# Phenotypic and Molecular detection of *Acinetobacter baumannii* Retrieved from the Environmental Surfaces of a Hospital in Rural Bangladesh

By

Homayra Zarin Noor Beva

19126071

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

Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University

August 2023

## Declaration:

It is hereby declared that,

1. The thesis submitted "Phenotypic and Molecular detection of *Acinetobacter baumannii* Retrieved from the Environmental Surfaces of a Hospital in Rural Bangladesh" is a project work with the collaboration of "International Centre for Diarrhoeal Disease Research, Bangladesh" while completing a degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, accepted, or submitted, for any other degree or diploma at a university or other institution.

3. This thesis data could not be published anywhere without the consent of icddr,b and mine.

4 I have acknowledged all main sources of help.

**Student's Full name and Signature:**

**Homayra Zarin Noor Beva**

**ID: 19126071**

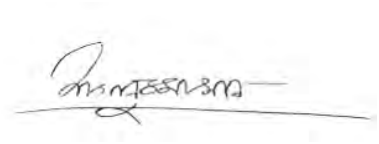
**Microbiology, MNS Department**

**BRAC University**

## Approval:

This research study was funded by core donors who provide unrestricted support to icddr,b for its operations and research. We gratefully acknowledge our core donors' support and commitment to icddr, b's research efforts. The study titled “Phenotypic and Molecular detection of *Acinetobacter baumannii* Retrieved from the Environmental Surfaces of a Hospital in Rural Bangladesh’’ submitted by Homayra Zarin Noor Beva (Student ID: 19126071) has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on 17 August 2023.

## Examining Committee:



**Internal Supervisor**

**Md. Hasanuzzaman**

Senior Lecturer

Department of MNS

BRAC University

**External Supervisor**

**Muntasir Alam, PhD**

Assistant Scientist

Infectious disease division (IDD)

Virology laboratory

icddr,b

**Program Coordinator**

**Nadia Sultana Deen, PhD**

Associate Professor

Department of MNS

BRAC University

**Department Head**

**A F M Yusuf Haider, PhD**

Professor and chairperson

Department of MNS

BRAC University

## Acknowledgment:

First of all, I would like to express my gratitude to the Almighty Allah for providing me an opportunity to complete my research work with patience and strength, under the supervision of promising individuals. After that, I want to express my gratefulness towards **Dr. Mustafizur Rahman**, Senior Scientist and Head of Virology laboratory, icddr,b and **Dr. A.F.M Yusuf Haider**, Chairperson of Mathematics and Natural Science Department, Brac University for providing me with the opportunity of completing my thesis under icddr,b.

Secondly, I would like to express my deepest gratitude towards my external supervisor, **Dr. Muntasir Alam**, Assistant Scientist from Virology laboratory, for allowing me to be a part of this current study that reflects his contribution of Ideas and knowledge. I am also grateful for his guidance and supervision regarding this current study which I believe has helped me to polish my research skills and wet lab skills.

Thirdly, I would like to mention my internal supervisor, **Md. Hasanuzzaman**, Senior Lecturer, MNS Department, Brac University, who has always checked on my progress regarding the thesis project and advised me to make right decisions. He has always encouraged me to perform my best. I will be forever grateful to him for his immense support and kindness.

Finally, I would like to thank **Afsana Rashed**, Research Assistant, Virology Laboratory, icddr,b for helping me shape my overall thesis by utilizing the available materials and providing me with necessary suggestions. I would also like to thank, **Md. Saiful Islam**, Senior Research Assistant, Virology Laboratory, icddr,b for guiding me with proper constructive suggestions and also for helping with the 16s sequencing based work of my thesis.

Moreover, I would like to thank my other team members, **Md. Fakhruddin,** Research Officer, Virology Laboratory, icddr,b; **Arpita Shyma Deb,** Research Officer, Virology Laboratory, icddr,b**; Nairita Ahsan Faruqui,** Research assistant**,** Virology Laboratory, icddr,b, for helping me achieve required skills and techniques to complete this project.

Also, I would like to thank **Shaket Md. Abul Hasan**, Medical Technologist, Virology Laboratory, icddr,b and **Md**. **Aminul Islam,** Field assistant, Virology Laboratory, icddr,b for their contribution.

Most Importantly, I want to express my unconditional love towards my best friends and family members, who have supported me throughout this whole journey.

**Homayra Zarin Noor Beva**

**August 2023**

## Abstract:

**Background:** Hospital Associated Infections (HAI) pose a great deal of risk to those with impaired immune system, especially Intensive Care Unit (ICU) and Neonatal Intensive Care Unit (NICU) patients. Among the important nosocomial pathogens, *Acinetobacter baumannii* has been marked as the "red alert" pathogen due to its resistance to major antibiotics including the last-resort antibiotics like carbapenems. *Acinetobacter baumannii* causes sepsis, pneumonia, urinary tract infection, surgical site infections etc. Here we focused on the identification, characterization and investigation of antibiotic susceptibility of *A.baumannii* isolated from the environmental surface of the neonatal intensive care unit of a charitable child hospital from rural Bangladesh.

**Methodology:** A total of 23 samples were collected from different neonatal wards in amies media and transported to Dhaka at 2-8°C. The samples were inoculated in the Chromagar *Acinetobacter* media. Phenotypic characterization was done via methods such as colony morphology, Gram's staining, oxidase test, catalase test. Identification and antimicrobial susceptibility testing was done using the VITEK-2 instrument. Finally, 16s rRNA sequencing was performed (PCR, Gel Electrophoresis, Sanger Sequencing) and a Maximum Likelihood phylogenetic tree was formed using BioEdit, Chromas, Blast,Mega, IQ-tree.

**Result:** A total of 23 environmental swab samples were collected. Bacterial growth was observed on 43% (10/23) environmental swab samples from which *A.baumannii* like colonies were observed on the Chromagar *Acinetobacter* media**.** VITEK-2 identification and antimicrobial susceptibility test on the suspected *Acinetobacter spp.* confirmed four of them to be *A.baumannii* (25% of 16) while confirming two (2/4) of the *A.baumannii* (50%) as MDR isolates. Both the MDR isolates were resistant against Cephalosporins, Carbapenems and Fluoroquinolones; intermediately resistant to Colistin and sensitive to Tigecycline and Cefoperazone/ sulbactam. The 16s rRNA sequencing supported the VITEK-2 identification of 75% (3/4) *A.baumannii correctly* while identifying 25% (1/4) as *A.nosocomialis* with percentage identity above 97%. The phylogenetic inference revealed that the *A.nosocomialis* didn't share much similarity with its comparison group whereas the three *A.baumannii* isolates were found to be more closely related to one another (bootstrap value 99.7) than other global comparison strains, indicating that the same region of isolation might have a connection with sharing similarity as well as spreading of these isolates across the hospital.

**Conclusion:** The study sheds light on the alarming situation created by the existence of MDR *A.baumannii* in the NICU. The pathogen is able to share MDR as well as strong survival genes by spreading in the hospital setting which is threatening to not only newborns, but also to other hospitalized patients. Therefore, strong measures need to be taken before this deadly bacteria becomes unstoppable.

## Table of Contents:


Declaration ..... 2
Approval ..... 3
Acknowledgment ..... 4
Abstract ..... 6
1. LITERATURE REVIEW: ..... 12
1.1 Healthcare-Associated Infections ..... 12
1.2 Impact of HAIs ..... 12
1.3 The burden of *A.baumannii* ..... 13
1.4 Identification of *A.baumannii* ..... 13
1.6 Global Prevalence of *A.baumannii* ..... 15
1.7 Clinical significance of *A.baumannii* ..... 15
1.8 A.baumannii infections on neonates: ..... 16
1.9 Multi-drug Resistant *A.baumannii* ..... 16
1.10 Objective of the study ..... 18
2. METHODOLOGY ..... 20
2.1 Study design and settings ..... 20
2.2 Flowchart of the Study design ..... 20
2.3 Sample collection ..... 21
2.4 Sample Processing ..... 21
2.5 Phenotypic Characterization ..... 21
2.5.1 Gram staining ..... 21
2.5.2 Catalase test ..... 22
2.5.3 Oxidase test ..... 22
2.6 Subculture on MacConkey agar ..... 22
2.7 VITEK-2 Identification and AST: ..... 22
2.8 Molecular characterization of the environmental isolates: ..... 23
2.8.1 DNA Extraction/ Preparation (Boiling Method) ..... 23
2.8.2 Conventional PCR ..... 24
2.8.2.1 Primer Details and Sequence of 16s rRNA gene ..... 24
2.8.2.2 Mastermix preparation ..... 24

2.8.2.3 PCR condition ..... 25
2.8.3 Gel Electrophoresis ..... 26
2.8.3.1 1.5% Agarose Gel Preparation ..... 26
2.8.3.2 Electrophoresis ..... 26
2.8.3.3 Visualization ..... 26
2.8.4 Sanger Sequencing ..... 27
2.8.4.1 PCR cleanup ..... 27
2.8.4.2 Cycle sequencing ..... 27
2.8.4.2.1 Mastermix preparation ..... 27
2.8.4.2.2 Cycle sequencing condition ..... 28
2.8.4.3 Sequencing cleanup ..... 28
2.8.4.4 Capillary electrophoresis ..... 29
2.8.4.5 Sequence analysis ..... 29
2.8.5 Phylogeny ..... 29
3. RESULT ..... 31
3.1 Environmental surface positive isolates ..... 33
3.2 Confirmation of the isolates via colony morphology, gram staining and biochemical tests ..... 34
3.2.1 Colony morphology of *Acinetobacter spp* suspects ..... 34
3.2.2 Gram stain result of *Acinetobacter spp* suspects ..... 34
3.2.3 Catalase and Oxidase tests result of *Acinetobacter spp* suspects ..... 35
3.3 Colony morphology on MacConkey agar ..... 36
3.4 VITEK-2 identification and Antimicrobial Susceptibility result ..... 36
3.5 Visualization of the bands of *A.baumannii* PCR products ..... 39
3.6 Sequence Analysis of all the VITEK-2 confirmed *A.baumannii* ..... 40
3.6.1 Generating Chromatogram of Sequences ..... 40
3.6.2 Query Sequence Formation ..... 40
3.6.3 BLASTN Result Analysis ..... 47
Table 14: BLASTN results ..... 48
4. DISCUSSION ..... 52
5. CONCLUSION ..... 57
7. REFERENCES ..... 58

## List of Figures:

Figure 1: Flowchart of the study methodology ..... 20
Figure 2: Positive growth of organisms from Environmental samples ..... 33
Figure 3: Colony of suspected *Acinetobacter spp* on Chromagar *Acinetobacter* media ..... 34
Figure 4: microscopic image of suspected *Acinetobacter* spp after Gram staining ..... 35
Figure 5: Catalase test result of suspected *Acinetobacter spp* ..... 35
Figure 6: Colony of suspected *Acinetobacter spp* on Chromagar *Acinetobacter* media ..... 36
Figure 7: Resistance data of *A.baumannii* isolates against antibiotics ..... 38
Figure 8: Visualization of the Bands of A.baumannii isolate from Gel-Image (green box) ..... 39
Figure 10: Phylogenetic tree comparing the obtained isolates of all four *Acinetobacter* (three *A.baumannii*, one *A.nosocomialis*) and global isolates based on 16srRNA sequence ..... 49

## List of Tables:

Table 1: Primer details for this PCR and 16S rRNA gene sequence ..... 24
Table 2: PCR reaction mixture for this reaction ..... 24
Table 3: Setting up the Thermal Cycler (Applied Biosystems ProFlex PCR System) ..... 25
Table 4: Agarose Gel preparation (1.5%) ..... 26
Table 5: Reagents and their amounts used for Cycle Sequencing Master-mix Preparation ..... 27
Table 6: Cycle sequencing thermal condition cycle ..... 28
Table 7: Presumptive Identification based on CHROMagarTM *Acinetobacter* ..... 31
Table 8: VITEK 2 Identification data of suspected *A.baumannii* ..... 36
Table 9: VITEK-2 Antibiotic Susceptibility Patterns of Environmental Isolates of *A.baumannii* 37
Table 10: Query sequence of isolate 1 ..... 40
Table 11: Query sequence of isolate 2 ..... 41
Table 12: Query sequence of isolate 3 ..... 43
Table 13: query sequence of isolate 4 ..... 45
Table 14: BLASTN Results ..... 48

# CHAPTER 1
# LITERATURE REVIEW

# 1. LITERATURE REVIEW:

## 1.1 Healthcare-Associated Infections:

Healthcare-associated infections (HAI), which are also known as nosocomial infection, are infections that appear while receiving treatment. They may appear in an array of healthcare delivery settings, including ambulatory settings, long-term hospitalization, exposure to hospital environment, healthcare staff, and other infected patients. (Szabó et al., 2022). WHO reports that, almost 15% of all hospitalized patients suffer from HAI (7% and 10% in developed countries and developing countries, respectively) (Khan et al., 2017)

Different studies have shown concerns about the increasing diversity of pathogens that can persist in different environmental surfaces of hospitals. They are not only colonizing but also gaining resistance to survive and spread their antimicrobial resistance genes (Fahy et al., 2023). The colonization of such organisms can cause infections that are difficult to cure and can contribute to morbidity or mortality.

