ANALYSIS OF BACTERIAL DIVERSITY FOUND IN DUST FILTER OF AIR-CONDITIONER IN HOSPITALS AND RESIDENTIAL BUILDINGS IN BANGLADESH

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

Mathematics and Natural Science

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing our 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. We have acknowledged all main sources of help.

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Abstract

Air conditioner (AC) is frequently used in hotter climates to stay cool indoors nowadays. However, Microbial contamination of air conditioners has emerged a public health concern owing to its increasing application in almost all building settings ranging from households, office to hospitals. Although the rising use of AC imposes risks, there is no comprehensive study conducted to evaluate bacterial profiles in these settings in Bangladesh. This research addressed the gap of limited data available on bacterial diversity of air conditioners and explored the prevalence of different microbial species present in the AC of hospitals and households adjacent to the respective hospitals in Bangladesh. The study was conducted by collecting swabs from the dust filter surface from hospitals and hospital adjacent households to compare the bacterial profiles between two different air conditioners of the same locality, observing higher bacterial levels in hospital, finding higher bacterial Colony Forming Unit (CFU) (approximately 10^5 in household and 10^9 in Hospital) and Extensive drug resistance in both hospital and household samples. Bacterial abundance was measured using CFU counts on Nutrient Agar followed by assessment on UTI selective Agar Bacterial identity was confirmed by MALDI-TOF, and evaluated risk through antimicrobial susceptibility testing (AST) revealed the antibiotic resistance profiles of respective bacterial species and potential risk associated with this. Among eight bacterial genera identified, *Pseudomonas* (50%), *Acinetobacter* (37.5%), *Staphylococcus* and *Enterobacter* (25%) were most. Moreover, in hospital AC, *Pseudomonas, Acinetobacter, Enterococcus, Staphylococcus* were predominant while in household AC *Enterobacter, Acinetobacter, Pseudomonas* genera are found to be significant. Bacteria identified from hospitals were found to differ significantly from households both in their abundance and diversity as well as Antibiotic Resistance Profiles. For instance, all bacteria isolated from household were Gram-negative, while those from hospitals were a mixed population of Gram-positive (42%) and Gram-negative (58%) bacteria. Moreover, 85% of bacteria in household are found to be extensively drug resistant and 100% of bacteria were extensively drug resistant in hospital AC. Among them, *Enterobacter cloacae* showed resistance to all antibiotics tested on AST panel. This study is expected to be the first of its kind in our country to annotate the microbial diversity in air conditioners, probable transmission and risk factors and finally help in policy making for increasing awareness and take necessary steps to prevent potential health hazards and suggest identification methods in future.

Keywords: Air Conditioner, Bacterial Diversity, Hospital in Bangladesh, Antimicrobial Resistance, MALDI-TOF.

Acknowledgement

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List of Acronyms

AST	Antibiotic Susceptibility test
AC	Air Conditioner
AMR	Antimicrobial Resistant
HAI	Healthcare-Associated Infection
HCCA	alpha-cyano-4-hydroxycinnamic acid
NI	Nosocomial Infection
NGS	Next Generation Sequencing
MDR	Multidrug resistance
MH	Mymensingh Medical Hospital
MB	Mymensingh Building (Nearby Residential household)
RTI	Respiratory Tract Infection
UTI	Urinary Tract Infection
UH	Uttara Adhunik Hospital
UB	Uttara Building (Nearby Residential household)
WHO	World Health Organization
XDR	Extensively drug resistant

Chapter 1

Introduction

In this modern time, people spend most of their time indoors. On one hand, Air conditioning has become a part of daily lives for people living in warm climates to replace the hot indoor air with cooler air. On the other hand, Contamination of Indoor air through Microorganisms progressively becoming a public health concern since Microorganisms are a major source of poor air quality. In a tropical country like Bangladesh, with the increasing efficiency and decreasing price of air conditioners, use and demands are also commensurately on the rise. Although there's been several efforts worldwide to determine the microbial profile in air conditioners and characterize them, Bangladesh is yet to have a comprehensive study to reveal a practical scenario describing the microbial abundance, diversity and risks associated with them.

1.1 Literature Review

Air conditioner is a device that cools and dehumidifies indoor air to provide a comfortable living environment. It works by circulating refrigerant through a system of coils, absorbing heat from the air inside and releasing it outside While operating, bacteria can circulate through air-conditioned air by being present in dust, debris and moist collected in the system's filters and ducts. Keeping this in mind, several studies have been performed worldwide. Anas et al. (2016) for instance, conducted research in which they used cotton swabs for the AC filter from different laboratories of their institutes and found several *Bacillus, Providencia and Aspergillus spp*. after incubating for several days. Khalfallah et al., 2016 had also observed *Staphylococcus aureus, Micrococcus spp., Flavobacterium spp., Bacillus spp., Klebsiella pneumonia, Pseudomonas spp.* according to their morphological characteristics from the cooling coil and drained water of the AC of Brack and Two March Hospital of Libya. However, Watanabe et al., 2022 had taken molecular-based diagnosis by taking cotton swab from the AC air filter, cooling coil, air inlet, air outlet, fan and inner wall of 17 residential buildings and had found highest relative abundance of *Proteobacteria, Firmicutes, Acinetobacter, Cyanobacteria,* and Bacteroidetes according to their Next Generation Sequencing (NGS) result. Hatayama et al., 2017 had also taken a molecular NGS approach where they had collected samples from air filters and evaporators (heat exchanger fins) and detected *Methyl bacterium* genus and *Sphingomonadaceae* family from the evaporator and *Enterobacteriaceae* from the filter. Moreover, Bakker et al., 2019 had investigated microbial ecology through molecular analysis

(PCR) by using polyurethane foam swabs of AC filters and cooling coils from 10 commercial buildings of the USA in different climates and found a diverse ecosystem of microbes including most of the bacteria mentioned above.

1.2 Healthcare Associated Infection

Healthcare Associated Infection (HAI) or Nosocomial infections (NI) are those infections patients primarily do not have before getting healthcare. So, these infections are obtained after or while receiving it. It can be of many different types such as central line associated bloodstream infection, catheter associated urine infection, surgical site infection, respiratory infection and so on. Some common pathogens responsible for HAI are but not limited to: Methicillin-resistant *Staphylococcus Aureus* (MRSA), Vancomycin-resistant *Enterococci* (VRE), *Clostridium difficile (C. diff), Pseudomonas aeruginosa* etc. (Current HAI progress report (CDC) 2024). Since conditioned air can transmit and circulate various pathogens, patients with exposed and vulnerable skin sites through which pathogens can enter—such as those with urinary catheters, venous catheters, dialysis catheters, or surgical wounds—as well as patients inhaling this air, are a matter of medical concern.

1.3 AC and Healthcare-Associated Infections

Microbial contamination is prevalent in various components of air conditioning (AC) systems, often harboring pathogenic bacteria that pose significant health risks. The sections of the AC responsible for air circulation can act as reservoirs for these microorganisms. A study conducted by Uduman *et. al.* (2002) reported an outbreak in a special care baby unit in the UAE, linked to *Serratia marcescens* contamination among infants, including bacteremia and meningitis. Nonetheless, following a comprehensive cleaning of the AC unit, no further infections from that strain were reported for nearly five months. Despite highlighting the critical role of environmental samples in HAI, the implications of these findings and studies examining the presence of pathogens in AC systems remain limited. The potential for AC systems to serve as reservoirs and transmitters of pathogens underscores the urgent need for further investigation into microbial contamination in these environments.

1.4 AC Diagram and Function

There are several important parts of an AC that need to be described to understand the importance of performing this research and the basic functionality of AC (**Figure 1.1**).

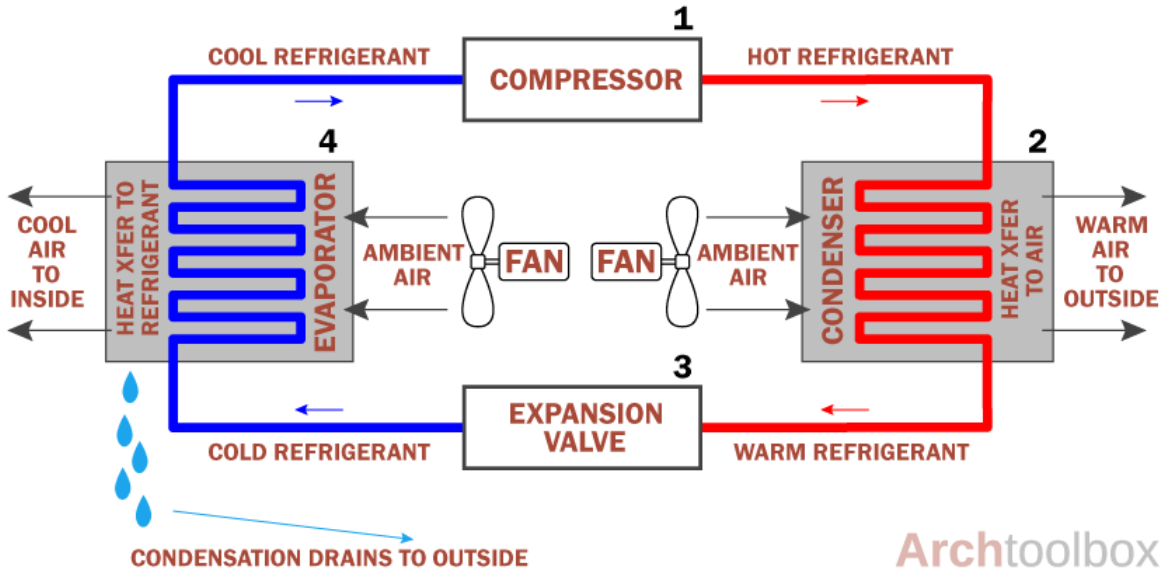


Figure 1.1: Air Conditioner Diagram. (How air conditioners work 2021)

(1) Compressor, (2) Condenser, (3) Expansion Valve (4) Evaporator. Here, the refrigerant flows through the coils to cool the indoor air through the evaporator and pass the warm air to the outdoor through the condenser.

1.4.1 Refrigerant: One of the most important components of an AC is its refrigerant. It is the chemical that constantly converts into gas-liquid-gas to absorb the heat from indoor to expel outside by circulating within the AC in a cycle of evaporation and condensation. Some common refrigerants are HCFCs (hydrochlorofluorocarbons) such as R-22 and HFCs (hydrofluorocarbons) such as R-32 (Tiwari, 2023).

1.4.2 Evaporator: An evaporator is a heat exchanger that receives heat from indoor by the use of liquid refrigerant. Here Cold liquid refrigerant receives heat from the room and transforms into gas. The metals used to construct evaporators are usually copper or aluminum coils through which refrigerant flows, because they have excellent heat conduction properties (Tiwari, 2023).

1.4.3 Compressor: Compressor receives cool refrigerant gas from evaporator and compresses it into a high-pressure and high-temperature state. The compressor works as a pump that takes

refrigerant gas from the evaporator located inside the room, compressing it to increase both pressure and temperature, then transferring the high-pressure and high-temperature gas to the condenser located outside the room (Tiwari, 2023).

1.4.4 Condenser: A condenser is another heat exchanger like the evaporator, but it receives heat from indoor as high-pressure and high-temperature gas. It earns its name by being the site where hot gas undergoes condensation into a liquid. Here the high-pressure and high-temperature gas enters into the condenser coil where a fan surrounding it blows air to cool down the refrigerant gas and transform it into liquid. By this method, hot gas converts into warm liquid to release the heat it absorbed from the room. The metals used to construct condensers are also copper or aluminum coils through which refrigerant flows (Tiwari, 2023).

1.4.5 Expansion Valve: An expansion valve is one of the main components of an air conditioner because of two reasons. First it regulates how much refrigerant will flow toward the evaporator to maintain the desired temperature. Second it converts high-pressure warm liquid coming from the condenser into low-pressure and low-temperature liquid. Then the low-pressure and low-temperature liquid goes to the evaporator where the cycle repeats until the desired room temperature is established (Tiwari, 2023).

1.4.6 Dust Filter: The main function of the Dust filter is to prevent dirty air from reaching different parts of the AC to maintain the functionality of this machine. However, it does not clean the air that enters into or exits from the AC by entirely removing delicate air particles, allergens and microbes. Therefore, heavy dusts containing undesirable air particles like infectants and allergens get deposited in the dust filter and require a regular scheduled cleaning program.

1.5 Sampling Site

In our research, dust filter was chosen as a sampling site because AC doesn’t generate cooled air on its own, rather, it only moves heat from the room to outside by using some laws of physics and chemistry in a highly effective manner. Thus, the air it blows into the room through the outlet is nothing but the same air it sucks from the room through the inlet. This recirculation of air in the same room might pose a risk to the residents, which can be easily understood by observing the dust assembled in the dust filter covering evaporator coils, which is usually

cleaned routinely. Therefore, the dust filter of AC, where dust containing microbes are trapped, is chosen as the sampling site for our research to represent the circulating air quality.

1.6 Aims and Objectives of the Research

Since there is a critical link between AC and HAI, this study will address a significant gap in research as there is limited information available regarding the microbial composition of bacteria present in AC systems of hospitals and residential buildings in Bangladesh. Moreover, our research not only aims to investigate and compare the presence and abundance of pathogenic bacteria in the AC systems but also will analyze the antibiotic resistance pattern. Microbiological and molecular techniques will be employed to identify the types of bacteria present and assess their potential threat. Additionally, Antibiotic Susceptibility Test (AST) will be performed to determine the resistance pattern of the identified microbes to antibiotics. By focusing on this aspect, we aim to provide valuable insights into the potential health risks associated with these systems in both hospital and residential settings.

Our sampling strategy will reflect the typical conditions found in hospitals of Bangladesh considering residential buildings as control, ensuring that our findings are representative of the broader scenario. By employing advanced microbiological and molecular methods, we will accurately distinguish between different types of bacteria present in the air conditioning systems. Overall, our research will contribute to a better understanding of the microbial ecology of air conditioning systems in Bangladesh and provide important data for improving indoor air quality and public health standards.

