bacteriological Analytical Manual (BAM) by Food and Drug Administration (FDA) (Feng P. et al.,2020), where the following steps were done-

2.5.1 Presumptive Test

For the presumptive test, a certain amount of lactose broth was taken into ten double strength (40 ml) and twenty single strength test tubes (80 ml), where 4ml (for double strength) & 400 microliter (for single strength) of grinded sample was mixed accordingly, vortexed and incubated for 24-48 hours at 37°C.

2.5.2 Confirmed Test

Once the incubation period was complete, ten test tubes with the highest proportion of positive results were chosen among the single strength and double strength presumptive tests, from where certain number of samples were then inoculated into ten test tubes containing 4 ml of Brilliant Green Lactose Bile (BGLB) broth in each, vortexed and incubated for 24-48 hours at 37°C.

2.5.3 Completed test

After proper incubation period, highest number of positive results were chosen from among the confirmed test results and streaked into 10 separate EMB agar plates using sterile loops, which were kept for incubation for 24-48 hours at 37°C & 44°C respectively in order to identify fecal and non-fecal organisms.

2.6 Bacterial culture

For bacterial cultures, several methods were applied, that includes- serial dilution, spread plate method, streak plate method and stock culture.

In case of serial dilution, once the enrichment step and the MPN method was complete minimum 8-fold dilution was fixed, for which 72 ml of sterile saline was prepared and poured up to 8 separate sterile test tubes. After that, 1000µl of sample was transferred to each test tube, which was then

followed by transferring 100μl of suspension into separate culture media plates from the tubes including the direct sample. Then, the spread plate method was performed using a sterile glass spreader, which was then labeled and incubated at 37℃ for 19-24 hours.

After complete incubation, growth of desired organisms was observed, counted and then they were transferred to separate NA plates using a sterile loop for further procedures.

2.7 Stock culture:

In order to keep the isolated colonies alive and to use for further proceedings a preliminary stock was prepared for the isolates using the T1N1 media, so that they can be used for other culture techniques like Gram-Staining, Biochemical test, DNA extraction, AST and so on.

2.8 Gram Staining:

Gram staining procedure requires the reviving of selected isolates in NA.

Here, the method required certain dyes including- Crystal violet(ml), Gram's Iodine(ml), Safranin and decolorizer(ethanol). Then they were stored in a cool dry place for further usage.

First of all, the fresh bacterial isolates were heat-fixed on microscopic slides through smearing using a sterile loop and sterile saline. Then, crystal violet drops were added on the smear, waited for 60 seconds and then washed using distilled water. The same steps were followed in case of safranin and gram's iodine also, but the decolorizer was added very carefully as long as the purple color dye was almost washed off.

Finally, the results were observed and interpreted under a microscope machine in order to determine whether the bacteria are gram negative or gram positive and whether their morphology is rod shape or coccus shaped. Additionally, the most important part is that the gram-positive bacteria have a higher peptidoglycan layer that retains the crystal violet and shows violet color, whereas the gram negative possess a higher lipid content not taking up the primary dye instead takes up the safranin and give red or pink stain.

2.9 Biochemical tests:

For the research purpose, three separate types of biochemical tests were performed which includes- Triple Sugar Iron test, Citrate utilization test and Motility Indole Urease test.

2.9.1 Triple Sugar Iron test

In case of TSI, freshly cultured isolates were used, which were then stabbed in freshly prepared TSI agar media in 9 ml sterile screw cap test tubes and then incubated at 37℃ for 24 hours.

2.9.2 Citrate utilization test

A similar procedure was followed for the citrate tests, only difference was that, in this case the citrate media was poured in 5 ml sterile vials where the fresh isolates were stabbed using a sterile needle and then they were incubated at 37℃ for 24 hours.

2.9.3 Motility Indole Urease test

However, in this case, initially the MIU agar media was prepared and then the Urea solution was made, as the urea solution does not require any extra heating. So, once the Urea solution was prepared based on the no. of isolates, it was then mixed in the MIU media which was prepared in sterile glass vials. After that, they were incubated at 37℃ for 24 hours.

Additionally, two separate tables of biochemical test interpretation were followed which were referred to by Al-Rhman and Alaubydi (2015) & Usman et al. (2021) respectively in two separate articles. Both the tables have been mentioned below-

Tests	Interpretation		
Citrate	+		
TSI	H_2S (-)	G (+)	A/A (+/+)
Motility	-		
Indole	-		
Urease	+		

Table 1: Biochemical tests for *K. pneumoniae* Al-Rhman and Alaubydi (2015)

Tests	Interpretation
Citrate	+
TSI	A/A(acid/acid)
Motility	+
Indole	-
Urease	+

Table 2: Biochemical tests for *Enterobacter spp.* (Usman et al. ;2021)

2.10. Detection of *Enterobacter spp. a*nd *K. pneumoniae* by Polymerase chain reaction (PCR):

The PCR method was followed by retrieval of fresh cultures, DNA extraction & finally Agarose Gel Electrophoresis

2.10.1 DNA Extraction:

Before starting the DNA Extraction procedures, fresh bacterial isolates were retrieved in NA plates, which were then inoculated in Eppendorf tubes containing 250µl NB (Nutrient broth), vortexed and stored in the shaker incubator for 18-24 hours at 37℃. The next day, growth was observed and the tubes were centrifuged for 15 minutes at 13rpm to get our desired pallet. Then, 250µl of TE (Tris-EDTA) buffer was added into the tubes carrying the pallet right after discarding the supernatant. After that, the boiling method was applied where the buffer mixed tubes were placed in a floater, put into a beaker containing a certain amount of water placing the beaker on an electric heater for a few minutes. Once the heating was complete, the heated tubes were again sent to centrifugation for 15 minutes at 13rpm. Finally, after centrifugation the supernatants were discarded and the tubes containing the DNA were stored in -20℃ culture fridge for further proceedings.

2.10.2 PCR:

PCR was performed in order to identify and confirm the presence of DNA bands of the desired microorganisms.

