Can Bacteria fly? -Bacteriological Assessment of Common Fly (*Musca domestica)* As a Potential Carrier for Public Health Epidemics

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A thesis submitted to the Department of Mathematics and Natural Sciences, BRAC University in partial fulfillment of the requirements for the Degree of
Bachelor of Science in Microbiology

DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES

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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing a 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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Ethics Statement

Throughout the process, no animal and human were harmed or disturbed to run the study. In addition, the samples were collected from different sites in fish markets or roadside food vendors by asking permission from sellers or buyers. Also all precautions were maintained during collecting the samples and further laboratory activities were done in BRAC University thesis lab after complete approval from the department.

Abstract

The primary aim of the research work is to analyze the diverse bacterial presence in the houseflies (*Musca domestica*) found from the fish markets and street food in some major areas of Dhaka city. Common house flies (*Musca domestica*) are familiar phenomena in the environment as they get suitable conditions to survive amidst harsh environmental conditions than any other pests.

During the study, 30 housefly samples obtained from different locations in Dhaka from which 148 isolated colonies were recovered from various selective and non-selective media plates (MSA, XLD, TCBS, Cetrimide, MacConkey agar and nutrient agar). Most expected organisms from the samples were *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Salmonella typhi*, and *Staphylococcus aureus*. For the identification, biochemical testing and conventional PCR methods were used. On the basis of the biochemical testing, isolates were thought to be different organisms, including *K.pneumoniae* (7.43%), *Pseudomonas aeruginosa* (7.43%), *Serratia marcescens* (14%), *Proteus spp.* (5%), and others which cause various enteric and nosocomial diseases in humans. In addition, PCR confirmation included 7.43% *Pseudomonas aeruginosa*, 4.73% *Vibrio* genus, 7.43% *Klebsiella pneumoniae* and others. However antibiotic susceptibility tests using Kirby-Bauer Disk Diffusion method were followed to overview ongoing conditions of antibiotics. Among all the antibiotics vancomycin showed almost 73.68% sensitivity to most of the organisms and 10.53% resistance. Following that ciprofloxacin showed 94.55% sensitivity to most organisms. On the contrary, erythromycin (58.18%) and Tetracycline (28.18%) gave small zones that indicated resistance against those organisms. Besides, Amoxicillin showed a high range of resistant zones. It was assumed that such resistance towards necessary antibiotics can create severe public health concerns adding that house flies can transmit the pathogens containing resistance genes with them acting like a carrier in the environment.

Keywords: Houseflies, Antibiotic Resistance, Pathogen, Resistant, Sensitive, Resistance genes.

Dedication

To us…

-Nuzhat & Tonuka

Acknowledgement

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List of Acronyms

PBS	Phosphate-buffered saline
MSA	Mannitol salt agar
XLD	Xylose lysine deoxycholate
TCBS	Thiosulfate citrate bile salts sucrose
NA	Nutrient agar
PCR	Polymerase chain reaction
MIC	Minimum inhibitory Concentration
MHA	Mueller Hinton Agar
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
TE	Tris-EDTA
LB	Luria broth
bp	Base pair
EtBr	Ethidium bromide

Chapter 1:
Introduction

Background:

In developing and under-developing countries, diseases associated with food have become a threat to public health. Food can get polluted with diverse contaminants, such as infectious pathogens or harmful chemicals, which can be life-threatening if ingested. Ingestion of such polluted food can cause many enteric and diarrheal diseases (Ranjbar et al., 2016). In an article published in 2015, it states that around 600 million cases of foodborne illnesses, along with 420,000 deaths were reported in 2010 (Havelaar et al., 2015). Numerous mechanical vectors such as flies, mosquitoes, mites, ticks, and bugs can spread a high number of diseases that can be due to bacteria, viruses, or others(Parvez et al., 2016) (WHO). Among the vectors, houseflies can interact with humans in a high number and spread diseases easily. Houseflies are found frequently near garbage areas or food areas where they can transmit serious diseases such as diarrhea, cholera, typhoid fever, and others (Parvez et al., 2016).

Housefly (Musca domestica), being a synanthrope, has the capability to spread illnesses to both humans and animals. They belong to the Muscidae family and Diptera order (Khamesipour et al., 2018). Being one of the most well-known and prevalent species of flies, they are found in tropical climates, especially in Central Asia. An adult housefly is a carrier of many pathogenic bacteria as it is mainly frequent in locations such as animal wastes, dustbins, street food-selling vendors, fish markets, hospitals, etc. By reproducing and continuing their lifecycle in these areas, flies transmit diseases with their body (mouth, wings, spewing of the gut) (Ugbogu et al., 2006). Among all the pathogenic bacteria, houseflies are identified as significant carriers of *Pseudomonas spp.*, *Staphylococcus aureus*, *Vibrio cholerae*, *Enterobacteriaceae*, *Klebsiella* spp., and others (Bahrndorff et al., 2017; Davari et al., 2010).

Klebsiella pneumoniae, gram-negative bacteria, is one of the most notorious organisms that imposes significant public health threat due to the emerging Multidrug resistant and difficult to treat strains. Being a common and potential community acquired pathogen, *K.pneumoniae* causes nosocomial pneumonia, urinary tract infection and others (Ko et al., 2002). According to a countrywide survey in Bangladesh, it is the third most frequent organism from clinical samples after *E.coli* and *Pseudomonas* species (Hussain et al., 2023). One of the major bacteria that is responsible for diarrheal diseases is *Vibrio cholerae.* It can be highly fatal without any proper

treatment and transmit through polluted water-sources and human to human contact. Furthermore, other *Vibrio* species such as *Vibrio parahaemolyticus* cause vibriosis infections (Baker-Austin et al., 2018). According to research, every one person in six was contaminated with *Vibrio Cholerae* O1 in the year 2014 (Azman et al., 2020). Around 589854 cases of cholerae were reported along with 7816 deaths in 58 countries in the year 2011 (Paul et al., 2016). Beside these, Pseudomonas aeruginosa is also a concerning pathogenic bacteria that caused 12.6 % of all bloodstream infections in the period of 2004 - 2015 and it is the second most prevalent bacteria in Bangladesh (Saha et al., 2022). Another bacteria, Salmonella typhi causes typhoid fever and is responsible for causing 21.7 million diseases with 216000 deaths worldwide (Ashurst et al., 2023).

One of the most concerning global issues that is increasing rapidly is the occurrence of antimicrobial resistance. Because of this significant problem WHO stated that the diagnosis and treatment of infectious disease is getting expensive and time-consuming. Various antibiotics are used to treat different types of infections. For instance, the β-lactamase antibiotic is used to treat *Staphylococcus aureus* in humans. However, with the rapid increase in the resistance of bacteria, it is becoming impossible to treat the infection with this antibiotic (Mamza et al., 2010). Similarly, it has turned into a common scenario to obtain bacterial isolates resistant to the tetracycline resistance gene (*tetA, tetB, tetC, tetD*) in Bangladesh (Sobur et al., 2019).
Antibiotic-resistant bacteria can be transmitted from one host to another with the help of houseflies (*Musca domestica*) (Marshall et al., 1990). In 2017, a study performed at Hamadan University of Medical Sciences found that house flies carry an abundant number of pathogenic bacteria. Based on the study, 22.9 percent of *Staphylococcus aureus* and 11.6 percent *of Escherichia coli* were found from 394 bacterial isolates (Nazari, 2017). Recently, in 2020, an article published by Akter *et al.* discovered the presence of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella spp*. in houseflies in Mymensingh, Bangladesh. They also found antibiotic-resistant bacteria in that region (Akter et al., 2020).

Literature review:

Common house flies (*Musca domestica*) are common phenomena in the environment as they get suitable conditions to survive amidst harsh environmental conditions than any other pests. Their

ability to move easily, reproduce, fly long distances as well as get enticed to any organic or decaying substances or places upgrades their survival chances (Barreiro et al., 2013). Now-a-days flies are found in almost all places in surroundings which have become a major concern. Some flies may even be responsible for economic loss and can also harm animals or plants (Campbell et al., 2001). Flies are easily attracted to humans and animals; more often to filthy conditions. Muscidae (housefly) and Calliphoridae (blowfly) are filthy flies that are mostly found in domestic areas and are related to act as vectors for food contamination (Fukuda et al, 2019). Houseflies get nutrition source from garbage, animal or human feces, foods or decaying matters so it is an obvious reason to get in constant close attachment in domestic area like kitchen, animal farm or slaughtering area; in addition, flies can fly a large range of distance that make them a carrier for microorganisms (Gioia et al., 2022). Houseflies contain bristles and hairs on their legs, so when they rest or attach on a surface microorganisms adhere to them which causes them to transfer microorganisms (Graczyk et al., 2001). Moreover houseflies can transfer bacteria through their mouth, wings, saliva etc (Vasan et al., 2008). According to a report by World Health Organization (WHO), almost 17% of diseases can be caused by insects carrying parasites, bacteria or viruses with them and also this can lead to the death of more than 700000 worldwide in a year. In addition, hundreds of isolates can be transmitted through a single fly interacting with different surfaces or sites.

Bangladesh is an overpopulated country where common or rare bacterial infections or diseases are ongoing problems. Recently cholera, dysentery, pneumonia, diarrhea, and shigellosis are causing rapidly in infants as well as adult or older persons. Houseflies are common in every corner of Bangladesh because of pollution, mismanagement of waste or sewage systems and also open foods on the street. Farag et al. (2013) stated that house flies play a common vector to cause shigellosis, in contrast, removing house flies from the environment can reduce the level of shigellosis in Bangladesh. Additionally he said that children less than five year's age are more prone to Shigella-associated illness that can lead to severe diarrhea because of houseflies transmitting such kinds of pathogens. Flies can carry fungi, yeast, *Aspergillus niger, Penicillium, Fusarium* along with bacteria or protozoa, thus it can cause severe food borne diseases and food contamination (Parvez et al., 2016). Houseflies around hospital area can carry *Staphylococcus aureus, Salmonella* spp and *Escherichia coli* adding that these bacteria can show resistance against erythromycin, tetracycline, penicillin and amoxicillin (Akter et al., 2020).