Chapter 2

2. Methodology

2.1. Study Design

This study was designed as hospital and household-based case-control study.

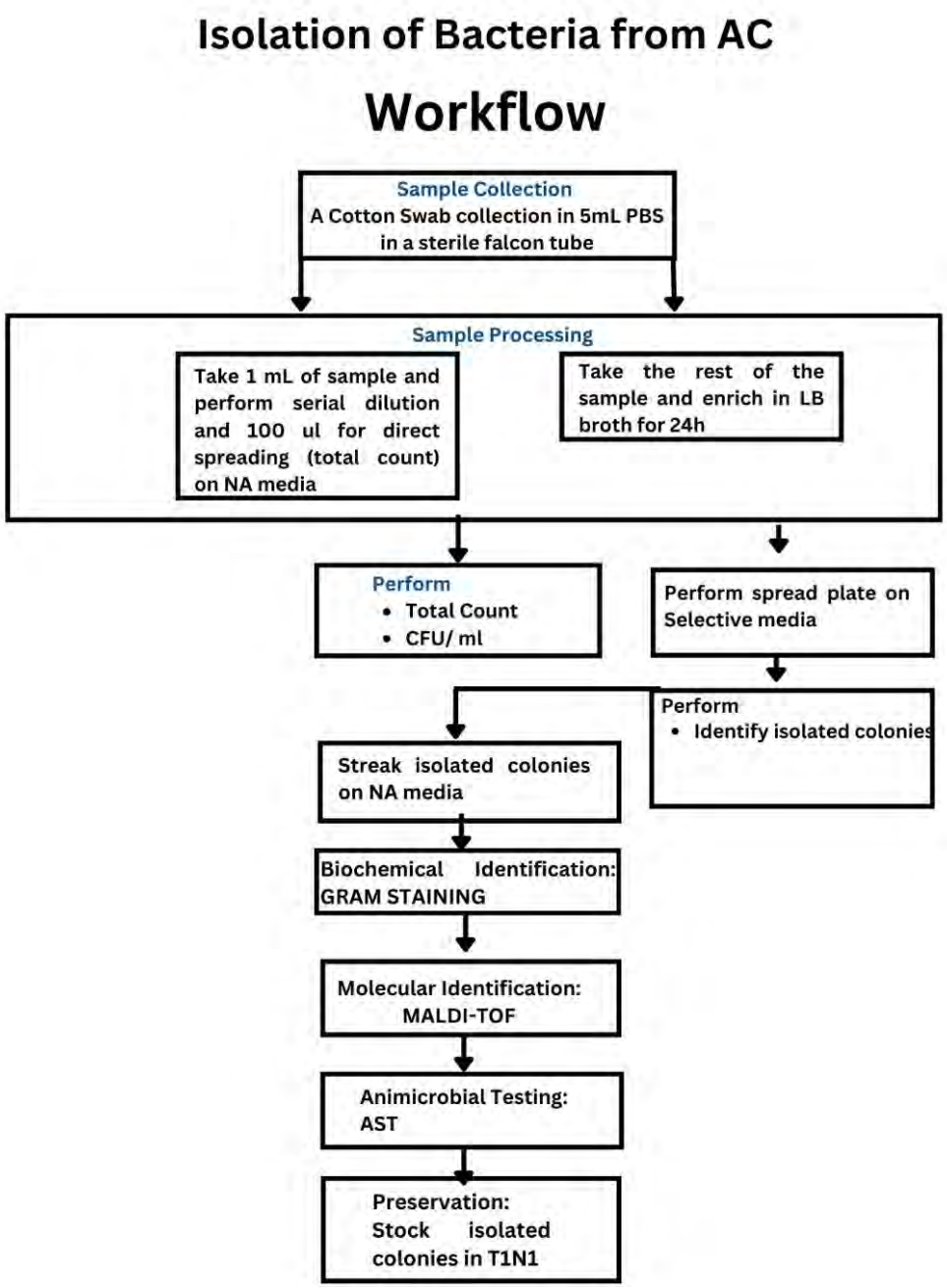


Figure 2.1: Schematic representation of study design

2.2 Selection of the Cases and Controls

Total of two ACs from the hospital (Uttara Adhunik Hospital in Dhaka and Mymensingh Medical Hospital in Mymensingh) emergency room were chosen as case because all kinds of patients enter the emergency room first for the medication. Hence, the ACs of those rooms has high chance of being contaminated with various pathogens which might spread through the cooled air and cause the patients and the attendance nosocomial infection. On the counterpart, two household ACs near those hospitals were chosen as control because households aren't as severely exposed as hospitals **(Table 1).**

2.2.1. Table 1: Inclusion and Exclusion Criteria

Inclusion criteria		**Extrusion criteria**
Hospital	**Household**	**1.** Irregularly operated AC. **2.** ACs that had been serviced within the last couple of days.
1. Hospital AC **2.** Split AC. **3.** Regularly operated AC.	**1.** Household AC within a 100-meter range from the respective hospital. **2.** Regularly operated AC. **3.** Homes where residents hadn't been ill recently.	

2.2.2. Questionnaire

A detailed questionnaire was structured into four sections-

2.2.3. Air Conditioner Details: This section includes information about the type of the air conditioner, servicing details, operation frequency, last servicing date, frequency of regulars servicing.

2.2.4. Hospital Specific Information: It summarizes the information about location, beds in the emergency room, daily footfall, typical patient stay duration, types of severity of cases handled daily and common case types.

2.2.5. Environmental and Usage Data: This section comprises information about the presence of air purifiers, frequency of surface cleaning, ventilation details in the emergency room and the household room, presence of a toilet inside of the room by which fecal contaminants get stuck in the dust filter and spread pathogens via the cooled air.

2.2.6. Consent and Ethical issues

As this study is not involve with any human sample, there is no ethical issues. However, the hospital authorities were fully informed of the purpose of the study, administrators who were responsible for emergency room operations, participated in the structured questionnaire by providing the accurate answers. The study was approved by the Institutional Review Board (IRB) of the Department of Mathematics and Natural Sciences, BRAC University

2.3. Media and Reagents

Nutrient Agar Medium (NA), Luria broth (LB), Phosphate Buffer Solution (PBS), UTI Agar Media (UTI), T1N1 agar media, 0.9% Sodium Chloride (NaCl) buffer solution, Paraffin oil, 70% Ethanol, NaCl were purchased from Mark Chemicals and HIMedia Laboratories Pvt. Ltd, India. And Glycerol ($C_3H_8O_3$) to stock the isolates was purchased from FUJIFILM Wako Pure Chemical Corporation, Japan.

2.4. Sampling Location

Between February and April 2024, two samples were aseptically collected from two hospitals located in different cities in Bangladesh: Uttara Adhunik Hospital in Dhaka and Mymensingh Medical Hospital in Mymensingh. In addition to the hospital samples, two household samples were taken as controls. Each household was situated within a 100-meter range from the respective hospital. The sampling focused on the dust filters of split-type air conditioners, which trap an abundance of microorganisms from the air in the specific hospital or household rooms (**Figure 2.2**).

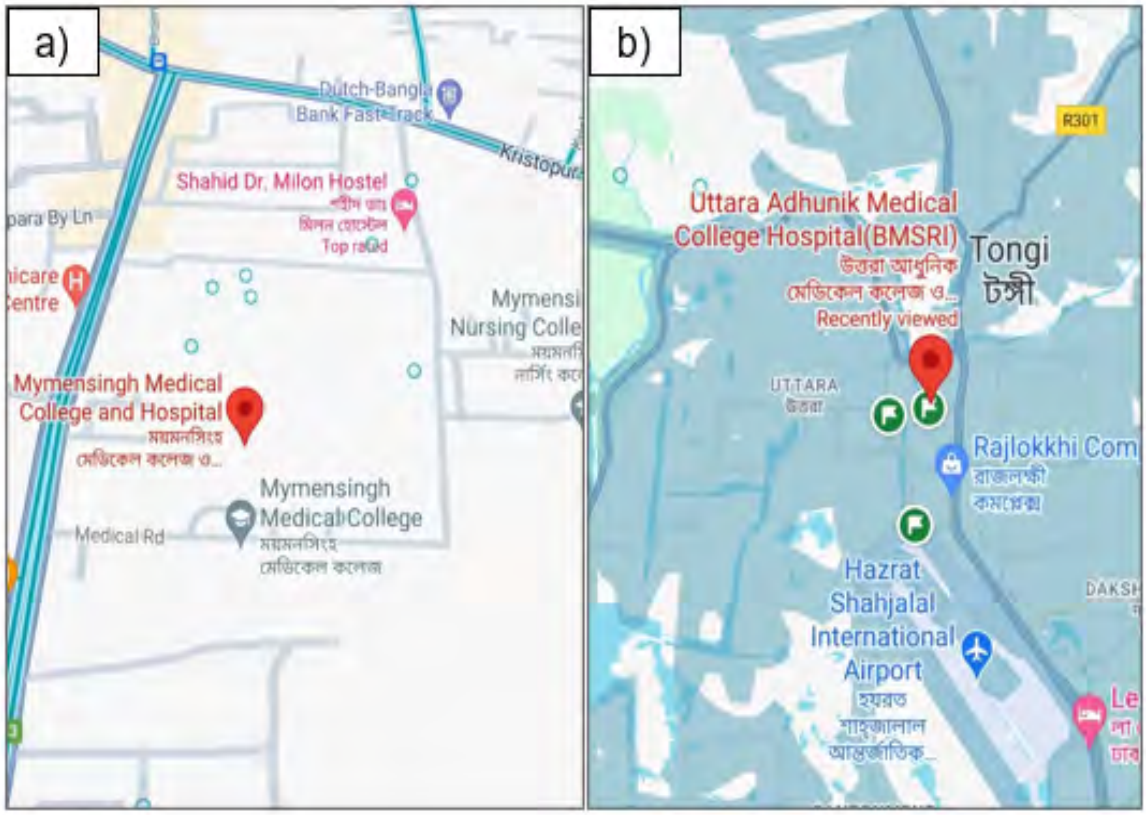


Figure 2.2: Sample collection site a) Mymensingh Medical College Hospital, Mymensingh. Sample collection site b) Uttara Adhunik Medical College Hospital (BMSRI), Dhaka.

2.5. Sample Collection

Total 4 samples were obtained from the four different regularly operated AC in spring. In brief, sterile cotton swab was used to wipe the bacteria off the 288 square inches surfaces of the dust filter and dipped into the PBS buffer until it turned turbid. Here, samples were collected from each hospital and household air conditioning unit and those underwent bacteriological analysis which included biochemical, microbiological and molecular tests. Samples were taken from the surfaces of the air inlet's dust filter of air conditioners.

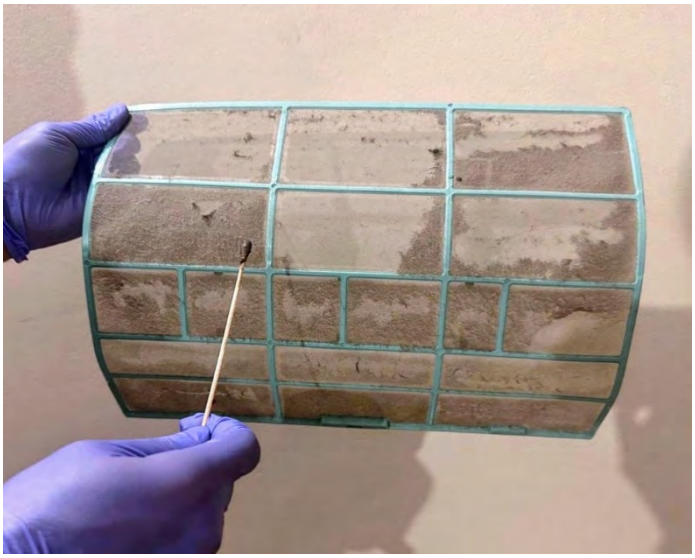


Figure 2.3: Aseptic collection of samples from dust filter of AC using sterile cotton swab in Uttara Hospital.

2.6. Sample Processing and Storage

The samples were stored in 5 ml Phosphate buffer solution (PBS) and transported immediately to the laboratory within 2hrs. The samples were kept in 4 ℃ and then used for experiments on the following day.

Figure 2.4: Collection of AC samples in 5ml PBS

2.7. Bacterial Culture and Count

To identify the bacteria, a traditional cultural method was performed and Colony Forming Unit/replicate (CFU/replicate) were calculated for quantitative analysis. In brief, the sample were vortexed to be mixed uniformly and 10-fold dilution was performed up to 10^8 (Almoffarreh, Alsaleh, & Alruwaili, 2016). After dilution, a 100µl spread plate was performed on NA media incubated at 37°C for 24 hrs. After incubation, visible colonies were found and the total count of each plate was taken.

On the other hand, after sample dilution, the rest of the main sample was mixed with 3ml of LB broth for enrichment and was incubated at 37°C for 24 hrs. After incubation, 100 µL of the enriched sample was spread plated onto UTI media by using a glass spreader and incubated at 37°C for 24 hrs. The next day, colonies were examined by looking at the colony morphology and got presumptive identification and also CFU/ml was performed. Then, the picked isolated colonies were streak plated on NA media and incubated for 24hrs at 37°C. Then they went for

the following molecular test to identify selected isolates and Antibiotic susceptibility test to find out their antimicrobial resistance towards antibiotics

2.8. Identification of Bacteria

To identify the bacteria, Matrix-Associated Laser Desorption/ Ionization Time-of-Flight (MALDI-TOF) mass spectrometry was performed which provided rapid and accurate results. Complimentary to MALDI- TOF, Gram staining was also performed to characterize bacteria based on their cell wall characteristics.

2.8.1. Gram Staining:

Gram staining is a differential staining technique that categorizes bacteria into gam-positive and gram-negative groups basis of divergence in their cell wall composition. It provides necessary information about bacterial cell wall characteristics, and facilitates initial bacterial identification and classification in microbiological studies (Tripathi & Sapra, 2023).