## 1.2 Impact of HAIs:

HAI poses a serious risk to those with impaired immune systems. The threat is increased significantly when it comes to patients in ICU. Patients in hospitals' ICUs who are critically ill and highly susceptible to infections are hospitalized there (Blot et al., 2022)Undoubtedly, this also threatens the neonates even further in the Neonatal Intensive care (NICU) due to their immature and weak immune system (Kajiyazdi et al., 2021).Studies have reported that, the NICU bloodstream infection rates connected with healthcare ranged from 5% to 32% (Sadowska-Krawczenko et al., 2012)UTIs were the second most common kind of HAI, accounting for 1.6% cases. Moreover, the study observed that 9.3% pneumonia and 18.6% bloodstream infections were the most common HAIs among 742 neonates admitted to the NICU. Added that, 1.3% cases of nosocomial urinary tract infection were also identified (Sadowska-Krawczenko et al., 2012)

Moreover, a study of Fahy et al. about 24% HAIs affected patients were ICU patients. Study also indicates pneumonia, surgical site infections, urinary tract infections and bloodstream infections as the most documented and most fatal nosocomial diseases where their prevalence were

respectively 28.9%, 18.1%, 1.5% and 9.9% among all HAIs. Most importantly, *Acinetobacter baumannii has* been identified to be highly responsible for these nosocomial diseases among other prevalent and dangerous MDR pathogens (Fahy et al., 2023)

## 1.3 The burden of *A.baumannii*

Because of an increased usage rate of broad-spectrum antibiotics, *A.baumannii* has become an emerging MDR nosocomial pathogen that has been reportedly more frequently identified in the last ten years. (Yadav et al., 2020) According to the Infectious Diseases Society of America (ISDA), *A.baumannii* is one of the "red alert" pathogens that seriously jeopardize the effectiveness of the current antibiotic arsenal. Reportedly, *A.baumannii* and *A.baumannii* strains having carbapenem -resistance have also been linked to 20.9% and 13.6% respectively, of all HAIs in ICUs. (Ayobami et al., 2019)

According to the National Healthcare Safety Network (2008), *A.baumannii* causes 8% of ventilator-associated pneumonia (VAP) and nearly 2% of all catheter-related bloodstream infections (BSIs), with death rates ranging from 13 to 30% (Hidron et al., 2008)

Moreover, *A.baumannii* infection-related mortality has been linked in numerous studies to bed rest, morbidity, catheterization, ICU stay, (Alrahmany et al., 2022) Therefore, *A.baumannii* can be identified as a burden to immunologically challenged individuals staying in the hospital.

## 1.4 Identification of *A.baumannii*

*A.baumannii* is an aerobic, non-lactose fermenting, gram-negative bacteria. It is also non motile oxidase negative, catalase positive. Due to the short rods shape, it is called cocco-bacillus(Yadav et al., 2020)

Both conventional and modern techniques (alone or combined) can be used to identify *A.baumannii*. colony morphology, gram staining, biochemical tests such as catalase, oxidase test, MIU test (motility indole urease test), TSI test(triple sugar iron test) etc. Moreover, methods like conventional or real time PCR, gel electrophoresis, Serology tests can also help to identify *A.baumannii*. Modern technologies such as the VITEK-2 system can efficiently and rapidly identify *A.baumannii*.

## 1.5 Possible Sources of *A.baumannii* in hospital environment:

*A.baumannii* can thrive in the hospital environment for a very long time, especially on inanimate surfaces. *A.baumannii* can be spread via direct contact with infected patients or indirectly through contact with contaminated inanimate objects. Due to the presence of different inanimate objects in the hospital setting, cross-colonization can take place frequently. The equipment and medical staff's attire, and their hands could contribute to the transmission of nosocomial infections which might lead to outbreaks (Pittet et al., 2006) Moreover, their ability to tolerate starvation, dehydration and form biofilms play a significant role in promoting its spread survival rate on dry surfaces. Their tolerance to temperature shifts also contribute to their ability to survive even in the most unfavorable environments.(Chapartegui-González et al., 2018)

*A.baumannii* can be found in hospital water, air, and inanimate surface samples, indicating that hospital settings may serve as a possible channel for the spread of *A.baumannii* infections, particularly in ICUs. Different studies also have highlighted the connection between *A.baumannii* outbreaks and environmental factors such as patient beds, air conditioners, and ventilation equipment. *A.baumannii* can be found on baby pacifiers, the bed wraps, and the wiper of the mobile aspirator, surgical tools, machines, catheters, cerebrospinal fluid shunts or Foley catheters (Rebic et al., 2018) medical equipment such as suctioning equipment, washbasins, bed rails, bedside tables, ventilators, pillows, mattresses, hygroscopic surgical bandages, resuscitation equipment, and trolleys, (Bernards et al., 2004) monitors, operation tables, anesthesia equipment, floors, tables , UV lamps, swabs on surfaces of, wash-hand basins,(Raka et al., 2013)curtains, door handle, drawer handle, bedside table, (Fournier et al., 2006)intravascular catheters, urinary catheters and drainage tubes etc. can act as potential sources of *A.baumannii*. Hospital food can also act as a possible source for acquiring *A.baumannii*,(Berlau et al., 1999) Also Bedrails play an important role in the survival and isolation of *A.baumannii* as a study mentioned about retrieving *A.baumannii* from Bed rail even after 9 days of discharge. (Jawad et al., 1996) Hands of Medical staff (Custovik et al., 2014), apron are also potential sources of *A.baumannii* (Kuczewski et al., 2022)

## 1.6 Global Prevalence of *A.baumannii*:

There are national, regional, and local prevalence-related data for *A.baumannii*.in June 2001, a prevalence study reflected that, *A.baumannii* infection was found to be 0.075% prevalent nationally in 1533 healthcare facilities with 305,656 patients where *A.baumannii* was the 15th most frequently isolated among other HAI causing bacteria.(Fournier et al., 2006)

*A.baumannii* is one of the important nosocomial pathogens in Bangladesh as well. The *A.baumannii* prevalence was about 14% in DMCH, Bangladesh, which is slightly higher than rates in underdeveloped nations like India, which were 9.5%. The prevalence rate was 18% in Japan, and it was greater in Kuwait and Saudi Arabia. Most international investigations that indicated a high percentage of *A.baumannii* isolated from ICU patients concur with this finding. According to their findings, the ICU superbug is *A.baumannii*. (Mahtab Uddin et al., 2022)

## 1.7 Clinical significance of *A.baumannii*:

Once *A.baumannii* is isolated in a medical facility, it poses a serious risk, especially in ICU units with chronically ill patients. These patients are at a greater risk of contracting *A.baumannii* infection because the majority of them have impaired immune systems and stay in hospitals for extended periods of time. Infections caused by *Acinetobacter spp*. that develop in hospitals include ventilator-associated pneumonia, skin and soft tissue infection, wound infection, UTIs, secondary meningitis, and bloodstream infection. The primary cause of these illnesses is *A.baumannii*. Patients who get artificial devices including catheters, sutures, ventilators, as well as those who have recently received dialysis or antibiotic medication, are also at risk of contracting *A.baumannii* infections.

According to a study, *A.baumannii* infections have increased by almost 30% over the years with mortality rates upto 36.5% (Appaneal et al., 2022) Numerous studies document elevated overall death rates in individuals with *A.baumannii* bacteremia or pneumonia. (Dijkshoorn et al., 2007)

## 1.8 A.baumannii infections on neonates:

According to (WHO), a child with the age under 28 days is identified as a neonate. A neonate has the highest susceptibility towards all kinds of infections due to its immature and weak immune system. Therefore, a deadly pathogen like *A.baumannii* if contracted leaves almost a low chance of survival for a child less than 28 days of age.

*A.baumannii* has become a significant pathogen that causes HAIs on a global scale. Infants with low birth weights admitted to the hospital, are immunocompromised and are more susceptible to nosocomial infections. Neonates admitted to NICU are at heightened risk of getting HAIs because to their underdeveloped immune systems and frequent invasive interventions, just as low birth weight infants referred to hospitals are immunocompromised and sensitive to nosocomial infections. *A.baumannii*-related bloodstream infections (BSIs) are most common in premature, low-birth-weight newborns.(Gramatniece et al., 2019)

26 newborns who had septicemia had blood samples taken, and 26 different *Acinetobacter* species were found. In other words, 26/240 or 10.8% of all neonatal septicemia cases were caused by *Acinetobacter*. 4 (15.4%) of these isolates were determined to be *Acinetobacter lwoffi,* and 22 (84.6%) were *A.baumannii* complex strains.

The mortality rate varies from 13.9% to 83% among all reports of *Acinetobacter* septicemias in newborns. Reportedly, 11.3% of people had died (ages 3-26). (Shete et al., 2009)

## 1.9 Multi-drug Resistant *A.baumannii*:

One of the top three threats to human health, according to the WHO, is antibiotic resistance due to the emergence of MDR bacteria. Among MDR bacteria, "ESKAPE," have been feared the most due to their high prevalence and resistance to antibiotics. *A.baumannii* has become more prominent MDR bacteria amongst ESKAPE group that has been documented more frequently during the past ten years due to the rising usage of broad-spectrum antibiotics in patients settling in the ICUs. (Towner, 2009)

Moreover, *A.baumannii* is receiving a lot of attention due to the rise in antimicrobial resistance and having strains that are MDR or XDR. The majority of regularly used antibiotics, such as aminopenicillins, 1$^{st}$ and 2nd generation cephalosporins, and chloramphenicol, are intrinsically ineffective against this pathogen. Additionally, it has a remarkable capacity to develop defenses

against tetracyclines, broad-spectrum -lactams, aminoglycosides, and fluoroquinolones. (Dijkshoorn et al., 2007)

In a study done by Yadav et al, reported that the rate of MDR *A.baumannii* isolation was 91.0% which is extensively high. Moreover, in a study by Shrestha et al. and Mishra et al, MDR *A.baumannii isolation* rates were around 96% and 95% of respectively.(Yadav et al., 2020)

Carbapenems have come to be thought of as the last resort antibiotics, due to its ability to effectively treat severe MDR infections after other conventional antibiotics like penicillin have failed. But, due to the emergence of carbapenem resistant *A.baumannii* , potentially toxic antibiotics such polymyxin B and colistin sulfate have been considered as last resort antibiotics. Unfortunately, Clinical strains of *A.baumannii* have also been found to be resistant to colistin lately.(Ilsan et al., 2021)Therefore, tigecycline can act as the final trump card against both carbapenem and colistin resistant*A.baumannii*.(Lu et al., 2023))

Reportedly, the mechanisms of carbapenem resistance are the synthesis of the enzyme carbapenemase, ESBL or AmpC β-lactamase producing capability. According to a study by Yadav et al. (2020), 19.9% of MDR *A.baumannii* produced ESBLs. A similar prevalence of ESBL was discovered in ICU patients in a prior investigation by Parajuli et al..The prevalence of metallo-β-lactamase (MBL) producing isolates was shown to be more common than ESBL and AmpC producing isolates in the particular investigation, where 67.7% of MDR *A.baumannii* were MBL producers (Yadav et al., 2020). Moreover. *A.baumannii* 's reduced sensitivity to third- and fourth-generation cephalosporins can also be a possible cause .

There are many known mechanisms of antimicrobial resistance of *A.baumannii* .Some other mechanisms are also being researched and some even are yet to be discovered. Firstly, One of the known approaches are the alteration of the antibiotic target site where *A.baumannii* reduces permeability or increases efflux to prevent the antibiotic to reach to the target site. Secondly, another approach is the enzymatic modification, which includes protecting the target site through genetic alterations to escape the effect of the antibiotic**.** Thirdly, one more approach requires the direct inactivation of antibiotics via hydrolysis or alterations. Lastly, the most important strategy of *A.baumannii* to defend itself from antibiotics is its genetic fluidity, rapid mutations and proper handling of foreign substances. (Kyriakidis et al., 2021)

"WHO" classified carbapenem resistant *A.baumannii* also known as "CRAB" , a high-priority target for antibiotic research and development in 2017 due large resistance report against antibiotics and report of causing large scale mortality and morbidity. Similarly, the Infectious Diseases Society of America (IDSA) designated it as a pathogen that poses a problem. *A.baumannii* that is multi-drug resistant poses a severe concern as well, the Centers for Disease Control and Prevention (CDC) reported in 2013; it is responsible for 7000 illnesses and 500 fatalities annually in the United States. (Alrahmany et al., 2022)

## 1.10 Objectives of the study:

The objective of this study is isolating such *A.baumannii* isolates from environmental surfaces of NICU, study their resistance pattern, phylogenetic relationship and locate the contaminated surfaces to prevent future outbreaks, or spreading of MDR genes.