Isolates such as *Enterococcus faecalis*, *E. faecium*, *E. casseliflavus* collected from house flies in restaurant area may contain resistant gene against tetracycline, erythromycin, streptomycin, ciprofloxacin and kanamycin (Macovei et al., 2006). However tetracycline resistance genes in bacteria are *tetA, tetB, tetC* and *tetD*; on contrary, methicillin-resistant *S. aureus* (MRSA) known as superbug contains resistance gene like *mecA* and *mecC* (Akter et al., 2020). Antibiotic susceptibility tests assess the sensitivity and resistance pattern of a bacteria; hence it can ensure further analysis for resistance genes. In a study by Khamesipour et al., (2018), houseflies in both urban and rural areas can spread almost 130 pathogenic bacteria that carry antibiotic resistance genes within them. According to a data analysis, female fly harbored $1.03 \pm 0.59 \times 10^6$ CFU/fly whereas male fly had bacterial load $3.45 \pm 1.37 \times 10^5$ CFU/fly from an urban site which concludes that female flies can transmit more bacterial CFU than male flies due to heavy contamination of bacteria in its ovipositor (Neupane et al., 2020).

Objective:

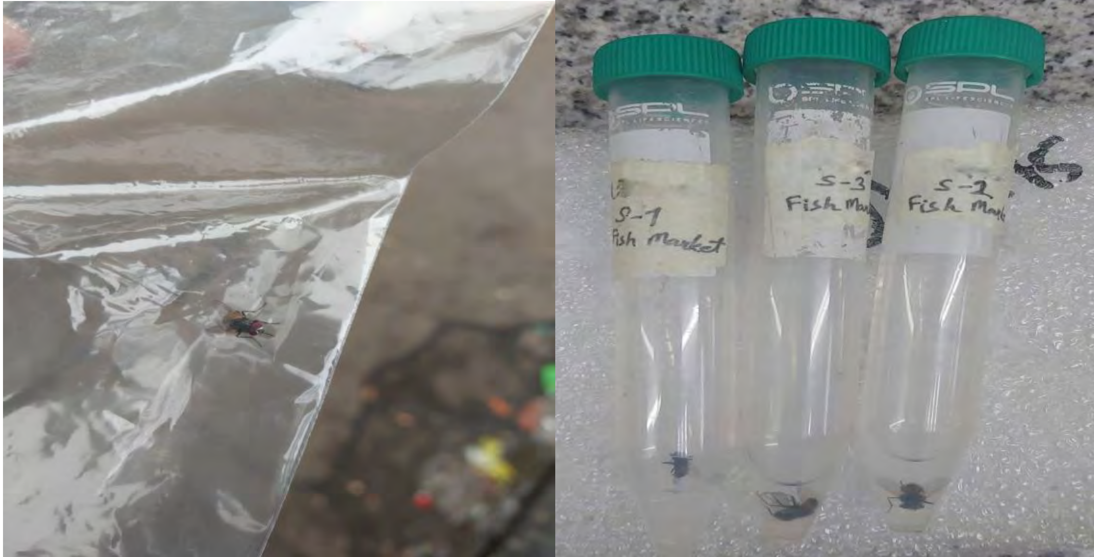
The fundamental objective of the research work is to analyze the diverse bacterial presence in the houseflies found from the fish markets and street food areas. Moreover, detection of antibiotic resistance genes present in Houseflies (*Musca domestica*) in Dhaka, Bangladesh is also another objective. Additionally, the ongoing situation of Antibiotic resistant bacteria to antibiotics will be evaluated.

Chapter 2:

Materials and methods

2.1 Sample collection and processing:

Using an aseptic nylon net, a total number of 30 houseflies were accumulated separately from three different locations including, Mohakhali (10), Kawran Bazar (10), and Mirpur (10) which were situated in Dhaka, Bangladesh (23.8041° N, 90.4152° E). The study locations were a part of Dhaka City Corporation. The targeted sample collection regions were mainly fish markets and street food selling areas due to the plenitude of houseflies as well as increased human interaction with the flies. Over the period of October 2022 to March 2023, the samples were obtained from the locations. As soon as the samples were collected into the net, they were placed in a sterilized zip lock bag and kept in an icebox before reaching the laboratory. To avoid any sort of contamination between the flies, they were kept in separate zip lock bags. Within 1 hour, the samples are transported to the microbiology laboratory of BRAC University, Dhaka, Bangladesh. The samples were then stored in a -20^0 C refrigerator until further processing. The sample house flies were visualized by observing the shape, size, and color for morphological identification to confirm that they were indeed *Musca domestica* species. Using disinfected forceps, each of the houseflies was then collected separately inside a laminar airflow and placed in an autoclaved sterile falcon tube containing 10 ml of PBS (Phosphate-buffered saline) solution. After that, the vortex mixer machine is used to blend the sample with the buffered solution to extract the bacteria from the body of the flies. The falcon tubes were then incubated at 37^0 C for 6-8 hours to ensure bacterial growth.



1. Single housefly in ziplock bag 2. Flies in PBS solution containing falcon

Figure 2.1 Sample collection and processing in PBS solution

2.2 Isolation of bacteria:

The incubated samples in PBS solution were processed further to obtain bacterial growth. Samples were used in three different ways including the raw version (sample in PBS solution), 1000 fold serial diluted (three 10 fold dilution), and 10000 fold serial diluted (four 10 fold dilution). Using various selective agar plates such as MacConkey agar, Cetrimide agar, TCBS (Thiosulfate citrate bile salts sucrose) agar, MSA (Mannitol salt agar), and XLD (Xylose lysine deoxycholate), all the samples are spread on the plates (30-50 microliter of sample) using a sterile glass spreader. These aseptic procedures were performed under a disinfected environment inside the laminar air flow. The spread plates were then kept in incubation for 24 hours at 37^0 C. The purpose of using these various media plates was to observe growth of both gram-positive and gram-negative bacteria. From these plates, presumptive identification (based on morphology of colonies) of bacteria was conducted and colonies were selected for further tests (biochemical and molecular detection) for confirmation. All the selected colonies from these selective media agar were streaked in a Nutrient Agar (NA) plate for isolation of pure bacterial culture.

The presumptive identification of bacteria for each of these plates include-

Table 2.1. Name of selective media and their presumptive identification

Agar media	Presumptive identification
Cetrimide Agar (HiMedia, Mumbai, India)	*Pseudomonas aeruginosa* display fluorescence underneath UV light
TCBS Agar (Himedia, Mumbai, India)	*Vibrio cholerae* is yellow with a dense center and glowing edges (Routh et al., 2018).
MSA agar (HiMedia, Mumbai, India)	*Staphylococcus aureus* gives golden yellow color colonies
MacConkey Agar (HiMedia, Mumbai, India)	*Klebsiella pneumoniae* is pink mucoid colony *Escherichia coli* is red or pink non-mucoid
XLD agar (Oxoid, UK)	*Salmonella typhi* gives black centered red colony *Shigella* spp has red colony

2.3 Identification of Isolates: Biochemical test:

Morphological analysis of the isolated colonies were performed for further detection of specific organisms. Various biochemical testing was completed for each of the isolates following gram-staining. The biochemical tests that are conducted for this purpose include-

Table 2.2. List of the biochemical tests

Biochemical test list
1. Catalase test
2. Oxidase test
3. Triple sugar Iron (TSI) test
4. Methyl Red (MR) test
5. Voges-Proskauer (VP) test
6. Citrate test
7. MIU test
8. Indole test
9. Gram-Staining

On the basis of these biochemical tests and gram-staining, samples are categorized into gram-positive and gram-negative bacteria. Furthermore, molecular identification is confirmed using the polymerase chain reaction (PCR).

2.4 Antibiotic susceptibility testing

Antibiotic susceptibility test is used to check the sensitivity or resistance pattern of a microorganism against certain antibiotics. It is a qualitative method that is used to determine the activity of antibiotics against bacterial infections as well as to evaluate the resistance pattern. Different methods were developed to assess antibiotic susceptibility test; for instance, broth dilution or Minimal Inhibitory Concentration (MIC) method, Kirby-Bauer disk diffusion method, Etest method or automated instrument system (Reller, L. B, & et al, 2009). However, among all these methods, the Kirby-Bauer disk diffusion method is universally used because of its easy to go procedure and not time consuming like other methods.

The Mueller-Hinton Agar (MHA) plate was used to perform a disk diffusion method by preparing bacterial suspension. Mueller-Hinton agar contains loose agar to diffuse properly and starch that inhibits the toxins produced by the bacteria so that it cannot interfere with antibiotic agents also it supports the growth of non-fastidious microorganisms (Lab Test Guide, 2023). At first, bacterial suspension in 0.9% saline solution was used by taking loopful colonies from bacterial culture. The bacterial suspension was compared with 0.5 McFarland standards to maintain 1.5 x 10^8 cells/ml. After that, the suspension was swiped from different angles on the plate using a sterile cotton swab. Then the antibiotic disks were placed on the media and incubated for 24 hours at 37ºC. When the incubation period was over, the zone of inhibition was measured in diameter by following Clinical and Laboratory Standards Institute (CLSI) guidelines. The susceptibility of antibiotics was then marked as sensitive, intermediate or resistant observing CLSI guidelines.

Table 2.4: The following chart is a list of antibiotics that were used to analysis the sensitivity or resistant activity of bacteria (HiMedia, Mumbai, India):

Antibiotic name	Antibiotic symbol	Disc content (µg)
Amikacin	AK	30
Amoxicillin	AMX	30
Amoxyclav	AMC	30
Azithromycin	AZM	15
Cefepime	CPM	30
Cefixime	CFM	5
Ceftriaxone	CTR	30
Ciprofloxacin	CIP	5
Erythromycin	E	15
Imipenem	IPM	10
Kanamycin	K	30
Meropenem	MRP	10
Tetracycline	TE	30
Vancomycin	VA	30

2.5 Molecular detection of isolates

After morphological identification, the isolates were further proceeding to molecular identification. This identification process confirms the presence of bacteria based on their presence of DNA or specific gene. For this reason, the extraction of DNA was done and then the PCR method was applied.