Initially, a loopful of bacterial culture was uniformly spread onto a glass slide and allowed to air dry for fixation. Heat fixation was then achieved by passing the slide through a flame intermittently. Subsequently, a 2% crystal violet solution was applied for one minute, followed by rinsing with running distilled water. Then, gram's iodine solution was applied on the smear for one minute, followed by another round of rinsing by distilled water. Moreover, decolorization was accomplished by tilting the slide in 45℃ and applying 95% ethanol as decolorizer, until the purple color stopped to flow from the slide. Later, counterstaining with Safranin solution for one minute was performed, and the slide was again rinsed with running distilled water. Finally, excess water was removed by gentle shaking and air dried before microscopic examination (Tripathi & Sapra, 2023).

2.9. Identification of Bacteria Using MALDI Biotyper by Bruker

MALDI Biotyper is a microbial identification system that is grounded on MALDI-TOF mass spectrometry which allows unbiased microbial identification down to the species level within a few minutes. It identifies the distinctive proteomic fingerprint of an unknown microorganism and matches the specific pattern against a comprehensive reference library to determine the identity of organisms. The reference library is being continuously updated which ensures a broad range of microbial identification easily, accelerates identification, providing rapid results that save valuable time in critical scenarios.

2.9.1. Reagent and Bacterial Sample Processing

A small isolated bacterial colony was initially selected NA plate and then mixed with a matrix solution. In this study, α-Cyano-4-hydroxycinnamic acid (HCCA) was used as a matrix which buffers the sample, facilitating the ionization and desorption of analytes, protect the damage, enable the transformation of laser light into heat, and providing H+ ions to the sample and facilitate signal quality of the mass spectrometry analysis and total sensitivity.

Figure 2.5: HCCA- Matrix (Bruker,2024)

The prepared sample-matrix mixture was spotted onto MBT Biotarget 96- a disposable MALDI target plate, featuring 96 sample positions that eliminates the hassle of target cleaning for reusable steel plates and costly validation procedures. Then 1 microliter of 70% formic acid was added to the sample for cell wall degradation and intracellular biomolecules like protein extraction, and waited until the liquid completely evaporated, leaving a dry and solid spot. Then, as matrix 1 microliter of HCCA standard solution provided by Bruker was added and waited until the liquid completely evaporated, leaving a dry and solid spot.

2.9.2. Analysis of Bacterial Sample

After that, MALDI target plate was inserted into the MALDI-TOF mass spectrometer. Here, the irradiation with a laser beam on the sample caused the matrix absorbing the laser energy and ionizing the bacterial proteins. These ionized proteins were desorbed from the sample surface and accelerated into the mass spectrometer's flight tube. (Welker, 2011).

Figure 2.6: MBT Biotarget 96 being placed into a MALDI Biotyper® sirius one system (Bruker,2024)

2.9.3 Data Acquisition

Ionized proteins were analyzed as they traveled through the flight tube, with their time-of-flight (TOF) measured. It is directly proportional to their mass-to-charge ratio (m/z). This process generated a mass spectrum that provided a proteomic fingerprint of the microorganism, focusing on ribosomal proteins (Van Belkum et al., 2017). MALDI-TOF utilized a soft ionization technique in order to maintain the structural integrity of the biomolecules. After ionization, the positively charged ions were directed through the flight tube towards a detector. Due to the inverse relationship between ion velocity and mass-to-charge ratio, smaller ions

reached the detector more quickly than larger ions. The collected data generated a mass spectrum, and it represented as a line graph (Clark et al., 2013; Seng et al., 2009).

2.9.4 Identification

By using specialized software, the acquired mass spectrum graph was compared against an extensive reference library of spectra from known microorganisms by matching the detected m/z values. The software compared the unique protein pattern of unknown microorganism to those in the database by employing pattern-matching algorithms.

2.9.5 Reporting

To ensure accuracy and dependability, the identification results were reviewed and interpreted by a microbiologist in order to ensure accuracy and reliability. This process was cost effective, accurate, adaptable, user friendly, versatile, rapid. It significantly enhanced the efficiency and reliability of identifying bacteria in microbiological research and diagnostics field. Furthermore, it could serve as an alternative of 16S rRNA sequencing for many applications depending on the requirements of accuracy, speed, cost, and the nature of the bacterial samples being studied.

2.10. Antibiotic Susceptibility Testing (AST):

After identification of bacterial isolates by MALDI Toff, AST was performed. The Kirby-Bauer disk diffusion test is the most commonly used method to determine the antibiotic resistance or susceptibility of microorganisms. MHA is used in AST as MHA media has higher diffusion rate compared to conventional growth media and also it incorporates starch as an energy source, which absorbs toxins produced by bacteria, which prevent them from interfering with drugs. Microorganisms are categorized as Resistant (R), intermediate (I), or susceptible (S) depending on their zone diameter (mm) values, following the guidelines set by the Clinical and Laboratory Standards Institute (CLSI, 2021). Twelve different antibiotic discs were used to determine which bacterial isolates are resistant to particular antibiotics and which are sensitive. The table stated below represents the lists of the antibiotics used in the research (**Table 1**).

2.10.1. Preparation of Inoculum and Compare with McFarland Solution:

The bacterial isolates were picked from sub-cultured NA plates by using an inoculating loop. One or two colonies were taken up and suspended in a 5ml saline solution. After proper vertexing, the turbidity of the inoculums was compared to the McFarland 0.5 standard, which is used to standardize microbiological testing by providing an approximate cell count density of $1.5x10^8$ CFU/ml within a particular range.

2.10.2. Protocols for AST:

Organisms' colonies were picked based on the colony size, the standardized inoculum of 0.5 McFarland (cell count density is ~$1.5x10^8$) turbidity standard was created. By using sterile cotton swabs, each bacterial isolate was individually lawned on MHA plates. After finishing the lawn culture, antibiotic discs were carefully removed from the stacks by using sterile forceps and then, the antibiotic disks were carefully placed on top the lawn culture. In order to prevent inhibition zones from overlapping, antibiotic discs were placed in such a way that there would be adequate space among the discs.

After 24 hours of incubation at 37℃, the zone of inhibition was measured following CLSI guidelines (**Table 2**). Isolates that showed resistance to at least one agent in three or more antibiotic categories were defined as multidrug-resistant strains (MDR) (Basak et al., 2016)

Table 2: List Of Antibiotic Disks, Their Group and Concentrations

S. No.	Antibiotic	Antibiotic group	Disc code	Disc potency (µg)
1	Azithromycin	Macrolide	AZM	15
2	Ampicillin	β-lactam	AMP	10
3	Vancomycin	Glycopeptide	VAN	30
4	Tetracycline	Tetracycline	TET	30
5	Cefepime	Cephalosporin	CPM	30
6	Chloramphenicol	Phenicols	C	30
7	Imipenem	Carbapenem	IPM	10
18	Linozoid	Oxazolidinones	LZ	30
9	Cefixime	Cephalosporins(3^{rd}gen)	CFM	5
10	Aztreonam	Monobactams	ATM	30
11	Kanamycin	Aminoglycosides	K	30
12	Amoxicillin+ clavulanic acid	Penicillin (beta-lactamase inhibitors)	AMC	30

AZM: Azithromycin, AMP: Ampicillin, VA: Vancomycin, TET: Tetracycline, CPM: Cefepime, C: Chloramphenicol, IPM: Imipenem, LZ: Linezolid, CFM: Cefixime, ATM: Aztreonam, K: Kanamycin, AMC: Amoxicillin-Clavulanic Acid.

Chapter 3

3. Results

3.1. Total Bacterial Counts

NA was utilized to observe the total counts and calculate CFU/AC filter from four different samples (**Figure 3.1**). A higher number of CFU was observed in dust filters of hospital compared to dust filters of residential buildings. The CFU/ml, CFU/per replicate and Average CFU/AC filter sample is represented on **Table 3**

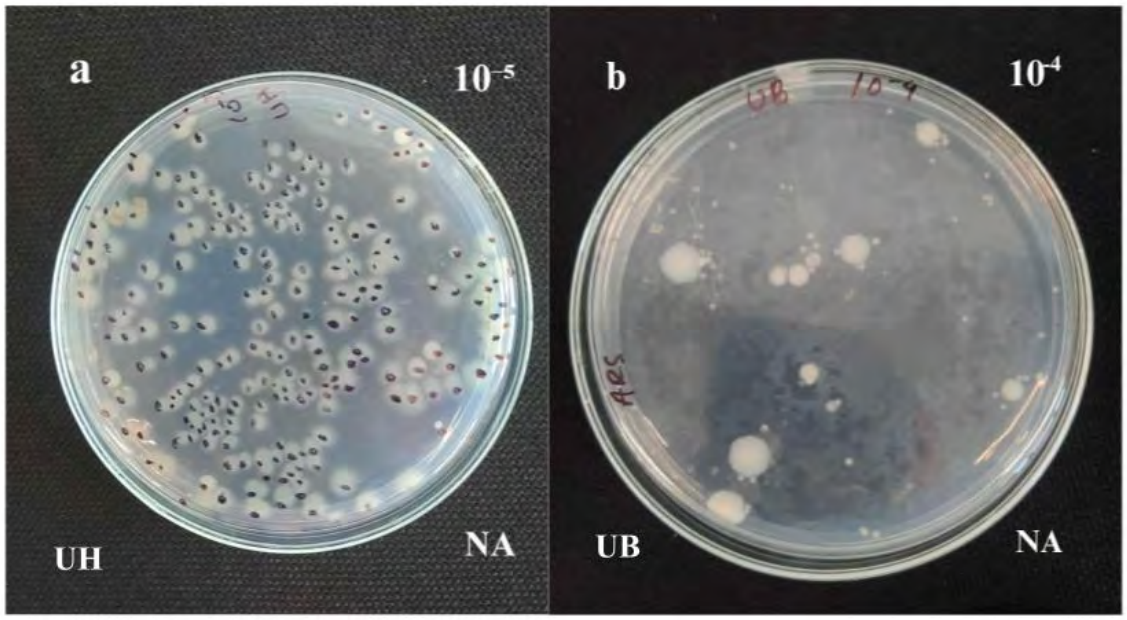


Figure 3.1: Total bacterial colony counts on NA. a) Total 223 colonies were observed on NA from diluted (10^{-5}) sample of Uttara Hospital (UH). b) Total 85 colonies were detected on NA from 10^{-4} dilution of sample collected from Uttara Households (UB).

Table 3: Average CFU Count of Samples

Sample ID No.	Replicate ID No.	Colony Number	Dilution factor	CFU/ml	CFU per replicate (5ml)	Average CFU per Dust Filter
UH	RUH1	223	10^{-5}	2.23×10^{8}	1.115×10^{9}	$1x10^{9}$
	RUH2	180	10^{-5}	$1.80x10^{8}$	9.0×10^{8}	
	RUH3	198	10^{-5}	$1.98x10^{8}$	9.9×10^{8}	
UB	RUB1	92	10^{-4}	$9.2x10^{5}$	4.6×10^{6}	$4.8x10^{6}$
	RUB2	110	10^{-4}	$1.1x10^{6}$	5.5×10^{6}	
	RUB3	85	10^{-4}	$8.5x10^{5}$	4.25×10^{6}	
MH	RMH1	230	10^{-5}	$2.3x10^{8}$	1.15×10^{9}	$1.06x10^{9}$
	RMH2	190	10^{-5}	$1.9x10^{8}$	9.5×10^{8}	
	RMH3	215	10^{-5}	$2.15x10^{8}$	1.075×10^{9}	
MB	RMB1	48	10^{-2}	$4.8x10^{5}$	2.4×10^{6}	$9.57x10^{5}$
	RMB2	55	10^{-2}	$5.5x10^{4}$	2.75×10^{5}	
	RMB3	39	10^{-2}	$3.9x10^{4}$	1.95×10^{5}	

UH-Uttara Hospital, UB- Uttara Household, MH-Mymensingh Hospital, MB- Mymensingh Household

Table 3 illustrates average CFU/dust filters along with colony number, dilution factor, CFU/ml of each replicate from four different sample sites including Uttara Hospital (UH), Uttara Household (UB), Mymensingh Hospital (MH), Mymensingh Household (MB). To ensure the accuracy, each site has been triplicated. Here, the result indicated that samples from MH and UH exhibit the highest average CFU/dust filter compared to UB and MB. UH showed approximately 1×10^{9} CFU/dust filter where MH had 1.06×10^{9} CFU per dust filter in average. On the other hand, UB and MB hold the lowest microbial concentration averaging 4.8×10^{6} and 9.57×10^{5} respectively. Dust filter surface : 288 $inch^2$

3.2. Graphical Comparison of Average CFU Counts of Per Dust Filter

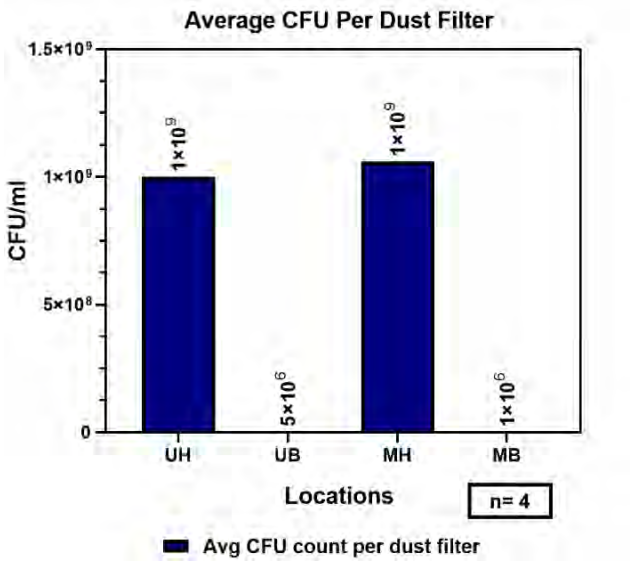


Figure 3.2: Representation of Average CFU counts per dust filter across four sampling sites.

