Isolation of *Klebsiella pneumoniae* from water and hand swabs of vegetable vendors collected from the wet markets of Dhaka city

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

It is hereby declared that

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

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Abstract

Klebsiella pneumoniae (K. pneumoniae) is considered one of the major causes of human hospital infections which is found in human intestine. This bacterium belongs to the Enterobacteriaceae family, and this is a gram-negative, encapsulate and non-motile bacterium. *K. pneumoniae* can be potential health hazard through increasing the antibiotic resistance. Virulence factors of *K. pneumoniae* allows higher adaptation to stress and can survive even after food processing. Vegetables are at higher risk of contamination because of poor sanitization procedures. Especially, water which is used to wash raw vegetables and vendor's personal hygiene can be the reason of contamination. This study was anticipated to investigate the possible source of *K. pneumoniae* from water sample and hand swabs of the vendor from wet market as well as to know the antibiotic-resistant *K. pneumoniae* from those samples. From 7 areas, total 32 samples were collected (water & hand swab) where 27 samples (87.5%) gave positive results for *K. pneumoniae*. Antimicrobial resistance was examined for Ampicillin (100%), Erythromycin (100%), and Imipenem (100%). While high susceptibility was observed to Nalidixic acid (100%), Gentamicin (92%), Amikacin (92%), Meropenem (95%), Ciprofloxacin (91%), Amikacin (87%), Colistin (87%), and Tetracycline (74%). Ciprofloxacin (88.8%), Ceftriaxone (88.8%), Tetracycline (74%), Meropenem (70.37%), Tigecycline (51.85%) and Nitrofurantoin (35%). Among this, 3.7 % is Extensive Drug Resistance (XDR) and no Pan drug Resistance (PDR) strains were found. This study shows that water and hand samples contain some antibiotic-resistant *K. pneumoniae*. So, Therefore, the source of water and vendors themselves can be the possible transmission route for the vegetable consumers. Moreover, as some isolates were antibiotic-resistant hence it is a matter of concern. Therefore, proper sanitization and clean water should be ensured to prevent contamination with *K. pneumoniae*.

Dedication

To our family

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List of Acronyms

PCR - Polymerase Chain Reaction

MDR - Multidrug Resistance

XDR - Extensive Drug Resistance

PDR - Pan drug Resistance

MHA - Muller Hinton Agar

NA - Nutrient Agar

DNA - Deoxyribonucleic acid

CLSI - Clinical and Laboratory Standards Institute

Chapter 1

Introduction

Klebsiella pneumoniae was described by Carl Friedlander in 1882 as an encapsulated bacillus. In 1886 we got the name *"Klebsiella"* which is a gram-negative, encapsulated, non-motile bacterium. *K. pneumoniae* belongs to Enterobacteriaceae family. This bacterium can be found in the environment and a great source for causing disease (Ashurst & Dawson, 2023).

1.1 Etiology

Klebsiella pneumoniae is considered the most common cause for hospital-acquired pneumonia in USA as 3%-8% nosocomial infections is caused by this pathogen (Aghamohammad et al., 2020). The virulence factor which plays the vital role for disease causing is the polysaccharide capsule of the bacterium. There are almost 77 different types of capsules, and the noticeable thing is *Klebsiella* species which are not capsulated are less virulent. The coating of lipopolysaccharides also plays as a major virulence factor. Fimbriae and Siderophores are also considered as virulence factor. *K. pneumoniae* are now experiencing a high rate of antibiotic resistance. As a result, the mortality rate is increasing by this pathogen. Multi drug resistance *Klebsiella* has become a great concern worldwide in these days (Ashurst & Dawson, 2023).

1.2 Epidemiology

Humans are the great host for *K. pneumoniae*. A study was conducted in North America, South America, Europe and Asia in 2015 under National Institutes of Health. They showed the mortality rate of *K. pneumoniae* which are Carbapenem resistant. The mortality rate from

this pathogen was 47.66%. Geographically the mortality rate for North America, South America, Europe, Asia was 33.24%, 46.71%, 50.06%, 44.82%, respectively (Xu et al., 2017).