2.5.1 DNA extraction

To purify bacterial DNA from the collected isolates, DNA extraction by boiling method was performed. The isolates were cultured on nutrient agar plate and then after proper growth, the bacteria were inoculated in Luria broth (LB) or Luria-Bertani medium for overnight. After overnight incubation, 700 µl of broth was collected and centrifuged at 13000 rpm for 10 minutes. When centrifugation was done, supernatant was discarded and the pellet at the bottom was washed with 300 µl of phosphate-buffered saline(PBS). It was mixed by doing a vortex. Again the mixture was centrifuged for 14000 rpm for 5 minutes. Following the supernatant was discarded also 200 µl of TE buffer was added. After that, the heat shock was given at 100ºC for 15 minutes. It was given a cold shock with ice for 10 minutes immediately when the heating step was over. Next another centrifugation was followed at 14000 rpm for 5 minutes so that the precipitates were gone at the bottom. Finally the supernatant was collected and was stored at -20ºC.

2.5.2 PCR confirmation

PCR, the Polymerase Chain Reaction is a process to amplify DNA segments that means it can amplify billions of copies of target DNA by in vitro process. The whole process of PCR is continued by several steps within desirable conditions. Moreover Kadri (2019) stated that the whole PCR process can run when the PCR mixture is properly prepared. It was also added that the mixture must contain the right amount of DNA template, Taq polymerase, all dNTPs and both primers. The both primers forward and reverse primers bind with complementary DNA sequence from upstream 5'-3' sequence and complementary 3'-5' sequence respectively.

The isolates were suspected dependent on biochemical tests; but for further confirmation, PCR by specific primer were carried out. Mainly the PCR amplification was done for *Pseudomonas* spp.*, Vibrio* genus and *Klebsiella pneumoniae*. A total volume of 13µl PCR mixture was used where 6µl master mix (Thermofisher), 1µl forward and reverse primer in each, 3µl nuclease free water and 2µl template DNA was added. Here the master mix contains an adequate amount of dNTPs, $MgCl_2$ and Taq polymerase to run the reaction. After preparing the reaction mixture along with template DNA, the whole mixture was ready to run after adjusting specific conditions and cycles. This PCR product was observed by gel electrophoresis once the reaction was completed. In gel electrophoresis 1.5% agarose was used to prepare the gel by adding it with a buffer. Additionally ethidium bromide (EtBr) was used as an intercalating agent before setting the gel. The whole gel was run at 90-100 voltage for almost 40-45 minutes.

Table 2.5: The list of specific forward and reverse primers that were used:

Primer	**Primer sequence**	**PCR Condition**	**No. of cycles**	**Amplicon size**	**Reference**
PA-SS	F 5’GGGGGATCTTCGGACCTCA3’ R 5’TCCTTAGAGTGCCCACCCG3’	95ºC 2mins 94ºC 20 sec 58ºC 20 sec 72ºC 40sec 72ºC 5mins	30	956	(Spilker et al., 2004)
Vibrio genus	F 5’GTCARATTGAAAARCARTTYGGG3’ R 5’ACYTTRATRCGNGTTTCRTTRCC3’	94ºC 5mins 94ºC 30sec 60ºC 30sec 72ºC 30sec 72ºC 10mins	25	689	(Kim et al., 2015)
K. pneumoniae	F 5'-TGCAGATAATTCACGCCCAG-3' R 5'-ACCCGCTGGACGCCAT-3'	94ºC 10mins 94ºC 30sec 62ºC 45sec 72ºC 45sec 72ºC 10mins	30	133	(Dong et al., 2015)

Chapter 3:

Results

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3.1 Growth observation from sample

From the 30 housefly samples obtained from different locations, 148 isolated colonies were recovered from various media plates (MSA, XLD, TCBS, Cetrimide, MacConkey agar). The colonies were selected presumptively by observing their colony morphology and phenotypic characteristics on these selective media. Each of the media was particular for some distinct organisms that were targeted. For instance, Thiosulfate citrate bile salts sucrose (TCBS) agar is a selective media for isolating *Vibrio* species. Similarly, Cetrimide agar is also selective for *Pseudomonas* species. *Pseudomonas aeruginosa* is a fluorescence glow under Ultraviolet (UV) light that helps in determining the bacteria (Brown & Lowbury, 1965). Furthermore, colonies that had mucoid pink-yellow color, golden yellow color, black-centered red colonies were also carefully chosen from MacConkey agar (Presumptive *Klebsiella pneumoniae*), MSA agar (Presumptive *Staphylococcus aureus*), and XLD agar (Presumptive *Salmonella typhi)* respectively. (American Society for Microbiology. et al., 1957).

1.Cetrimide media 2. TCBS Media 3. MSA media

Figure 3.1 Various types of specific colonies on different selective media

The isolated bacteria from different locations was presumptive for various organisms. Mostly *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Salmonella typhi*, and *Staphylococcus aureus* were found in high numbers based on presumption. The number of isolates obtained from each location along with their presumptive identification is given in a table below.

Table 3.1 Presumptive identification of isolates based on location

Sample collection area	Sample number per location	Total isolate number	Suspected Organisms
Mohakhali	10	17	*Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Serratia marcescens, Proteus* spp.
Kawran Bazar	10	55	*Vibrio* spp. , *Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Salmonella typhi, Shigella* spp.
Mirpur	10	76	*Vibrio* spp. , *Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Salmonella typhi.*

Further biochemical tests and molecular detection was performed to identify the isolates accurately.

3.2 Biochemical analysis

The biological profiling of bacteria gives a presumptive idea about the bacteria. In this study, 10 biochemical tests, including gram staining were conducted to understand the morphology and

characteristics of the bacteria that helped in identification. Gram-staining is a vital part of the biochemical analysis as it gives the initial information on whether the isolated sample is a gram-positive or gram-negative bacteria. It also provides knowledge about bacterial shape, including cocci, bacillus, and others. In this study, the 148 isolates were gram-stained and observed under the microscope to identify the bacterial type and formation. The findings are presented in a bar graph below-

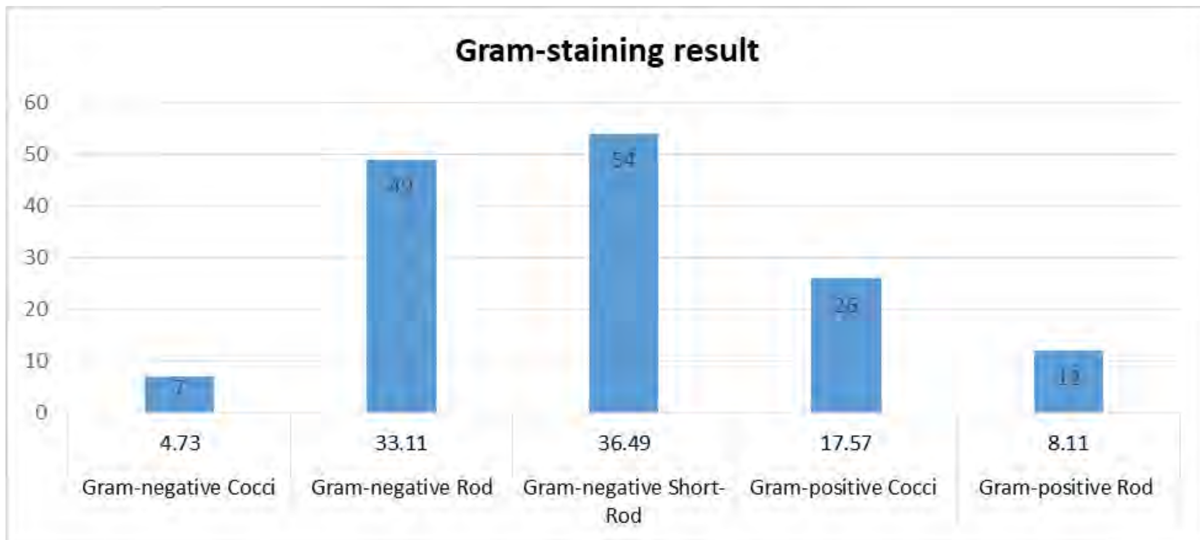

Figure 3.2.1 Bar graph representing Gram-positive and gram-negative bacteria

The bar data shows that around 74% of all isolates were gram-negative bacteria. Among them, 4.73% were gram-negative cocci, 33.11% were gram-negative rods, and 36.49% were gram-negative short rods. Similarly, the percentage of gram-positive bacteria was 25.67%. Here, the graph states that 17.57% is gram-positive cocci, which gives a presumption that the isolates can be *staphylococcus* species. Furthermore, the gram-negative bacilli can indicate the bacteria to be *Enterobacteriaceae,* such as *E.coli*, *Klebsiella pneumoniae*, etc.(Tripathi & Sapra, 2023).