Table 4: Distribution of Bacteria in Different Samples

Types of Bacteria	Bacteria Name	Sample ID			
		UH	UB	MH	MB
Gram Negative	*Pseudomonas putida*	+	-	-	+
	Pseudomonas monteilii	+	-	-	-
	Pseudomonas fulva	-	-	+	-
	Pseudomonas stutzeri	-	-	+	+
	Enterobacter cloacae	-	+	-	-
	Enterobacter hormachei	-	-	-	+
	Acinetobacter baumannii	-	-	+	+
	Acinetobacter variabilis	-	-	-	+
	Acinetobacter pittii	-	-	-	+
	Leclercia adecarboxylata	-	+	-	+
	Mixta calida	-	-	+	-
Gram Positive	*Staphylococcus haemolyticus*	+	-	+	-
	Staphylococcus arlettae	-	-	+	-
	Enterococcus hirae	+	-	-	-

	Bacillus cereus	-	-	+	-

UH-Uttara Hospital, UB- Uttara Household, MH-Mymensingh Hospital, MB- Mymensingh Household

Table 4 displayed the distribution of bacterial species and distinguished Gram negative and Gram-positive bacteria according to their specific sample sites. Amon fifteen bacterial species, eleven of them were Gram-negative - *Pseudomonas putida, Pseudomonas monteilii, Pseudomonas fulva, Pseudomonas stutzeri, Enterobacter cloacae, Enterobacter hormachei, Acinetobacter baumannii, Acinetobacter variabilis, Acinetobacter pittii, Leclercia adecarboxylata, Mixta calida*. The rest four bacterial species were Gram-positive bacteria- *Staphylococcus haemolyticus*, *Staphylococcus arlettae*, *Enterococcus hirae* and *Bacillus cereus*. Gram-negative bacteria were found in both hospital and household settings (UH, UB, MH, MB). However, Gram-positive bacteria were absent in household AC (UB, MB). Here, among Gram-negative bacteria, *P. putida and P. monteilii* were found in Uttara hospital AC, on the other hand, more diverse bacteria were found in Mymensingh hospital AC settings (MH) including- *P. fulva, P. stutzeri, A. baumannii, M. calida.* Among Gram-positive bacteria, *S. haemolyticus* were found in both hospital AC settings (UH, MH), *S. arlettae (*MH), E. hirae (UH), B. cerius (MH). To note, in household AC – ten Gram-negative species were fou*nd and E. cloacae, E.hormachei and L. adecarboxylata* were exclusively found on household AC.

3.3. Representation of Common and unique bacterial species across the location

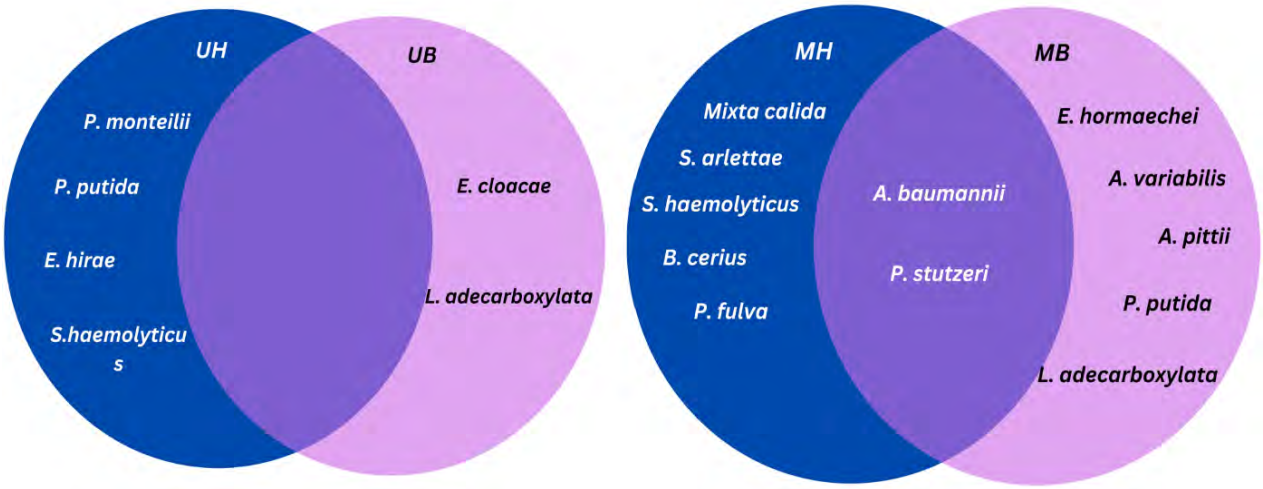


Figure 3.3: Venn diagram showing the shared and unique bacterial species across UH and UB; MH and MB.

Figure 3.3 represented the bacterial species across Uttara hospital and household; Mymensingh hospital and household. It showed no shared bacteria between UH and UB. However, *A. baumanni* and *P. stutzeri* was common in MH and MB.

3.4. Distribution of Gram-Positive vs Gram-Negative bacteria

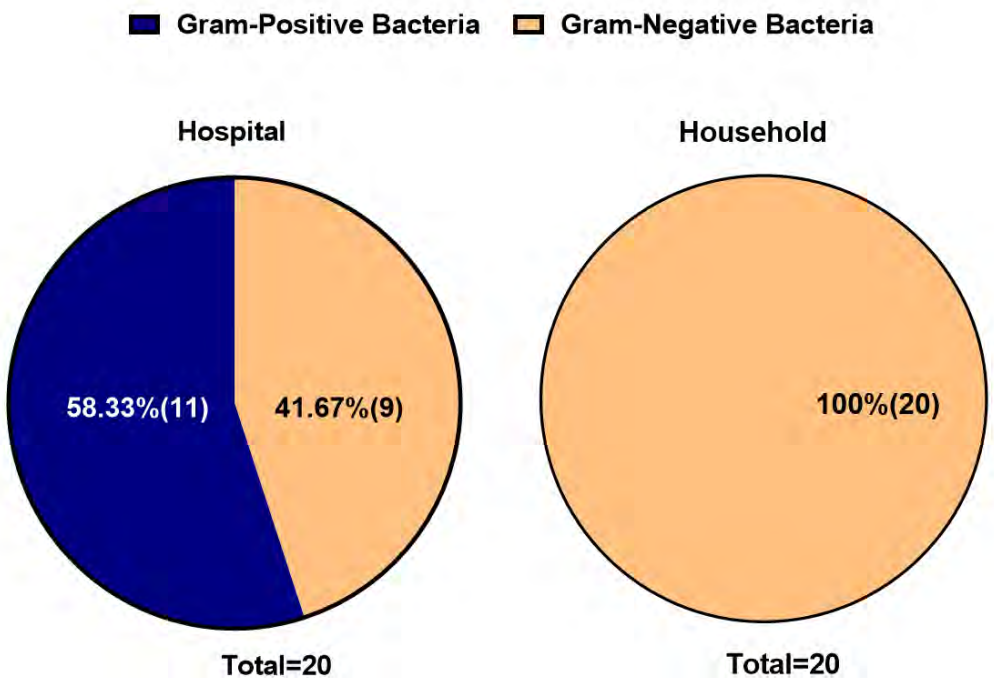


Figure 3.4: a) Gram-positive vs Gram-negative in hospital AC, b) Gram-positive vs Gram-negative in household AC.

The graph represents gram-positive and gram-negative bacteria isolated from dust filters of AC in hospital and household, it also highlighted the difference of bacterial population in gram positive and gram-negative bacteria between hospital and household. Here, a total 20 bacterial isolates were found and all of them were observed as gram-negative bacteria. 100% of bacterial population was gram negative in household environment indicating complete dominance of gram-negative bacteria in household ac. Conversely, in hospital settings, among 20 bacterial isolates, 9 of them (41.67%) were gram positive and the remaining 11 isolates (58.33%) were positive (**Figure 3.4)**.

3.5. Antibiotic Susceptibility Test

AST of each bacterial isolates has showed variable resistance and sensitivity patterns to twelve antibiotics tested.

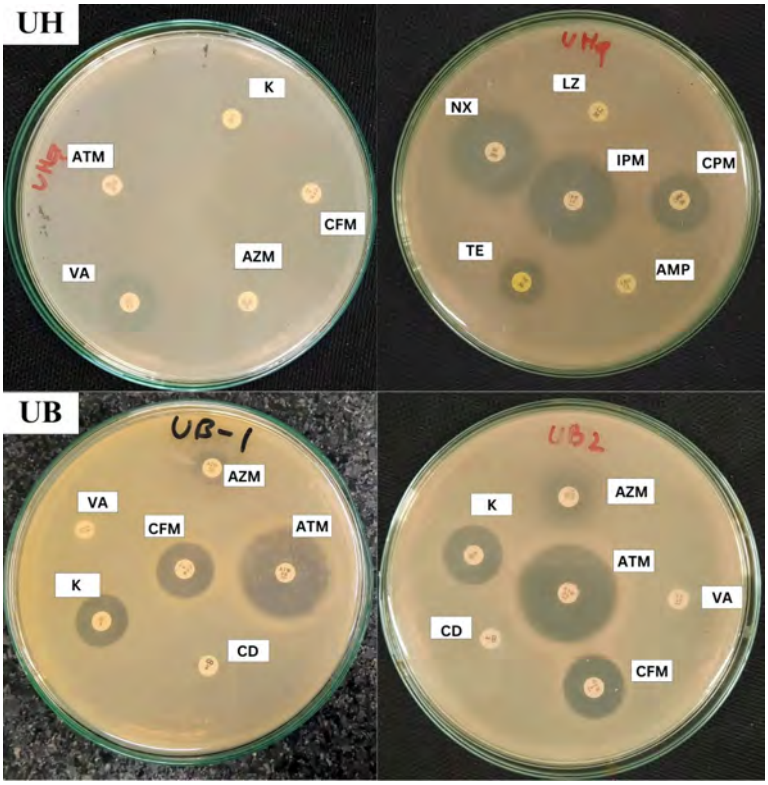


Figure 3.5: Antibiotic susceptibility test result performed in MHA media.

In **Figure 3.5**, Each plates represents different bacterial sample (UH5, UH7, UB1, UB3) and antibiotic discs were properly placed to observe the susceptibility. After 18-24h incubation, clear zones suggested bacterial susceptibility to particular antibiotic whereas minimal or absence of bacterial inhibition indicated resistance to corresponding antibiotic

3.6. Antibiotic Resistance Pattern of Bacteria-

Antibiotic resistance profile is crucial to determine the efficacy of difference treatments for bacterial infections. Here, the resistance patterns of *Staphylococcus* spp. and *Enterococcus* spp. were analyzed.

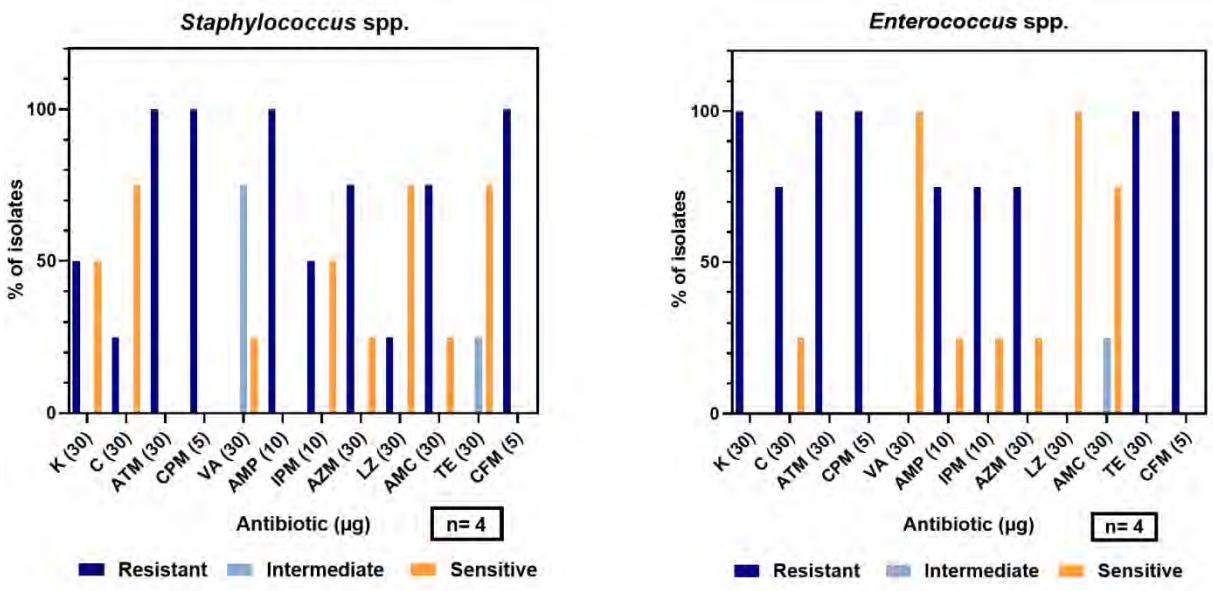


Figure 3.6: (a) The antibiotic susceptibility profiles of *Staphylococcus* spp. The graph illustrates their antibiotic-resistant pattern for *Staphylococcus* species, analyzed from four isolates (n=4). (b) The antibiotic susceptibility profiles of *Enterococcus* spp. Here, the graph depicts the antibiotic susceptibility of *Enterococcus* species, based on four isolates (n=4). n = number of bacterial isolates found in respected genus.

In figure 3.6 a, the data demonstrates that *all Staphylococcus* isolates were fully resistant to ATM, CPM, AMP, CFM antibiotics (100%). Here, resistance is prominently observed. A significant proportion of bacterial isolates (75%) were resistant to AZM and AMC, 50% of isolates were resistant to K and IPM, 25% of isolates were resistant to LZ and C. Remarkably, no isolates exhibited complete sensitivity to any of the antibiotics tested. However, 75% of isolates were sensitive to C, LZ and TE, 25% of isolates were sensitive to VA, AZM and AMC. On the other hand, intermediate susceptibility was in 75% of bacterial isolates for VA and 25% for TE.