# CHAPTER 2
# METHODOLOGY

## 2. METHODOLOGY:

### 2.1 Study design and settings:

The study was conducted at the International Center for Diarrhoeal Disease Research, Bangladesh, icddr,b. This study was carried out from September 2022-November 2022.

### 2.2 Flowchart of the Study design:

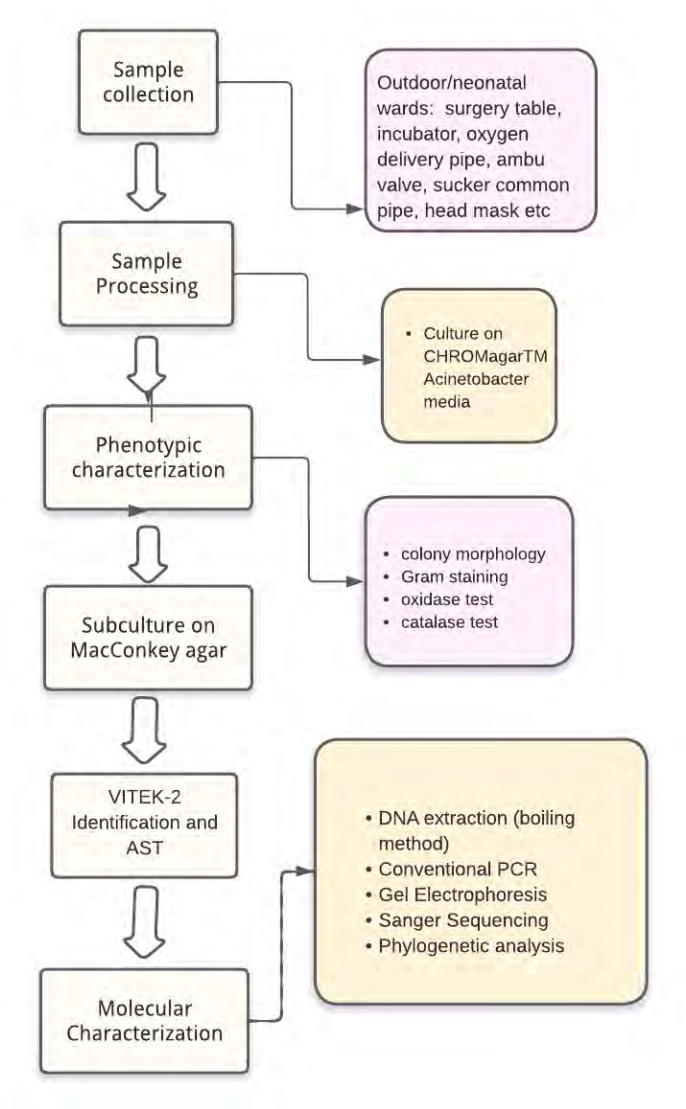



**Figure 1: Flowchart of the study methodology**

## 2.3 Sample collection:

A charitable Hospital from rural Bangladesh was selected from sample collection. Different objects from different sites were selected for swab sample collection. A sterile cotton swab was moistened with PBS and it was swiped over the targeted object. Afterwards, it was placed in the Amies transport media and transported at 2-8°C to Dhaka, within 24 hours of collection.

A total of 23 Swabs had been collected from Ambu valve, oxygen delivery pipe, Medical Suction device Inlet tube, nebulizer mask, procedure table, Medicine Tray, Nebulizer Mask, Head Mask, glass from places such as outdoor and several Neonatal words.

## 2.4 Sample Processing:

CHROMagar™ *Acinetobacter* was prepared according to the manufacturer's description. This is a highly selective media as it supports the growth of *Acinetobacter spp* along with a few other gram negative bacteria, inhibiting all other gram positive bacteria. According to the manual of ChromagarTM *Acinetobacter* media (instruction for use), *Acinetobacter spp.* provides red colonies and a few other gram negative bacteria provide blue colonies, even though mostly are inhibited along with all other gram positive bacteria.

The samples were inoculated on the ChromagarTM *Acinetobacter* media. The plates were kept in the incubator for 24 hours at 37°C.The plates were examined for the growth of red colonies of *Acinetobacter* species as earlier mentioned. Afterwards, biochemical tests were performed on the environmental isolates The isolates were subjected to gram staining, catalase and oxidase testing.

## 2.5 Phenotypic Characterization:

The red colonies from the media was selected for Gram staining and other biochemical tests. Firstly, the colony morphology was examined. Then, gram staining was performed on the chosen red colonies from the media.

### 2.5.1 Gram staining:

Gram staining allows differentiation between gram positive and gram negative organisms; therefore, it serves as a differential stain. Gram staining causes *A.baumannii* to show up as pink

color as it is a gram negative bacteria. Also gram staining helps to see the shape of the bacteria due to coloration. Under the microscope, *A.baumannii* is viewed as short, rounded or rod shaped for which they are called coccobacillus.

### 2.5.2 Catalase test:

After the gram negative test Oxidase test and catalase tests are also performed. As, *A.baumannii* is catalase positive it produces gas-bubbles while coming in contact with H2O2, breaking it down with catalase enzyme and producing water and oxygen.(H2O2 → H2O + O2)

### 2.5.3 Oxidase test:

*A.baumannii* being an oxidase negative microorganism, it appears colorless while coming in contact with Kovac's reagent, which means it does not contain cytochrome C oxidase enzyme.

## 2.6 Subculture on MacConkey agar:

The suspected isolates of *Acinetobacter spp*. were subcultured on MacConkey agar which is a selective media as it allows the growth of gram negative bacteria such as *Acinetobacter spp*. and inhibits other gram positive bacteria. It is a differential media as it allows color change to yellow of the media in the presence of lactose fermenters. As *Acinetobacter* spp are non-lactose fermenters, they can't cause the media to change color.

## 2.7 VITEK-2 Identification and AST:

VITEK 2 (bioMérieux) is a fully automated system that is able to perform bacterial identification and AST testing using its respective compatible software. VITEK-2 was performed on the suspected *A.baumannii* colonies produced from MacConkey agar medium. For the procedure, bacterial isolates were emulsified in 0.45% saline to the equivalent of a 0.5 McFarland turbidity standard to create suspension. The respective suspension was used in identification and AST for the VITEK 2 system. Instructions were followed according to the manufacturer's descriptions.

## 2.8 Molecular characterization of the environmental isolates:

After the phenotypic characterization, all the isolates were subjected to molecular characterization. PCR, Gel Electrophoresis, Sanger Sequencing, BLAST is performed. VITEK-2 identification was done on only the suspected *A.baumannii* isolates prior to performing other mentioned methods.

### 2.8.1 DNA Extraction/ Preparation (Boiling Method):

1. Pure culture was prepared for each isolate and single colony was inoculated in 5 ml LB broth .

2. The cultures were incubated overnight at 37°C.

3. 1 ml of bacterial culture in a micro centrifuge tube was taken and centrifuged at 10000 rpm for 5 mins.

4. The pellet was collected and the supernatant was discarded

5. Again, 1 ml of *A.baumannii* culture was added into the same micro centrifuge tube and centrifuged at 10000 rpm for 5 mins.

6. The supernatant was discarded and the pellet was collected.

7. 300 µL of nuclease free water was added and the pellet was resuspended. It was again centrifuged at 10000

rpm for 5 mins.

8. The supernatant was discarded and again the pellet was resuspended, into 300 µL of nuclease free water.

9. The tube was placed into a heat block, pre-heated at 100°C, for 10 mins.

10. Immediately the tube was transferred to ice for 10 mins.

11. Then the tube was centrifuged at 10000 rpm for 5 mins.

12. 250 µL of supernatant was carefully drawn without disturbing the pellet.

13. The total DNA was stored at -20°C until further use

## 2.8.2 Conventional PCR:

## 2.8.2.1 Primer Details and Sequence of 16s rRNA gene

**Table 1: Primer details for this PCR and 16S rRNA gene sequence**

| Gene | Primer sequence 5'-3' | Size |
|---|---|---|
| 16S rRNA | 27 F (AGAGTTTGATCCTGGCTCAG) | ~1450bp |
| | 1492R (TACGGTTACCTTGTTACGACTT) | |

## 2.8.2.2 Mastermix preparation:

**Table 2: PCR reaction mixture for this reaction**

| KIT: Promega Go Taq G2 Hot Star PCR | | | | |
|---|---|---|---|---|
| 16s RNA | Reagents | Volume (µL) | Number of sample | Volume (µL) |
| | MgCl2 | 1.0 | 14 | 14.0 |
| | Buffer (10X) | 4.0 | | 56.0 |
| | dNTP | 0.4 | | 5.6.0 |
| | 27F (20 uM) | 1.0 | | 14.0 |
| | 1492R (20 uM) | 1.0 | | 14.0 |

| | | | | |
|---|---|---|---|---|
| | Go Taq G2 Polymerase | 1.0 | | 14.0 |
| | Nuclease free water | 10.5 | | 147.0 |
| | Template | 2.0 | | 28.0 |
| | Total volume= | 20 µL | | 292.6 µL |

### 2.8.2.3 PCR condition:

**Table 3: Setting up the Thermal Cycler (Applied Biosystems ProFlex PCR System)**

| PCR condition | Temperature (◦C) | Time (min) |
|---|---|---|
| Initial denaturation | 95 | 5 |
| Denaturation | 95 | 1 |
| Annealing | 60 | 1 |
| Elongation | 72 | 1 |
| Final extension | 72 | 5 |

## 2.8.3 Gel Electrophoresis:

Gel electrophoresis is performed to evaluate the PCR product.

## 2.8.3.1 1.5% Agarose Gel Preparation:

**Table 4: Agarose Gel preparation (1.5%)**

| Components | Unit |
|---|---|
| Agarose **(InvitrogenTM)** | 0.6 g |
| TBE buffer **(0.5X)** | 40 mL |
| GelRed **(InvitrogenTM)** **(75bp - 20kb)** | 4 µL |

## 2.8.3.2 Electrophoresis:

1. The apparatus was filled with a 0.5X TBE buffer.
2. TBE solution is poured into a casting tray
3. the gel was cooled and solidified to create a gel slab with a row of wells at the top
4. The solid gel was placed into the TBE filled chamber.
5. The gel was positioned to ensure the closest contact of chamber well with the negative electrode
6. 4 µL of each sample were loaded into the wells
7. 3 µL ladder was loaded into the well
8. The electrodes were connected while setting the electricity at 100 volts.
9. This operation was conducted for 1 hour.

## 2.8.3.3 Visualization:

The bands of PCR products were visualized using the Gel DocTM XR+ **(BioRad, USA)**

## 2.8.4 Sanger Sequencing:

PCR products were cleaned and performed to remove the excess unincorporated primers and dNTP, ensuring a specific reaction. Sanger sequencing was performed using **The Applied Biosystems Genetic Analyzer (Thermo Fisher Scientific)**

### 2.8.4.1 PCR cleanup:

1. In an eppendorf tube 5 µL PCR product was taken.

2. 2 µL of ExoSAP-IT Reagent (exonuclease I and alkaline phosphatase) was added respectively to digest and deactivate excess unwanted primers and dNTP.

3. Centrifugation of the tubes to settle down the products in the bottom of the tube.

4. Finally, the tubes were incubated at 35◦C for 4 minutes followed by another period of incubation at 80◦C degree Celsius for 1 minute in **ProFlex PCR system**.

### 2.8.4.2 Cycle sequencing:

Using BigDye Chain Terminator version 3.1 Cycle Sequencing Kit,**(Applied Biosystems, USA)**PCR products were directly sequenced.and ABI PRISM 310 automated analyzer **(Applied Biosystems, USA)**, sequences were analyzed according to manufacturers' instruction.