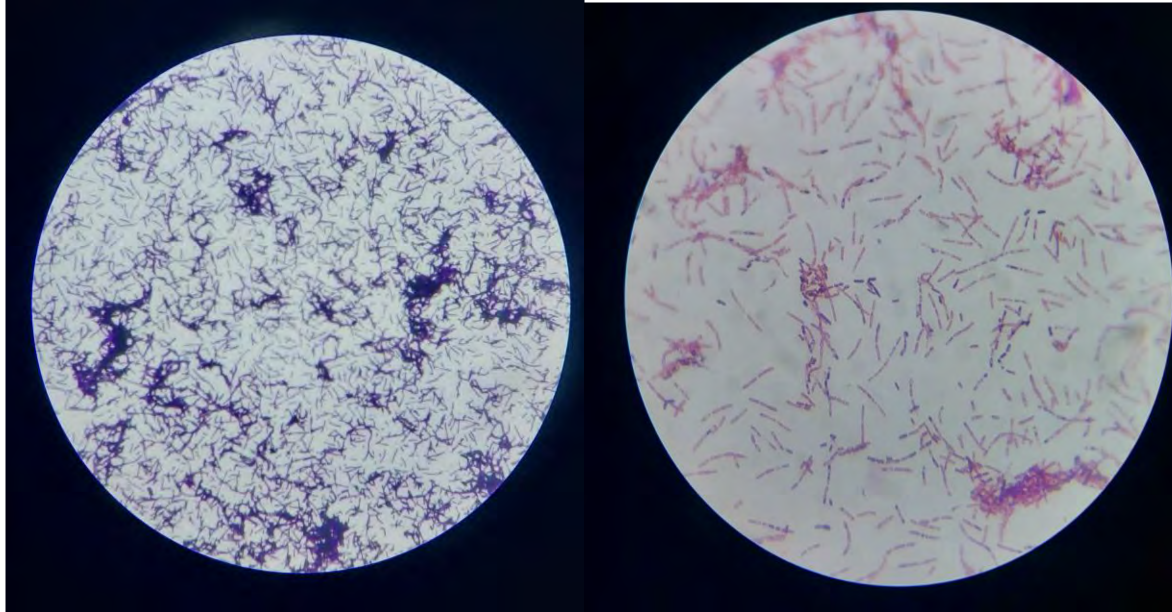

Figure 3.2.2 Gram-staining result

Followed by the gram-staining of the isolates, various biochemical tests were conducted to identify the isolates by their character and morphology. Among all the isolates, some of the results of these biochemical analysis is presented in a table below-

Table 3.2 Table of biochemical test of some of the isolates

Organism Code	Catalase Test	Oxidase test	Indole	MR test	VP test	Citrate test	MIU	TSI	Organism identification
KB5CET23	+	+	-	-	-	+	Motile; urease neg	Red butt and slant; no H2S; no gas	Pseudomonas aeroginosa
MK4MAC1R	+	-	-	-	+	+	Non-motile; urease POS (pink)	Yellow butt and slant; NO H2S; gas	Klebsiella pneumoniae
MK3MSA13	+	-	+	-	+	-	Motile; urease neg	Yellow butt and slant; NO H2S; No gas	Serratia marcescens : 100%
MK2CET13	+	+	-	-	-	+	Motile; urease neg	Red butt and slant; no H2S; no gas	Pseudomonas aeroginosa
KB7MAC15	+	-	-	-	+	+	Non-motile; urease POS(pink)	Yellow butt and slant; NO H2S; gas	Klebsiella pneumoniae
MP1TCBS13	-	+	+	-	+	-	Motile;urease POS (pink)	Red butt and slant; H2S; gas	vibrio genus
KB5CET12	+	+	-	-	-	+	Motile; urease neg	Red butt and slant; no H2S; no gas	Pseudomonas aeroginosa
MP6TCBS12	+	-	+	+	-	-	Non-motile; urease POS(pink)	Red butt and slant; H2S; no gas	vibrio genus
MP3 TCBS13	+	+	+	-	+	+	Motile; urease neg	Yellow butt and slant; NO H2S; No gas	Vibrio cholerae
MP2XLD12	+	-	-	+	-	+	Motile; urease neg	Red butt and slant; H2S; no gas	Salmonella typhi
KB7MAC35	+	-	-	-	+	+	Non-motile; urease POS(pink)	Yellow butt and slant; NO H2S; gas	Klebsiella pneumoniae
MP2XLD24	+	-	-	+	-	-	Motile; urease neg	Red butt and slant; H2S; no gas	Salmonella typhi
KB7MSA43	+	-	-	+	+	+	Non-motile; urease POS (pink)	Yellow butt and slant; NO H2S; No gas	Staphylococcus aureus
MP2XLD44	+	-	-	+	-	-	Motile; urease neg	Red butt and slant; H2S; no gas	Salmonella typhi
MP6MSA22	+	-	-	+	+	+	Non-motile; urease POS (pink)	Yellow butt and slant; NO H2S; No gas	Staphylococcus aureus
KB1MAC14	+	+	+	+	+	+	Motile; urease neg	Yellow butt and slant; NO H2S; gas	Serratia marcescens (96.15%)
KB7MSA14	+	-	-	+	+	+	Non-motile; urease POS (pink)	Yellow butt and slant; NO H2S; No gas	Staphylococcus aureus

Similarly, biochemical tests of other organisms were also conducted to give a presumptive identification of bacteria. On the basis of the biochemical testing, isolates were thought to be different organisms, including *Klebsiella pneumoniae*, *Vibrio* species, *Escherichia coli*, *Serratia marcescens, Salmonella typhi,* and others. The result of the biochemical tests were checked in reference to the biochemical characteristics of these organisms. The graph below represents the ration of the isolates-

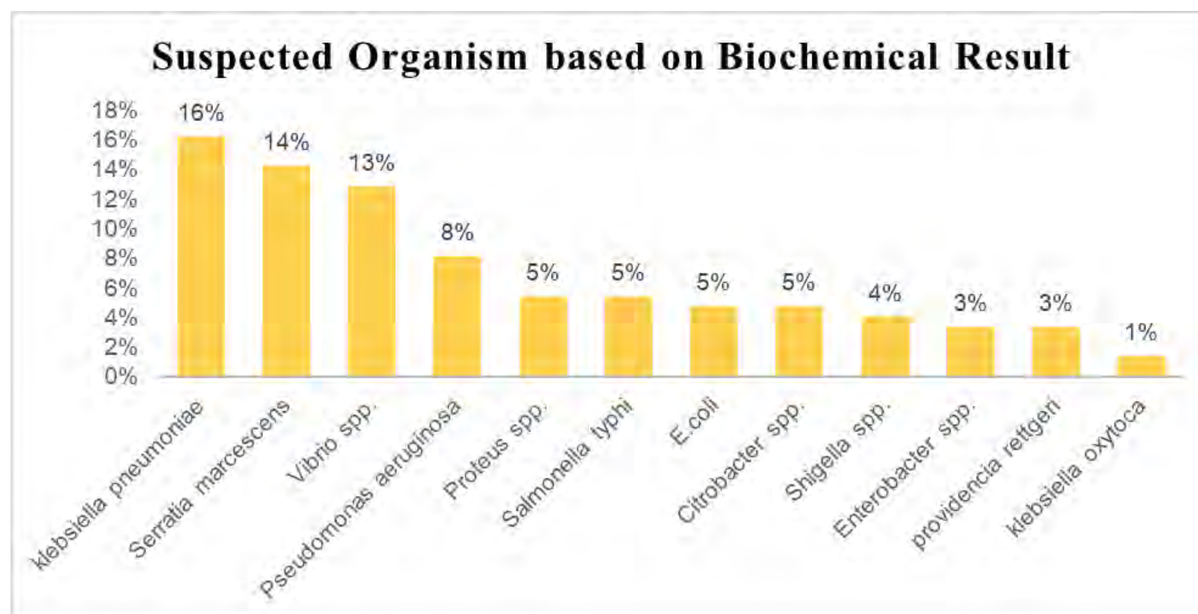

Figure 3.2.3 Graph of suspected organisms based on biochemical results

From this, it is visible that *Klebsiella pneumoniae* is predicted to be 16% from the total 148 isolates. Also around 13% of isolates are suspected to be of *Vibrio* species. However, confirmation of organisms cannot be done with molecular testing such as PCR.

1.MIU TEST 2. MR TEST 3. TSI TEST

4. Citrate Test 5. Oxidase Test

Figure 3.2.4 Some of the biochemical tests of the isolates.

Therefore, It is necessary to perform Polymerase chain reaction (PCR) to identify the isolates on the molecular level. It will allow not only genus level detection but also species identification.

3.3 Assessment of Antibiotic Susceptibility Testing

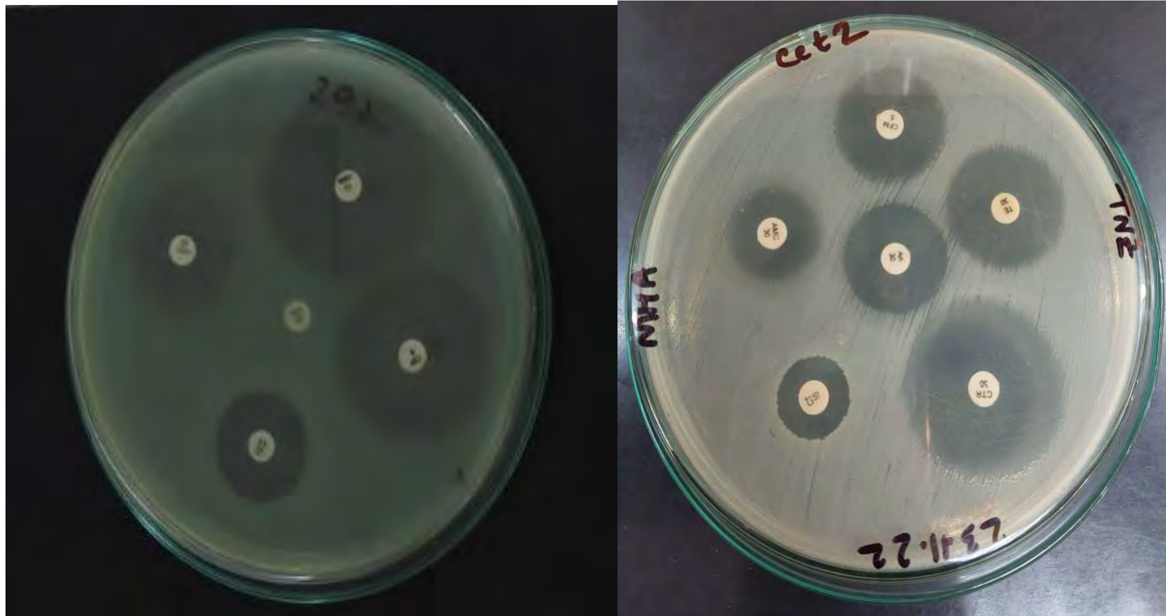

Figure 3.3.1: Kirby-Bauer Disk Diffusion method

The Antibiotic susceptibility test of all 148 isolates was conducted. Based on the graphical representation below shows almost all the isolates were resistant towards amoxicillin. Furthermore, isolates were also sensitive towards erythromycin, tetracycline, imipenem, amoxiclav and others.