To start with, two separate mixtures of PCR components (13µl and 15µl) were prepared in sterile PCR tubes for both the organisms, which consisted a mixture of- (7.5µl of Master Mix, 0.6µl of both Forward and Reverse primer, 4.3µl of Nuclease Free Water and 2µl of extracted DNA for each *Enterobacter spp.* suspected isolates) & (7.5µl of Master Mix, 0.5µl of both Forward and Reverse primers, 2.5µl of Nuclease Free Water and 2µl of extracted DNA for each *Klebsiella pneumoniae* suspected isolates) respectively, where the DNA templates were added right after mixing rest of the components.

Next, all the tubes were sent for short spin in a mini centrifuge machine to ensure the absence of bubbles and then they were put into the PCR machine by setting up the following conditions- (95°C for 5 mins; 95°C for 45 secs; 58°C for 30 sec; 72°C for 30 secs and 72°C for 5 mins, for *Enterobacter spp.*) & (94°C for 10 mins; 94°C for 30 secs; 60°C for 45 sec; 72°C for 45 secs and 72°C for 10 mins, for the amplifications of genes for *K. pneumoniae*) respectively to start the machine. Finally, after the completion of PCR, the PCR products were stored in a -20°C culture fridge for Agarose Gel Electrophoresis. (Include condition).

Primers	Primer sequence	Amplicon size	Reference
27F	5'-AGAGTTTGATCCTGGCTCAG-3'	1500 bp	(Alkhafaji & Radi, 2016)
1492R	5'-AAG GAG GTG ATC CAG CCG CA-3'		

Table 3: Universal primer

Primers	Target gene	Primer sequence	Amplicon size	Reference
KPNF	Diguanylate -cyclase	5'-TGCAGATAATTCACGCCCAG-3'	133 bp	(Mahmudunnabi et al., 2018)
KPNR		5'-ACCCGCTGGACGCCAT-3'		

Table 4: Primer for *K. pneumoniae*

2.10.3 Agarose Gel Electrophoresis:

Once the PCR cycles were completed, 1.3% agarose gel was prepared in sterile Tris-Borate-EDTA (TBE) Buffer and 4µl Ethidium Bromide dye (ETBR). After that, the gels were visualized under ultraviolet illumination machines.

2.10.4 Sequencing:

2 suspected isolates of *Enterobacter spp.* were selected and sent to a renowned company named DNA Solution for the 16S rRNA sequencing in order to identify and confirm the bacteria.

2.11 Antimicrobial Susceptibility Test (AST):

To perform the antibiotic susceptibility testing for this research, updated Clinical & Laboratory Standards Institutes (CLSI) guideline of 2020 was followed along with the Kirby-Bauer disc diffusion method. Initially, freshly cultured isolates were inoculated in sterile test tubes containing sterile saline which was then vortexed and compared with the McFarland 0.5 standard. After that sterile cotton swabs were used to dip in the saline solution and then lawn on the freshly prepared MHA agar plates. Next, 10 separate types of antibiotic discs were used and placed on the lawned plates carefully using a sterile forceps. However, the antibiotic disc placement on the agar was done by maintaining a proper distance so that the inhibition zones did not overlap each other. Finally, the plates were labeled and incubated for 18-24 hours at 37 °C.

Serial no.	Antibiotic groups	antibiotics	Disc amount (µg)	Resistant (mm)	Inter-mediate (mm)	Suscept ible (mm)
1	Tetracyclin e	Tetracycline	30µg	≤11	12-14	≥15
2	Carbapene m	Meropenem	10µg	≤19	20-22	≥23
3	Phenicols	Chloramphe nicols	30µg	≤12	13-17	≥18
4	Cephem	Cefixime	5µg	≤15	16-18	≥19
		Cefepime	30µg	≤18	–	≥25
5	Macrolides	Azithromyci n	15µg	≤12	–	≥13
6	Monobacta ms	Aztreonam	30µg	≤17	18-20	≥21
7	Aminoglyc osides	Gentamicin	10µg	≤12	13-14	≥15
		Kananycin	30µg	≤13	14-17	≥18
8	Quinolones & fluoroquino lones	Levofloxaci n	5µg	≤16	17-20	≥21
		Nalidixic acid	30µg	≤13	14-18	≥19
		Ciprofloxaci n	5µg	≤21	22-25	≥26
9	Penicillin	Ampicillin	10µg	≤13	14-16	≥17
10	β-lactams	Piperacillin - tazobactam	100/10µ g	≤17	18-20^	≥21

Table 5: Antibiotic Susceptibility testing according to CLSI guidelines (30th edition). The table contains the name of the antibiotics and their groups, zone measurement of inhibition & and their concentration

Chapter 3
Results

3.1 Bacterial isolation from frozen nugget from Hatirpool and New Eskaton area of Dhaka city:

3.1.1 Isolation of *Enterobacter spp.* from sample 1:

13 isolates of suspected *Enterobacter spp.* were isolated from sample 1 (Hatirpool, Dhaka), which were then confirmed and identified through- biochemical tests & Gram's staining

Figure 1: Isolation of *Enterobacter spp.* The first row shows the results of the following- MPN method, culture method, while the second one shows the biochemical tests, Gram's staining & AST.