In figure 3.6 b, the data reveals a concerning level of resistance (100%) in *Enterococcus* species, particularly to K, ATM, CPM, TE, CFM. A significant proportion of bacterial isolates

(75%) were resistant to C, AMP, IPM, AZM. However, all bacterial isolates were fully sensitive to VA and LZ and 75% of the isolates remained sensitive to AMC while a smaller fraction (25%) showed sensitivity to C, AMP, IPM, AZM. The intermediate susceptibility was minimal, observed only in 25% of isolates for AMC.

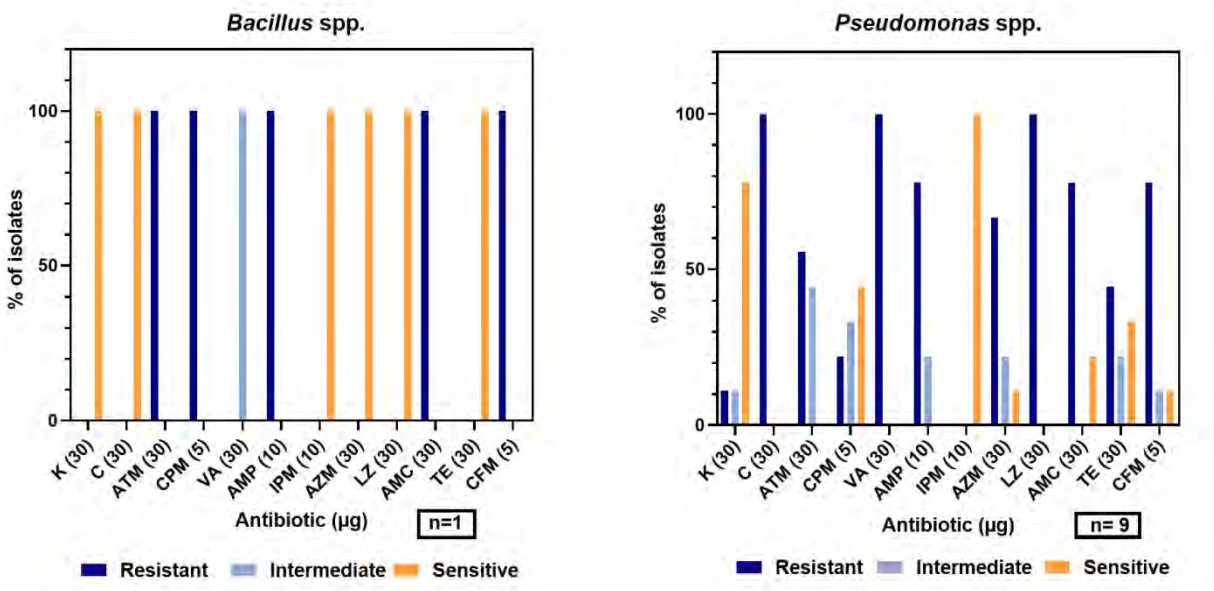


Figure 3.7: (a) The antibiotic susceptibility profiles of *Bacillus* spp. The graph represents the antibiotic susceptibility data for a single bacterial isolate (n=1) of *Bacillus* spp. (b) The antibiotic susceptibility profiles of *Pseudomonas* spp. The graph represents the antibiotic susceptibility data for nine bacterial isolates (n=9) of *Pseudomonas* spp. n = number of bacterial isolates found in respected genus.

In figure 3.7 a, the isolates demonstrated a notable resistance profile in 100% of isolates resistance to five antibiotics including ATM, CPM, AMP, AMC and CFM. Here, sensitivity is prominently observed in the isolates with 100% of susceptibility for K, C, IPM, AZM, LZ and CFM. The intermediate susceptibility was observed in 100% of isolates for VA exclusively.

In figure 3.7 b, the bacterial isolates showed notably high resistance rate to several antibiotics. Here, complete resistance was observed for C, VA and LZ, a significant proportion of bacterial isolates (77.78%) were resistant to AMP, AMC and CFM, 55.55% of isolates were resistant to ATM, 66.67% of isolates were resistant to AZM. Additionally, TE showed a considerable resistance at 44.44% and smaller proportion (22.22%) of isolates were resistant to CPM. In spite of overall high resistance, IPM was completely effective with 100% susceptibility among all isolates. A notable fraction of isolates (77.78%) was observed being sensitive to K.

However, a considerable proportion (44.44%, 33.33%) showed sensitivity to CPM and TE respectively. Comparatively, a lesser percentage of isolates (22.22%) were sensitive to AMC, 11.11% of isolates were sensitive to AZM and CFM. Conversely, intermediate susceptibility was in 44.44% of isolates for ATM, 33.33% for CPM, 22.22% of isolates for TE, AMP, AZM and the smallest portion (11.11%) of bacterial isolates for K and CFM.

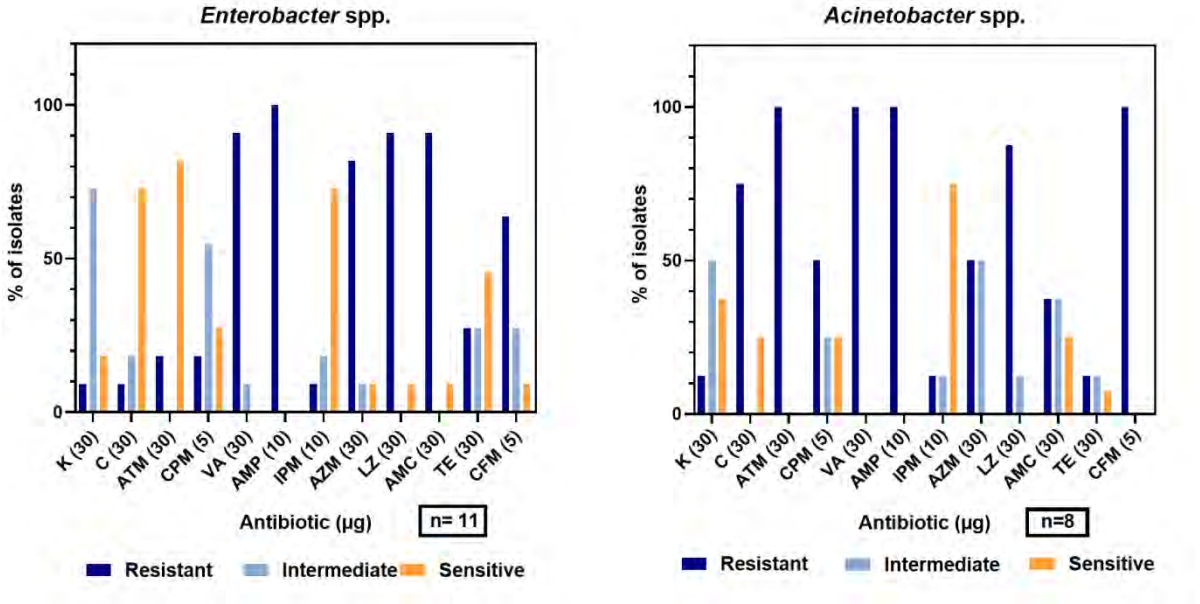


Figure 3.8: (a) The antibiotic susceptibility profiles of *Enterobacter* spp. The graph illustrates the antibiotic susceptibility of eleven isolates (n=11) of *Enterobacter* spp.(b) The antibiotic susceptibility profiles of *Acinetobacter* spp. The graph represents the antibiotic susceptibility data for nine bacterial isolates (n=8) of *Acinetobacter* spp. n = number of bacterial isolates found in respected genus.

In figure 3.8 a, the isolates demonstrated resistance profile with complete resistance (100%) to AMP only. Similarly, high resistance level (90.91%) is observed for VA, LZ, AMC and 81.82% isolates for AZM. Moreover, CFM showed a substantial resistance in 63.64% of isolates. A lesser portion of isolates (18.18%) were resistant to ATM and CPM, 27.27% of isolates were resistant to TE and only 9.09% of isolates were observed being resistant to K, C and IPM. On the other hand, the highest portion of isolates (81.82%) were sensitive to ATM, 72.73% of isolates exhibited sensitivity to C and IPM, 45.45%of isolates were found sensitive to TE only. Besides, lesser fraction of isolates (27.27%) was sensitive to CPM, a minor percentage (9.09%) of isolates observed as sensitive to CFM, AZM, LZ and AMC. On the contrary, intermediate susceptibility is most prominent with K where 72.73% of isolates exhibited intermediate response. Approximately, half of the isolates (54.55%) were

intermediate susceptibility to CPM, 27.27% of isolates showed intermediate response to TE and CFM, 18.18% of isolates were intermediately susceptible to C, IPM and a tiny portion of isolates (9.09%) exhibited intermediate susceptibility to VA and AZM both.

In figure 3.8 b, the data reveals a concerning level of resistance (100%) in *Acinetobacter* species, particularly to ATM, VA, CFM and AMP. Here, resistance is prominently observed among the isolates. A significant proportion of *Acinetobacter* species (87.5%) showed resistance to LZ, 75% of isolates were resistant to C, half of the isolates (50%) were resistant to CPM. Moreover, lesser portion of *Acinetobacter* species (37.5%) exhibited resistance to AMC, 12.5% of isolates showed resistance to TE and IPM. On the other hand, sensitivity was minimal where six of the antibiotics found to be sensitive towards *Acinetobacter* species. Here, IPM exhibited the highest sensitivity among 75% of isolates. However, 37.5% of isolates were sensitive to K, 25% of isolates showed sensitivity to C, CPM and AMC. Moreover, minimal portion of isolates (7.5%) were sensitive to TE. To add, intermediate susceptibility was nominal, observed in 50% isolates for K and AZM, 37.5% of isolates were observed as intermediately sensitive to AMC, 25% of isolates were showed the intermediate response for CPM and a small fraction of isolates (12.5%) observed intermediate susceptibility for TE, LZ and IPM.

The graph bellow illustrated the resistance patterns of *Leclercia* spp. and *Mixta* spp. (**Figure 3.9**)

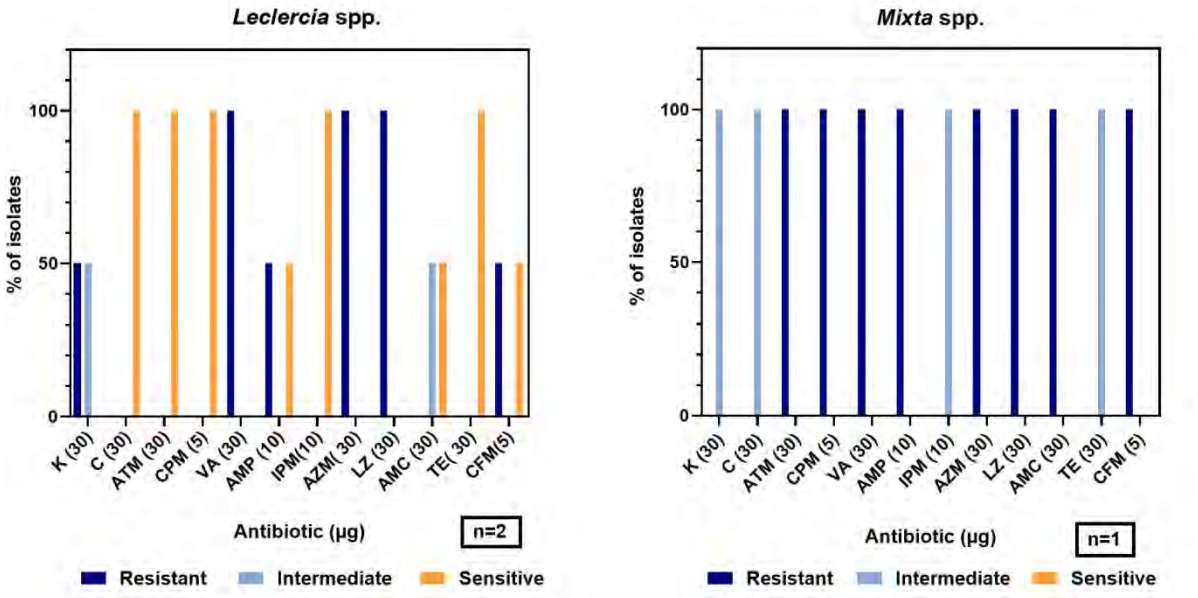


Figure 3.9: (a) The antibiotic susceptibility profiles of *Leclercia* spp. The graph illustrates their antibiotic-resistant pattern for *Leclercia* species, analyzed from two isolates (n=2). (b) The antibiotic susceptibility profiles of *Mixta* spp. Here, the graph depicts the antibiotic susceptibility of *Mixta* species, based on single isolate (n=1). n = number of bacterial isolates found in respected genus.

In figure 3.9 a, the graph indicated a heterogenous response with six antibiotics being effective while others not. The isolates exhibited resistance profile in 100% of isolates resistance to AZM, VA and LZ. Half portion of isolates (50%) were resistant to K, AMP and CFM. On the other hand, susceptibility was prominently observed. Here, all bacterial isolates (100%) were sensitive to five antibiotics including C, ATM, CPM, IPM and TE; 50% of *Leclercia* species were sensitive to AMP and AMC. Regarding intermediate susceptibility, K and AMC showed intermediate response in 50% of *Leclercia* isolates.