### 2.8.4.2.1 Mastermix preparation

**Table 5: Reagents and their amounts used for Cycle Sequencing Master-mix Preparation**

| Reagent | Concentration | Single reaction amount |
|---|---|---|
| Sequencing buffer | 5X | 2.0 µL |
| BigDye® Chain Terminator v3.1 RR mix | 2.5X | 0.5 µL |
| Forward primer or Reverse primer | 0.2 µM | 0,3 µL |

### 2.8.4.2.2 Cycle sequencing condition

**Table 6: Cycle sequencing thermal condition cycle**

| Stage | Step | Temperature (°C) | Denaturation | Cycle |
|---|---|---|---|---|
| 1 | initial denaturation | 94 | 1 min | 25 |
| 2 | Denaturation | 94 | 10s | |
| | Annealing | 58 | 5s | |
| | Extension | 60 | 4 min | |
| | final extension | 60 | 10 min | |
| 3 | Hold | 4 | Hold | |

### 2.8.4.3 Sequencing cleanup:

After the completion of cycle sequencing,

1. The products were centrifuged for 2 mins at 4100g.
2. Cycle sequencing products were mixed with 45 µL of SAM solution (Applied Biosystems, USA) and 10 µL of X-terminator solution (Applied Biosystems, USA) in an automated shaker for 30 minutes.
3. Before addition, the X-terminator solution was vortexed properly at maximum speed for at least 30 seconds, until homogenous.
4. The PCR strip was vortexed for 30 mins continuously (1500-2500 rpm).
5. Centrifugation at 4000 X g for 2 minutes,
6. 10 µL supernatants from each tube were transferred to a new 0.2 mL PCR tube strip.
7. The tubes were subjected to capillary electrophoresis through (denaturing) POP-6 TM polymer in an automated sequencing machine ABI PRISM® 310 Genetic Analyzer (Sequencing by Sanger Method)

### 2.8.4.4 Capillary electrophoresis:

Capillary array, polymer, Cathode and anode buffer was inserted. Afterwards, Spatial calibration was performed. Samples were placed, the automated protocol was followed. Finally, the fragment profile was analyzed.

### 2.8.4.5 Sequence analysis:

Sequencing data were produced in .ab1 file format. For each isolate one forward and one reverse sequence file was produced. The sequences were viewed in **Chromas (2.6.6**) and the chromatogram of the files were analyzed. For each sequence, the low quality sequences from both ends (left and right) were trimmed and deleted for a better output. then, the sequence was exported and saved as a FASTA file format.

Afterwards, the forward FASTA sequence was opened through **BioEdit (7.2)**. Then the reverse sequence was imported and reverse complement was produced respectively. Finally, performing pairwise alignment, consensus sequence was formed. The consensus sequence was used as the query sequence to perform BLASTN which is a part of Basic Local Alignment Search Tool used to find respective sequence's similarity with other sequences already submitted in the online databases.(BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi )

### 2.8.5 Phylogeny:

The evolutionary history was inferred using the Maximum Likelihood method. All the sequences were aligned via ClusalW using MEGA 11 (Tamura et al., 2021). The software IQ-tree 1.6.12. (Nguyen et al, 2015) was used. For the analysis, tree construction + ultrafastbootstrap (1000 replicates) were chosen (Hoang et al, 2017). TN+F was chosen as the model of substitution.

# CHAPTER 3
# RESULT

## 3. RESULT

**Table 7: Presumptive Identification based on CHROMagarTM *Acinetobacter***

| Place | Specimen Site | Colony Appearance (Color, Size, Shape, Texture) on CHROM-agar *Acinetobacter* Base (Without MDR supplement) | Growth on MDR *Acinetobacter* Media | Catalase Test | Oxidase Test | Tentative Interpretation (Based on *Acinetobacter* Chromagar an Biochemical tests) |
|---|---|---|---|---|---|---|
| Outdoor | Medical Suction device Inlet tube | Red, Large, Smooth, Circular | NO | Positive | Negative | *Acinetobacter spp* |
| | | Red, pinpoint, Circular | YES | Positive | Negative | *Stenotrophomonas spp.* |
| | Nebulizer Mask | Red, Small, Circular | YES | Negative | Positive | Gram Negative other than *Acinetobacter* |
| Neonatal ward A | Ambu Valve | Red, pinpoint, Circular | YES | Positive | Negative | *Stenotrophomonas spp.* |
| | | Red, pinpoint, Circular | YES | Positive | Negative | *Stenotrophomonas spp.* |
| | Medical Suction device Inlet tube | Red, Small, Circular, Smooth | NO | Positive | Positive | Gram Negative other than *Acinetobacter* |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Head Mask | Red, Small, dry, irregular, Rough | YES | Positive | Positive | *Pseudomonas spp.* |
| Incubator A | Oxygen Delivery Pipe | Red, Small, Circular | NO | Negative | Positive | Gram Negative other than *Acinetobacter* |
| | Medical Suction device Inlet tube | Red, Large, Smooth, Circular | YES | Positive | Negative | *Acinetobacter spp* |
| | | Light Red, Small, smooth , circular | YES | Positive | Positive | Gram Negative other than *Acinetobacter* |
| Neonatal ward B | Procedure table | Large, Red, Circular | NO | Positive | Negative | *Acinetobacter spp* |
| | | pinpoint, Red, Circular | YES | Positive | Negative | *Stenotrophomonas spp.* |
| | Medical Suction device Inlet tube | Light Red, Medium, Circular, smooth | YES | Positive | Negative | *Acinetobacter spp* |
| | nebulizer | Red, Small, circular, smooth | NO | Positive | Negative | Gram Negative other than *Acinetobacter* |
| | | Blue, small, Circular, Smooth | NO | Positive | Negative | Enterobacteriaceae |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Light Red, Small, smooth , circular | NO | Positive | Negative | Gram Negative other than *Acinetobacter* |

## 3.1 Environmental surface positive isolates:

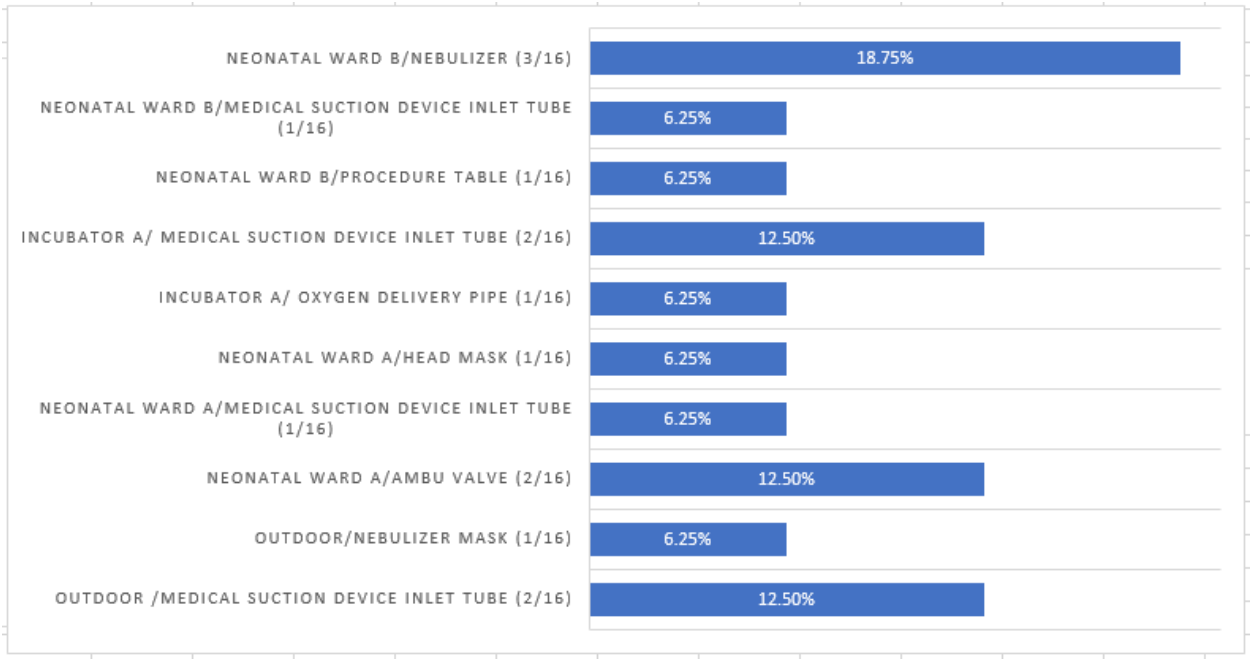



**Figure 2: Positive growth of organisms from Environmental samples**

Out of 23 environmental samples, 10 samples (43%) showed positive growth of 16 isolates on the selective media. The data from **Table 7** and **Figure 2**, reveal that the highest percentage of positive isolate growth is seen from neonatal ward (606/607/608) which is about (5/16) 31.25%, the second highest specimen site responsible for high availability microorganism from Neonatal ward (306) which is (4/16) 25%, and lastly, both incubator and outdoor show (3/16) 18.75% positive growth of isolates, each.

Among all the sites, (6/16) 37.5% isolates were retrieved from Medical Suction device Inlet tube, which is higher compared to other sample collection sites.

## 3.2 Confirmation of the isolates via colony morphology, gram staining and biochemical tests:

The 16 isolates obtained were identified as gram negative bacteria. In this study, 4 of the isolates (25%) were suspected to be *Acinetobacter* species according to their colony morphology, gram staining and biochemical tests such as oxidase and catalase test.

### 3.2.1 Colony morphology of *Acinetobacter spp* suspects:

According to the ChromagarTM *Acinetobacter* Media manual (Instruction For Use) the selective media produces medium to large red, circular, smooth colonies for *Acinetobacter spp*.

Similar colonies appeared on the ChromagarTM *Acinetobacter* media of the suspected *Acinetobacter* isolates.

**Figure 3: Colony of suspected *Acinetobacter spp* on Chromagar *Acinetobacter* media**

### 3.2.2 Gram stain result of *Acinetobacter spp* suspects:

The gram staining result showed that the bacteria were short rod in shape and pink in color which means it's a gram negative cocco bacillus microorgaism like *Acinetobact spp.*

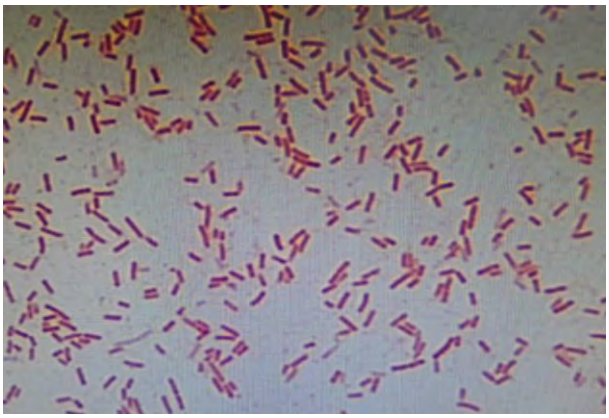


**Figure 4: microscopic image of suspected *Acinetobacter* spp after Gram staining**

### 3.2.3 Catalase and Oxidase tests result of *Acinetobacter spp* suspects:

Gas bubbles were produced during the catalase test and during the oxidase test no color change was seen which indicated that the organisms were catalase positive and oxidase negative, like *Acinetobacter spp*.

**Figure 5: Catalase test result of suspected *Acinetobacter spp***

Additionally, According to the ChromagarTM *Acinetobacter* media manual (Instruction For Use), bacteria with very small or pinpoint, rough, reddish colonies after 18-24 hr, are suspected *Stenotrophomonas spp*. Also, they showed catalase positive and oxidase negative results.

Moreover**,** the manua lalso mentions that *Pseudomonas spp* is identical to *Stenotrophomonasspp* in colony morphology, even though a positive oxidase test differentiates them. Therefore, the data from **Table 7** supports the presumption of *Stenotrophomonasspp* and *Pseudomonas spp* being possible suspects.

## 3.3 Colony morphology on MacConkey agar:

The colonies appeared on the MacConkey agar were medium sized, pink, circular, smooth. The organism did not change the color of the media which means the isolates were non-lactose fermenters. These characteristics also matches with the appearance of *Acinetobacter spp.* on MacConkey agar.

**Figure 6: Colony of suspected *Acinetobacter spp* on Chromagar *Acinetobacter* media**

## 3.4 VITEK-2 identification and Antimicrobial Susceptibility result:

As this study focuses on only *Acinetobacter Spp*, other suspected gram negative bacteria except *Acinetobacter* were eliminated from VITEK-2 identification and further steps.