3.1.2 Isolation of *klebsiella pneumoniae* from sample 2:

21 or 13 isolates of suspected *klebsiella pneumoniae* were isolated from sample 2 (New Eskaton, Dhaka), which were then confirmed and identified through- biochemical tests & Gram's staining
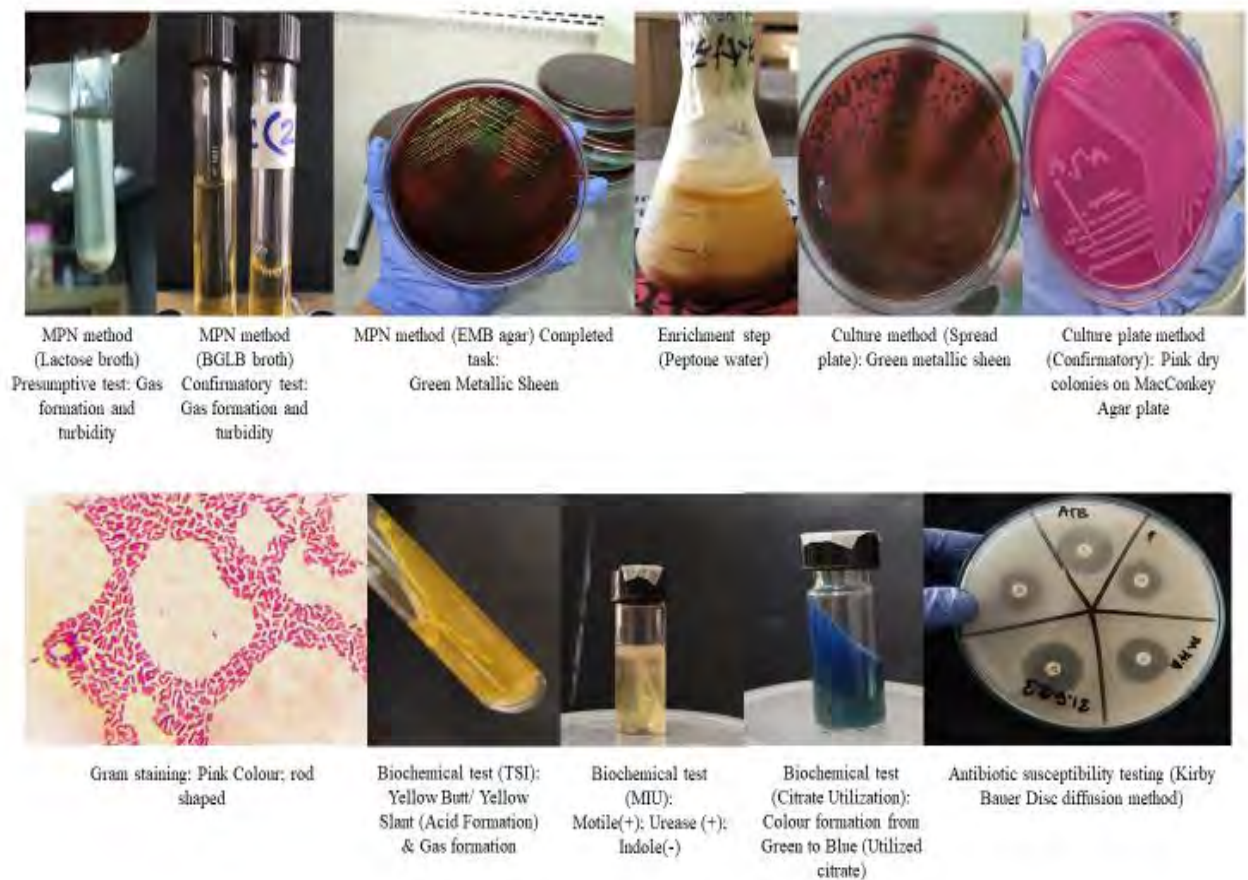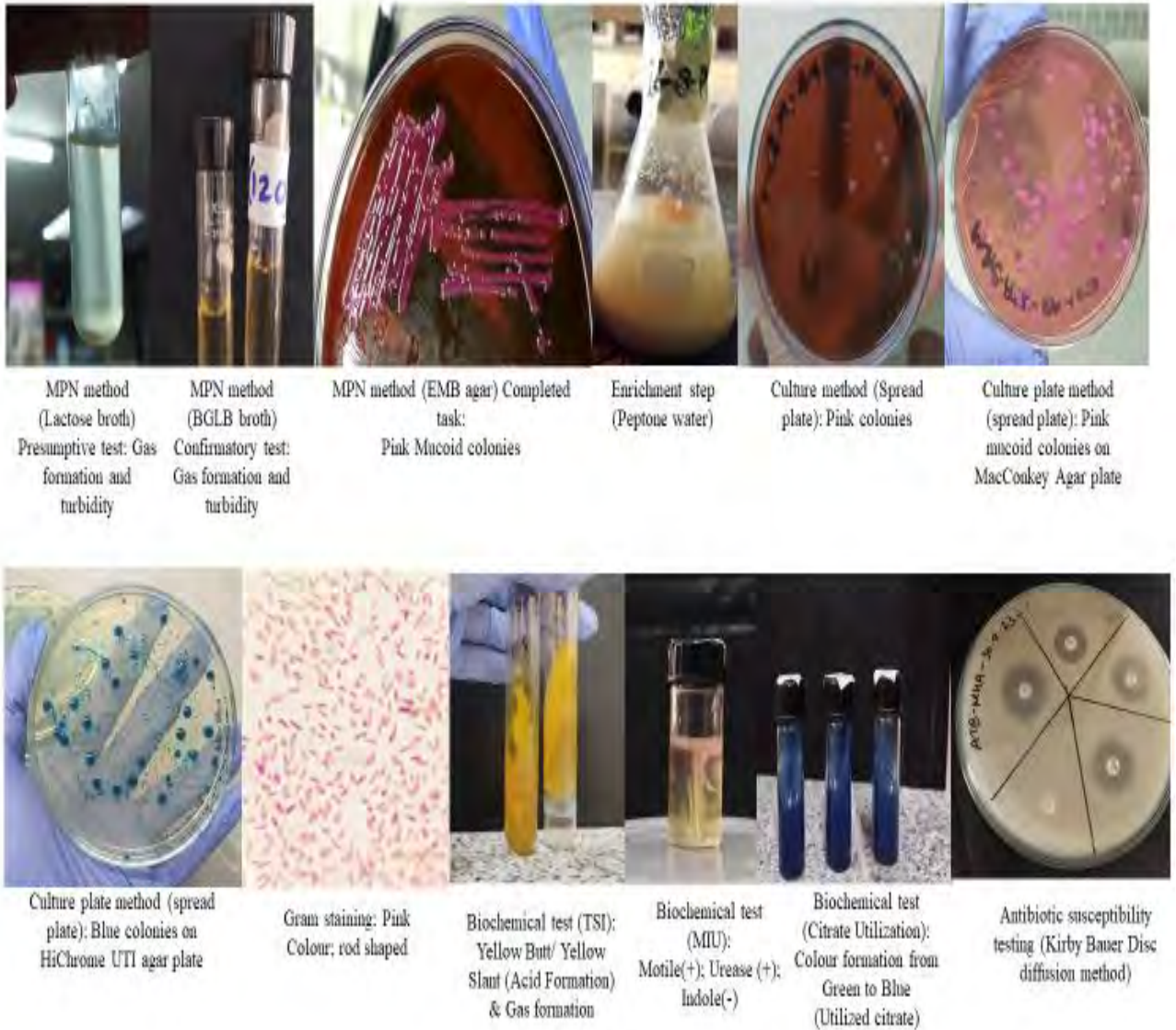

Figure 2: Isolation of *K. pneumoniae.* The first row shows the results of the following- MPN method, culture method, while the second one shows the biochemical tests, Gram's staining & AST.