In figure 3.9 b, the graph reveals a high level of resistance to most antibiotics tested in the research. Here, complete resistance was observed (100%) in the single isolate for ATM, CPM, VA, LZ, AMP, AZM, CFM and AMC. Remarkably, *Mixta* species exhibited zero sensitivity to any of the antibiotic tested. However, complete intermediate susceptibility was observed in the isolate for K, C, IPM and TE. (**Appdx. T1**)

3.6.1 Graphical Comparison of Bacterial Resistance Patterns in Hospital and Households-

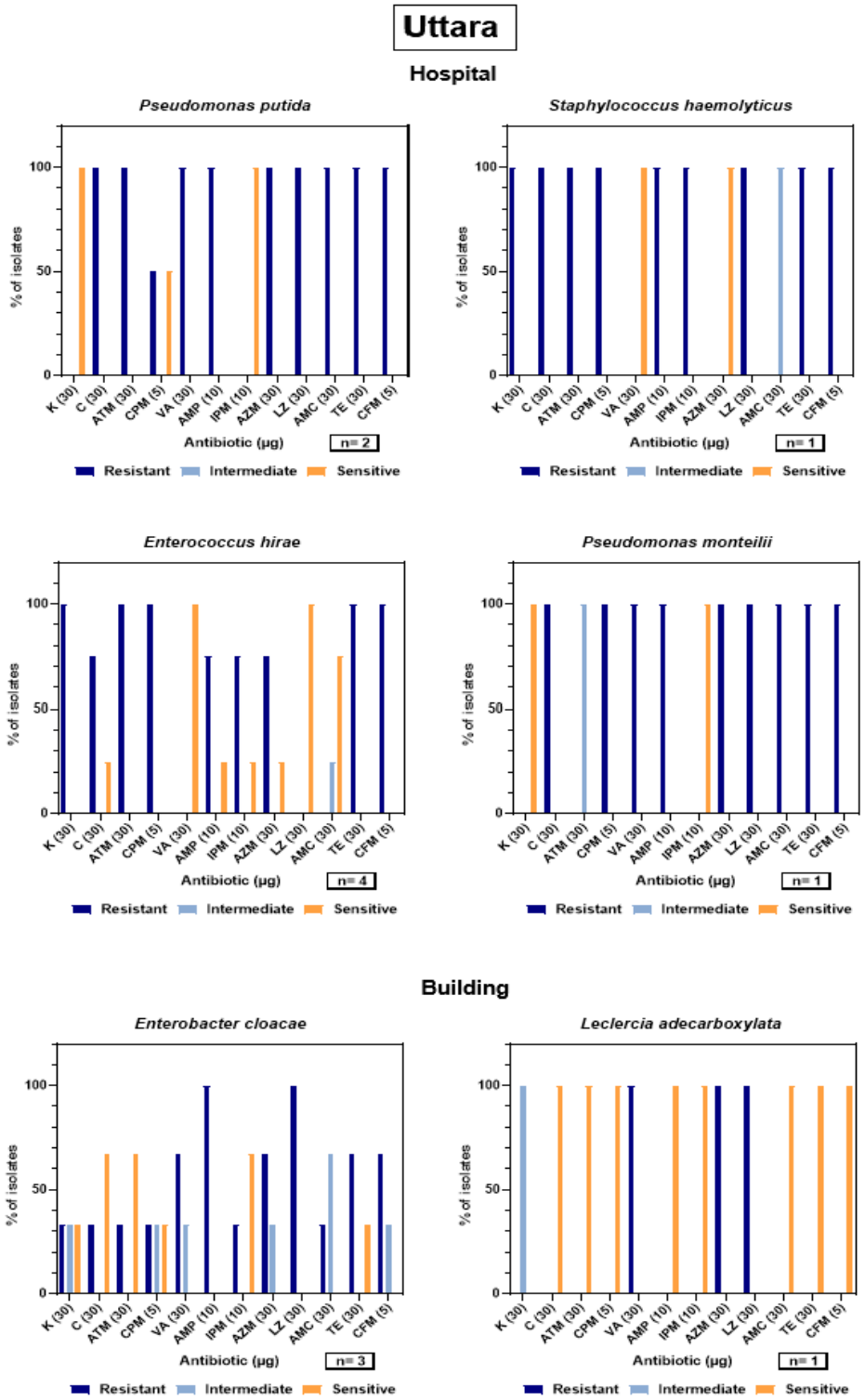


Figure 3.10: Comparison of antibiotic resistance patterns in Uttara hospital and household AC.

3.6.2 Graphical Comparison of antibiotic resistance patterns in Mymensingh hospital and household AC.

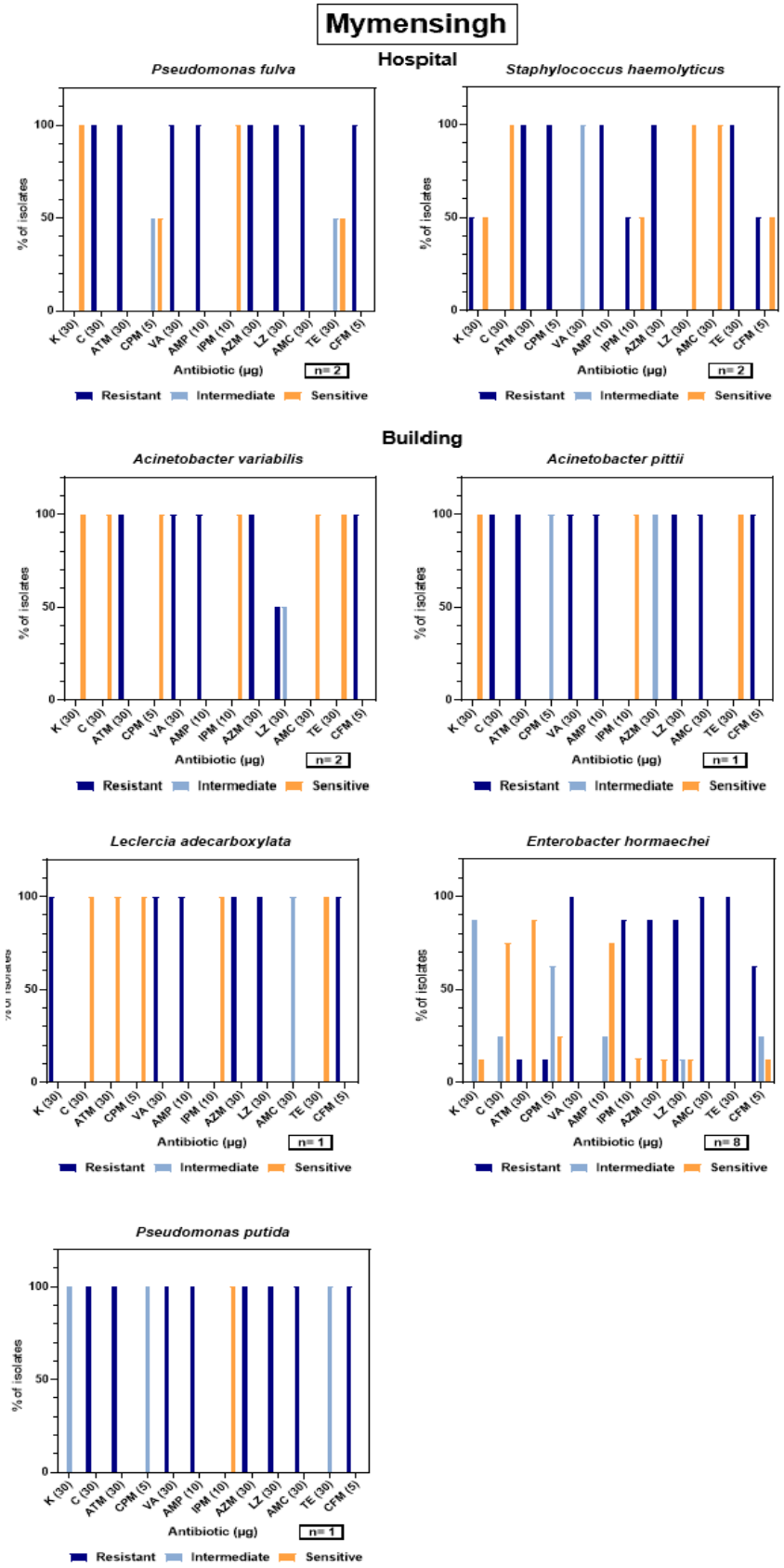


Figure 3.11: Comparison of antibiotic resistance patterns in Mymensingh hospital and household AC.

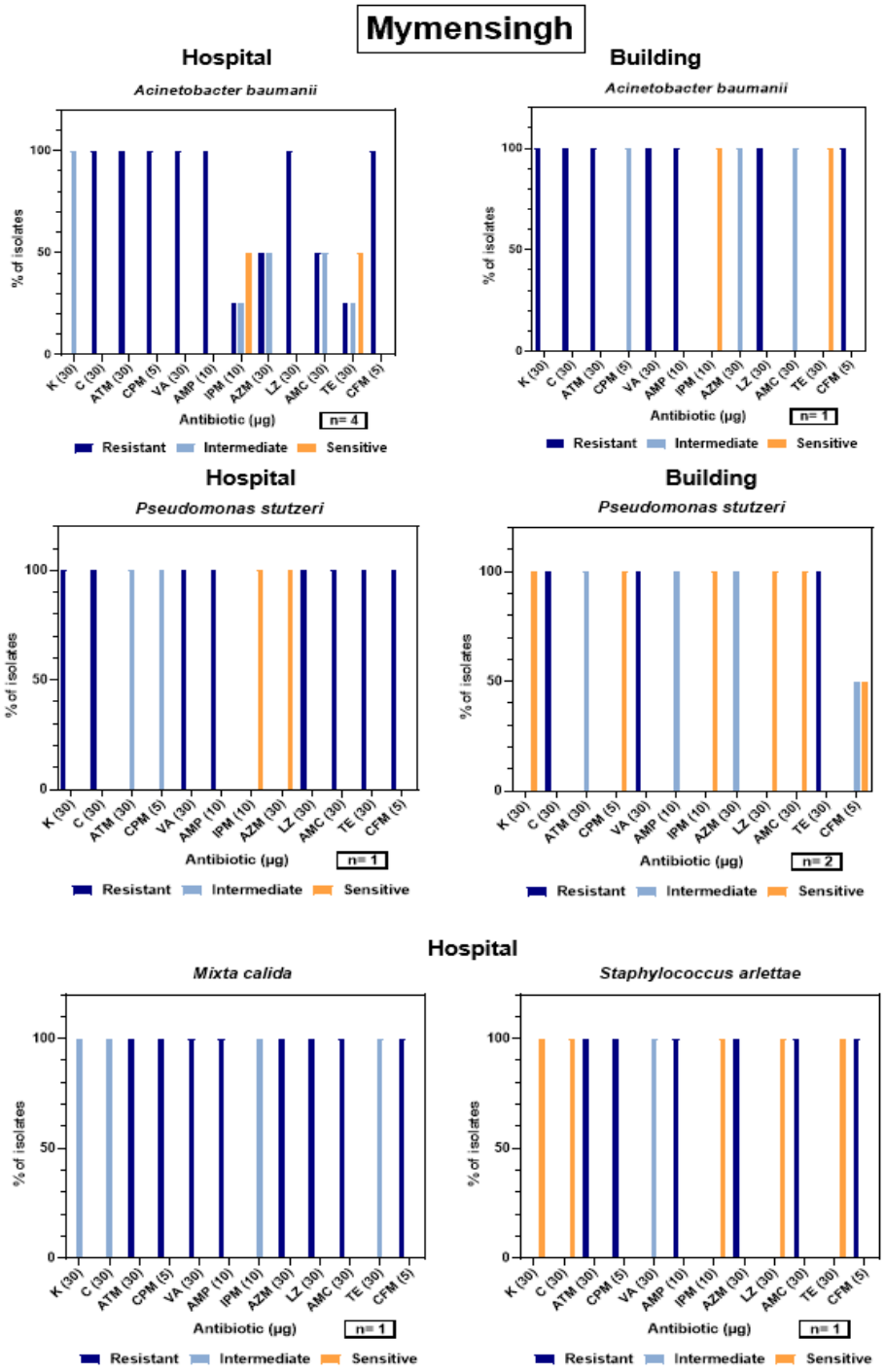


Figure 3.12: Comparison of antibiotic resistance patterns in Mymensingh hospital and household

3.6.3: Graphical Representation of Multiple Antibiotic Resistance (MAR) Index of Bacterial Isolates in Hospital and Household

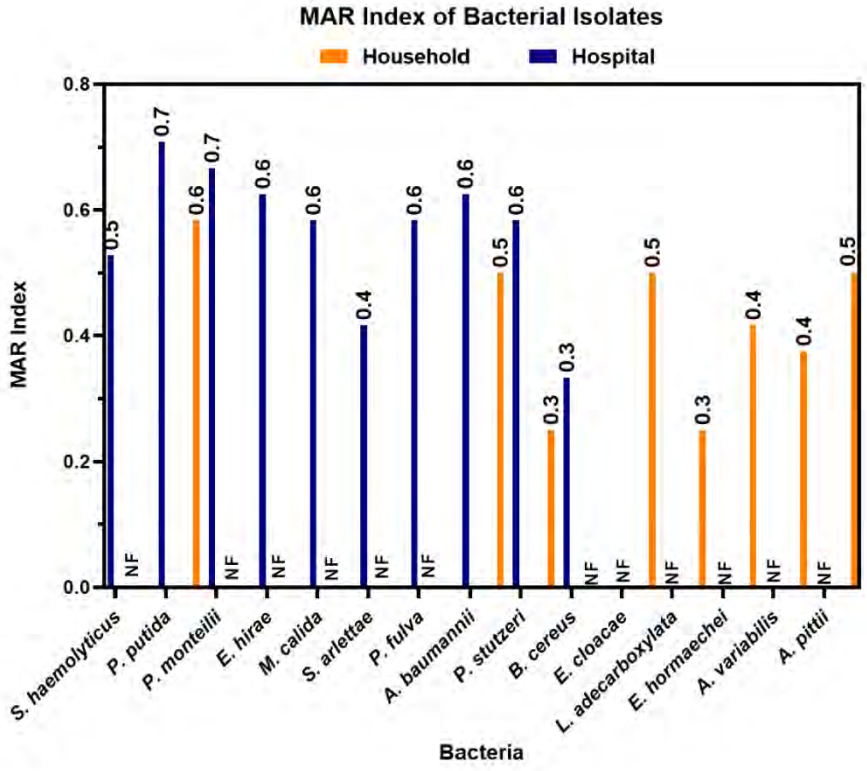


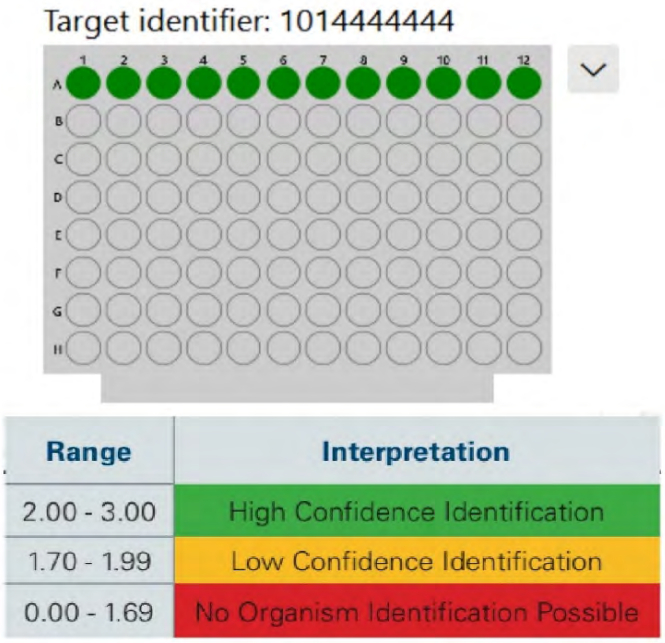
Figure 3.13: MAR Index of Bacterial Isolates in Hospital and Household.