**Table 8: VITEK 2 Identification data of suspected *A.baumannii***

| isolate no. | place | Specimen Site | VITEK2 Identification |
|---|---|---|---|
| isolate 1 | Outdoor | Medical Suction | *Acinetobacter baumannii* |

| | | | |
|---|---|---|---|
| | | device Inlet tube | |
| isolate 2 | Incubator A | Medical Suction device Inlet tube | *Acinetobacter baumannii* |
| isolate 3 | Neonatal ward B | Procedure table | *Acinetobacter baumannii* |
| isolate 4 | Neonatal ward B | Medical Suction device Inlet tube | *Acinetobacter baumannii* |

**Table 9: VITEK-2 Antibiotic Susceptibility Patterns of Environmental Isolates of *A.baumannii***

| | Antibiotic Class | antibiotic drug | isolate 1 | isolate 2 | isolate 3 | isolate 4 |
|---|---|---|---|---|---|---|
| | **Cephalosporin** | **Cefepime** | S | R | S | R |
| | | **Ceftriaxone** | I | R | S | R |
| | **Beta lactamase inhibitor** | **Cefoperazone/ Sulbactam** | S | S | S | S |
| | | **Piperacillin + Tazobactam,** | S | R | S | R |
| | **Fluoroquinolone** | **Ciprofloxacin** | S | R | S | R |
| | **Polymyxin** | **Colistin** | I | I | I | I |
| | **Sulfonamide** | **Cotrimoxazole** | S | S | S | R |
| | **Aminoglycoside** | **Gentamicin** | S | S | S | R |
| | **Carbapenem** | **Imipenem** | S | R | S | R |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | **Meropenem** | S | R | S | R |
| | **Glycylcycline** | **Tigecycline** | S | S | S | S |
| **MDR status** | | | – | **MDR** | – | **MDR** |

Antibiotic susceptibility pattern for four of the molecularly confirmed isolates against 11 antimicrobial agents such as Cefepime,Cefoperazone/Sulbactam, Ceftriaxone, Ciproffoxacin, Colistin, Cotrimoxazole, Gentamicin, Imipenem, meropenem, Piperacillin + Tazobactam, Tigecycline were performed. In The **figure 7,** the X axis represents number of isolates andY axis represents antibiotics.

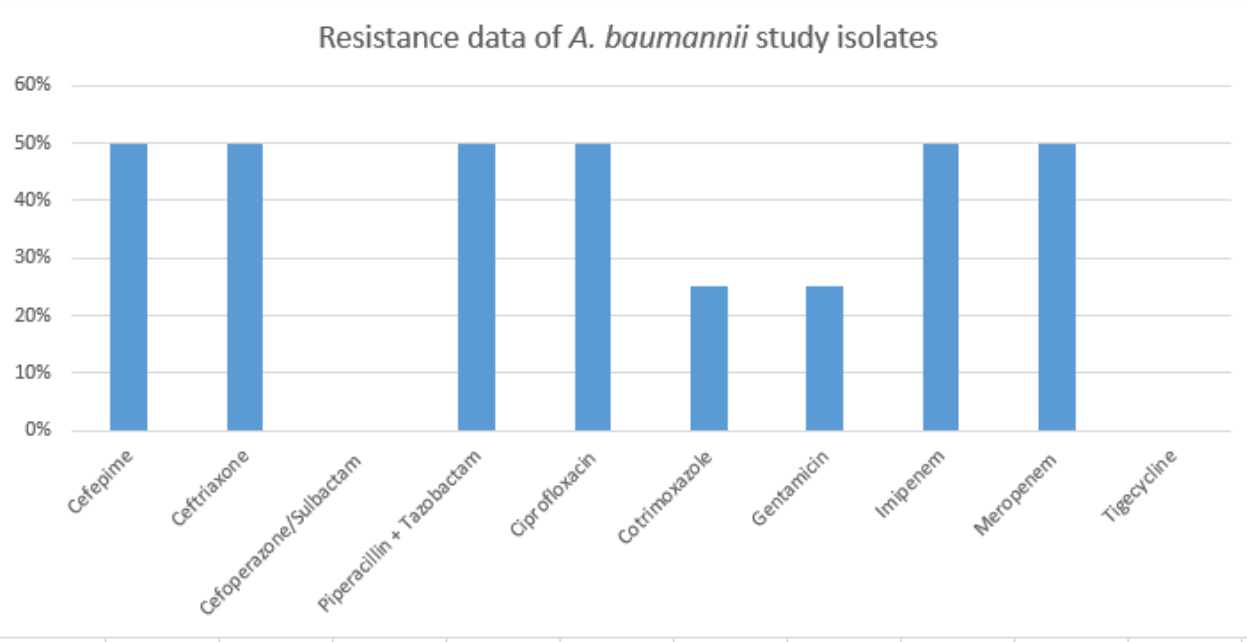



**Figure 7: Resistance data of *A.baumannii* isolates against antibiotics**

From the **Table 9** and **Figure 7** it can be interpreted that all four of the *A.baumannii* isolates were intermediately resistant to Colistin. Moreover, 100% isolates are sensitive to both Cefoperazone/Salbactum and Tigecycline whereas 50% isolates were resistant to Cefepime, ciprofloxacin, imipenem, meropenem and Piperacillin + Tazobactam.

Among them, 75% of the isolates were sensitive to both cotrimoxazole and gentamicin. Respectively, 50% of the isolates were resistant, 25% were intermediately resistant and 25% were Sensitive against Ceftriaxone.

Isolate 2 and Isolate 4(2/4) *A.baumannii* isolates (50%) were marked as MDR as according to Magiorakos et al, isolates that are non-susceptible(resistant or intermediately resistant) to at least 1 agent in at least 3 antimicrobial categories, are identified as MDR isolates. and, Isolate 2 and 4 fulfill the criteria. (Magiorakos et al., 2012)

Similarly, the data from **Table 6** indicates the growth of the respective isolates in the MDR Chromagar *Acinetobacter* Media, proving its reliability.

## 3.5 Visualization of the bands of *A.baumannii* PCR products:

All four isolates of *A.baumannii* (VITEK-2 confirmed) showed positive results by matching with the positive control band size which is ~1450bp.

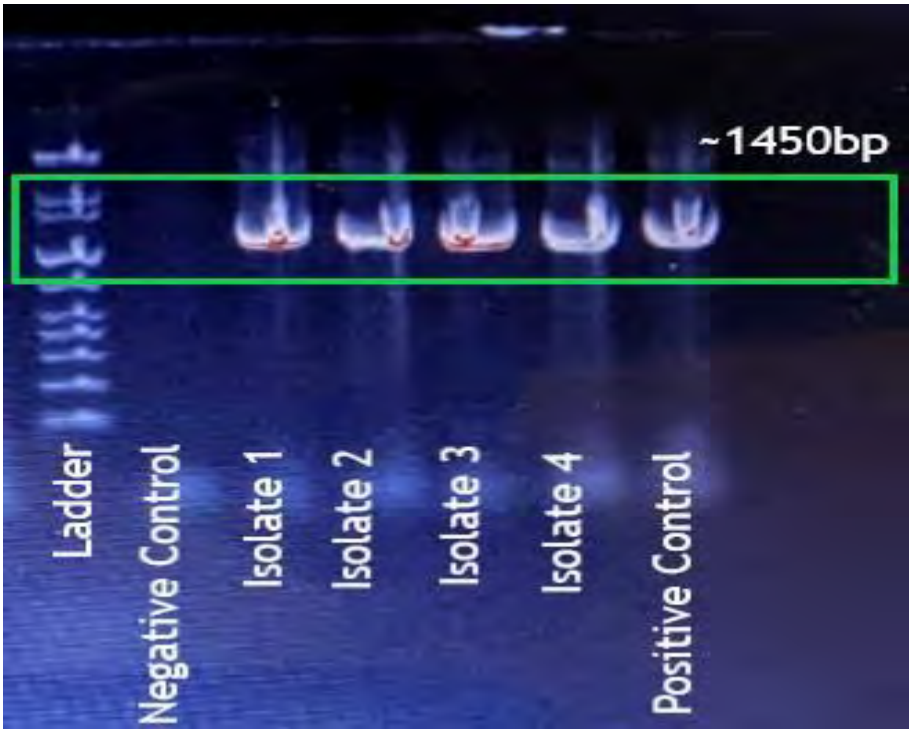



**Figure 8: Visualization of the Bands of A.baumannii isolate from Gel-Image (green box)**

## 3.6 Sequence Analysis of all the VITEK-2 confirmed *A.baumannii*:

Sequencing was done using purified PCR products. 27F and 1492R primers were used for the sequences. Sequencing data of four VITEK-2 confirmed *A.baumannii* isolates were analyzed by the **Chromas 2.6.6** tool which generated a four color chromatogram showing the result of the sequencing run.

In the chromatogram, the colors green, black, blue and red represent adenosine, guanosine, cytosine, and thymine respectively.

### 3.6.1 Generating Chromatogram of Sequences:

Forward and reverse Sequences were viewed via **Chromas 2.6.6.** Low quality sequences were trimmed. All the chromatograms showed readable peaks with distinctive single color. Acceptable level of noise was observed.

### 3.6.2 Query Sequence Formation:

BioEdit (7.2) software was used to create consensus sequences and to retrieve the particular FASTA sequence.

**Table 10: Query sequence of isolate 1**

| | |
|---|---|
| QUERY SEQUENCE<br><br>(1411 bp) | CGAGCGGGGGAAGGTAGCTTGCTACCGGACCTAGCGGCGG<br>ACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGG<br>ACAACATCTCGAAAGGGATGCTAATACCGCATACGTCCTA<br>CGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGA<br>TGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCC<br>TACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATC<br>CGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG<br>AGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTG<br>ATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGT<br>AAAGCACTTTAAGCGAGGAGGAGGCTACTTTAGATAATAC<br>CTAGAGATAGTGGACGTTACTCGCAAAATAAGCACCGGCT<br>AACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAG |

| | |
|---|---|
| | CGTTAATCGGATTTACTGGGCGTAAAGCGCGCGTAGGCGG<br>CTAATTAAGTCAAATGTGGATAATGCCYCGATGGCTTAAA<br>CCTTGGGAATTTGCATTTCGATAACTGGTTAGCTAGAGTGT<br>GGGAGAGGGATGGTAGAATTCCAGGTGTAGCGGTGAATAT<br>GCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCA<br>TCTGGCCTAACACTGACGCTGAGGTGCGAAAGCATGGGGA<br>GCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAGA<br>CGATGTCTACTAKCCGTTGGGGCCCTTTGGAGGCTTTTAGT<br>GGCCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTAC<br>GGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCG<br>CACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCG<br>AAGAACCTTACCTGGCCTTGACATAGTAAGAACTTTCCAGA<br>GATGGATTGGTGCCTTCGGGAACTTACATACAGGTGCTGCA<br>TGGCTGTCGTCAGCTCGTGTCTTGAGATGTTGGGTTAAGTC<br>CCGCAACGAGCGCAACCCTTTTCCTTATTCGCCAGCGAGTA<br>ATGTCGGGAACCTTAAGGATACTGCCAGTGACAAACTGGA<br>GGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGG<br>CCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGT<br>TGCTACACAGCGATGTGATGCTAATCTCAAAAAGCCGATC<br>GTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCG<br>GAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATA<br>CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGA<br>GTTTGTTGCACCAGAACGTAGCTACGCCTAACTGCAAAGA<br>GG |

**Table 11: Query sequence of isolate 2**

| | |
|---|---|
| QUERY SEQUENCE<br>(1411 bp) | TCGAGCGGGGGAGGTAGCTTGCTACCG<br>GGACCTAGCGGCGGACGGGTGAGTAA<br>TGCTTAGGAATCTGCCTATTAGTGGGG |

| | |
|---|---|
| | GACAACATCTCGAAAGGGATGCTAATA<br>CCGCATACGTCCTACGGGAGAAAGCA<br>GGGGATCTTCGGACCTTGCGCTAATAG<br>ATGAGCCTAAGTCGGATTAGCTAGTTG<br>GTGGGGTAAAGGCCTACCAAGGCGAC<br>GATCTGTAGCGGGTCTGAGAGGATGAT<br>CCGCCACACTGGGACTGAGACACGGC<br>CCAGACTCCTACGGGAGGCAGCAGTG<br>GGGAATATTGGACAATGGGGGGAACC<br>CTGATCCAGCCATGCCGCGTGTGTGAA<br>GAAGGCCTTATGGTTGTAAAGCACTTT<br>AAGCGAGGAGGAGGCTACTTTAGTTAA<br>TACCTAGAGATAGTGGACGTTACTCGC<br>AAAATAAGCACCGGCTAACTCTGTGCC<br>AGCAGCCGCGGTAATACAGAGGGTGC<br>GAGCGTTAATCGGATTTACTGGGCGTA<br>AAGCGTGCGTAGGCGGCTTATTAAGTC<br>GGATGTGAAATCCCCGAGCTTAACTTC<br>GGGTAACTYGCATTCGATATYTGGTGA<br>GCTAGAGTATGGGAGACGGATGGTAG<br>AATCTMCAGGTGTAGCGGTGAAATGC<br>GTAKAGATCTGGAGGARTACCGATGG<br>GCGAAKGCAGCCATCTGGSCTAATACT<br>GACGCTGAGGTACGAMAGCATGGGGA<br>GCAMACAGGATTAGATACCCTGGTAG<br>TCCATGCCCGTAWACGATGTSTACTTA<br>GCCCGCTKGGGGCCCTGTTGAKGCTTT<br>TAGTGGCGCAGCTAACGCGATAAGTAG<br>ACCGCCTGGGGAGTACGGTCGCAAGA<br>CTAAAACTCAAATGAATTGACGGGGGC |

| | |
|---|---|
| | CCGCACAAGCGGTGGAGCATGTGGTTT<br>AATTCGATGCAACGCGAAGAACCTTAC<br>CTGGCCTTGACATACTAGAAACTTTCC<br>AGAGATGGATTGGTGCCTTCGGGAATC<br>TAGATACAGGTGCTGCATGGCAGTCGT<br>CAGCTCGTGTCGTGAGATGTTGGGTTA<br>AGTCCCGCAACGAGCGCAACCCTTTTC<br>CTTACTTGCCAGCATTTCGGATGGGAA<br>CCTTAAGGATACTGCCAGTGACAAACT<br>GGAGGAAGGCGGGGACGACGTCAAGT<br>CATCATGGCCCTTACGGCCAGGGCTAC<br>ACACGTGCTACAATGGTCGGTACAAAG<br>GGTTGCTACACAGCGATGTGATGCTAA<br>TCTCAAAAAGCCGATCGTAGTCCGGAT<br>TGGAGTCTGCAACTCGACTCCATGAAG<br>TCGGAATCGCTAGTAATCGCGGATCAG<br>AATGCCGCGGTGAATACGTTCCCGGGC<br>CTTGTACACACCGCCCGTCACACCATG<br>GGAGTTTGTTGCACCAGAACGTAGCTA<br>GCCTAACTGCAAAGAGGGCGT |