3.2 Biochemical Test Results

In order to acquire a bit more confirmation 3 types of biochemical tests were performed including-TSI, MIU and Citrate utilization test for both *Enterobacter spp.* and *Klebsiella pneumoniae*. The results for both the organisms are charted below.

3.2.1 Biochemical Test results for *Enterobacter spp.*:

Bacteria	TSI (slant/butt/gas)	Motility	Indole	Urease	Citrate
Enterobacter spp.	Yellow (Acid)/Yellow (Acid)/+	Positive (+)	Negative (-)	Positive (+)	Positive (+)

Table 6: Biochemical tests results of 13 isolates for *Enterobacter spp*. Yellow in the TSI test indicates gas production. Positive citrate test indicates color change from green to Persian blue and the positive motility test indicates that the bacteria is motile.

3.2.2 Biochemical Test results for *Klebsiella pneumoniae*:

Bacteria	TSI (slant/butt/gas)	Motility	Indole	Urease	Citrate
Klebsiella Pneumoniae	Yellow (Acid)/Yellow (Acid)/+	Negative (-)	Negative (-)	Positive (+)	Positive (+)

Table 7: Biochemical tests results of 7 isolates for *Klebsiella pneumoniae* Yellow in the TSI test indicate gas production. Positive citrate test indicates color change from green to Persian blue and the negative motility test indicates that the bacteria is non-motile.

3.3 Polymerase Chain Reaction or PCR confirmation through Agarose Gel Electrophoresis

Finally, PCR for both the desired isolates were determined by the Polymerase Chain Reaction.

3.3.1 PCR and Agarose Gel electrophoresis for the identification of *Enterobacter spp.:*

For the suspected *Enterobacter spp.* isolates, PCR was performed and desired bands were found. Which was then visualized and confirmed through Agarose Gel Electrophoresis, where the actual size of the band was determined, which is 1500bp.

3.3.2 PCR & Agarose gel electrophoresis for the identification of *Klebsiella pneumoniae*:

Similarly, PCR method was performed for the confirmation of the suspected *Klebsiella pneumoniae* isolates and among 28 isolates 25 was found to be positive.

Once the PCR was completed Agarose Gel Electrophoresis was performed to visualize and identify target DNA bands, from which it was also determined that the targeted DNA band size was 133 base pairs as it was mentioned in the primer's properties.
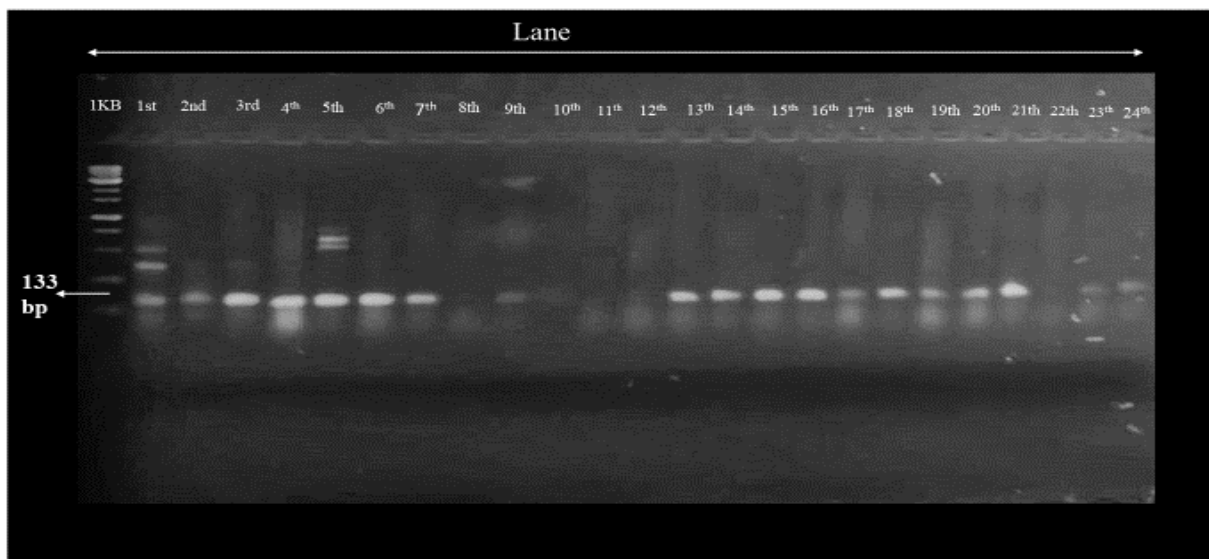

Figure 3: Agarose Gel electrophoresis & visualization of *Klebsiella pneumoniae* DNA bands. 133bp is the band size. The first lane contains the 1kb ladder. Then from lane 1 to lane 25 contains the sample products.

3.4 16S rRNA sequencing

With the help of 16S rRNA sequencing, it was made clear that the suspected isolates from the sample 1 are *Enterobacter spp.* However, even though the suspected bacteria showed DNA bands at 1500 bp through the PCR and Agarose Gel Electrophoresis, after the completion of the sequencing process the isolates were confirmed through running the FASTA sequences in the NCBI Blast, where it was found that, the two isolates that were sent for sequencing can be *Enterobacter mori & Enterobacter asburiae as* they display the closest of percent identity and query coverage, as it was not possible to confirm the complete identity of the isolates. The alignments and the blast search results of both the isolates, along with a distance tree of results are displayed below-

