Comparison of Automated System D2-MINI with Conventional Method and VITEK-2 for Identification and Antibiotic Susceptibility Pattern of Gram-Negative Non-Fermentative Bacteria

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

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Declaration

It is hereby declared that

1. The thesis submitted is my/our original work while completing the 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 that has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I/We have acknowledged all main sources of help.

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Abstract:

This study evaluates and compares the performance of the automated D2-MINI system with conventional microbiological methods and the VITEK-2 system for the identification and antibiotic susceptibility testing (AST) of gram-negative non-fermentative bacteria (NFGNB). A total of 30 clinical isolates were analyzed, including *Burkholderia cepacia* (n=6)*, Burkholderia pseudomallei* (n=6)*, Pseudomonas aeruginosa* (n=6)*, Acinetobacter baumannii complex* (n=4)*, Stenotrophomonas maltophilia* (n=4*), Pseudomonas fluorescens* (n=1)*, Pseudomonas stutzeri* (n=1)*, Achromobacter xylosoxidans* (n=1)*, and Rhizobium radiobacter* (n=1). The isolates were recovered from blood (43.3%), pus (23.3%), wound swabs (20.0%), catheter tips (10.0%), and urine (3.3%). Identification was performed using conventional methods (Gram staining, biochemical tests, and AST by disk diffusion) and compared with automated systems (VITEK-2 and D2-MINI).

The results showed that both the VITEK-2 and conventional methods achieved 100% concordance for genus and species identification. However, the D2-MINI system demonstrated high genus-level concordance (100%) for common pathogens like *Burkholderia* spp., *Pseudomonas aeruginosa,* and *Stenotrophomonas maltophilia* but failed to identify *Rhizobium radiobacter* at both genus and species levels. Additionally, it misidentified *Pseudomonas fluorescens* as *Pseudomonas putida* and *Achromobacter xylosoxidans* as *Brucella maltose* and showed 83.3% species-level accuracy for *Acinetobacter baumannii complex*. Antibiotic susceptibility testing revealed that VITEK-2 exhibited 100% concordance with the disk diffusion method for key antibiotics like ceftazidime, meropenem, and piperacillin-tazobactam (TZP). However, it failed to generate susceptibility profiles for *Burkholderia pseudomallei* and had limited data for *Stenotrophomonas maltophilia*. The D2-MINI system showed 100% concordance for ceftazidime and trimethoprim-sulfamethoxazole but performed poorly for meropenem and TZP. Both systems had database limitations, with D2-MINI unable to test amoxicillin-clavulanate, doxycycline, and ciprofloxacin for *Burkholderia* spp., while VITEK-2 lacked results for ceftazidime and doxycycline in *Pseudomonas* spp.

This study highlights the strengths and limitations of automated systems in diagnosing NFGNB. While VITEK-2 offers higher accuracy and broader antibiotic panels, the D2-MINI provides a cost-effective alternative for common pathogens but struggles with rare species and certain

antibiotics. The findings underscore the need for database improvements in automated systems, particularly for non-fermentative bacteria and last-resort antibiotics, to enhance clinical utility in resource-limited settings.

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Chapter 1

Introduction and Objectives

Introduction

Gram-negative bacteria are a diverse group of microorganisms characterized by their unique cell wall structure. Unlike gram-positive bacteria, they have a thin layer of peptidoglycan, which is surrounded by an outer membrane containing lipopolysaccharides (LPS). This outer membrane serves as a protective barrier, making gram-negative bacteria more resistant to antibiotics, detergents, and enzymes. During Gram staining, they do not retain the crystal violet dye and instead take up the counterstain, appearing red or pink under the microscope (Public Library of Science, 2002; NCBI, 2023).

Among gram-negative bacteria, non-fermenting species are a distinct subgroup. These bacteria generally do not break down carbohydrates to produce acid, a feature that differentiates them from fermenting bacteria. Selective media, such as MacConkey agar, are commonly used to identify and differentiate them. In this medium, non-fermenting bacteria do not ferment lactose, resulting in colorless colonies, while fermenting bacteria typically produce colored colonies (Bose, Tiwari, and Thakur, 2020; Gopi, 2009).