**Table 12: Query sequence of isolate 3**

| | |
|---|---|
| QUERY SEQUENCE<br>(1405 bp) | CGAGCGGGGGAAGGTAGCTTGCTACC<br>GGGACCTAGCGGCGGACGGGTGAGTA<br>ATGCTTAGGAATCTGCCTATTAGTGGG<br>GGACAACATCTCGAAAGGGATGCTAAT<br>ACCGCATACGTCCTACGGGAGAAAGC<br>AGGGGATCTTCGGACCTTGCGCTAATA<br>GATGAGCCTAAGTCGGATTAGCTAGTT |

| | |
|---|---|
| | GGTGGGGTAAAGGCCTACCAAGGCGA<br>CGATCTGTAGCGGGTCTGAGAGGATGA<br>TCCGCCACACTGGGACTGAGACACGGC<br>CCAGACTCCTACGGGAGGCAGCAGTG<br>GGGAATATTGGACAATGGGGGGAACC<br>CTGATCCAGCCATGCCGCGTGTGTGAA<br>GAAGGCCTTATGGTTGTAAAGCACTTT<br>AAGCGAGGAGGAGGCTACTTTAGTTAA<br>TACCTAGGGATAGTGGACGTTACTCGC<br>AAAATAAGCACCGGCTAACTCTGTGCC<br>AGCAGCCGCGGTAATACAGAGGGTGC<br>GAGCGTTAATCGGATTTACTGGGCGTA<br>AAGCGTGCGTAGGCGGCTTATTAAGTC<br>GGATGTGAAATCCCCGAGCTTAACTTC<br>GGGTAACTYGCAYTCGATACTGGTGAG<br>CTAGAGTATGGGAGACGGATGGTAGA<br>ATTAMCAGGTGTAGCGGTGAAATGCG<br>TAKAGATCTGGAGGAATACCGATGGC<br>GAAGGCAGCCATCTGGCCTAATACTGA<br>CGCTGAGGTACGAAAGCATGGGGAGC<br>AAACAGGATTAGATACCCTGGTAGTCC<br>ATGCCGTAAACGATGTCTACTAGCCCG<br>TTGGGGGCCTTKTGAAKGCTTTAGTCG<br>KCGCAGCTAACGCGATAAGTAGACCG<br>CCTGGGGAGTACGGTCGCAAGACTAA<br>AACTCAAATGAATTGACGGGGGCCCG<br>CACAAGCGGTGGAGCATGTGGTTTAAT<br>TCGATGCAACGCGAAGAACCTTACCTG<br>GCCTTGACATACTAGAAACTTTCCAGA<br>GATGGATTGGTGCCTTCGGGAATCTAG |

| | |
|---|---|
| | ATACAGGTGCTGCATGGCAGTCGTCAG<br>CTCGTGTCGTGAGATGTTGGGTTAAGT<br>CCCGCAACGAGCGCAACCCTTTTCCTT<br>ACTTGCCAGCATTTCGGATGGGAACCT<br>TAAGGATACTGCCAGTGACAAACTGGA<br>GGAAGGCGGGGACGACGTCAAGTCAT<br>CATGGCCCTTACGGCCAGGGCTACACA<br>CGTGCTACAATGGTCGGTACAAAGGGT<br>TGCTACACAGCGATGTGATGCTAATCT<br>CAAAAAGCCGATCGTAGTCCGGATTGG<br>AGTCTGCAACTCGACTCCATGAAGTCG<br>GAATCGCTAGTAATCGCGGATCAGAAT<br>GCCGCGGTGAATACGTTCCCGGGCCTT<br>GTACACACCGCCCCGTCACACCATGGG<br>AGTTTGTTGCACCAGAACGTAGCTACG<br>CCTAACTGCAAAGAGG |

**Table 13: query sequence of isolate 4**

| | |
|---|---|
| QUERY SEQUENCE<br>(1416 bp) | AGTGCAGTCGAGCGGGGGAGGTAGCT<br>TGCTACCGGACCTAGCGGCGGACGGGT<br>GAGTAATGCTTAGGAATCTGCCTATTA<br>GTGGGGGACAACATCTCGAAAGGGAT<br>GCTAATACCGCATACGTCCTACGGGAG<br>AAAGCAGGGGATCTTCGGACCTTGCGC<br>TAATAGATGAGCCTAAGTCGGATTAGC<br>TAGTTGGTGGGGTAAAGGCCTACCAAG<br>GCGACGATCTGTAGCGGGTCTGAGAGG<br>ATGATCCGCCACACTGGGACTGAGACA<br>CGGCCCAGACTCCTACGGGAGGCAGC |

| | |
|---|---|
| | AGTGGGGAATATTGGACAATGGGGGG<br>AACCCTGATCCAGCCATGCCGCGTGTG<br>TGAAGAAGGCCTTATGGTTGTAAAGCA<br>CTTTAAGCGAGGAGGAGGCTACTTTAG<br>TTAATACCTAGAGATAGTGGACGTTAC<br>TCGCAAAATAAGCACCGGCTAACTCTG<br>TGCCAGCAGCCGCGGTAATACAGAGG<br>GTGCGAGCGTTAATCGGATTTACTGGG<br>CGTAAAGCGTGCGTAGGCGGCTTATTA<br>AGTCGGATGTGAAATCCCCGAGCTTW<br>ACKYGGKAACTYGCACTTCCGATATCT<br>GGGTGAGCTAGGAGTATGGGAGACGG<br>ATGGTAGCAATTACCAGGTGTAGCGGT<br>GAAATGCGTAGAGATCTGGAGGAATA<br>CCGATGGCGAAGGCAGCCATCTGGSCT<br>AATACTGACGCTGAGGTACGAAAGCAT<br>GGGGAGCAMACAGGATTAGATACCCT<br>GGTAGTCCATGCCGTAAWCGATGTSTA<br>CTTAGCCCGTTGGGGGCCTTKTGAKGC<br>TTTTAGTCGKCGCAGCTAACGCGATAA<br>GTAGACCGCCTGGGGAGTACGGTCGC<br>AAGACTAAAACTCAAATGAATTGACG<br>GGGGCCCGCACAAGCGGTGGAGCATG<br>TGGTTTAATTCGATGCAACGCGAAGAA<br>CCTTACCTGGCCTTGACATACTAGAAA<br>CTTTCCAGAGATGGATTGGTGCCTTCG<br>GGAATCTAGATACAGGTGCTGCATGGC<br>AGTCGTCAGCTCGTGTCGTGAGATGTT<br>GGGTTAAGTCCCGCAACGAGCGCAAC<br>CCTTTTCCTTACTTGCCAGCATTTCGGA |

| | |
|---|---|
| | TGGGAACCTTAAGGATACTGCCAGTGA<br>CAAACTGGAGGAAGGCGGGGACGACG<br>TCAAGTCATCATGGCCCTTACGGCCAG<br>GGCTACACACGTGCTACAATGGTCGGT<br>ACAAAGGGTTGCTACACAGCGATGTGA<br>TGCTAATCTCAAAAAGCCGATCGTAGT<br>CCGGATTGGAGTCTGCAACTCGACTCC<br>ATGAAGTCGGAATCGCTAGTAATCGCG<br>GATCAGAATGCCGCGGTGAATACGTTC<br>CCGGGCCTTGTACACACCGCCCCGTCA<br>CACCATGGGAGTTTGTTGCACCAGAAC<br>GTAGCTAGCTCTAACTGCAAAGAGG |

### 3.6.3 BLASTN Result Analysis:

The results of the BLAST analysis of the partial sequences of isolates 1, 2, 3 and 4 were analyzed in the table below. The criteria of the analysis were based on highest percentage identity and query coverage along with lowest E value. The query coverage percentage is critical as it represents the involvement of alignment against the respective query sequence, and the percentage identity represents hits and similarities against query sequence. Moreover, the Lowest E value represents that the chances of any random alignment matching the query sequence is lowest which represents good alignment results.

From the BLASTN results, uncultured bacteria were filtered out. From the results **(Table 14)** it is seen that three of the isolates were shown to be most close to *Acinetobacter baumannii* whereas one of them were close to *Acinetobacter nosocomialis*.

The particular strains showed 0.0 E values, 100% query coverage and percentage identity above 97% against all of the four isolates.

## Table 14: BLASTN results

**Table 14: BLASTN Results**

| isolate no. | 16s rRNA Identification (Forward/Reverse) | strain (16S, partial sequence) | percentage identity | max score | total score | Query coverage | E-value |
|---|---|---|---|---|---|---|---|
| isolate 1 | *Acinetobacter nosocomialis* | *Acinetobacter nosocomialis* strain Ed2 | 98.23% | 2453 | 2453 | 100% | 0.0 |
| isolate 2 | *Acinetobacter baumannii* | *Acinetobacter baumannii* strain ECAn10 | 97.73% | 2431 | 2431 | 99% | 0.0 |
| isolate 3 | *Acinetobacter baumannii* | *Acinetobacter baumannii strain* HNXY33P | 98.36% | 2459 | 2459 | 100% | 0.0 |
| isolate 4 | *Acinetobacter baumannii* | *Acinetobacter baumannii* strain ECAn10 | 97.74% | 2435 | 2435 | 99% | 0.0 |

## 3.7 Phylogenetic Analysis:

A total of 26 sequences were used. 4 sequences were from this current study, 20 sequences were from top 5 blast result of each isolate, and 2 sequences were used as Bangladeshi environmental isolates of *A.baumannii* and *A.nosocomialis*. Each sequence was named by their isolate name, strain name, country location and sample source.

The Phylogenetic tree from **FIG 9** below consists of all four *Acinetobacter* isolates (three*A.baumannii*, one *A.nosocomialis*) in comparison with the global isolates based on 16S rRNA sequence (top 5 blast results)

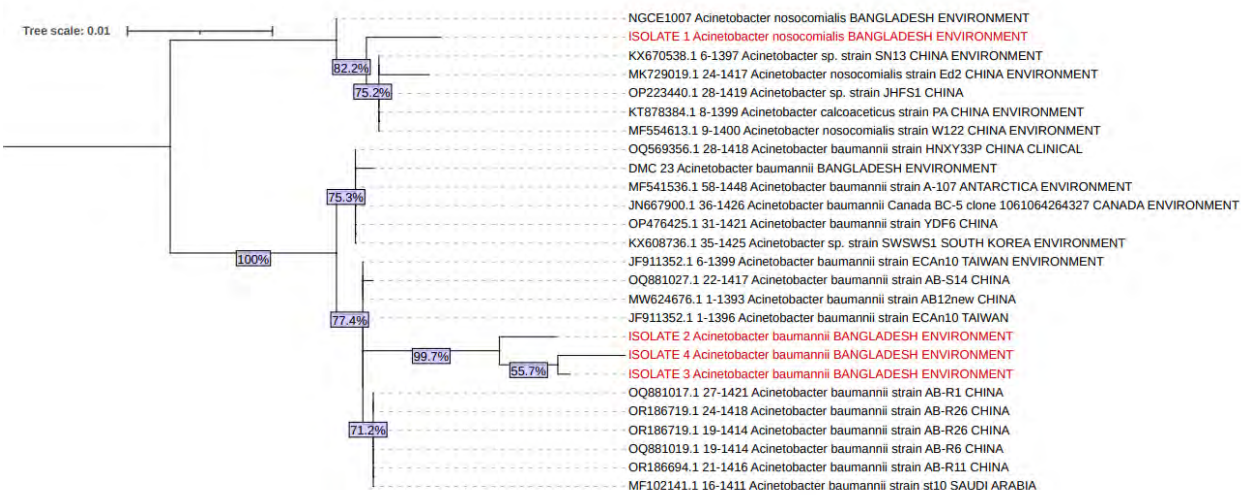



**Figure 9: Phylogenetic tree comparing the obtained isolates of all four *Acinetobacter* (three *A.baumannii*, one *A.nosocomialis*) and global isolates based on 16srRNA sequence**

Here, the tree is divided into two different clades. One clade includes *A.nosocomialis* with its *A.nosocomialis* strains from blast results and the NGCE1007*A.nosocomialis* reference strain. Whereas, another clade includes *A.baumannii*, DMC23*A.baumannii* reference strain and other strains of *A.baumannii* from blast result. This indicates that these two differentspecies and do not share much similarity.