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑ Enterobacter quasiroggenkampii strain WCHECL1060 16S ribosomal RNA, partial sequence	Enterobacter quasiroggen...	1299	1299	99%	0.0	96.24%	1538	NR_179166.1
☑ Enterobacter mori strain YIM Hb-3 16S ribosomal RNA, partial sequence	Enterobacter mori	1288	1288	99%	0.0	95.98%	1522	NR_146667.2
☑ Enterobacter asburiae strain JM-458 16S ribosomal RNA, partial sequence	Enterobacter asburiae	1232	1232	99%	0.0	94.64%	1422	NR_145647.1
☑ Enterobacter chengduensis strain WCHECI-C4 16S ribosomal RNA, partial sequence	Enterobacter chengduensis	1232	1232	99%	0.0	94.73%	1538	NR_179167.1
☑ Enterobacter wuhouensis strain WCHEs120002 16S ribosomal RNA, partial sequence	Enterobacter wuhouensis	1225	1225	99%	0.0	94.60%	1536	NR_180450.1
☑ Enterobacter kobei strain CIP 105566 16S ribosomal RNA, partial sequence	Enterobacter kobei	1219	1219	99%	0.0	94.48%	1451	NR_028993.1
☑ Enterobacter kobei strain JCM 8580 16S ribosomal RNA, partial sequence	Enterobacter kobei	1216	1216	99%	0.0	94.35%	1468	NR_113321.1

Fig 4: NCBI BLAST search results 1. Obtained from 27F primer of isolate 1(AE6).

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑ Enterobacter sichuanensis strain WCHECL1597 16S ribosomal RNA, partial sequence	Enterobacter sic...	1513	1513	100%	0.0	99.05%	1528	NR_179946.1
☑ Enterobacter chengduensis strain WCHECI-C4 16S ribosomal RNA, partial sequence	Enterobacter che...	1513	1513	100%	0.0	99.05%	1538	NR_179167.1
☑ Enterobacter quasiroggenkampii strain WCHECL1060 16S ribosomal RNA, partial sequence	Enterobacter qua...	1513	1513	100%	0.0	99.05%	1538	NR_179166.1
☑ Enterobacter mori strain YIM Hb-3 16S ribosomal RNA, partial sequence	Enterobacter mori	1507	1507	100%	0.0	98.94%	1522	NR_146667.2

Fig 5: NCBI BLAST search results 2. Obtained from 1492R primer of isolate 1(A.E6).

Fig 6: NCBI BLAST search results 3. Obtained from 27F primer of isolate 2(A.E5).

Fig 7: NCBI BLAST search results 4. Obtained from 1492R primer of isolate 2(A.E5).

3.5 Antimicrobial Susceptibility Test (AST):

Antibiotic Susceptibility Testing was performed for both *Enterobacter spp.* and *Klebsiella pneumoniae* following the Kirby Bauer disc diffusion method and also the updated CLSI guideline (13th Edition; 2020).

3.5.1 AST for *Enterobacter spp.:*

The results of AST for 13 *Enterobacter spp.* isolates are stated below:

Antibiotics	Resistant	Intermediate	Sensitive	SDD
CIP	6(46.2%)	3(23%)	4(30.8%)	0
GEN	0	1(7.7%)	12(92.3%)	0
CPM	1(7.7%)	0	11(84.6%)	1(7.7%)
LE	2(15.4)	6(46.2%)	5(38.5%)	0
AK	2(15.4)	1(7.7%)	8(76.9%)	0
PIT	0	9(69.2%)	4(30.8%)	0
MRP	2(15.4)	1(7.7%)	10(76.9)	0
AZM	1(7.7%)	0	12(92.3%)	0
AMP	13(100%)	0	0	0

Table 8: Antibiotic Susceptibility Testing of 13 *E. spp.* isolates
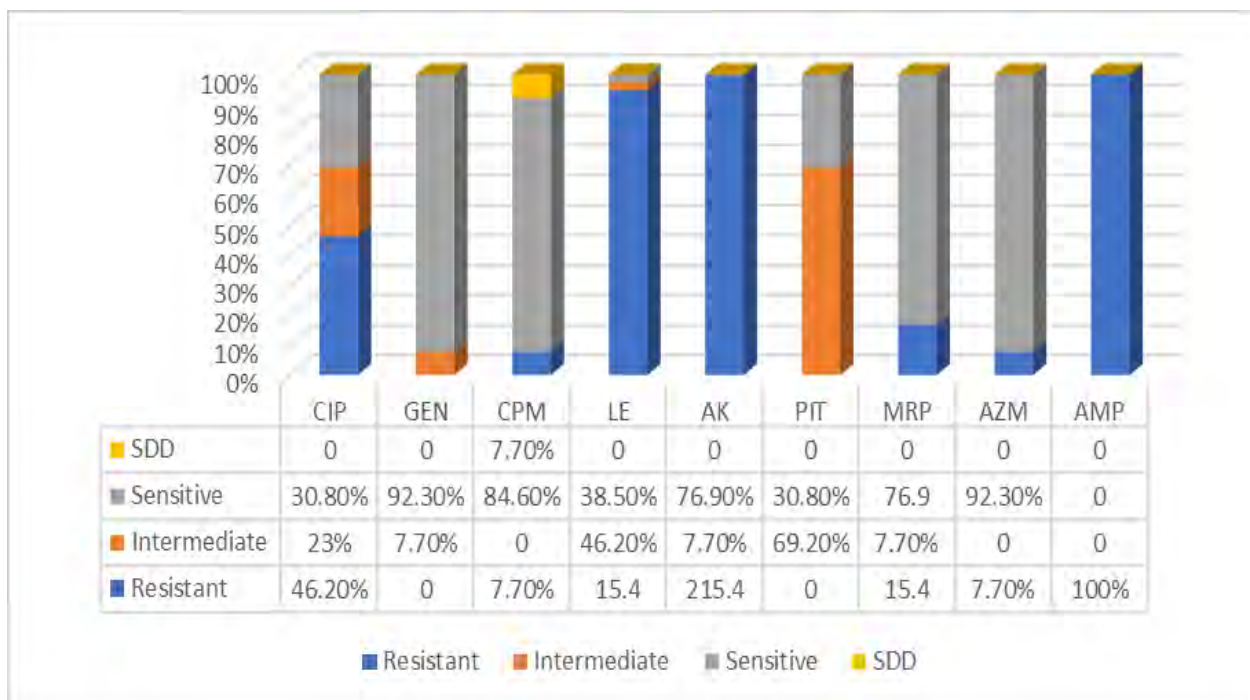
Figure 8: Bar chart of the percentage of Antibiotic Susceptibility Testing for 13 *E. spp.* Isolates

3.5.2 AST for *Klebsiella pneumoniae*:

Antibiotics	Resistant isolates and percentage	Intermediate isolates and percentage	Sensitive isolates and percentage
AMP	7(100%)	0	0
NA	0	5(71.43)	2(28.6%)
LE	1(14.29%)	3(42.86%)	3(42.86%)
GEN	0	0	7(100%)
ATM	0	2(28.6%)	5(71.43)
AZM	1(14.29%)	0	6(85.71%)
CFM	1(14.29%)	0	6(85.71%)
C	0	1(14.29%)	6(85.71%)
MEM	0	3(42.86%)	4(57.14%)
TE	0	0	7(100%)

Table 9: Antibiotic Susceptibility Testing for **7** *K. pneumoniae* isolates.