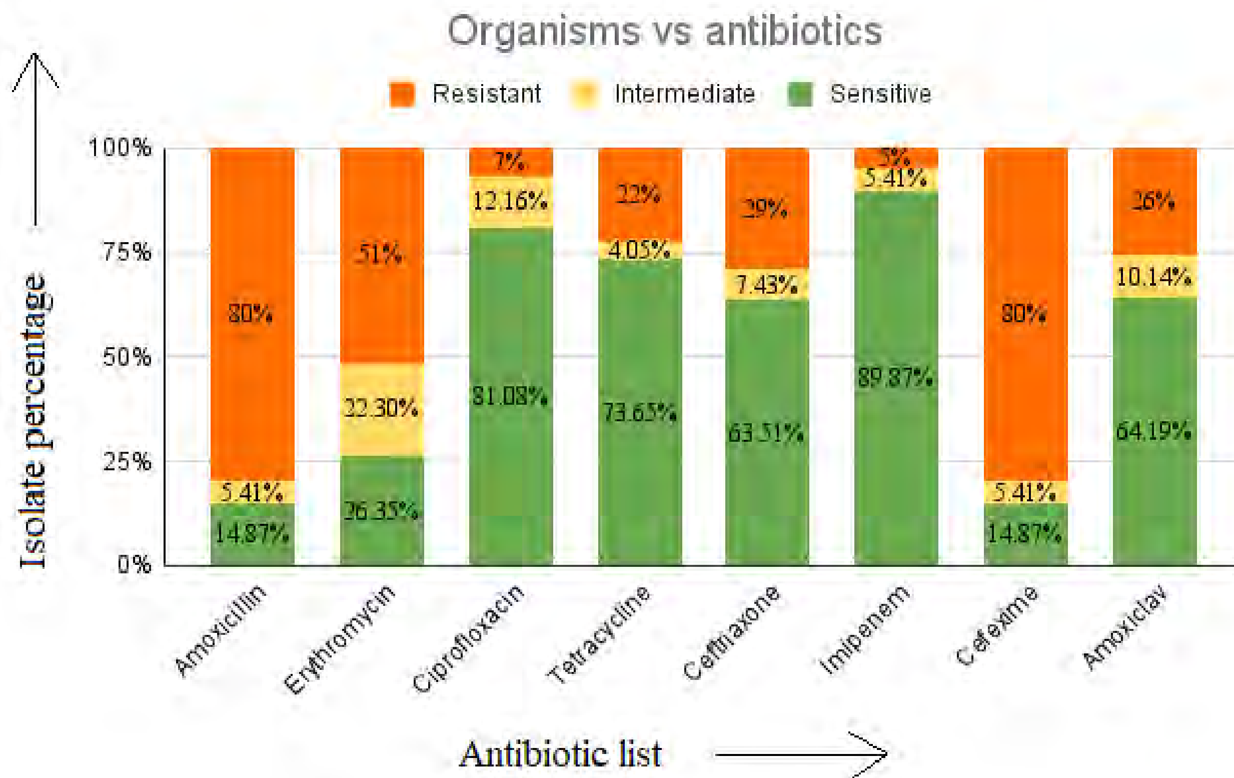

Figure 3.3.1 Antibiotic susceptibility of isolates against antibiotic

Figure 3.3.1 shows the graphical representation of all 148 isolates antibiotic susceptibility towards different antibiotics. It shows that 80 % of all isolates were resistant towards amoxicillin and cefixime. Following that, 51 percent had erythromycin resistance with 26.35% sensitivity and 22.3% intermediate zone. Furthermore, a good range of sensitivity was seen in case of ciprofloxacin, tetracycline, ceftriaxone, amoxiclav and imipenem which are 81.08%, 73.65%, 63.51% , 64.19%, and 89.87% respectively.

Among all the collected isolates from all the sample sites, 38 isolates were positive bacteria according to their morphology. Generally vancomycin (30µg) is used against gram positive bacteria. After performing an antibiotic susceptibility test, vancomycin showed more sensitivity against the gram positive organisms rather than being resistant nevertheless a very small amount of isolates showed intermediate.

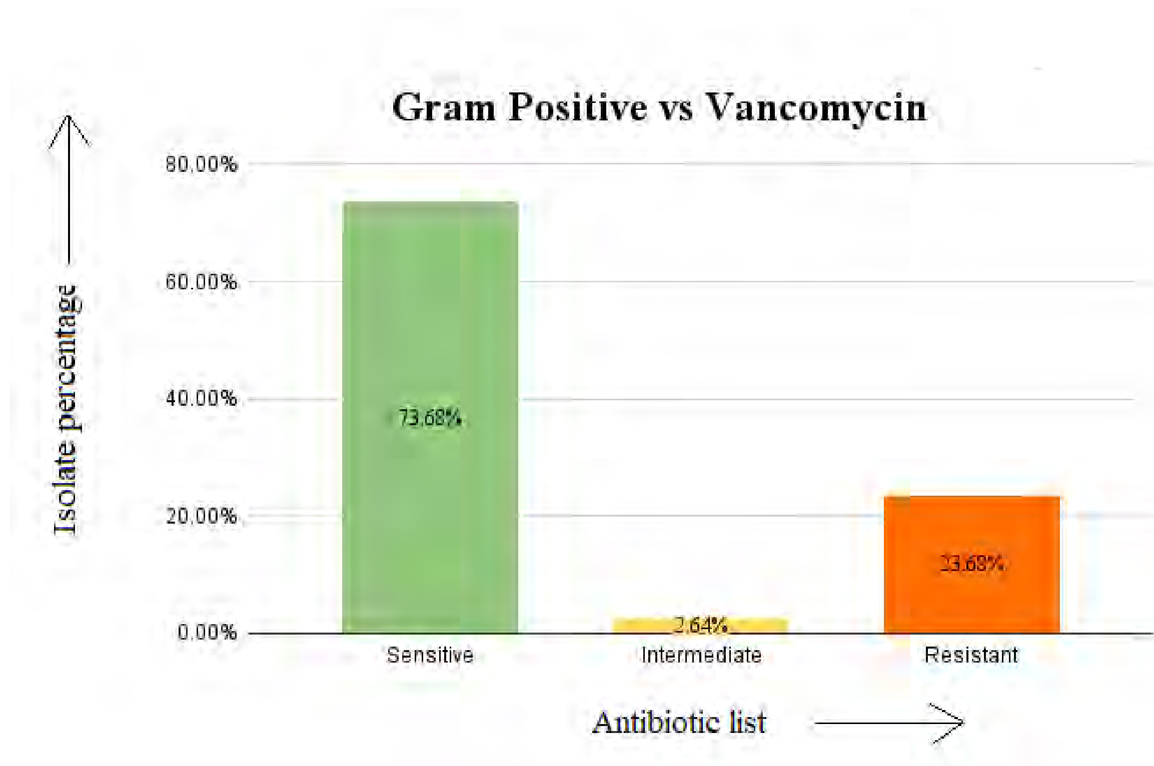

Figure 3.3.2: Vancomycin activity against Gram Positive bacteria

Figure 3.3.2 shows the susceptibility of vancomycin against almost all isolates. To elaborate, vancomycin showed almost 73.68% sensitivity to most of the organisms and 10.53% resistance which is much lesser than sensitivity whereas only 2.64% were intermediate in this case.

The following graph represents an overview of using antibiotics against *Pseudomonas aeruginosa*.This graph elaborates the present situation of antibiotics' activity against this gram negative bacterium. At first, *P. aeruginosa* was mostly resistant to Amoxicillin, Erythromycin and Cefixime. Moreover it was completely resistant to Vancomycin as this antibiotic is mainly used against gram positive organisms. Secondly, it showed a positive result in case of Imepenem, Ciprofloxacine, Tetracycline and Kanamycin. On the contrary, it was equally sensitive and resistant to Amoxiclav with a little portion for intermediate.

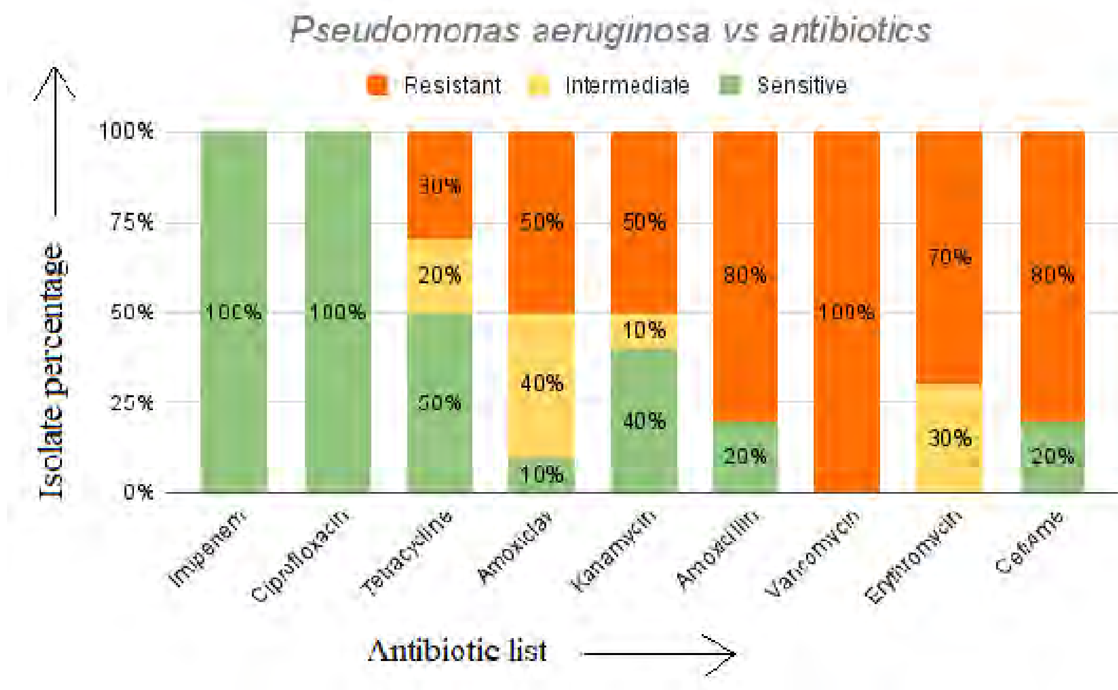

Figure 3.3.4: Antibiotic susceptibility test ratio of *Pseudomonas aeruginosa*

Figure 3.3.3 focuses on the susceptibility condition of antibiotics against *P. aeruginosa*. Firstly it was highly resistant to Amoxicillin and Cefixime which was 80% with no intermediate phase. Additionally Erythromycin was also shown 70% resistant by this organism but no sensitivity rather 30% intermediate.