According to another study of National Institutes of Health, conducted in Italy in 2018, 5%-38% people carry them in their stools and 1% to 6% in their nasopharynx. In the hospitalized patients carrier rate was greater than the people in community. Study also showed that, in western countries approximately 3%-5% patients acquired infections due to *K. pneumoniae*, whereas in developing countries of Africa the rate was 15%. Lifestyle, safety measurement, hygiene could be the reason behind this (Ashurst & Dawson, 2023).

According to World Health Organization, in 2017 800,000 children died from Pneumonia. Report says that in Bangladesh 186 children (4.6%) died during hospitalization. Antibiotic resistance increases the mortality rate. Furthermore, out of the 4,007 pneumonia patients 83 were the most positive blood cultures. And maximum of those were gram-negative bacteria. Out of 83 samples 11 *K. pneumoniae* were detected (McLernon, 2021).

1.3 *K. pneumoniae* in non-clinical environment

Most of studies of *K. Pneumoniae* are based on clinical samples. That is why, non-clinical samples like veterinary and environmental health are neglected. The infection sources from foods are barely investigated. There are a lot of aspects of research for molecular epidemiology and resistance development of *K. pneumoniae* in environment. Foodborne bacteria are extensively studied, unfortunately research on *K. pneumoniae* is rare. Awareness should be raised of the fact that non-human sources can act as a reservoir for this pathogen. The study in Germany, 2021 revealed that vegetable like tomatoes, salads, carrots, cauliflower, mushrooms are the carriers of *K. pneumoniae* strains. Also, those were multi drug resistant bacteria (Wareth & Neubauer, 2021)

Another research was done in our lab (BRAC University, Dhaka, 2022) where 252 commercially available raw vegetable samples in 12 different places from Dhaka city were collected and 100 (39.7%) of them tested positive for *K. pneumoniae* (Unpublished data).

Vegetables undergo microbial spoilage by a different group of pathogenic bacteria, fungi, viruses and parasites. Contaminated foods can carry pathogens which may harbor different virulence factors like toxins, enzymes or virulent gene which further evoke the pathogenesis. As a result, outbreaks of food-borne diseases associated with vegetables are a usual occurrence (Ahmed et al., 2014).

As a developing country Bangladesh is also at a high risk. As *K. pneumoniae* can be found in environment easily so wet market can be the possible way to cause infection for a mass population. Food borne illness is very common in Bangladesh. During food processing there are a lot of chances of contamination. In case of vegetable, sellers always sprinkle water on vegetables to keep those in fresh condition. They do not use clean water in most cases which can be the route for *K. pneumoniae*. Moreover, in local markets vendors do not maintain proper hygiene and their hands are not properly clean while handling the vegetables. As a consequence, this might be a big reason of contamination and foodborne illnesses.

In 2014 "American Journal of Agriculture and Forestry" published an article was conducted in Dhaka, Bangladesh which showed the presence of different pathogens in salad vegetables. They founded the presence of *K. Pneumoniae* (104 cfu/g) in onion (Ahmed et al., 2014). This study clearly showed that food-borne illness from vegetables which is a matter of concern.

1.4 Aims & Objectives

The aim of this study was to know what the possible way of presence of *K. pneumoniae* in vegetable. So, we have examined the water that vendors use for washing vegetables and their hand swabs sample to confirm the presence of the pathogen.

Chapter 2

Methods & Materials

2.1 Study Area

The study was done in Dhaka, Bangladesh. A total 7 different wet markets of this city were included in this study. Those areas were Farmgate, Rampura, Khilgaon, Dhanmondi, Gulshan 2, Mohammadpur and Mirpur. Total 42 samples (21 water samples & 21 hand swabs) were collected from those following areas from May 2023 to July 2023.

2.2 Samples Collection & Processing

- The water samples were collected in autoclaved tubes and the hand swabs samples were collected from vegetable sellers.
- Firstly, 5-ml autoclaved falcon tubes containing sterile saline water were used to collect hand samples. Samples were taken from vendor's hand by the help of a sterile cotton swab. Then, the cotton swab was dipped into saline water to preserve it.
- After that, water which they use to wash those vegetables were also collected into falcon-tubes (10 ml).
- One set of sterile gloves and a sterile Ziplock bag were used to avoid cross-contamination. Finally, samples were transferred into the laboratory by maintaining cold chain for further analysis (within 2 hours). The whole process was done by maintaining sterility so that no contamination occurs.