Non-fermenting gram-negative bacteria are widely distributed in nature and can be found in various environments, including water systems, soil, plants, healthcare settings, animals, and humans. They play diverse roles in ecosystems, but some species have significant implications for human health. Understanding the differences between fermenting and non-fermenting bacteria is critical for diagnosing infections and selecting effective antimicrobial regimens accordingly. (Bassetti et al., 2019).

In healthcare settings, non-fermenting gram-negative bacteria are of particular concern because they are opportunistic pathogens that frequently cause infections in hospital settings. They are commonly associated with severe infections in immunocompromised individuals, such as those in intensive care units (ICUs). Notable pathogenic species include *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, and the *Burkholderia cepacia* complex. These bacteria are often linked to respiratory tract infections, bloodstream infections, and wound infections (Abdelraouf *et al.*, 2018). In addition to these well-known pathogens, other non-fermenting species, such as *Acinetobacter lwoffii*, *Sphingomonas species*, and *Ochrobactrum*

anthropi, are typically opportunistic or non-pathogenic but can still cause infections under specific circumstances (Bassetti et al., 2019; Abdelraouf *et al.*, 2018).

A major challenge with non-fermenting gram-negative bacteria is their remarkable ability to resist antibiotics. They often exhibit intrinsic and acquired mechanisms of resistance to antibiotics, making them highly adaptable and difficult to treat. This resistance contributes to prolonged hospital stays, increased treatment costs, and higher morbidity, and mortality rates (Gopi, 2009). Their role in nosocomial (hospital-acquired) infections highlights the urgency of addressing the global issue of antibiotic resistance. Overall, non-fermenting Gram-negative bacteria are a significant concern in infectious disease management. Their ability to thrive in diverse environments, coupled with their association with severe infections and high antibiotic resistance, underscores the importance of continued research and the development of effective prevention and treatment strategies (Rajkumari and Roy, 2023; Public Library of Science, 2002).

In Bangladesh, it has become increasingly important to identify and monitor infections caused by non-fermentative gram-negative bacteria. These organisms have been linked to various infections, including meningitis, septicemia, pneumonia, urinary tract infections (UTIs), and surgical site infections (SSIs). Proper management of these infections requires understanding the resistance patterns of the causative agents and implementing effective treatment strategies (Bassetti et al., 2019; Afsana, Dey & Akhter, 2023).

Recent studies have highlighted the role of *Sphingomonas paucimobilis* in outbreaks, particularly in pediatric infections and neonatal intensive care units (NICUs). This organism can be isolated from clinical specimens and distilled water as well emphasizing its importance as an environmental contaminant in healthcare settings (Afsana, Dey & Akhter, 2023). *Pseudomonas aeruginosa* is another critical pathogen frequently associated with infections such as wound and burn infections, eye infections, and respiratory tract infections. It also contributes to bone and joint infections alongside *Acinetobacter baumannii*. Respiratory tract infections (RTIs) especially ventilator-associated pneumonia (VAP) caused by *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Stenotrophomonas maltophilia* are a growing concern in hospital environments (Gopi, 2009).

Additionally, bloodstream infections (sepsis) caused by *Acinetobacter baumannii* and *Burkholderia cepacia* are increasingly reported, presenting significant challenges due to these bacteria' resistance to numerous antimicrobial drugs (Gopi, 2009; Ingle, 2024).

The rising incidence of infections caused by non-fermentative gram-negative bacteria in Bangladesh underscores the need for comprehensive monitoring systems, better infection control measures, and ongoing research to understand their resistance mechanisms. This will aid in improving patient outcomes and reducing the burden of these infections in healthcare facilities (Rajkumari and Roy, 2023).

In our research, we collected 30 clinical samples from a reputable laboratory to identify non-fermenting gram-negative bacteria (NFGNB). The samples underwent standard microbiological diagnostic procedures. Initially, they were cultured on MacConkey agar and incubated at 37°C for 18–24 hours. Once colonies developed, traditional biochemical tests were conducted. These included oxidase, catalase, Gram staining, and metabolic assays using Triple Sugar Iron (TSI) agar, Simmons Citrate Agar, Bile Esculin Agar, and MIU (Motility, Indole, Urease). This step-by-step approach helped identify bacterial species. Additionally, an antimicrobial susceptibility test (AST) using Kirby-Bauer disc diffusion method, guided by CLSI (Clinical and Laboratory Standards Institute) recommendations, was performed to determine effective treatment options (NCBI, 2023; Bose, Tiwari and Thakur, 2020).