On the contrary, *P.aeruginosa* showed susceptible activity (100%) towards both Imepenem and Ciprofloxacin without any resistance or intermediate pattern. Further it showed a good sensitivity zone in the case of Tetracycline and Kanamycin which was 50% and 40% respectively. However Amoxiclav showed an opposite pattern of kanamycin while showing sensitivity and intermediate zone which 10% and 40% consecutively.

The next graph represents an overview of antibiotics against *Klebsiella pneumoniae*. Firstly it indicates maximum resistance towards amoxicillin and erythromycin. On the other hand, it presents the highest sensitivity of imipenem and amoxiclav. However among all the antibiotics only amoxicillin shows little portion for intermediate phase.

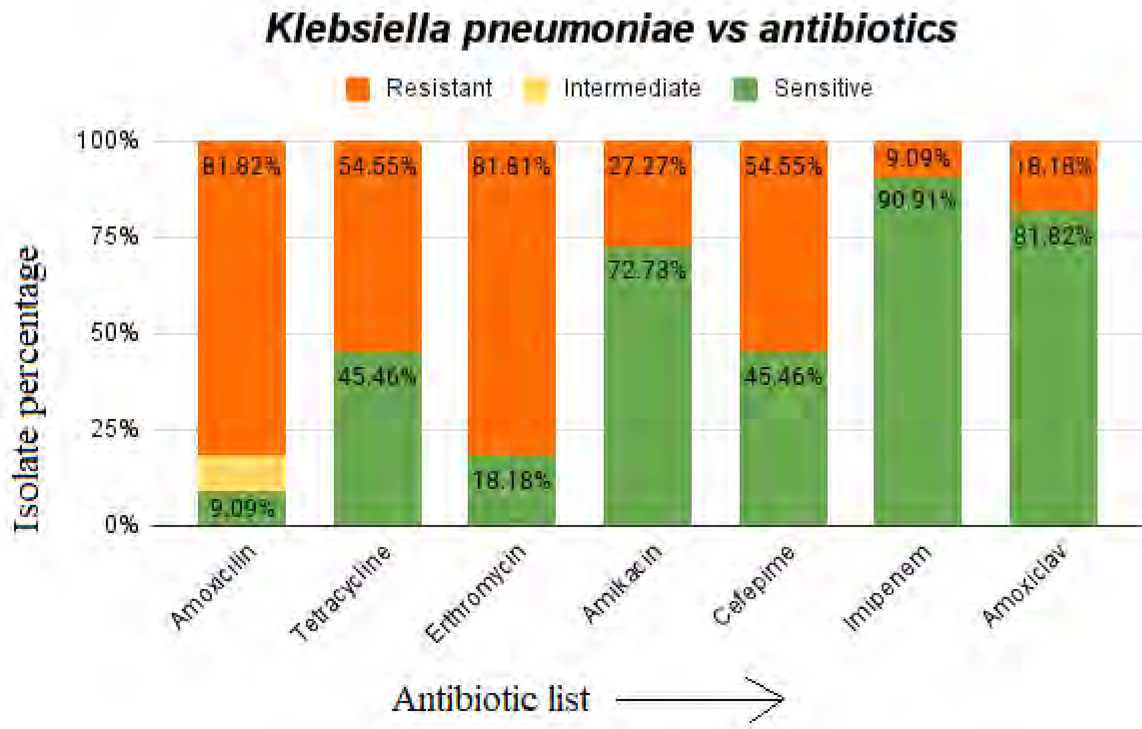

Figure 3.3.5: Antibiotics susceptibility test ratio by *Klebsiella pneumoniae*

Figure 3.3.5 shows almost 81% resistance of amoxicillin and erythromycin by *Klebsiella pneumoniae.* In case of amoxicillin, it contains a 9.09% intermediate and sensitivity ratio. Moreover, in imipenem and amoxiclav, it indicates 90.91% and 81.82% sensitivity respectively.

Additionally, bacteria show equal amounts of resistance and sensitivity without any intermediate zone in both tetracycline and cefepime which is 54.55% and 45.46% consecutively. Besides, in amoxiclav, isolates show 81.82% sensitivity and 18.18%

resistance in total. However all the antibiotics contain several amounts of both sensitive and resistance zone without any intermediate zone.

The following chart overviews data on antibiotic susceptibility tests by Vibrio. Among the isolated Vibrio, it shows maximum resistance for amoxicillin. Then it comes to erythromycin to give the second highest resistance. In contrast, imipenem, ciprofloxacin, amikacin, tetracycline, amikacin and amoxiclav give higher sensitivity than resistance. In contrast, all isolates contain intermediate zones except amoxicillin and amikacin.

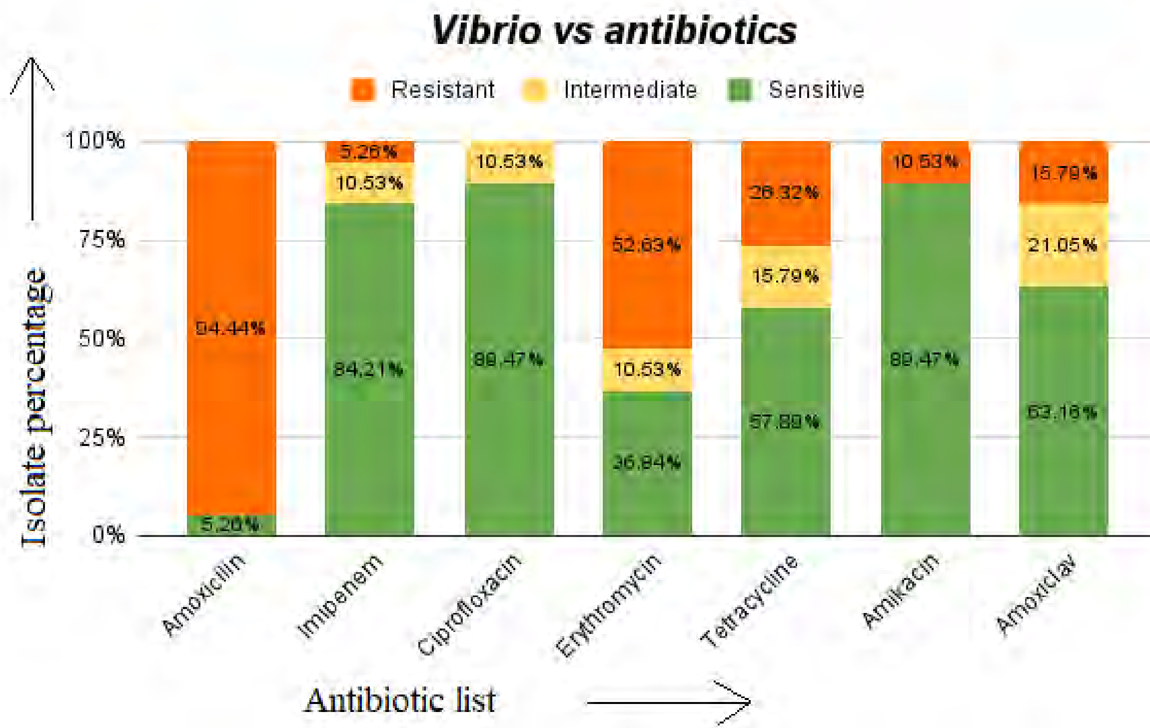

Figure 3.3.6 Antibiotic susceptibility test ratio by *Vibrio*

Figure 3.3.6 indicates the resistance and sensitivity condition by *Vibrio*. First of all, it gives

94.44% resistance by almost all organisms with a sensitive result of 5.26% for amoxicillin. Secondly, 52.63% resistance towards erythromycin was seen whereas it has 36.84% sensitivity and 10.53% intermediate.

Next it comes to sensitivity outcomes by the organism. In this case,both amikacin and ciprofloxacin have the same sensitivity zone by the bacteria which is 89.47%. Then 84.21% sensitivity is shown by the bacteria for imipenem. After that tetracycline and amoxiclav indicate a good portion of sensitivity with both resistance and intermediate. However in both ciprofloxacin and amikacin, bacteria shows same sensitivity (89.47%) but in ciprofloxacin, it contains no resistant zone but intermediate whereas amikacin has a resistance portion.

3.4 Bacterial prevalence based on molecular detection

Morphological identification was done based on several biochemical tests including gram staining. To have the confirmed identification of the isolated colonies using their DNA or specific gene sequence, PCR method was used. In this method, the reaction was run under certain conditions to check the exact base pair size of the suspected isolates compared with the given primer sets.

From the isolates, almost 12 isolates were suspected as *Pseudomonas spp*. For the final confirmation of having *Pseudomonas aeruginosa*, the PCR was run directly using PA-SS primer sets with annealing temperature at 58ºC for 30 cycles. When the PCR reaction was completed, the products were visualized under UV light after gel electrophoresis. The gel electrophoresis must be run using a DNA ladder to compare the base pair size of the products. The expected band size for *Pseudomonas aeruginosa* is 956 bp.

In the figure, 12 suspected isolates were given for PCR reaction then gel electrophoresis. But the observed band size at 956bp was 11 isolates. Hence it confirmed the presence of *Pseudomonas aeruginosa* among the total suspected isolates.