Figure 1: Sample collection

2.3 Isolation of *K. pneumoniae*

Initially, each collected water and hand swab sample were taken for further experiment. For isolating *K. pneumoniae* spread plate method was used from the collected water and hand swab samples. Those samples were spread on MacConkey agar plates then incubated at 37°C for 24 hours. The growth of *K. pneumoniae* was distinguished by its mucoid growth which appeared as pink color single colonies. After that, those obtained *K. pneumoniae* isolates were streaked on HiCrome KPC Agar Base (CHROMagar, n.d.). KPC is a selective as well as differential culture medium which is specially used for qualitative direct detection of colonization resistant Enterobacteria (Sayed et al., 2020). The streaked plates were incubated at 37°C for 24 hours. On KPC agar the isolates of *K. pneumoniae* appeared in metallic blue color. Then again, from HiCromeTM KPC Agar Base few colonies were picked up and were sub-cultured on Nutrient agar (NA) plates using the streak plate method and incubated at 37°C for 24 hours. Pure single creamy white colonies appeared after incubation period. Finally, *K. pneumoniae* isolates were stored for further use.

2.4 DNA extraction of *K. pneumoniae*

DNA Extraction was done by Boiling Method. Firstly, 1000µL (1ml) of bacterial cell suspension (24hr culture) was taken. Secondly, it was centrifuged at 4,500 rpm for 5min at 4°C. Thirdly, the supernatant was discarded and 50µL nuclease-free water was added to that right after that. Then, it was boiled at 100°C for 5 min. Again, centrifuged at 3000g for 10 min. The supernatant was taken in a fresh Eppendorf tube, 600µL of cold 100% ethanol was added into it and was centrifuged at 3000g for 10 min. After centrifugation the supernatant was discarded, and the pellet was washed in 600µL cold 70% ethanol. Then, it was centrifuged at 3000g for 5 min. After that, the supernatant was discarded carefully and the pellet was air dried for 10-15 min. Lastly, it was re-suspended in 120µL TE buffer and stored at -20°C for further processing.

2.5 PCR detection of *K. pneumoniae*

K. pneumoniae isolates were verified by PCR as previously published (Yigrem et al., n.d.) using Master mix (12), Forward primer (2 µL), Reverse primer (2 µL), DNA template (4 µL), Nuclease free water (5 µL). Two pairs of *K. pneumoniae*-specific primers, Pf (5′-ATT TGA AGA GGT TGC AAA CGA T3′)/Pr1 (5′-TTC ACT CTG AAG TTT TGT GTT C-3′) and Pf/Pr2 (5′-CCG AAG ATG TTT CAC TTC TGA TT-3′), were allocated. The PCR detection was performed using these two primer pairs. (The PCR program was set at) cycling conditions were 35 cycles of 30 s at 94 °C, 20 s at 57 °C, and 20 s at 72 °C, followed by a 10-min hold at 72 °C. (Liu et al., 2008) Later PCR products were kept at -20 °C.

2.6 Agarose gel electrophoresis

For making agarose gel 98 ml distilled water, 2 ml buffer and 1.2 g agarose were used. 0.5 ml etbr was also added. After gel forming the sample (7 µL) was loaded to the well. Similarly, 7 µL ladder (1 kb) was used for detection. Finally, the electrophoresis process was done at 100 volt and the result was visible under UV light. The product size was 130 bp.

2.7 Antibiotic susceptibility test

This study was done using Kirby-Bauer disk diffusion method to test antimicrobial susceptibility on Mueller-Hinton agar (MHA) with 14 antibiotics. In this process, bacterial suspension was adjusted to a McFarland standard of 0.5. Total 14 antibiotic agents which were classified into 10 categories were tested at specific concentrations per disk. Critical value of each antibiotic is showed in (Table 1). The selected antibiotics have great significance for both human and animal medicine. Nevertheless, isolates were classified as susceptible, moderately resistant & resistant by using the breakpoints designated by the CLSI. Those isolates which are moderately resistant also considered as resistant (Zhang et al., 2018). Moreover, antibiotic resistance has become the most challenging part for treating any patient. As a consequence, this study is required to check multidrug-resistant (MDR), extensive drug-resistant (XDR) & pandrug-resistant (PDR) of bacterial isolates. MDR indicates non-susceptibility to minimum one agent in three or more antimicrobial categories. Whereas XDR was explained as non-susceptible to at least one agent in all and two or fewer antimicrobial classes. Lastly, non-susceptibility to all agents in all antimicrobial groups is defined as PDR. (Basak et al., 2016)