In recent years, microbiology has witnessed transformative advancements, particularly in diagnosing and identifying non-fermentative gram-negative bacteria (NFGNB). These bacteria demand precise identification for effective treatment and infection control. Diagnostic methods have evolved to address the challenges posed by these pathogens, encompassing both traditional and modern approaches (Petersen and Becker, 2022). Traditional manual techniques, such as biochemical tests, and antimicrobial susceptibility tests (AST) remain widely used in many laboratories. Despite their widespread use, these conventional methods have several challenges. Firstly, they are time-intensive, often requiring up to 24 to 48 hours or more for results, delaying clinical decision-making. They also struggle with detecting emerging drug resistance and possess limitations in differentiating between rare or closely related bacterial species. With the rise in

multidrug-resistant bacterial infections, the need for faster and more precise diagnostic tools has become critical (Rajkumari and Roy, 2023).

The AST method is simple and cost-effective but time-consuming and laborious. The result depends on visual interpretation which can cause faulty reporting owing to human error. Furthermore, these methods cannot identify an organism's genetic basis of resistance, which is important for identifying multidrug-resistant bacteria (Petersen and Becker, 2022).

To address these challenges, advanced diagnostic technologies such as VITEK 2 and D2-MINI have been introduced. VITEK 2 is an automated diagnostic method that is widely used for bacterial identification and antimicrobial susceptibility testing (AST) by biochemical analysis using colorimetry. This high-throughput technique for bacterial identification and AST mainly focuses on the clinical microbiology laboratory, especially in the area of providing increased levels of automation and increased capacity for higher-volume laboratories. VITEK 2 offers significant improvements, including faster results (within 10 hours), reduced manual workload, human error, and the ability to generate detailed bacterial resistance profiles. It is particularly useful for smaller laboratories due to its compact design. However, it has some drawbacks, such as the high cost of testing cards, its focus on bacteria and yeast, and occasional misidentification due to incomplete reference databases. Moreover, it might not effectively detect low-level resistance patterns (Gupta and Sood, 2021; Bose, Tiwari, and Thakur, 2020)

The D2-MINI represents another advanced approach. It provides rapid and precise identifications, requires minimal sample preparation, and has a lower cost per test compared to VITEK 2. Additionally, it handles a wide range of microorganisms, including bacteria and fungi, while simultaneously offering antimicrobial susceptibility results. It can identify a wide range of organisms, including rare pathogens, making it an extremely useful tool in microbiology. The system can be combined with other diagnostic tools to improve the detection of resistance mechanisms (Rajkumari and Roy, 2023; Ingle, 2024).

However, like VITEK 2, it has limitations, including high initial setup, maintenance costs, the need for specialized training of lab personnel to operate the device effectively, and the accuracy of identifications is heavily reliant on the comprehensiveness of the reference database. Though The D2-MINI system has limited ability to detect antimicrobial resistance directly, combining

the D2-MINI with other systems, such as molecular diagnostic tools or automated AST systems like VITEK 2, can result in a more complete diagnostic profile. This integrated approach enables rapid identification and detailed resistance profiling, essential for effective patient management (Petersen and Becker, 2022; Bose, Tiwari, and Thakur, 2020). Finally, overcoming the lengthy learning process associated with the D2-MINI necessitates extensive training programs for laboratory personnel. These should include both technical training and troubleshooting to ensure that laboratory technologists can effectively use the system. Working with manufacturers or academic institutions to provide workshops and certifications can help standardize the use of the system across labs (Rajkumari and Roy, 2023).

The choice of diagnostic tools in clinical settings depends on various factors, including the laboratory's infrastructure, cost considerations, and the need for quick and reliable results. By leveraging advanced technologies while complementing them with traditional methods, healthcare systems can enhance diagnostic accuracy and contribute to better patient outcomes. Consequently, this study was conducted to evaluate the diagnostic performance of the D2-MINI system specifically for non-fermenting gram-negative bacteria (NFGNB). By examining its accuracy and efficiency, we aimed to determine whether it could serve as a reliable and cost-effective alternative for diagnosing these challenging bacterial infections, which are increasingly implicated in multidrug-resistant and nosocomial infections.