The clade consisting of *A.nosocomialis* strains, reference strain NGCE1007 is somewhat distant from the other strains. Even Though, the strains from top blast results of isolate 1 show high similarity (Bootstrap value 75.2) with one another by forming a cluster, isolate 1 *A. nosocomial* shows somewhat more similarity compared to the reference strain NGCE1007 as the isolate 1 and blast strains share a sub-clade (bootstrap value 82.2). In terms of evolutionary time line, Isolate 1 *A.nosocomialis* is closest to *Acinetobacter nosocomialis* strain Ed2.

Interestingly, the other clade of *A.baumannii*, two nodes are created. The reference strain DMC23 and *A.baumannii* strains A-107, Canada BC-5, YDF6, *Acinetobacter* sp strain SWSWS1 split and form cluster, indicating high level of similarity (bootstrap value 75.2%)

Moreover, among the three isolates from this study from the neonatal ward, *Acinetobacter baumannii* (Isolate 2,3,4)form sub-clade sharing a lot of similarity with each other (Bootstrap value 99.7). Moreover, in the evolutionary timeline, Isolate 2 and 3 are closer than they are to isolate 4.

On the contrary, isolate 2,3 and 4 share lower level of similarity with the strains AB-R6, AB-R26, AB-R1, AB-R11, AB-S14, ECAn10, AB12new, st10 and the reference strain DMC23 and are distant in term of evolutionary timeline even though they appear from the same node.

# CHAPTER 4
# DISCUSSION

## 4. DISCUSSION:

Nosocomial infections also known as Hospital Acquired Infections have increasingly become a great matter of concern worldwide affecting about 1.4 million people every year. Infact, *A.baumannii* is responsible for majority of the nosocomial infections to ICU or NICU patients who are the most vulnerable targets due to having weak immune systems.(Shamsizadeh et al., 2017)

*A.baumannii* is responsible for mostly bloodstream infections and ventilator associated pneumonia, which have been linked to high death frequency and morbidity. Moreover, infections such as wound infections, skin or soft tissue infections, urinary tract infection, secondary meningitis are results of the *Acinetobacter calcoaceticus-baumannii* (ACB) complex which represents *Acinetobacter* species such as *A. calcoaceticus*, *A.baumannii*, *A.nosocomialis*, *A. pittii*, *A. seifertii*, and *A. dijkshoorniae* altogether (Bergogne-Bérézin & Towner, 1996)

Not only the emergence of *A.baumannii* as nosocomial pathogens, but also the emergence of *A.baumannii* as MDR or multi drug resistant pathogens have significantly caused a lot of chaos in the current world scenario. MDR *Acinetobacter baumannii* have been marked as "red alert" pathogens for gaining resistance against last resort antibiotics such as carbapenem and colistin. Such MDR have been reported all over the world including our country, which is why this study focuses on the MDR *Acinetobacter baumannii* retrieved from the NICU of the respective Bangladeshi hospital.

In the study, 23 environmental surface samples were collected from neonatal wards and 10 samples (43%) showed significant growth of 16 isolates where 16 of them were confirmed to be gram negative bacteria. Among the sites, Medical Suction device Inlet tube (37.5%) was responsible for the existence of most isolates. 4 suspected *Acinetobacter spp* were confirmed to be *A.baumannii* via VITEK-2 Identification, whereas 16s rRNA sequencing revealed that one of the isolates (1/4) showed more similarity to the sequences of *A.nosocomialis* rather than *A.baumannii*. Few other organisms like *pseudomonas spp*, *Stenotrophomonas spp.* were also suspected to be present.

In this study (according to the VITEK-2 identification), 25% *A baumannii* were found from a total of 23 environmental surface samples. Among the sites, Medical Suction device Inlet tubes from different neonatal wards were not only responsible for the highest rate of isolates, but also 75% responsible for the growth of (3/4) *A.baumannii* isolates that were found. In another study by

Shamsizadeh reported that out of 42 hospital environmental surface samples, 17% (7/42) *A.baumannii* isolates were found from ICU. This particular study also highlighted the importance of *A.baumannii* isolates from mechanical ventilators as they could possibly act as air contaminants causing ventilator associated pneumonia. Another study from Cutovic et al. Also reported that out of 175 hospital surfaces, medical equipment, hospital staff, and other objects where (51.6%) *A.baumannii* identified at a higher rate compared to other HAI causing bacteria. Similarly, a study from Aygun et al. Also reported the isolation of 39.3% *A.baumannii* from 22 out of 59 environmental samples. (Shamsizadeh et al., 2017) .Another study from Kuczewski et al. revealed that only 4.9% *A.baumannii* (11/137 samples) were found (Kuczewski et al., 2022)

On the contrary, one study from Ali et al. regarding environmental surface samples from Ramadi Teaching Hospital revealed that there were no *Acinetobacter* Spp retrieved from any of the surface samples. Infact, isolates like *Kocuria sp, Staphylococcus sp, Rothia sp, Bacillus sp, pseudomonas sp*, were reportedly isolated.("Phylogenetic Tree of Some Nosocomial Bacteria Isolated from Ramadi Teaching Hospital," 2020) The study from Kuczewski et al reported the growth of gram negative bacteria such as *Stenotrophomonas maltophilia, klebsiella pneumoniae, pseudomonas aeruginosa, Staphylococcus aureus* and so on.(Kuczewski et al., 2022).

Few other gram negative bacteria were also found from this study as well. Some of the gram negative bacteria were suspected to be *Strenotrophomonas, Pseudomonas*. *Stenotrophomonas maltophilia* has been reported as an emerging global opportunistic pathogen. This bacteria is typically being more frequently isolated from hospital settings which poses great threat to the critically ill patients of ICU and NICU and almost caused similar infections like *A.baumannii*. WHO also has marked it as most underrated MDR organisms as they have been reported to be resistant against carbapenems, aminoglycosides, fluoroquinolones.(Brooke, 2014) Moreover, Pseudomonas aeruginosa is also known as a very problematic isolate causing 10-11% nosocomial infections. Bacteremia, ventilator associated pneumonia, urinary and wound infections are some of the diseases caused by the pathologen. Moreover, the enzyme carbapenemase makes them resistant against most of the antibiotic classes such as Carbapenems along with aminoglycosides, cephalosporins.

This study also reveals that 50% (2/4) of the *A.baumannii* isolates were MDR isolates, showing resistance to higher class antibiotics such as piperacillin+ tazobactam , fluoroquinolones such as

Ciprofloxacin , Carbapenem such as imipenem and meropenem. Gentamicin and cotrimoxazole resistance was also seen in only 1 of the MDR bacteria (isolate 4). All the isolates showed Sensitivity to Tigecycline and cefoperazone+ sulbactam and intermediate resistance to polymyxin such as Colistin. Similarly, in the study of Joseph et al and Al-Sweih et al, respectively, 20% and 12% *Acinetobacter* spp. showed resistance to colistin sulfate, (Yadav et al., 2020)In a similar study by Shamsizadeh et al, shows highly antibiotic resistant *A.baumannii* that were isolated from hospital surfaces of and ICU. 70% and 81% of *A.baumannii* isolates from surface samples showed resistance to gentamicin, imipenem respectively. Overall the study reported 67.5% of *A.baumannii* as MDR isolates But this study shows that 50% of the isolates were resistant against gentamicin and imipenem which were also MDR. In another study, almost all MDR isolates were found to be resistant to piperacillin and cephalosporins, 93.8% to gentamicin, and 89.4% to meropenem, Whereas, in this study, all MDR isolates were also found to be resistant against piperacillin, cephalosporins, meropenem and 50% resistant to gentamicin. (Yadav et al., 2020)

This study also focuses on the isolates identified via 16S rRNA sequencing. From the BLASTN result of the four isolates, 75% (3/4) isolates were identified as *A.baumanni* while one of them was identified as *A.nosocomialis.* According to a study from (Nithichanon et al., 2022), even though *A.baumannii* is more prevalent in comparison with *A.nosocomialis*, the isolate should not be ignored. The respective study in Northeast Thailand revealed that both *A.baumannii* and *A.nosocomialis* were responsible for 33% and 36% mortality rates, respectively. Moreover, along with 70.83% *A.baumannii* 29.17% *A.nosocomialis* isolates were also identified. The study also highlighted and compared the infection rates caused by both of the *Acinetobacter* species. Reportedly, *A.nosocomialis* had been responsible for 36% pulmonary, 7% septicemia and 14% wound infections whereas *A.baumannii* infections rates were much higher (47%, 32% and 29% respectively) having MDR rates such as 68% and 43% respectively for *A.baumannii* and *A.nosocomialis*. Another Chinese report showed concerns due to high MDR rates of *A.baumannii* (86%) but comparatively lower *A.nosocomialis* (29%). The study strongly suggests to take *A.nosocomialis* into serious consideration as a lot of wrong diagnosis can take place due to the similarity of *A.nosocomialis* and *A.baumannii*.

In this study, Phylogenetic relationship was also analyzed among the study isolates comparing with their strains from top last result along with 2 reference strains from Bangladesh based on the 16s rRNA sequence.

The isolate 1 *A.nosocomialis* showed a little similarity with its top blast results. Moreover, among the three *A.baumannii* isolates from the neonatal ward, (Isolate 2, 3, 4) form subclade sharing a lot of similarity with each other rather than the other comparison strains. The shared similarity among themselves indicates that the region of isolate identification also played an important role. Among the other *A.nosocomialis* and *A.baumannii* strains from the phylogenetic inference, were not found to cause any significant diseases, one strain stood out, that is the strain isolates from Saudi Arabia which was *Acinetobacter baumannii* strain ST10. A Bangladeshi Study by, shows that different strains such as ST2, ST10, ST149, ST575, ST1063 and ST1065 were found from clinical and environmental sample of a hospital. And though st2 was dominant, st10 strain revealed to have*armA* + $bla_{OXA-23}$ + $bla_{PER-7}$ antibiotic resistance genes and biofilm forming genes such as bap, csuE, pgaB(Farzana et al., 2022)Another study by Abhari et al including a hospital from Iran, revealed that st10 strain contained blaOXA-68 along with ampC-10 or ampC-20 genes which are also antibiotic resistance genes (Abhari et al., 2019)

Finally, from **Table 7** it can be seen that all of the study isolates were actually retrieved from different specimen sites, which indicates the possible spread of the *A.baumannii* in the respective hospital in the study. Spreading of these isolates also means spreading of antimicrobial resistance genes along with few other strong survival genes that may help to increases their tolerance against harsh and dry hospital environments. Studies report that hospital environment promotes survival of *A.baumannii* by challenging it with obstacles such as dry, nutrient-less environment and frequent temperature shifts, to which *A.baumannii* responds by rapid genetic mutations and modification due to impressive genetic plasticity and adapts quite quickly to harsh environment colonize (Kyriakidis et al) Biofilm formations and colonization in the hospital surface might also lead to chronic and persistent infections along with prolonged survival and spreading of antibiotic resistance (Fahy et al., 2023) According to the study of Tena et al. and Shamsizadeh et al. they have referred the hospital environment as a reservoir of *A.baumannii* which increases the risk of a deadly and prolonged outbreak.(Shamsizadeh et al., 2017)

# CHAPTER 5
# CONCLUSION

## 5. CONCLUSION:

*A.baumannii* has become a matter of great concern due to its spread in the hospital setting, threatening immunocompromised individuals, especially the neonates as they have weak and immature immune systems. The spread of these MDR *A.baumannii* could lead to antimicrobial resistance and which could cause all antibiotics to fail in the treatment of infections caused by *A.baumannii*. Therefore, the respective hospital faces a huge risk of outbreak and increased chance of antimicrobial resistance of deadly pathogens such as *A.baumannii*. Thus, necessary measures need to be taken by the hospital authority urgently to avoid such deadly consequences. The hospital surfaces should be cleaned thoroughly and cleaning surveys should be held more frequently. Moreover, the hospital staff should also practice hygiene and cleanliness as they might act as the transmission mode of the pathogens. After all of the safety measures, hopefully *A.baumannii* along with other potential pathogens can be stopped from creating disastrous situations.