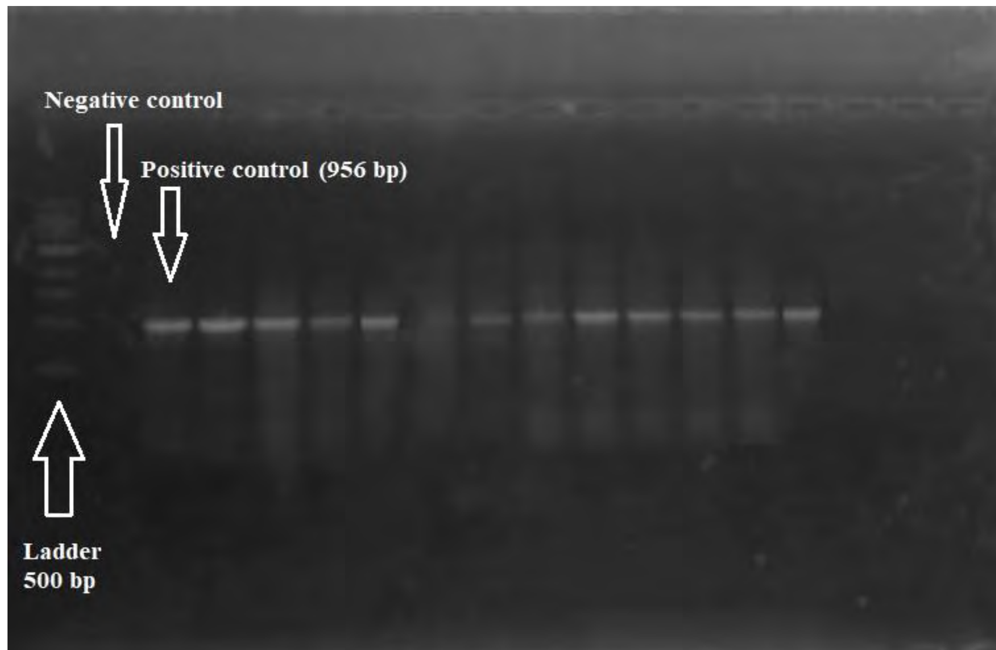

Figure 3.4.1: Gel electrophoresis result of PA-SS

Figure 3.4.1 shows the result after gel electrophoresis for *Pseudomonas aeruginosa*. The first line was a 500bp ladder (500, 1000, 1500 ... .bp) to confirm the size of DNA fragments. The next line is negative control, positive control and the PCR products were given serially. Here the shining fragments were indicating the similarity of 956bp for *Pseudomonas aeruginosa* that was confirmed as a positive result.

During the sampling process, TCBS agar plate was used to detect *Vibrio* genus. Among all the isolates, 19 isolates were suspected as *Vibrio* genus. During the PCR reaction, the annealing temperature was maintained at 60ºC for 25 cycles. The volume of the mixture was 13µl and *Vibrio* genus primer sets were mixed in it. The main purpose of this reaction was to have amplicon size at 689bp. This band size confirms the presence of *Vibrio* genus in the sample.

Gel electrophoresis was performed at 100v for 45 minutes. In addition, a 1kb ladder along with controls were used during the gel run. After the gel run, almost 7 positive bands were observed under UV light among 19 suspected. The observed bands for 7 isolates were in equal line to positive control which was 689bp. As a result, it was confirmed that those isolates were among the *Vibrio* genus.

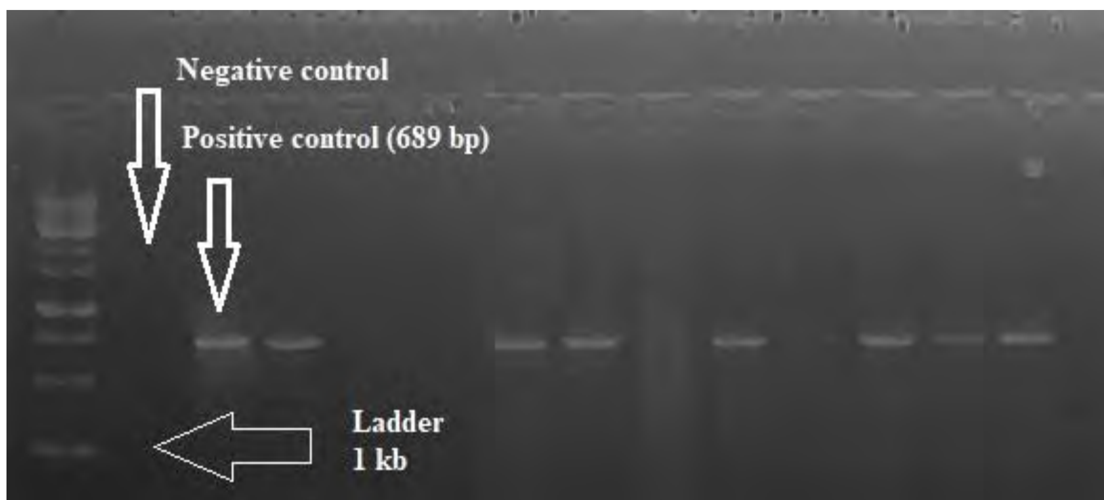

Figure 3.4.2: Gel electrophoresis result of *Vibrio* genus

Figure shows the gel run result of *Vibrio* genus. In the first row, 1kb (250, 500, 750, 1000…bp) DNA ladder was used and negative control as well as positive control was given respectively. In this case, the positive band size was showing a single band alongside with 689bp of ladder and positive control; therefore confirmation of presence of *Vibrio* genus.

The isolation of *Klebsiella pneumoniae* was given for PCR to assure the confirmation of this bacteria using *K. pneumoniae* primer sets. The annealing temperature of this was 62ºC for 30 cycles. The expected base pair size for this specific primer sequence was 133. The mixture volume was 15µl which included 0.5µl forward and reverse primers in each.

After the gel ran in 100V with 1.5% agarose in it, the gel was visualized under UV. However it showed some positive band size according to ladder and positive control. Though it contained 27 suspected isolates, 11 isolates confirmed the band size of 133bp. Therefore these 11 positive band size isolates had confirmed identification of *Klebsiella pneumoniae*.

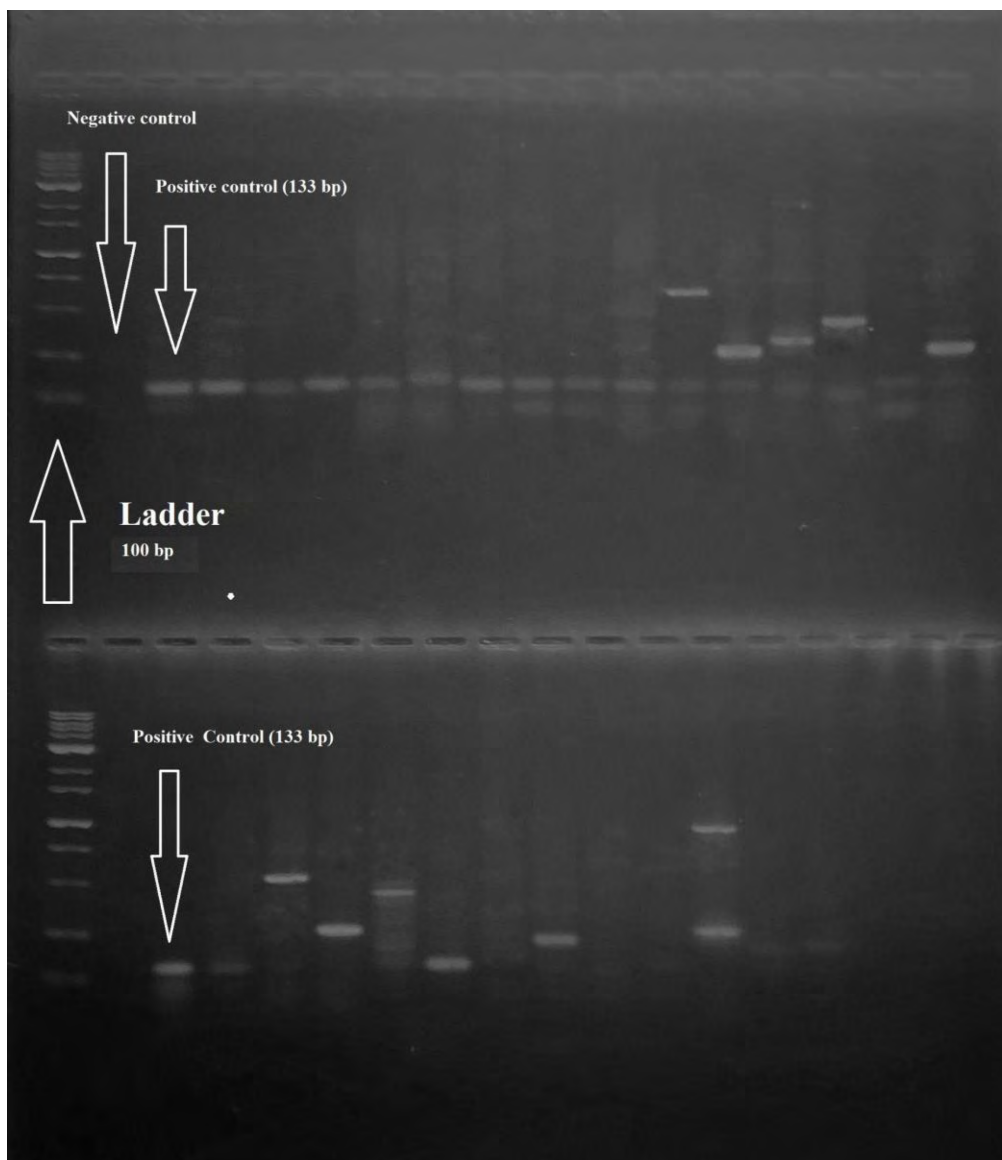

Figure 3.4.3: Gel electrophoresis result of *Klebsiella pneumoniae*

Figure shows the band size of *Klebsiella pneumoniae* by gel electrophoresis. In this gel, 100bp DNA ladder (100, 200, 300…bp), negative control and positive control was used consecutively. As the expected band size was 133bp, some fragments showed band size gradually in the first half and other bands were seen in the second half. Consequently, it confirmed the positive result of *Klebsiella pneumoniae* identification.