Figure 3.13 compared the MAR index of bacterial species from hospital and households. All of the species had MAR index higher than 0.2 and many of them even exceed 0.6. Here, hospital isolates showed higher MAR index compared to households. For example, *P. monteilli, P. putida* have highest MAR index (0.7); while in households *P. putida* had the highest score of 0.6. However, NF represented absent isolates e g., *S. haemolyticus, P. monteilii, E. hirae , M. calida* were absent (NF) in household AC.

3.7 MALDI-TOF

Total 40 isolates were analyzed in MALDI-TOF. Every sample was attributed with a score value that represents the confidence of identification. The results are listed under detected species accompanied by log score and proper traffic color (**Table 5**) The score from 2-3 indicates high confidence species level identification and it is colored as green. Scores between 1.70-1.99 indicates low confidence level identification which is highlighted with yellow and 0-1.69 score suggested no identification, this was highlighted as red. For instance, *S. haemolyticus* (MHC5) had score of 2.23 which confirmed itself as high confidence level identification (Table 7). Similarly, *A. baumannii* (MHC8) had score of 2.45 indicating high confidence level. Most of the isolates had high confidence level which confirmed the reliability of bacterial identification. However, some isolates showed lower level identification like *A. baumannii* (MHC12) – 1.78, *M. calida* (MHC1)- 1.97, *P. fulva (*MHC3) -1.97 which indicated occasional difficulties encountered to identify the isolate confidently. However, there is no red highlighted score found which suggested all isolates were identified. Moreover, the best match is the higher log score value, if first match acquires high score (2-3) indicating a trustworthy identification, the second-best match shows identical or a bit lower score. This supports a confident identification. Overall, 32 out of 40 isolates (80%) were identified with high confident score and remaining 8 isolates (20%) showed less confidence to identify bacteria in species level (**Appdx. T2**)**.**

3.7.1 Representation of Microbial Interpretation by MALDI-TOF



Range	Interpretation
2.00 - 3.00	High Confidence Identification
1.70 - 1.99	Low Confidence Identification
0.00 - 1.69	No Organism Identification Possible

Figure 3.7.1: Result Interpretation (Bruker,2024)

The figure represents microbial interpretation process based on MALDI-TOF results where green wells inferred successful identification with high confidence, yellow box indicated low confidence with range of 1.70-1.99 which indicated a tentative match. Red represented no organism detected due to insufficient bacteria for proper identification.

4. Discussion:

This study investigates the bacterial diversity and abundance in the air conditioning system's dust filter from hospitals and households in Bangladesh to explore the potential health risks associated with pathogens spread from ACs. The research addressed a crucial gap in understanding bacterial profiles specifically in the context of HAI or NI, their antibiotic resistance patterns evaluating the implications for public health.

4.1. Prevalence and Common Bacteria

In this study, the four most abundant genera detected on AC dust filters were *Enterobacter, Pseudomonas, Acinetobacter, Staphylococcus* species. Previous research on bacterial communities in AC units supports these findings, emphasizing the public health implications and impact on indoor air quality. For instance, study by Watanabe et al. (2022) and Khalafallah et al. (2016) examined various components of AC units in 17 Japanese homes and identified a diverse bacterial profile on AC filters including *Pseudomonas, Paracoccus, Acinetobacter,Enhydrobacter,Sphigomonas,Staphylococcus, Corynebacterium, Streptococcus ,Actinotignum, Bacillus* spp. and *Staphylococcus* spp. in hospital AC system, underscoring potential risk associated with airborne pathogens in healthcare settings.

On the other hand, in households, we identified *Pseudomonas, Acinetobacter, Enterobacter and leclercia* spp., with *Enterobacter* and *Leclercia* spp. found exclusively in household environment. Moreover, Enterobacter spp., an emerging opportunistic pathogen, which can be found in soil, water, sewage, plants, the gastrointestinal tract of humans and animals and many hospital settings. Although not commonly noted in AC systems, *Enterobacter* spp. is frequently found in healthcare environment and linked to nosocomial infection (Khajuria et al., 2014; Al-Hasan et al., 2011; Mahaluca et al., 2018).

Moreover, our study showed the predominance of gram-negative bacteria both in hospital and household AC. The observed predominance of gram-negative bacteria in AC aligns with a previous study done in Japan showed surface of ac filter was contaminated with 10 bacterial genera where 6 of them were gram negative and the remaining 4 genera were gam positive. (Wtanabe et al.,2021). Gram-negative bacteria are known to have unique structured lipopolysaccharide (LPS) which has additional membrane, efflux pumps and other resistance

mechanisms (Saxena D et., al 2023) which make them to persist in various environmental challenges, they are more difficult to treat (Saxena et al., 2023). Hence, resulting to dominance in both settings. Furthermore, it has endotoxins in their cell walls that might cause allergic sensitization in human and workplace-related illness (Wtanabe et al.,2021). As showed earlier, among gram – bacteria *P. putida, P. monteilli, P. fulva, P. stutzeri, E. cloacae, E. hormaechae, A. baumannii, A. variabilis, A. pitti, M. calida, L. adecarboxylata* – cause severe infections like bacteremia, respiratory infections, pneumonia, UTI, wound infections etc. (Price et al., 2017; Venkatasubramani & Viswanathan, 2019). Among them *A. baumannii, E. cloacae, E. hormaechei* are known for its high mortality rate and significant morbidity (Lemos et al., 2014; Logan & Weinstein, 2017). However, we have observed that *Enterobacter* is found exclusively in household ac and enterococcus is found exclusively in hospital. Hence, these findings suggest that *Enterobacter* favors to grow in household AC and *Enterococcus* favors growing in hospital ac which does not align with other studies because these bacteria are frequently found in hospital settings as well. (D'Souza et al., 2019).

Gram-positive bacteria are often part of normal skin flora in human and it sheds direct human contact (Ardelt et al., 2014; Viktorovs et al., 2022). Our findings observed both gram-positive (25%) and gram-negative bacteria (75%) in hospitals which aligns with other previous study by Morfín-Otero et al. (2012). We found only 4 types of bacteria gram-positive *(S. haemolyticus, S. arlettae, E. hirae, B.cerius)* where *(S. haemolyticus, S. arlettae* could cause bacteremia with venous catheters or invasive devices, endocarditis (Ogawa et al., 2010). Though, *E. hirae* is commonly associated with infections in animals but can cause cerebral abscess, wound infection, bacteremia, post-neurosurgical meningitis, neonatal meningitis, skin and soft tissue infections, pneumonia, septicemia, UTIs and intestinal / abdominal cavity infections (Davin-Regli et al.,2019). In immunocompromised patients, *E. hirae* bacteremia could lead to death (Nakamura et al., 2021). On the other hand, *Bacillus cerius* is another opportunistic pathogen that's commonly associated to foodborne illness and it is found in various hospital environments and patients which caused cross contamination and nosocomial infections. (Glasset et al., 2018).

Furthermore, several highly pathogenic bacteria such as *A. baumannii, E. cloacae, E. hormaechei* in households which is alarming. We also found comparatively less pathogenic but opportunistic pathogens – *A. vaiabilis, A.pittii, P. putida, P.stutzeri, L.adecarboxylata* which can lead to severe infections to immune-compromised people (Price et al., 2017;

Venkatasubramani & Viswanathan, 2019). To note, there was attached toilet in both hospital and households. We know, bacteria that are found in fecal matter, or toilets, they survive in high humidity, can be aerosolized when the toilet is flushed without covering the lid (Yano et al. (2017). They get stuck in ac dust filter and proliferate there and spread disease. (Price et al., 2017; Brown et al., 2012). Our investigated bacteria can also be found in toilets or fecal contaminants include *Pseudomonas putida*, *Pseudomonas monteilli*, *Pseudomonas stutzeri*, *Acinetobacter variabilis*, *Acinetobacter pittii*, *Acinetobacter baumannii*, *Enterobacter cloacae*, *Enterobacter hormaechei*, *Leclercia adecarboxylata*, *Enterococcus hirae*, *Bacillus cereus*, *Staphylococcus haemolyticus*, and *Staphylococcus arlettae*. (Flores et al., 2011; Hill et al., 2002; Miyauchi et al., 2008; Chan et al., 2011; Al-Sheridah et al., 2014; Beukers et al., 2017; Nakamura et al., 2021)

4.2. Uncommon Bacteria

However, specific studies about *Enterococcus* spp. residing in AC is not explicitly documented. There were a bunch of research that specified *Enterococcus* spp. Found in various surfaces, air dust, and water sources, in healthcare settings of hospitals. A study by Li et al. (2021) stated hospital ACs as hotspots as they can accumulate loads of resistance and pathogens over time and emphasized the importance of monitoring bacterial genera like *Enterococcus, Staphylococcus, Streptococcus* in hospital environments while indicating their crucial role in spreading antibiotic resistance. Another undocumented bacterium found in our study is *Leclercia adecarboxylata*. It is not commonly found in AC however it has its potential risk as an opportunistic pathogen (Zayet et al., 2021; Mauri et al., 2013; Yescas-Zazueta, 2024). Furthermore, *Mixta* spp. *(Mixta calida)* is less documented in research field, however some paper suggested this bacterium imposing risks for immunocompromised individuals which reinforced the potential for *Mixta. calida* act as an opportunistic pathogen (Abedi et al., 2021; Tsilifis & Pareja-Cebrian, 2021)

4.3. Proteome Identification by MALDI-TOF:

In our study, MALDI-TOF was able to identify all 40 bacterial isolates to the species level using the MBT compass HT RUO software to compare the acquired spectra. Here, 80% of the sample was recorded as high confidence level scored between 2-3, highlighted with green. The rest 20 % sample was recorded in score between 1.70-1.99 (yellow). Here, several factors could contribute to having a low confidence level. MALDI-TOF system finds it difficult to

identify closely related species (Seng et al., 2010). For example, *P. fulva* (MHC3) has a score with 1.91, *and P. putida in* MBC10 (1.76) resulting in a yellow range. It might happen that *P. fulva* or *P. putida* generated a mass spectrum which is closely related to other species on the same genus level. Having similar protein profiles would also contribute to the overlap of their mass spectra remarkably, which can lead to confusion in identifying the species efficiently, resulting in a lower log score. (Hodge et al., 2013). Moreover, inconsistent protein extraction or contamination might lead to spectra which fail to ensure clarity for a high confidence score. (Bittremieux et al., 2017). On the other hand, due to evident protein fingerprints, well-established reference spectra and consistency of optimal sample handling resulted in 80% isolates with as high confidence score.

4.4. Antibiotic Resistance:

Our findings indicated similar bacteria found in only Mymensingh hospital and household AC dust filters which included- *A. baumannii* and *P. stutzeri*. Both of them are highly resistant bacteria. For instance, *A. baumannii* found in hospitals were resistant to ten antibiotics (C, ATM, CPM, VA, AMP, IPM, AZM, LZ, AMC, CFM) and *A. baumannii* found in households (MB) were resistant to seven antibiotics including- K, C, ATM, VA, AMP, LZ and CFM. Likewise, *P. stutzeri* from hospital (MH) was resistant to eight antibiotics including- K, C, VA, LZ, APM, AMC and CFM. Moreover, *P. stutzeri* from households (MB) were resistant to three antibiotics – C, VA, and LZ. It is encouraging that household bacteria tend to have lower resistance patterns compared to hospitals. However, the presence of XDR bacteria in non-hospital settings imposes a potential threat to public health even if they have comparatively lower resistance levels. It can stem from various reasons such as antibiotic misuse, the spread of resistance strains, cross-contamination, environmental contamination etc. The misuse of antibiotics, especially through incomplete antibiotic treatments, self-medications which could be the primary driver of this resistance. (Bert et al., 2022; Morgan et al., 2011; Aleem et al., 2017).) The presence of such resistant strains suggest that these might evolve further, could become more resistant, and transfer resistant genes to other bacteria which would create community-wide resistance issues. Study shows that resistance can spread among bacterial populations via horizontal gene transfer (Svara & Rankin, 2011; Zurfluh et al., 2017). For instance, Gulberg et al. analyzed how antibiotics even at low concentrations could work for favor in resistant bacteria which could then proliferate and share resistance genes with susceptible strains (Gullberg et al., 2011). As the households are within 100 meters, there could

be a possibility of direct transmission from hospitals to households. However, it is premature to assert transmission from hospitals with low sample quantity in this study.

The overall prevalence of XDR bacteria found in hospital AC is 100% and in household AC is 85%. In hospital, the most resistant bacteria is *A. baumannii* which showed resistance to eleven different antibiotic classes out of twelve tested antibiotics. *S. haemolyticus, P. putida, P. monteilii* showed resistance to 10 different antibiotics. Additionally, *E. hirae, A. baumannii, P. fulva* showed resistance to nine different antibiotics. Furthermore, *P. stutzeri, M. calida*. displayed resistance to 8 distinct antibiotics.