Antimicrobial class	Antimicrobial agent (disk potency, µg)	Zone of inhibition		
		Sensitive	Intermediate	Resistant
Aminoglycosides	Gentamicin (10)	≥15	13–14	≤12
	Amikacin (30)	≥17	15-16	≤14
Fluoroquinolones	Nitrofurantoin (30)	≥17	15-16	≤14
	Ciprofloxacin (5)	≥21	16–20	≤15
Macrolides	Erythromycin (15)	≥23	14–22	≤13
Glycylcyline	Tigecycline (150	≥19	15–18	≤14
	Tetracycline (30)	≥19	15–18	≤14
Phenicols	Chloramphenicol (30)	≥18	13–17	≤12
Beta-lactamase	Ampicillin (10)	≥17	14-16	≤13
Carbapenem	Meropenem (10)	≥23	20-22	19
	Imipenem (10)	≥ 16	14-15	≤13
Polymyxin E	Colistin (10)	≥17	12-16	≤ 11
Cephalosporin	Ceftriaxone (30)	≥23	20-22	≥19
Quinolone	Nalidixic acid (30)	≥19	14-18	≤13

Table 1: List of the antibiotics, antibiotic disk potency and zone of inhibition (CLSI,….)

Chapter 3

Result

3.1 Isolation of *Klebsiella pneumoniae*

At first collected samples from the water and hand swab were lawned in MacConkey Agar media. Positive result from MacConkey media which gives mucoid colony were taken (figure a) and sub-cultured on HiCrome KPC agar media to see the positive result of *K. pneumoniae* (figure b).

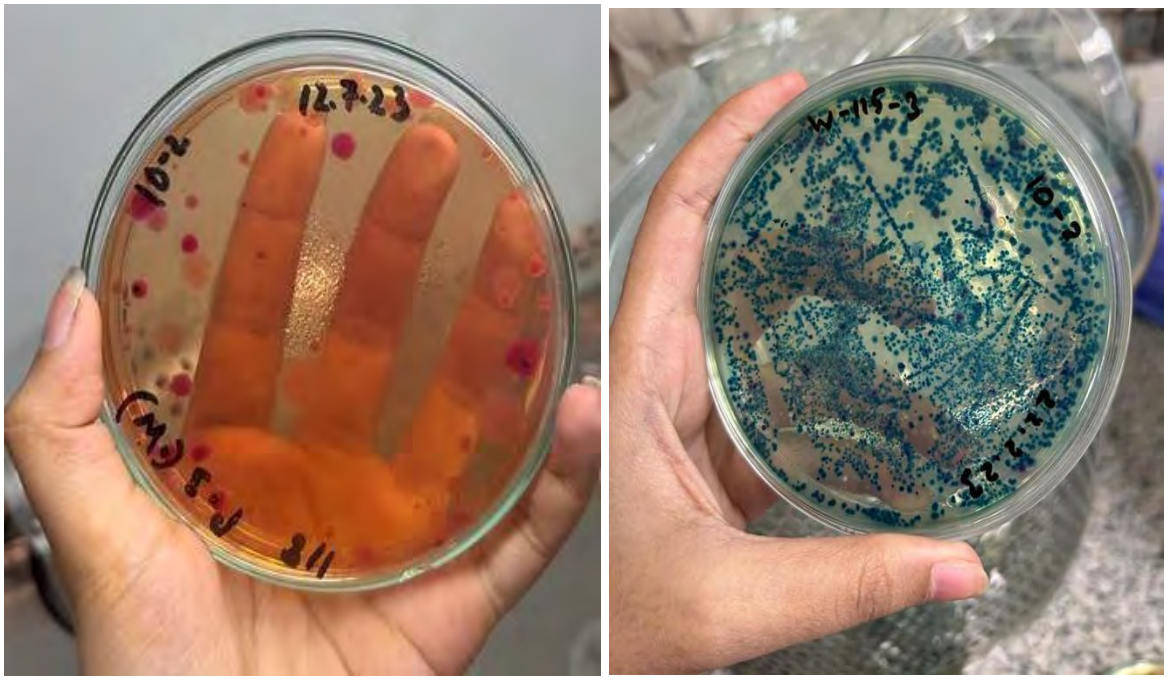

Figure 2: (a) K. pneumoniae mucoid colony on MacConkey agar media and (b) blue color colony in KPC media.