	AMP	NA	LE	GEN	ATM	AZM	CFM	C	MEM
▦ Sensitive	0	28.60%	42.86%	100%	71.43%	85.71%	85.71%	85.71%	57.14%
■ Intermediate	0	71.43	42.86%	0	28.60%	0	0	14.29%	42.86%
■ Resistant	100%	0	14.29%	0	0	14.29%	14.29%	0	0

■ Resistant   ■ Intermediate   ▦ Sensitive
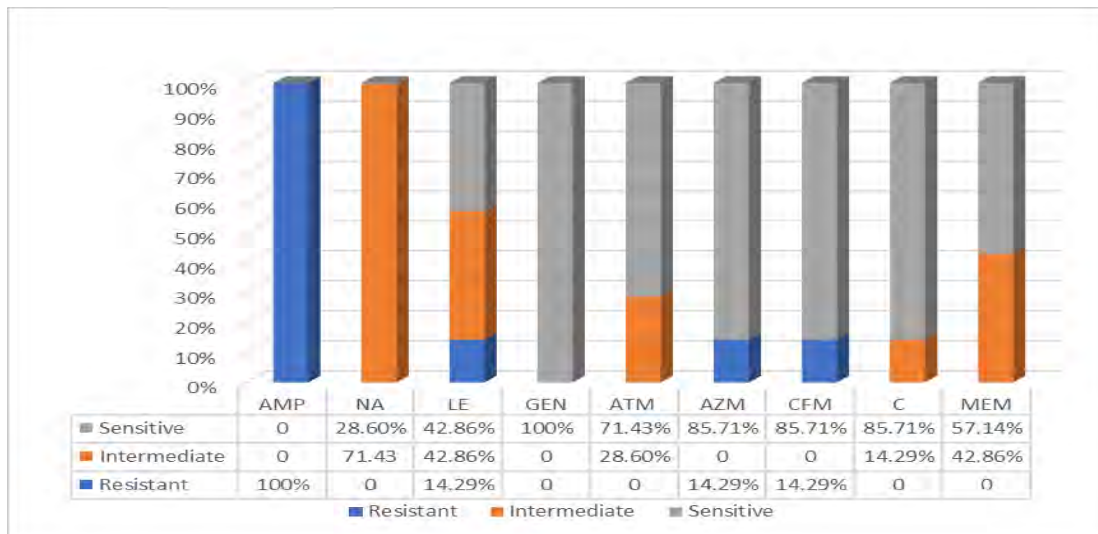
Figure 9: Bar chart of the percentage of Antibiotic Susceptibility Testing for 7 *K. pneumoniae* isolates.

3.6 Multidrug Resistant isolates:

According to the Centers for Disease Control and Prevention (CDC), the term Multi-Drug Resistance means an isolate being resistant to three or more classes of antibiotics.

Here, among 20 isolates, 4 isolates (20%) were found to be resistant to 3 or 4 categories of antibiotics making them multidrug isolates. The following isolates were found to be multidrug resistant- 4,10,13 & 3k. However, 3(23.07) out of 13 isolates of *Enterobacter spp.* were found to be multidrug resistant, on the contrary among 7 *klebsiella pneumoniae* isolates 1(10%) isolate was found to be multidrug resistant which means that particular was resistant to 3 or more antibiotics. The whole statement is stated in a graph below-
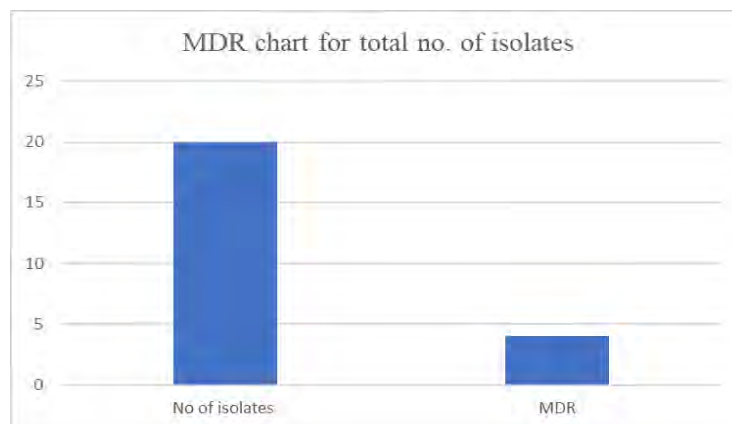
Figure 10: MDR for total isolates. Total isolates 20. Which includes 13 *E. spp.* isolates & 7 *K. pneumoniae* isolates

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3.6.1 MDR of *Enterobacter spp.*
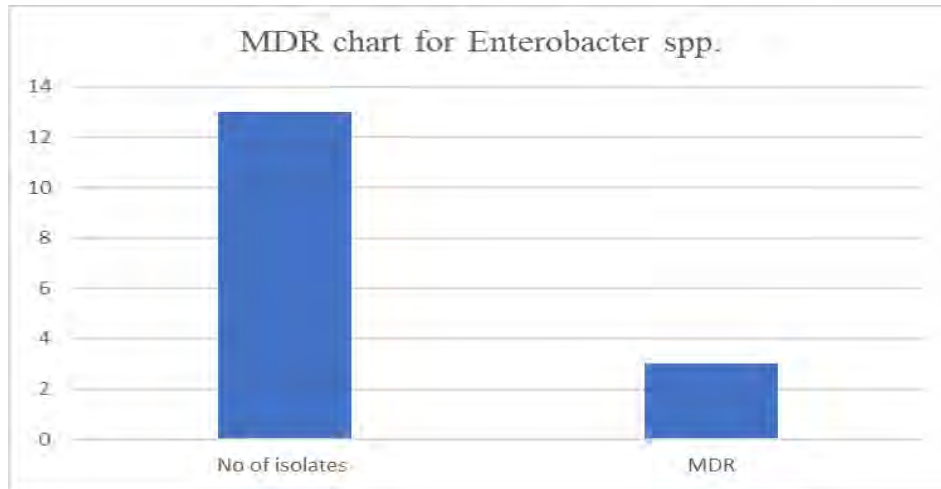
Figure 11: MDR for 13 *E. spp.* Isolates