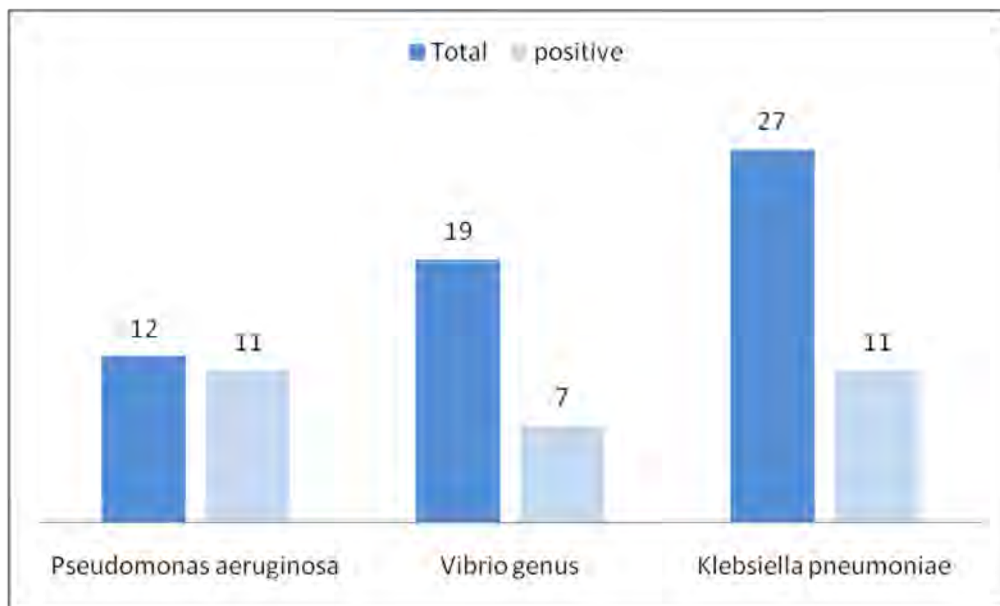

Figure 3.4.4: Overview of PCR confirmed isolates

The above graphical presentation gives an overall idea on confirmed molecular identification of *Pseudomonas aeruginosa*, *Vibrio* genus and *Klebsiella pneumoniae* among the suspected isolates.

Chapter 4:

Discussion

It is not uncommon for a housefly (*Musca domestica*) to carry various microorganisms and spread them in the environment. However, the issue can be a potential risk if the organism or bacteria is a harmful pathogen. It might get even worse if the pathogen turns out to be an antibiotic-resistant one or even a multidrug-resistant one. The primary concern is to identify which bacteria is being transmitted by the fly and how threatening can be.

It is established that houseflies are capable of carrying at least one type of bacteria (Nazari et al., 2017). This statement is comparable with the findings of this study. All sample flies tested in this study had various bacterial growth on different media. Houseflies can carry pathogenic bacteria both internally and externally. Externally pathogens are transmitted through their body surface areas, such as the mouth, wings, legs, and other body parts. Internally pathogens develop in the gut and transmit during feeding or regurgitating. Mostly, flies are habitat on animal feces, decaying substances, or manure, they take up pathogens from the surface which then reproduce on the fly's gut.(Van Gompel & Van Bortel, 2008). It is also found that the amount of pathogenic bacteria discovered on the body of the fly is adequate to spread the disease to another host (Khamesipour et al., 2018). The flies thus serve as a mechanical vector to carry and spread numerous disease-causing pathogens such as *Salmonella typhi*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Serratia marcescens*, *Proteus* spp., and *others*. (Parvez et al., 2016; Ranjbar et al., 2016; Sobur, Hasan, et al., 2019). This finding is in line with the study as the presence of different types of bacteria was observed based on both biochemical results as well as Polymerase chain reaction (PCR). Some of the bacteria that were found and also predicted in this research were *K.pneumoniae* (7.43%), *Pseudomonas aeruginosa* (7.43%), *Serratia marcescens* (14%), *Proteus spp.* (5%), and others. These pathogens are known to cause various enteric and nosocomial diseases in humans. From a canteen in Bangladesh, various pathogenic bacteria were obtained from flies, including *Salmonella typhi*, *Shigella*, *E.coli*, *Klebsiella pneumonia*e, and others (Parvez et al., 2016). Their study area mainly focused on the kitchen of the dormitories after preparing the food when the waste from food was in high quantity. It was also their finding that opportunistic pathogens such as *Providencia*, *Enterobacter*, *S.marcescens*, and others were found in these places. Similarly, in the current research, fish markets, as well as street food areas, had a high number of bacterial presence as decaying items allowed flies to carry and spread the pathogens.

Bacterial identification has both conventional and molecular detection methods. Though molecular detection such as PCR (Polymerase Chain Reaction) is a unique method, it gives the accurate identification of an isolate. In the article studied in Mymensingh, Bangladesh; 140 houseflies were collected and among them 78.6% *Staphylococcus aureus*, 66.4% *Salmonella* spp and also 51.4% *Escherichia coli* were found (Akter et al., 2020). The author confirmed that all these isolates were detected through the PCR method based on their DNA band size. Further bacterial presence was observed not only in adult houseflies but also in different stages of houseflies which includes *Schineria* and *Bacteroides* from maggots, *Neisseria* from pupae along with *Lactococcus* and *Macrococcus* in adult stage (Wei et al., 2012). In our study till now, a total 148 isolates were collected which was further given for PCR confirmation. After molecular identification, it was confirmed that *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Vibrio* genus were present in housefly samples. In another study in Libya, a great number of pathogenic isolates were found that includes 42% *Escherichia coli*, 70% *Klebsiella* spp, 2% *Aeromonas* spp from the hospital site. Moreover, street samples included 36% *Pseudomonas* spp, 12% *Staphylococcus* spp and 22% *Streptococcus* spp. (Rahuma et al., 2005). Likewise, this study on common houseflies (Musca domestica) in Dhaka city confirmed the presence in adequate ratio of bacteria. To be specific, *Pseudomonas aeruginosa* was 7.43% in total, *Vibrio* genus was 4.73%, *Klebsiella pneumoniae* was 7.43% and others. In the previous studies, authors worked with a bulk amount of houseflies samples whereas we did with 30 samples till now. Also some more PCR confirmation will be needed to identify other isolates also to overlook their resistance ability on specific antibiotics.

The antibiotic susceptibility result showed different patterns including sensitive, resistance or intermediate for different isolates. The collected isolates were from different sites in Dhaka city. Recently antibiotic resistance has become a serious issue in public health concern, so several antibiotics were tested with regards to all isolates to check the present activity of those antibiotics. In this study, isolates were divided based on their cellular morphology like gram positive (25.67%) and gram negative bacteria (74.33%). These isolates were tested for susceptibility of antibiotics depending on antibiotic groups and activity. Now-a-days penicillin, amoxicillin, tetracycline and erythromycin are most common antibiotics in the health sector but

these antibiotics are showing a great range of resistance (Akter et al., 2020). In our study, erythromycin and tetracycline showed a good range of resistance which are 58.18% and 28.18% respectively. But amoxicillin showed the most resistance than other antibiotics, which was almost 90%. To note that bacteria has become most resistant to cefixime and amoxicillin which was also retrieved in our study; whereas ciprofloxacin, chloramphenicol and gentamicin are still sensitive enough to treat diseases (Parvez et al., 2016). Moreover a study in Nigeria, *Pseudomonas aeruginosa*, *Salmonella* spp and *Proteus* spp were found from flies in a greater number also all these bacteria showed amoxicillin resistance (Odetoyin et al., 2020). Alongside, the study also revealed that ciprofloxacin was less resistant when it was tested against *Klebsiella pneumoniae* and *Staphylococcus aureus*. In our study we also observed that 94.55% of organisms showed sensitivity towards ciprofloxacin which was more accepted from previous articles. In a study in Nigeria, *Pseudomonas aeruginosa*, *Salmonella* spp and *Proteus* spp were found from flies in a greater number also all these bacteria showed amoxicillin resistance (Odetoyin et al., 2020). Alongside, the study also revealed that ciprofloxacin was less resistant when it was tested against *Klebsiella pneumoniae* and *Staphylococcus aureus*. During our study on antimicrobial resistance of houseflies, *Pseudomonas aeruginosa* showed varieties of zones which lead to sensitivity, intermediate or resistance. To illustrate, it shows 100% sensitivity towards ciprofloxacin and imepenem which was certain information according to previous literature reviews. On the other hand, it was mostly resistant to amoxicillin, cefixime along with erythromycin which is an alarming sign as stated by previous authors.Houseflies carry pathogenic bacteria while flying here and there and also can transmit to other places while sitting on the surface (Yin et al., 2022). The author also claimed that flies spread resistance genes while transmitting the bacteria, thus it plays as a vector to transmit bacteria as well as antimicrobial resistance genes through their external or internal body parts.Houseflies produce an early antimicrobial compound in their larvae phase to protect them in the environment and this compound can be an initial way to increase antimicrobial resistance issue by houseflies (Niode, 2022). However the isolates did show highly resistant to some specific antibiotics, so it can be said that they may contain some resistance gene which has high possibility to be transferred through houseflies (*Musca domestica*).

Conclusion

This study mainly interprets the possible microorganism's transfers via common house fly (*Musca domestica*). Recently houseflies are considered as potential carriers to transmit microorganisms either pathogenic or non-pathogenic through their internal or external body parts. Large amounts of microorganisms can spread through house flies which may carry resistance or virulence genes.

From this study, the confirmed isolates were *Pseudomonas aeruginosa*, *Vibrio* genus and *Klebsiella pneumoniae* by using molecular detection method PCR from different samples. In addition, a presumptive identification was made based on morphology and biochemical tests. For instance, *Vibrio cholarae*, *Staphylococcus aureus*, *Salmonella* spp. and *Serratia marcescens.* Moreover some of the isolates had shown antibiotic susceptibility where antibiotic resistance was also observed. As this is an ongoing project and some isolates are still unidentified, further molecular analysis will be needed for the confirmation of other isolates and also to understand the mechanism of resistance genes.

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