## 7. REFERENCES:


1. Abhari, S. S., Badmasti, F., Modiri, L., Aslani, M. M., & Asmar, M. (2019). Circulation of imipenem-resistant *Acinetobacter* baumannii ST10, ST2 and ST3 in a university teaching hospital from Tehran, Iran. *Journal of Medical Microbiology*, *68*(6), 860–865. https://doi.org/10.1099/jmm.0.000987
2. Alrahmany, D., Omar, A. F., Alreesi, A., Harb, G., & Ghazi, I. M. (2022). *Acinetobacter* baumannii Infection-Related Mortality in Hospitalized Patients: Risk Factors and Potential Targets for Clinical and Antimicrobial Stewardship Interventions. *Antibiotics*, *11*(8), 1086. https://doi.org/10.3390/antibiotics11081086
3. Appaneal, H. J., Lopes, V. V., LaPlante, K. L., & Caffrey, A. R. (2022). Treatment, Clinical Outcomes, and Predictors of Mortality among a National Cohort of Admitted Patients with *Acinetobacter baumannii* Infection. *Antimicrobial Agents and Chemotherapy*, *66*(3). https://doi.org/10.1128/aac.01975-21
4. Ayobami, O., Willrich, N., Harder, T., Okeke, I. N., Eckmanns, T., & Markwart, R. (2019). The incidence and prevalence of hospital-acquired (carbapenem-resistant) *Acinetobacter baumannii* in Europe, Eastern Mediterranean and Africa: a systematic review and meta-analysis. *Emerging Microbes & Infections*, *8*(1), 1747–1759. https://doi.org/10.1080/22221751.2019.1698273
5. Bergogne-Bérézin, E., & Towner, K. J. (1996). *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clinical Microbiology Reviews*, *9*(2), 148–165. https://doi.org/10.1128/CMR.9.2.148
6. Berlau, J., Aucken, H. M., Houang, E., & Pitt, T. L. (1999). Isolation of *Acinetobacter* spp including *A.baumannii* from vegetables: implications for hospital-acquired infections. *Journal of Hospital Infection*, *42*(3), 201–204. https://doi.org/10.1053/jhin.1999.0602
7. Bernards, A. T., Harinck, H. I. J., Dijkshoorn, L., van der Reijden, T. J. K., & van den Broek, P. J. (2004). Persistent *Acinetobacter baumannii?* Look Inside Your Medical Equipment. *Infection Control & Hospital Epidemiology*, *25*(11), 1002–1004. https://doi.org/10.1086/502335

8. Blot, S., Ruppé, E., Harbarth, S., Asehnoune, K., Poulakou, G., Luyt, C.-E., Rello, J., Klompas, M., Depuydt, P., Eckmann, C., Martin-Loeches, I., Povoa, P., Bouadma, L., Timsit, J.-F., & Zahar, J.-R. (2022). Healthcare-associated infections in adult intensive care unit patients: Changes in epidemiology, diagnosis, prevention and contributions of new technologies. *Intensive & Critical Care Nursing*, *70*, 103227. https://doi.org/10.1016/j.iccn.2022.103227
9. Brooke, J. S. (2014). New strategies against *Stenotrophomonas maltophilia* : a serious worldwide intrinsically drug-resistant opportunistic pathogen. *Expert Review of Anti-Infective Therapy*, *12*(1), 1–4. https://doi.org/10.1586/14787210.2014.864553
10. Chapartegui-González, I., Lázaro-Díez, M., Bravo, Z., Navas, J., Icardo, J. M., & Ramos-Vivas, J. (2018). *Acinetobacter* baumannii maintains its virulence after long-time starvation. *PloS One*, *13*(8), e0201961. https://doi.org/10.1371/journal.pone.0201961
11. Diep Thi Hoang, Olga Chernomor, Arndt von Haeseler, Bui Quang Minh,and Le Sy Vinh (2017) UFBoot2: Improving the ultrafast bootstrapapproximation. Mol Biol Evol, in press.https://doi.org/10.1093/molbev/msx281
12. Dijkshoorn, L., Nemec, A., & Seifert, H. (2007). An increasing threat in hospitals: Multidrug-resistant *Acinetobacter* baumannii. In *Nature Reviews Microbiology* (Vol. 5, Issue 12, pp. 939–951). https://doi.org/10.1038/nrmicro1789
13. Fahy, S., O'Connor, J. A., Lucey, B., & Sleator, R. D. (2023). Hospital Reservoirs of Multidrug Resistant *Acinetobacter* Species—The Elephant in the Room! *British Journal of Biomedical Science*, *80*. https://doi.org/10.3389/bjbs.2023.11098
14. Farzana, R., Swedberg, G., Giske, C. G., & Hasan, B. (2022). Molecular and genetic characterization of emerging carbapenemase-producing *Acinetobacter* baumannii strains from patients and hospital environments in Bangladesh. *Infection Prevention in Practice*, *4*(2), 100215. https://doi.org/10.1016/j.infpip.2022.100215
15. Fournier, P. E., Richet, H., & Weinstein, R. A. (2006). The Epidemiology and Control of *Acinetobacter* baumannii in Health Care Facilities. *Clinical Infectious Diseases*, *42*(5), 692–699. https://doi.org/10.1086/500202
16. Gramatniece, A., Silamikelis, I., Zahare, Ie., Urtans, V., Zahare, Ir., Dimina, E., Saule, M., Balode, A., Radovica-Spalvina, I., Klovins, J., Fridmanis, D., & Dumpis, U. (2019). Control of *Acinetobacter* baumannii outbreak in the neonatal intensive care unit in Latvia:

whole-genome sequencing powered investigation and closure of the ward. *Antimicrobial Resistance & Infection Control*, *8*(1), 84. https://doi.org/10.1186/s13756-019-0537-z

17. Hidron, A. I., Edwards, J. R., Patel, J., Horan, T. C., Sievert, D. M., Pollock, D. A., & Fridkin, S. K. (2008). Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Annual Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006–2007. *Infection Control & Hospital Epidemiology*, *29*(11), 996–1011. https://doi.org/10.1086/591861
18. Jawad, A., Heritage, J., Snelling, A. M., Gascoyne-Binzi, D. M., & Hawkey, P. M. (1996). Influence of relative humidity and suspending menstrua on survival of Acinetobacter spp. on dry surfaces. *Journal of Clinical Microbiology*, *34*(12), 2881–2887. https://doi.org/10.1128/jcm.34.12.2881-2887.1996
19. Kajiyazdi, M., Dasdar, S., Kianfar, N., & Kaveh, M. (2021). Nosocomial Infection Surveillance in Neonatal Intensive Care Units of Bahrami Children's Hospital. *Archives of Pediatric Infectious Diseases*, *9*(3). https://doi.org/10.5812/pedinfect.108840
20. Khan, H. A., Baig, F. K., & Mehboob, R. (2017). Nosocomial infections: Epidemiology, prevention, control and surveillance. *Asian Pacific Journal of Tropical Biomedicine*, *7*(5), 478–482. https://doi.org/10.1016/j.apjtb.2017.01.019
21. Kuczewski, E., Henaff, L., Regard, A., Argaud, L., Lukaszewicz, A.-C., Rimmelé, T., Cassier, P., Fredenucci, I., Loeffert-Frémiot, S., Khanafer, N., & Vanhems, P. (2022). Bacterial Cross-Transmission between Inanimate Surfaces and Patients in Intensive Care Units under Real-World Conditions: A Repeated Cross-Sectional Study. *International Journal of Environmental Research and Public Health*, *19*(15), 9401. https://doi.org/10.3390/ijerph19159401
22. Kyriakidis, I., Vasileiou, E., Pana, Z. D., & Tragiannidis, A. (2021). Acinetobacter baumannii Antibiotic Resistance Mechanisms. *Pathogens*, *10*(3), 373. https://doi.org/10.3390/pathogens10030373
23. Lam-Tung Nguyen, Heiko A. Schmidt, Arndt von Haeseler, and Bui Quang Minh(2015) IQ-TREE: A fast and effective stochastic algorithm for estimatingmaximum likelihood phylogenies. Mol Biol Evol, 32:268-274.https://doi.org/10.1093/molbev/msu300
24. Lu, C., Wang, J., Pan, L., Gu, X., Lu, W., Chen, D., Zhang, C., Ye, Q., Xiao, C., Liu, P., Tang, Y., Tang, B., Huang, G., Fang, J., & Jiang, H. (2023). Rapid detection of multiple

resistance genes to last-resort antibiotics in Enterobacteriaceae pathogens by recombinase polymerase amplification combined with lateral flow dipstick. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.1062577

25. Magiorakos, A.-P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., & Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, *18*(3), 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x
26. Mahtab Uddin, B. M., Akhand, F., Bari, S., Suchi, S. E., Saha, R., & Ahmed, M. U. (2022). Prevalence and Resistance Profile of *Acinetobacter* baumannii Isolated from Clinical Specimens at a Tertiary Care Hospital in Bangladesh. *Journal of National Institute of Neurosciences Bangladesh*, *7*(2), 177–180. https://doi.org/10.3329/jninb.v7i2.58117
27. Nithichanon, A., Kewcharoenwong, C., Da-oh, H., Surajinda, S., Khongmee, A., Koosakunwat, S., Wren, B. W., Stabler, R. A., Brown, J. S., & Lertmemongkolchai, G. (2022). *Acinetobacter* nosocomialis Causes as Severe Disease as *Acinetobacter* baumannii in Northeast Thailand: Underestimated Role of *A.nosocomialis* in Infection. *Microbiology Spectrum*, *10*(6). https://doi.org/10.1128/spectrum.02836-22
28. Phylogenetic Tree of Some Nosocomial Bacteria Isolated from Ramadi Teaching Hospital. (2020). *International Journal of Pharmaceutical Research*, *12*(04). https://doi.org/10.31838/ijpr/2020.12.04.366
29. Raka, L., Mulliqi-Osmani, G., Begolli, L., Kurti, A., Lila, G., Bajrami, R., & Jaka-Loxh, A. (2013). Acinetobacte. In *Infection Control*. InTech. https://doi.org/10.5772/55618
30. Rebic, V., Masic, N., Teskeredzic, S., Aljicevic, M., Abduzaimovic, A., & Rebic, D. (2018). The Importance of *Acinetobacter* Species in the Hospital Environment. *Medical Archives*, *72*(3), 330. https://doi.org/10.5455/medarh.2018.72.330-334
31. Sadowska-Krawczenko, I., Jankowska, A., & Kurylak, A. (2012). Healthcare-associated infections in a neonatal intensive care unit. *Archives of Medical Science*, *5*(5), 854–858. https://doi.org/10.5114/aoms.2012.31412

32. Shamsizadeh, Z., Nikaeen, M., Nasr Esfahani, B., Mirhoseini, S. H., Hatamzadeh, M., & Hassanzadeh, A. (2017). Detection of antibiotic resistant *Acinetobacter* baumannii in various hospital environments: potential sources for transmission of *Acinetobacter* infections. *Environmental Health and Preventive Medicine*, *22*(1), 44. https://doi.org/10.1186/s12199-017-0653-4
33. Shete, V. B., Ghadage, D. P., Muley, V. A., & Bhore, A. V. (2009). *Acinetobacter* Septicemia in Neonates Admitted to Intensive Care Units. *Journal of Laboratory Physicians*, *1*(02), 073–076. https://doi.org/10.4103/0974-2727.59704
34. Szabó, S., Feier, B., Capatina, D., Tertis, M., Cristea, C., & Popa, A. (2022). An Overview of Healthcare Associated Infections and Their Detection Methods Caused by Pathogen Bacteria in Romania and Europe. *Journal of Clinical Medicine*, *11*(11). https://doi.org/10.3390/jcm11113204
35. Tamura K., Stecher G., and Kumar S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution https://doi.org/10.1093/molbev/msab120
36. Towner, K. J. (2009). *Acinetobacter*: an old friend, but a new enemy. *Journal of Hospital Infection*, *73*(4), 355–363. https://doi.org/10.1016/j.jhin.2009.03.032
37. Yadav, S. K., Bhujel, R., Hamal, P., Mishra, S. K., Sharma, S., & Sherchand, J. B. (2020). Burden of Multidrug-Resistant *Acinetobacter* baumannii Infection in Hospitalized Patients in a Tertiary Care Hospital of Nepal . *Infection and Drug Resistance*, *Volume 13*, 725–732. https://doi.org/10.2147/IDR.S239514