3.6.2 MDR of *Klebsiella pneumoniae*
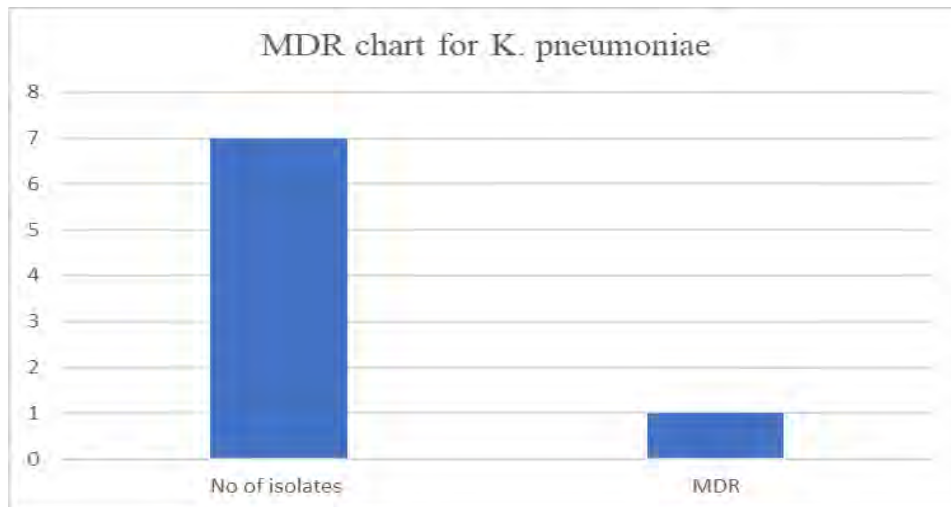
Figure 12: MDR for 7 *K. pneumoniae* isolates

3.7 MAR Index:

Multiple Antibiotic Resistance (MAR) Index is an efficient method for the identification of the bacterial infections and the drug resistance of those pathogens. It is basically calculated based on the ratio of the number of the antibiotics to which a particular bacterium is resistant to the overall number of antibiotics the bacteria that is exposed (Afunwa et al., 2020). In this research, the MAR Index of both *Enterobacter spp. & Klebsiella pneumoniae* are stated below-

Samples	Resistant	Total no of antibiotics	MAR
1	1	9	0.1
2	2	9	0.2
3	2	9	0.2
4	3	9	0.3
5	1	9	0.1
6	2	9	0.2
7	2	9	0.2
8	1	9	0.1
9	1	9	0.1
10	3	9	0.3
11	2	9	0.2
12	2	9	0.2
13	5	9	0.5
9p	2	10	0.2
17k	1	10	0.1
3k	3	10	0.3
7k	1	10	0.1
5k	1	10	0.1
6k	1	10	0.1
11k	1	10	0.1

Table 10: MAR Index of *E. spp. & K. pneumoniae*

Chapter 4

Discussion

The main purpose of this study was to isolate and identify the presence of pathogenic bacteria like *Enterobacter spp.* & *K. pneumoniae* from frozen RTC foods such as nuggets from around several areas of Dhaka Bangladesh. However, despite being very popular, there has not been any distinctive research on the bacteriology of frozen nuggets. Instead, the focus mostly has been put on raw food products or freshly cooked products. In recent reports, it has been found that frozen foods of animal origin are also responsible for causing foodborne diseases though emergence of several antimicrobial resistant pathogens. Even though the rapid increase in antimicrobial resistance is a global concern, there is not much of any evidence of such incidence in Bangladesh (Sultana et al., 2014).

In this study branded frozen nuggets samples were collected from two locations of Dhaka city and then bacterial pathogens were identified. Here, only one organism was isolated from each of the two samples. Among them, 13 isolates of *Enterobacter spp.* were isolated from sample 1, for which a number of isolation and identification methods were performed along with PCR and specially 16S rRNA sequencing. Even though *Enterobacter spp.* was the suspected organism, after sequencing it became quite clear that the probable strains of *Enterobacter* can be- *E. mori* & *E. asburiae*. However, even after the completion of the sequencing it couldn't be confirmed that which exact strain of *Enterobacter* it would be as in the result there were a lot of closely related strains of that organism, for which only two particular strains that were closely related were taken into account and the rest of the isolates were considered as *Enterobacter spp.* Additionally, it will be really tough to infer a particular isolate of this sample to be confirmed as any one of the *Enterobacter* strains, as the sample size here is really limited rather than a bulk amount of sample. That is why, only two closely related strains of *Enterobacter* had to be taken into account. Unfortunately, there have been quite a few researches on the isolation of *Enterobacter spp.* or any of its strains from food samples, especially on frozen foods like nuggets. For instance, in China there was a study conducted by Ye et al. (2018), where among the total number of isolates (1024) obtained from retail foods, only 14 of them were identified as the *Enterobacter* strains, which also included 2 ESBL producing strains. Similarly, Messaoudi et al. (2009) conducted comparative research on meat products in Tunisia, where he found a relatively higher prevalence of