3.2 PCR and confirmation by gel electrophoresis

PCR products were analyzed by electrophoresis in 1.2% agarose gel. Here (figure3) from 2–12 lanes, 130 bp DNA fragment of *K. pneumoniae* was amplified by two sets of primer Pf and Pr1 primer. At lane 1 DNA ladder (1kb) is given.

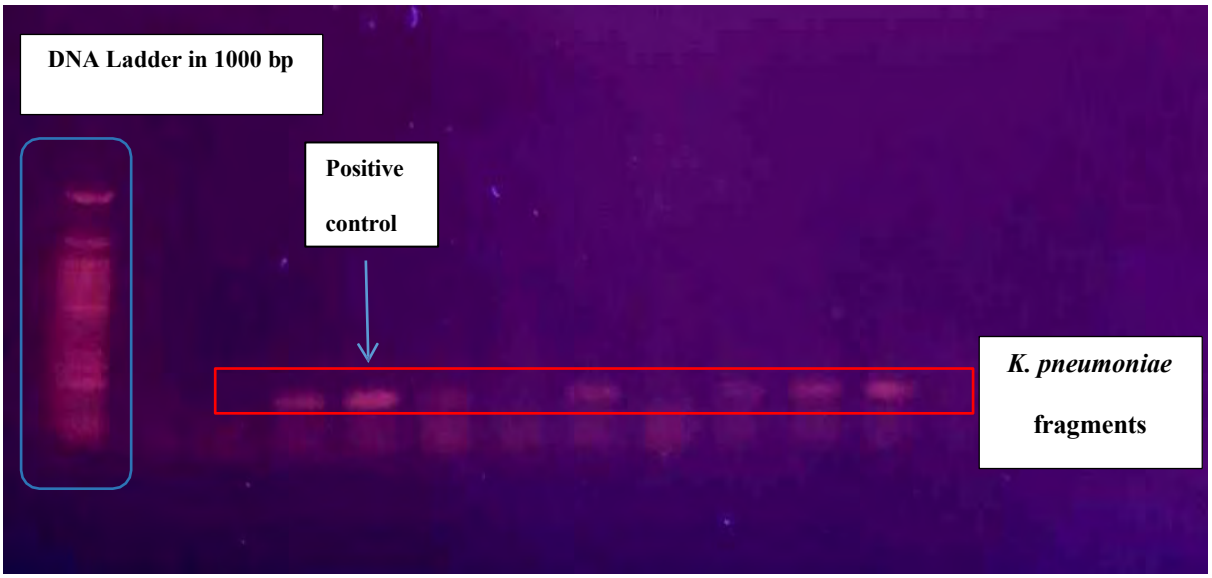

Figure 3: Detection of K. pneumoniae by PCR

3.3 *Klebsiella pneumoniae* in Water and Hand swab Samples

Out of the 42 samples collected, consisting of 21 water and 21 hand swabs, a total of 10 samples (23.8%) exhibited positive growth. Notably, two areas - Rampura and Mirpur - yielded no growth in any sample. The Gulshan 2 area exhibited the highest positivity rate among all water samples, with every water sample testing positive and a sole hand swab sample yielding a positive result. Following Khilgaon and Dhanmondi, Mohammadpur and Farmgate had an identical ratio (table 2.)

Area	Number of collected sample		Growth found of *k. pneumoniae*	
	Water (a)	Hand swab (b)	Water (a)	Hand Swab(b)
Farmgate	3	3	1(33.3%)	0
Rampura	3	3	0	0
Khilgaon	3	3	2 (66.6%)	0
Dhanmondi	3	3	2 (66.6%)	0
Gulshan 2	3	3	3 (100%)	1 (33.3%)
Mohammadpur	3	3	1(33.3%)	0
Mirpur	3	3	0	0
Total	21	21	9 (42.85%)	1 (4.76%)

Table 2: K. pneumoniae isolates from different water samples and hand swabs.