Contrary to expectations, household AC units harbored a substantial proportion of XDR bacteria (85%) and a smaller fraction of MDR bacteria (15%). The most resistant bacteria identified is *E. cloacae* which showed resistance to all antibiotics which have tested in the study. *E. hormaechei* demonstrated resistance to 9 antibiotics, *P. putida* was resistant to 8, while *A. baumannii* and *A. pittii* were resistant to 7 antibiotics and both *A. variabilis* and *L. adecarboxylata* showed resistance to six antibiotics.

4.4.1. Potential Transmission Pathways

In household, the significant resistance of e. cloacae might arise from various sources, The presence of *A. baumannii, P. putida, P. stutzeri* proves that these extremely resistance bacteria aren't isolated in hospitals only. Though pseudomonas showed different antibiotic resistance patterns in hospital and household, *A. baumannii* showed similar resistant patterns both in house and hospital. As the households were near to the hospitals, bacteria could spread through cross contamination pathways for instance patients, hospital workers clothing, contaminated equipment, and also air could be a route of transmission (Harrison, 2003). As some of our observed bacteria that are common in hospital and household can be aerosolized and spread. (Munoz-Price et al., 2013; Noble & Overman, 1994)

However, we found *Enterobacter* solely in households with extremely high resistant pattern which is quite alarming. High resistance of *e. cloacae and E. hormachei* have emerged as a threat as the infection has caused many deaths in immunocompromised patients. (Seng et al., 2010). As, *E. cloacae*, and *E. hormaechei* are common bacteria in gut flora, animal gastrointestinal tract (Davin-Regli et al.,2019). Hence, spreading through aerosol from toilets could be a source. Moreover, high moisture in households could be another reason for

Enterobacter to proliferate there (Jayaprakash et al., 2017). Unlike hospitals, households are not exposed to extensive usage of antibiotics (Goodman et al., 2020). Hence, it was unexpected to observe this extensive resistance pattern of *Enterobacter*, *Pseudomonas*, *Acinetobacter* in households. However, the presence of the XDR and MDR pattern suggests that households are also exposed to antibiotic misuse, which lead them to become resistant to many antibiotics. Antibiotic misuse multiplies the issue as the spread of these pathogenic-resistant bacteria will complicate and reduce the available treatment efficiency (Ventola, 2015). The presence of these bacteria is dangerous for immunocompromised patients as they undergo various antibiotic treatments often. The observed opportunistic bacteria can proliferate and infect easily in them and cause suffering.

4.5. Strength and Limitation:

The study employed MALDI-TOF for bacterial identification which saved time and labor and also could be the alternative of PCR, eliminating complex of primer designing, DNA amplification. It addressed an overlooked area in by discovering the bacterial diversity in dust filters of AC in hospital and household in Bangladesh.

Gram staining couldn't be performed fully because the safranin dye wasn't functioning. The bacteria failed to obtain safranin dye during the gram staining, even when the safranin dye absorption was being performed alone. Therefore, all bacteria gave colorless appearance in the microscope when dyed with safranin alone. Moreover, PCR for all the bacteria could not be performed due to the absence of primers. However, in the beginning of this study, some PCR and gel electrophoresis were successfully performed. Furthermore, due to restrictions, full procedure of MALDI-TOF could not be observed and the main machine wasn't available to study.

4.6. Conclusion

AC is the reservoir of various bacteria which could spread to the indoor environment and cause illness. The discovery of XDR bacteria imposes potential threat to public health in hospital as well as household. Our study focused on the bacterial diversity in dust filter of the AC. We found *Pseudomomas, Acinetobacter, Staphylococcus, Enterobacter* in high abundance where 3 genera were Gram- positive *(Staphylococcus, Enterococcus, Bacillus*) and the rest five genera were gram negative (*Pseudomonas, Acinetobacter, Enterobacter, Leclercia, Mixta)*. Our study found highly pathogenic and XDR bacteria like *Acinetobacter, Enterobacter, Staphylococcus, Enterococcus, Bacillus, Pseudomonas* which infections raise alarms about the efficacy of treating immunocompromised people, elderly and patients in hospitals. In hospital, due to high traffic of patients, attendance and healthcare individuals combined with the contamination of XDR bacteria multiplies the risk of nosocomial infections. *A. baumannii* being resistant to 11 classes of antibiotics imposes direct threat to the public health and especially immunocompromised individuals, patients in ICU, dialysis room, and cancer patients, newborns etc. On the other hand, we have found few bacteria that have bit less antibiotic resistance pattern but it does not suggest that households impose less threats. We found XDR and MDR bacteria here, including *e. cloacae* which was resistant to all antibiotics tested, *Acinetobacter, Pseudomonas, Leclercia* with high resistant patterns. Factors like cross contamination, aerosolization from toilets, and visitors, humidity, moisture take part in spreading these pathogens. Our study also suggested that bacteria like *A. baumannii* could spread to near households via air, cross contaminations etc. As bacteria in the dust filter of AC contaminates the inside parts of the AC and spreads through the cooled air, it is crucial to clean and maintain the proper cleanliness protocols accordingly depending on the location and health of individuals. In this study, the findings underscore an urgent call for the regimented surveillance of air conditioners, enhanced infection control measures not in hospital but also in households. Also, public awareness programs to reduce the risk of antibiotic resistance and maintaining personal hygiene. Failing to address this problem would bring considerable burden to public health with widespread outbreak of extensively resistant bacterial infection, higher cost for the treatment and cost of losing more lives.

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Appdx Table 1

MDR and XDR Pattern of Bacterial Isolates at Hospitals and Households

Source	B**acterial isolate**	**Isolate number**	R**esistant to**	X**DR/ MDR/ AMR**
MH	*Mixta calida*	1	ATM, CPM, VA, AMP, AZM, LZ, AMC, CFM	XDR
	Staphylococcus arlettae	1	ATM, CPM, AMP, AZM, AMC, CFM	XDR
	Pseudomonas fulva	1	C, ATM, VA, AMP, AZM, LZ, AMC, CFM	XDR
		2	C, ATM, VA, AMP, AZM, LZ, AMC, CFM	XDR
	Staphylococcus haemolyticus	1	K, ATM, CPM, AMP, IPM, AZM, CFM, AMC	XDR
		2	ATM, CPM, AMP, AZM, CFM,	XDR
	Acinetobacter baumannii	1	C, ATM, CPM, VA, AMP, IPM, AZM, LZ, AMC, CFM	XDR
		2	C, ATM, CPM, VA, AMP, AZM, LZ, TE, CFM	XDR
		3	C, ATM, CPM, VA, AMP, LZ, AMC, CFM	XDR

		4	C, ATM, CPM, VA, AMP, LZ, CFM	XDR
	Bacillus cereus	1	ATM, CPM, AMP, AMC, CFM	XDR
	Pseudomonas stutzeri	1	K, C, VA, AMP, LZ, AMC, TE, CFM	XDR
MB	*Enterobacter hormaechei*	1	AMP, AZM, LZ, TE, AMC, VA, CFM	XDR
		2	VA, AMP, AZM, LZ, AMC, TE, CFM	XDR
		3	VA, AMP, AZM, LZ, AMC, CFM	XDR
		4	VA, AMP, AZM, LZ, AMC, CFM	XDR
		5	ATM, CPM, VA, AMP, AMC, CFM	XDR
		6	VA, AMP, AZM, LZ, AMC	XDR
		7	VA, AMP, AZM, LZ, AMC	XDR
		8	VA, AMP, AZM, LZ, AMC	XDR
	Acinetobacter variabilis	1	ATM, VA, AZM, AMP, CFM	XDR
		2	ATM, VA, AMP, AZM, LZ, CFM	XDR

	Acinetobacter baumannii	1	K, C, ATM, VA, AMP, LZ, CFM	XDR
	Acinetobacter pittii	1	C, ATM, VA, AMP, LZ, AMC, CFM	XDR
	Pseudomonas stutzeri	1	C, VA, LZ	MDR
		2	C, VA, LZ	MDR
	Pseudomonas putida	1	C, ATM, VA, AMP, AZM, LZ, AMC, CFM	XDR
	Leclercia adecarboxylata	1	K, VA, AMP, AZM, LZ, CFM	XDR
UH	*Staphylococcus haemolyticus*	1	K, C, ATM, CPM, AMP, IPM, LZ, AMC, CFM	XDR
	Pseudomonas putida	1	C, ATM, CPM, VA, AMP, AZM, LZ, AMC, TE, CFM	XDR
		2	C, ATM, VA, AMP, AZM, LZ, TE, AMC, CFM	XDR
	Pseudomonas monteilii	1	C, CPM, VA, AMP, AZM, LZ, AMC, TE, CFM	XDR
	Enterococcus hirae	1	K, C, ATM, CPM, AMP, IPM, AZM, TE, CFM	XDR

		2	K, C, ATM, CPM, IPM, AZM, TE, CFM	XDR
		3	K, C, ATM, CPM, AMP, IPM, AZM, TE, CFM	XDR
		4	K, ATM, CPM, AMP, TE, CFM	XDR
UB	*Enterobacter cloacae*	1	VA, AMP, AZM, LZ, AMC, CFM	XDR
		2	C, VA, AMP, AZM, LZ, AMC	XDR
		3	K, ATM, CPM, AMP, IPM, LZ, TE, CFM	XDR
	Leclercia adecarboxylata	1	VA, AZM, LZ	MDR

H: Hospital, B: Community, AZM: Azithromycin, AMP: Ampicillin, VA: Vancomycin, TET: Tetracycline, CPM: Cefepime, C: Chloramphenicol, IPM: Imipenem, LZ: Linezolid, CFM: Cefixime, ATM: Aztreonam, K: Kanamycin, AMC: Amoxicillin-Clavulanic Acid.

R- Resistant, MDR- isolate is resistant to at least ≥ 3 antibiotic classes, XDR- isolate is resistant to at least ≥ 5 antibiotic classes

Appdx Table 2

Bacterial Identification and Interpretation Using MBT Compass

Sample ID	Organism (Best Match)	Score Value	Organism (2nd Best Match)	Score Value
MH C1	*Mixta calida*	1.97	*Mixta calida*	1.97
MH C2	*Staphylococcus arlettae*	2.00	*Staphylococcus arlettae*	2.00
MH C3	*Pseudomonas fulva*	1.97	*Pseudomonas fulva*	1.82
MH C4	*Staphylococcus haemolyticus*	2.22	*Staphylococcus haemolyticus*	2.12
MH C5	*Staphylococcus haemolyticus*	2.23	*Staphylococcus haemolyticus*	2.19
MH C6	*Acinetobacter baumannii*	2.38	*Acinetobacter baumannii*	2.18
MH C7	*Acinetobacter baumannii*	2.30	*Acinetobacter baumannii*	2.22
MH C8	*Acinetobacter baumannii*	2.45	*Acinetobacter baumannii*	2.07
MH C9	*Pseudomonas fulva*	1.91	*Pseudomonas putida*	1.77
MH C10	*Bacillus cereus*	2.24	*Bacillus cereus*	2.19
MH C11	*Pseudomonas stutzeri*	2.14	*Pseudomonas stutzeri*	2.08
MH C12	*Acinetobacter baumannii*	1.78	*Acinetobacter baumannii*	1.72
MB C1	*Enterobacter hormaechei*	2.36	*Enterobacter hormaechei*	2.33
MB C2	*Acinetobacter variabilis*	2.05	*Acinetobacter variabilis*	2.02
MB C3	*Leclercia adecarboxylata*	2.25	*Leclercia adecarboxylata*	2.20
MB C4	*Enterobacter hormaechei*	2.46	*Enterobacter hormaechei*	2.41
MB C5	*Enterobacter hormaechei*	2.29	*Enterobacter hormaechei*	2.28
MB C6	*Acinetobacter variabilis*	1.96	*Acinetobacter variabilis*	1.96

Sample ID	Organism (Best Match)	Score Value	Organism (2nd Best Match)	Score Value
MB C7	*Acinetobacter baumannii*	2.36	*Acinetobacter baumannii*	2.30
MB C8	*Pseudomonas stutzeri*	1.97	*Pseudomonas stutzeri*	1.95
MB C9	*Pseudomonas stutzeri*	2.01	*Pseudomonas stutzeri*	1.98
MB C10	*Pseudomonas putida*	1.76	*Pseudomonas putida*	1.76
MB C12	*Acinetobacter pittii*	2.43	*Acinetobacter pittii*	2.36
MB C13	*Enterobacter hormaechei*	2.41	*Enterobacter hormaechei*	2.38
MB C14	*Enterobacter hormaechei*	2.43	*Enterobacter hormaechei*	2.43
MB C16	*Enterobacter hormaechei*	2.05	*Enterobacter hormaechei*	2.01
MB C18	*Enterobacter hormaechei*	2.35	*Enterobacter hormaechei*	2.35
MB C21	*Enterobacter hormaechei*	2.45	*Enterobacter hormaechei*	2.38
UH1	*Staphylococcus haemolyticus*	2.11	*Staphylococcus haemolyticus*	1.99
UH2	*Pseudomonas putida*	2.14	*Pseudomonas monteilii*	1.90
UH3	*Pseudomonas putida*	2.07	*Pseudomonas putida*	2.07
UH4	*Pseudomonas monteilii*	1.92	*Pseudomonas putida*	1.88
UH5	*Enterococcus hirae*	2.20	*Enterococcus hirae*	2.16
UH6	*Enterococcus hirae*	2.28	*Enterococcus hirae*	2.26
UH7	*Enterococcus hirae*	2.34	*Enterococcus hirae*	2.30
UH8	*Enterococcus hirae*	2.42	*Enterococcus hirae*	2.37
UB1	*Enterobacter cloacae*	2.36	*Enterobacter asburiae*	2.21
UB2	*Enterobacter cloacae*	2.23	*Enterobacter cloacae*	2.11
UB3	*Enterobacter cloacae*	2.35	*Enterobacter cloacae*	2.33

Sample ID	Organism (Best Match)	Score Value	Organism (2nd Best Match)	Score Value
UB4	*Leclercia adecarboxylata*	2.53	*Leclercia adecarboxylata*	2.41