Objectives

The study aimed to compare the efficacy of the automated D2 MINI system with conventional methods and the VITEK-2 system for the identification and antibiotic susceptibility pattern of gram-negative non-fermentative bacteria.

Specific Objectives:

The specific objectives of this study were to:

1. isolate, identify, and then determine the antibiotic susceptibility pattern of gram-negative non-fermentative bacteria by conventional methods.

2. identify and determine the antibiotic susceptibility pattern by the automated methods D2 MINI and VITEK-2.

3. compare the percentage of identification concordance of D2 MINI with conventional methods and VITEK-2.

4. compare the percentage of susceptibility concordance of D2 MINI with conventional methods and VITEK-2.

Chapter 2

Materials and Methods

This study was conducted to evaluate and compare the performance of the automated system D2-MINI with the conventional method and VITEK-2 for the identification and antibiotic susceptibility testing (AST) of gram-negative non-fermentative bacteria. For this purpose, a total of 30 isolates were obtained from different clinical specimens. These organisms were tested by conventional methods. The process began with the isolation of bacterial cultures, followed by Gram staining to confirm morphology and gram reaction. Biochemical tests, including oxidase, catalase, triple sugar iron (TSI), motility-indole-urease (MIU), citrate utilization, and bile esculin hydrolysis, were performed for preliminary identification. Antibiotic susceptibility testing (AST) was carried out using the disk diffusion method as per CLSI guidelines. Subsequently, the same isolates were compared with the automated systems VITEK-2 and D2-MINI to evaluate their efficacy in identification and sensitivity.

Study design: It was a cross-sectional, analytical type of observational study.

Study place: Laboratory procedures were carried out at the K.A. Monsur Research Laboratory, Department of Microbiology, Ibrahim Medical College.

Study period: This study was conducted from March 2024 to November 2024 (9 months).

Laboratory procedure:

2.1 Isolation, identification and antibiotic susceptibility test by conventional method:

Culture:

MacConkey Agar distinguishes gram-negative bacteria by their ability to ferment lactose. Lactose fermentation produces acid, lowering the pH and imparting a pink hue to the medium (Tille, 2021).

After the initial growth, bacterial colonies were carefully transferred onto freshly prepared MacConkey agar plates, following the manufacturer's instructions.

Inoculation Procedure:

A sterile inoculating loop was used to streak the bacterial sample across the MacConkey agar plate in a zigzag motion. The plates were then incubated at 37°C for 24 hours (Tille, 2021). After incubation, lactose fermenters appeared as pink colonies, while non-lactose fermenters formed colorless colonies. The selective nature of MacConkey Agar effectively inhibited gram-positive bacterial growth, allowing the isolation of gram-negative organisms (Tille, 2021).

Gram staining:

Gram staining is a fundamental differential staining technique in microbiology, used to classify bacteria into gram-positive and gram-negative groups based on their cell wall properties, while also allowing for the observation of bacterial morphology under a microscope. The procedure involves the use of specific reagents: crystal violet as the primary stain, iodine as a mordant to form a complex with the stain, alcohol or acetone as a decolorizer to remove the stain from Gram negative cells, and safranin as a counterstain for decolorized cells.

A thin smear of bacterial culture was prepared on a clean glass slide, heat-fixed, and stained with crystal violet for one minute, followed by iodine for another minute. The slide was then decolorized dropwise with alcohol, which removed the primary stain from Gram-negative bacteria, while Gram-positive bacteria retained the stain due to their thick peptidoglycan layer. Safranin was subsequently applied for 30 seconds to counterstain the Gram-negative cells. The slide was rinsed, air-dried, and examined under a microscope using oil immersion at 1000x magnification, where Gram-positive bacteria were observed as purple and Gram-negative bacteria as pink or red. This method was used to observe the shapes and arrangements of the bacteria. (Moyes et al., 2009).