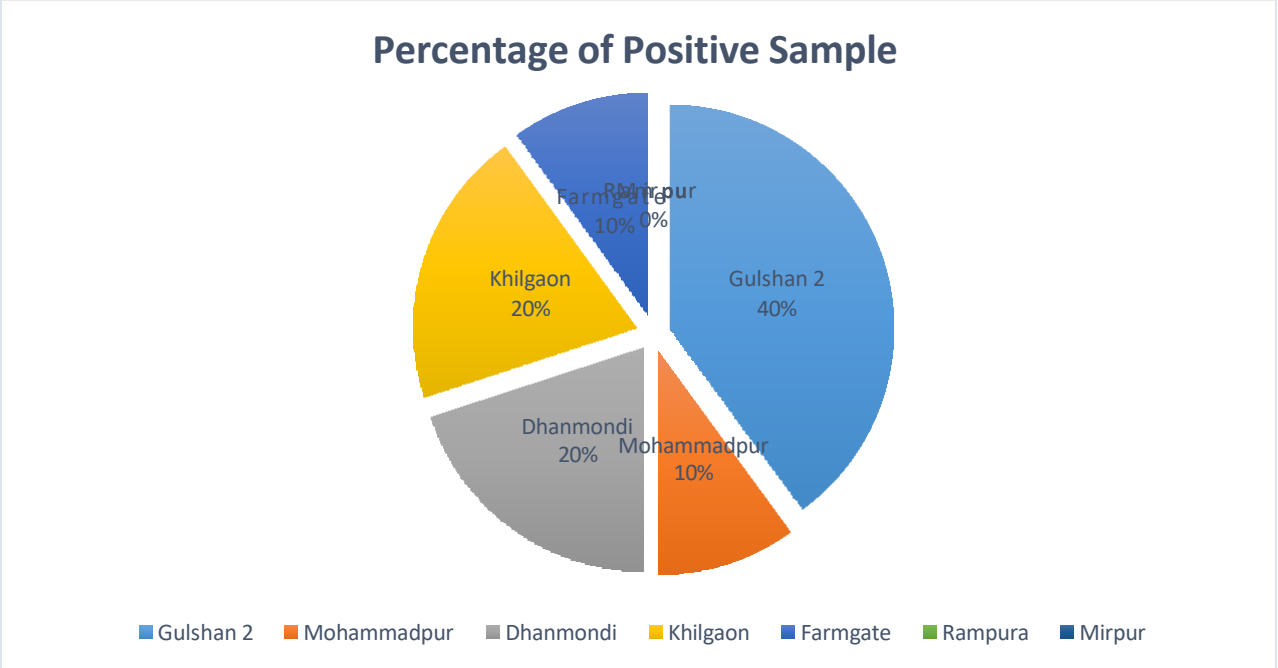

Figure 4: Percentage of positive K. pneumoniae per sample area.

3.4 Antimicrobial Susceptibility Test

From the positive isolates 27 isolates were randomly selected, and antibiotic susceptibility were performed. Here are the results are shown in Table 3.

In this study, 85.1% is chloramphenicol resistance and 62.9% is Colistin resistance. On the other hand, all of them are Nalidixic acid sensitive with other strains giving sensitivity to given antibiotics at Gentamicin and Amikacin at 92 %, Ciprofloxacin and Ceftriaxone 88.8%, Tetracycline at 74%, Meropenem 70.37 % Tigecycline 51.85% and Nitrofurantoin 35%.

Antimicrobial class	**Antimicrobial agent (disk potency, µg)**	**Sample numbers (n) and (%)**		
		Sensitive	**Intermediate**	**Resistant**
Aminoglycosides	Gentamicin (10)	25 (92%)	0	2 (7.4%)
	Amikacin (30)	25 (92%)	0	2 (7.4%)
Fluoroquinolones	Nitrofurantoin (30)	10 (37%)	14 (51.85%)	3 (11.1%)
	Ciprofloxacin (5)	24 (88.8%)	2 (7.4%)	1 (3.7%)
Macrolides	Erythromycin (15)	0	0	27 (100%)
Tetracyclines	Tigecycline (15)	14 (51.85%)	10 (37%)	3 (11.1%)
	Tetracycline (30)	20 (74%)	3 (11.1%)	4 (14.81%)
Phenicols	Chloramphenicol (30)	0	4 (14.81%)	23 (85.1%)
B-Lactamase	Ampicillin (10)	0	0	27 (100%)
Carbapenem	Meropenem (10)	19 (70.37%)	5 (18.51%)	3 (11.11%)
	Imipenem (10)	0	0	27 (100%)
Polymyxin E	Colistin (10)	0	10 (37%)	17 (62.9%)
Cephalosporin	Ceftrioxone (30)	24 (88.8%)	2 (7.4%)	1 (3.7%)
Quinolone	Nalidixic acid (30)	27 (100%)	0	0