Enterobacter spp. strains (49.01%) than the obtained isolates of *K. pneumoniae* strains (33.3%), which expresses an alarming concern in the public health of that particular country. In another research, Splittstoesser et al. (1980) conducted a study on 575 packages of vegetables, where they found the prevalence of *Enterobacter spp.* in the following rates: 23% (111) of Enterobacter *cloacae*, 4% (19) of *Enterobacter agglomerans* and 2.9% (13) of *Enterobacter aerogenes* among the total number of isolates. In fact, 2 isolates of *Enterobacter asburiae* were isolated from among 125 PIF (Powdered Infant Formula Milk) samples, which were distributed in a Neonatal Intensive Care Unit (NICU) of Iran (Mardaneh & Dallal 2016). From another research on Batavia lettuce in Malaysia, it was found that among 4 separate *Enterobacteriaceae* isolates 2 of them were identified to be the strain of *Enterobacter*, which is *Enterobacter asburiae* (Lau et al., 2014). Similarly, a research conducted in Congo found that, among their 6 samples of fermented peppers there was presence of 2 separate types of *Enterobacter* species including- *E. mori* & *E. mori* in 3 of their samples, while another study lead by La Anh (2015), found the presence of *Enterobacter mori* in sauce paste among some other type of fermented foods. Thus, from the above discussion it can be said that, even though there are several researches of the bacteriology of any particular food samples all over the world, there are not much of reports on the bacteriology of frozen nuggets specifically the isolation of *Enterobacter spp.* from frozen nuggets in Bangladesh.

Another organism that was isolated from sample 2 was *K. pneumoniae,* for which almost similar types of identification and isolation methods were performed except the sequencing. Because, in this case, the desired pathogen was easily isolated from the sample using the existing methods. However, there have been noticeable researches on the isolation and identification of *K. pneumoniae* from any type of foods all around the globe. For instance, in a study of 998 food samples, a total of 99 isolates of *Klebsiella pneumoniae* was obtained from among which 4 of them was from raw seafood, 26 of them was from raw chicken samples, 34 of them were from frozen raw food samples and lastly 35 were from the cooked food samples (Guo et al., 2016). Another study led by Hartantyo et al. (2020), found that, among his total number (97) of foodborne *K. pneumoniae* isolates, only 10% (10 out of 97) of them were resistant to multiple antibiotics, where a half of them came from raw foods and the other half came from Ready-to-Eat foods (RTE) or chilled foods. However, in Bangladesh, a study lead by Sultana et al. (2014) found that, among 57 isolates of different bacterial pathogens from among 20 types of food samples, 12.28% of them were *Klebsiella spp.* and only 3.5% of them were *Enterobacter spp.* Another study conducted in

Bangladesh found that, among 90 street food samples, prevalence of *Enterobacter sakazakii* strains in Nan was 98% according to their Analytical Profile Index (API) results (Banik et al., 2021). Similarly, Nipa et al. (2011) conducted a study on salad vegetables in Chittagong, Bangladesh, where 9.04% of the total isolates were *Klebsiella spp.* and 21.8% of the total bacterial count was *Enterobacter spp.* So, finally, it can be said that, even though there are a lot of bacteriological studies based on any type of food samples, there are only a few studies which are only based only on one particular bacterium or only one particular frozen food like nuggets.

Here for the research purpose, 14 different types of antibiotics of 10 different groups were used for both the organisms including- Tetracycline, Carbapenem, Phenicol etc. from which it was found that Penicillin group of antibiotics had the highest percentage of resistance in both cases, whereas Tetracycline & Aminoglycoside groups of antibiotics showed highest percentage of susceptibility in *K. pneumoniae*, along with Macrolide & Aminoglycosides showing highest percentage of susceptibility in *Enterobacter spp.* Among the total no. of isolates, only one *K. pneumoniae* isolate (10%) and three *Enterobacter spp.* isolates (23.07%) were found to be resistant to multiple drugs of antibiotics. Which means, till now from this research it can be hypothetically said that the *Enterobacter spp.* isolates possess more threat to antimicrobial resistance than the isolates of *K. pneumoniae.*

So, from the comparative evidences and antimicrobial analysis stated above, it can be easily said that, even though there are several reports on the isolation and identification of *Enterobacter spp.* or any of its species like *E. mori* or *E. asburiae* from any type of samples, whether it is clinical, dairy, fresh raw, fresh cooked or frozen foods, there is a very little evidence of study on bacteriology of frozen nuggets, which is why this study on the bacteriology of frozen nuggets, specially the identification of *Enterobacter spp.* strains can be an important prospect to conduct more distinctive studies in the near future in order to assess more different factors like probable incidence of any type of diseases from such pathogenic bacteria etc.

Last of all, food contamination may occur at any time, any place or any particular way through several factors like- microbial, chemical, environmental, physical etc. (NeríN et al., 2016). According to the World Health Organization (WHO), there were more than 2.3 lakhs deaths and over 20 million disabilities occurred in 2010, which mostly emerged from some serious foodborne illness (Bick et al., 2020). That is why in order to prevent or eradicate food contamination and the possibility of different foodborne outbreaks, it has become a must to follow some fundamental

food safety protocols like Hazard Analysis & Critical Control Points (HACCP) to assess and control the prevalence of several food hazards that may come in contact with food and contaminate it (Bick et al., 2020). Moreover, some food hygiene measures like- safe packaging, proper handling, use of surface disinfectants, reduction of environmental pollutants etc. should also be followed strictly all over the world (Faille et al., 2018). Additionally, the law enforcing authorities related to food sectors of all the countries, should be more strict in providing and monitoring food hygiene and safety related rules with the help of Government interventions in order to reduce the rapid emergence of foodborne diseases.

Chapter 5
Conclusion

To conclude, factors affecting food safety have become a growing concern all over the world, as the emergence of antimicrobial resistance is increasing day by day. Especially when it comes to such rare pathogens like *Enterobacter spp.* strains that have been found in this study, as there is no complete evidence of prevalence of these bacteria in the frozen nuggets. But there are a good number of studies carried out around the world based on the identification of *K. pneumoniae* from frozen foods or nuggets, which also have been identified in this current study. In fact, both the pathogenic bacteria in this study showed antimicrobial resistance to a certain level, which may create a greater concern for the consumers, which may lead to the emergence of more serious types of foodborne diseases not only in this country but also all over the world. That is why some necessary steps like- proper maintenance of food hygiene, strict monitoring in all the prevailing food sectors, proper food handling and sterile-suitable environment for food production and supply etc. must be followed for the betterment of global public health. Additionally, more extensive research on the bacteriology of foods specially frozen foods like nuggets is a must to identify the underlying factors related to them, specifically on the rare bacterium like *Enterobacter spp.*

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