Table 3: Antibiotic resistance profile of isolate

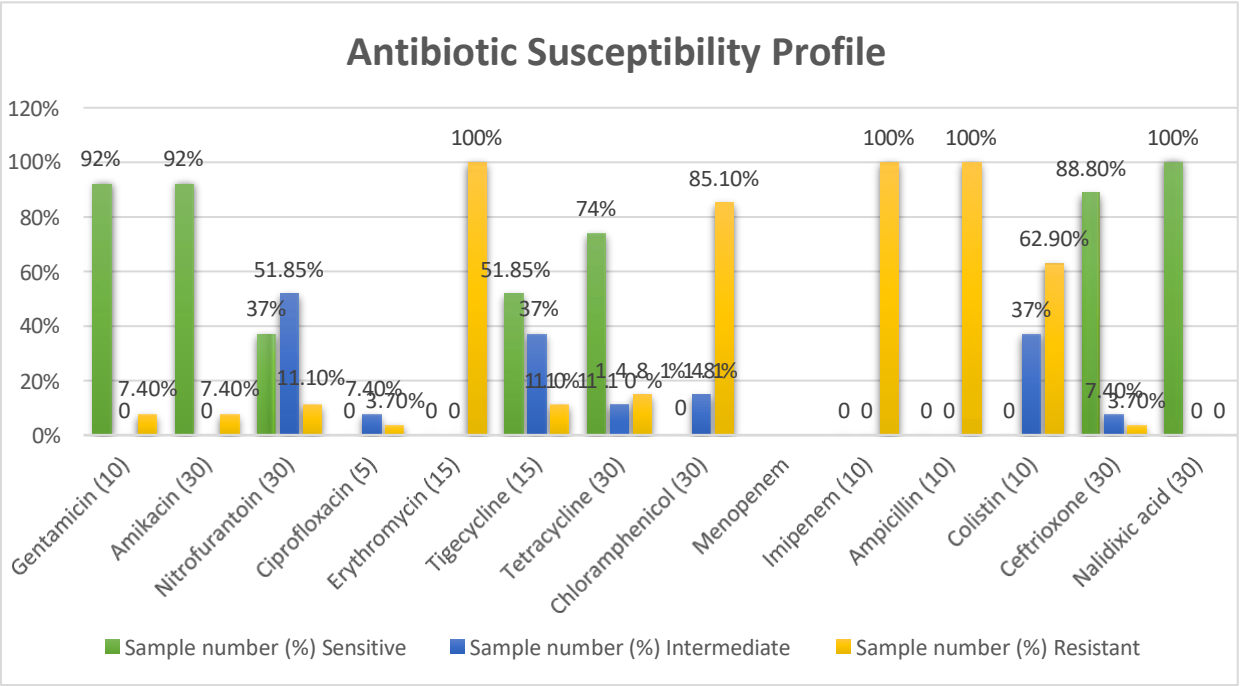

Figure 5: Antibiotic susceptibility profile

3.5 Multi-drug resistance

Among 27 isolates all of them were multi-drug resistant bacteria because they are 100 % resistant to Ampicillin, Erythromycin, and Imipenem. Also, 3.7 % is Extensive Drug Resistance (XDR) but no Pan drug Resistance (PDR) strains were seen.

Chapter 4

Discussion

This study focused on the characterization and isolation of virulent and antibiotic-resistant *Klebsiella pneumoniae*, is an emerging reason for pneumonia. (*Klebsiella Pneumoniae* in Healthcare Settings | HAI | CDC, 2023). Food may operate as a reservoir for multi drug-resistant bacteria like *K. pneumoniae* (‘Resistant Bugs,’ n.d.) with transmissible antibiotic-resistance genes due to the overuse of antibiotics (Soliman et al., 2021). However, there is very little data available for identifying and characterizing *K. pneumoniae* from water sample and hand sample of Bangladesh, limiting the possible risk assessment and the development of safety protocols. In this study, we isolated 10 positive samples (9 water & 1 hand) from 42 samples of *K. pneumoniae* from vegetable vendors’ hands and water samples (23.8 % of total sample). No research was found like ours but some information was collected from a study conducted in Malaysia’s vegetable market where 200 sample the raw vegetable samples collected . From that 65% was contaminated with *K. pneumoniae* which is more from our founding (Pearson & Gash, n.d.). (Puspanadan et al.,2012). Another study conducted in Botswana where dried vegetables were collected as sample and percentage of *K. pneumoniae* positive was 86.4% (Pearson & Gash, n.d.). Therefore, research and study say that raw vegetables can a one of the main resources of any direct *K. pneumoniae* diseases and precautions.

Likewise, the isolates' susceptibility to 14 antimicrobial drugs which is categorized into 10 groups where results seen of Ampicillin had the highest rate of resistance in this study (100%) along with Erythromycin and Imepenem. The study conducted in 1979 found that *K. pneumoniae* had moderate resistance to ampicillin due to the presence of b-lactamase genes (Labia et al., 1979). However, by 2004, *K. pneumoniae* had become fully resistant to ampicillin

(Struve & Krogfelt, 2004). So, Imepenem like antibiotic which was beta lactamase inhibitor antibiotic used to work on *K. pneumoniae* to 2021, giving 100% susceptibility (Mashaly & Mashaly, 2021). It is alarming that only after 2 years, we are seeing its resistance in *K. pneumoniae*. Also, erythromycin antibiotic resistance has increased from 70% (Bokaeian et al., 2014) to 100% from 2014 to 2023.

Detecting MDR and possibly virulent *K. pneumoniae* in food that can be eaten uncooked is horrifying (Hartantyo et al., 2020). A study which was done on raw vegetables, fruits in 2016 in Saudi Arabia, found only resistance against Erythromycin and Penicillin with 72% multi drug resistance in *K. pneumoniae* (Abu-Zaid et al., 2016) and study in Spain described, isolates from raw fresh vegetables had antibiotic resistance *K. pneumoniae* which has resistance against amoxicillin/clavulanic acid and ampicillin (Falomir et al., 2013). But unfortunately, our samples are 100% multi drug-resistant (MDR) to Carbapenem, beta-lactamase, and Macrolide types of antibiotics (table 3).

Although in good side, 100% of isolates were sensitive to Nalidixic Acid, which ranges down to Gentamicin and Amikacin at 92 %, Ciprofloxacin and Ceftriaxone 88.8%, Tetracycline at 74%, Meropenem 70.37 %. So, we can say Nalidixic Acid is a more likely susceptible antimicrobial substance that may be able to treat infections brought on by *K. pneumoniae*. In the detection of the strains of our sample two sets of primers were used (Pf/Pr1 and Pf/Pr2) from which Pf/Pr1 delivered an improved output compared with Pf/Pr2.

Finally, these findings demonstrate the need for other alternative antimicrobial drugs in the future to prevent *K. pneumoniae* from developing multi drug resistance. The widespread usage of antibiotics and non-compliance with the recommended dosage regimen may cause this growing resistance pattern. The mechanism of resistance and pathogenicity of *K. pneumoniae* in raw vegetables, therefore, requires in-depth study.

Chapter 5

Conclusion

In conclusion, 10 sample positive samples collected from 7 different area vendor was *Klebsiella pneumoniae* positive. They were grown into MacConkey and then HiCrome KPC Agar media to see the positive result and showed antibiotic resistance in MHA being 100% MDR, although 3.7% was XDR. Thus, the results suggest that raw vegetables can be a potential route of transmission of *K. pneumoniae* infection in consumers posing a threat to public health safety and the best antibiotics will work on it is nalidixic acid.

As a result, proper sanitation of raw vegetables is the utmost priority when it comes to preventing *K. pneumoniae* contamination in vegetables and saving lives.

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