Biochemical tests:

Biochemical tests are fundamental tools in microbiology, designed to identify and characterize microorganisms based on their metabolic and enzymatic activities. These tests exploit the ability

of bacteria to produce specific enzymes that catalyze chemical reactions, breaking down. Substrates like carbohydrates, proteins, or lipids into detectable end-products such as acids, gases, or alcohols. The resulting biochemical reactions are visualized using color changes, gas formation, or other physical indicators facilitated by reagents and specialized media (Cheesbrough,2000)

Oxidase test:

The oxidase test detects the presence of cytochrome c oxidase, an enzyme in the electron transport chain. The reagent used is tetramethyl-p-phenylenediamine dihydrochloride, which turns purple in the presence of the enzyme.

A piece of filter paper was placed in a clean Petri dish, and 1-2 drops of freshly prepared oxidase reagent (1% solution of Tetramethyl-p-phenylenediamine dihydrochloride) were added. Using a wooden stick, a colony was picked up from the culture and smeared on the soaked filter paper. The development of a deep purple color within 10 seconds indicates a positive test.

Catalase Test:

This test determines the ability of bacteria to break down hydrogen peroxide into water and oxygen, using the enzyme catalase. Hydrogen peroxide (3%) is used as the reagent.

A few isolated colonies having the same morphology from the culture to be tested were picked up from the plate by a sterile toothpick and clipped into 1 mL of 3% hydrogen peroxide (H_2O_2) contained in a small clear test tube. A positive reaction was evidenced by the immediate production of gas bubbles from the surface of the bacterial colonies dipped with the toothpick.

Triple Sugar Iron (TSI) Test:

The TSI test differentiates bacteria based on their ability to ferment glucose, lactose, and sucrose, as well as their ability to produce hydrogen sulfide. The test provides diagnostic information through changes in color and gas production.

The bacterial culture was inoculated by stabbing the agar's butt and streaking the slant. The inoculated TSI slants were incubated at 37°C for 24 hours. Changes in color and gas production

were then observed (Fawzi, 2021). The red slant and yellow butt (Red/Yellow or Alkaline (K)/Acidic (A)) were interpreted as only glucose having been fermented. The yellow slant and yellow butt (Yellow/Yellow or Acidic (A)/Acidic (A)) were interpreted as lactose and/or sucrose, or all three sugars, having been fermented. The red slant and red butt (Red/Red or Alkaline (K)/Alkaline (K)) indicated that none of the three sugars had been fermented. The blackening of the media or the formation of black-colored spots was recorded as indicating H_2S production and cracking of the media, the presence of gas bubbles, or gaps within the media were recorded as indicators of gas production.

Motility Indole Urease test:

The MIU test is used to determine bacterial motility, indole production, and urease activity. The test results are interpreted based on color changes and appearance in the media.

Using a sterile straight wire loop, an isolated colony of the test organism was inoculated into MIU agar media by stabbing the media with a sterile straight wire. The tube was incubated at 37°C overnight. For the indole reaction, 1 mL of Kovacs reagent was added to the culture tube. A red color in the surface layer within 10 minutes indicated positive indole production. Motility was shown by spreading turbidity throughout the medium. Urease production was shown by a pink color in the medium.

Citrate Test:

The citrate utilization test determines whether a bacterium can use citrate as its sole carbon source. Simmons' citrate agar, containing bromothymol blue as an indicator, is used.

The Simmons Citrate agar slants were prepared by dissolving the dehydrated medium, autoclaving, and allowing it to cool. After inoculating the slant by streaking, the plate was incubated at 37°C for 24 hours. Color changes in the media were observed to determine if the bacteria could utilize citrate. A positive result was demonstrated by growth with a color change from green to intense blue along the slant, and a negative result was demonstrated by no growth and no color change, with the color of the slant remaining green.

Bile Esculin Test:

The bile esculin test detects the ability of bacteria to hydrolyze esculin in the presence of bile. The medium contains bile salts and ferric citrate, which reacts with hydrolyzed esculin to form a black precipitate.

The slant surface is streaked with the bacterial culture and incubated at 37°C for 24 hours. A positive test is marked by blackening of the medium, indicating bile tolerance, while no blackening denotes a negative result.

Antibiotic susceptibility testing:

The identified bacterial species were tested for antimicrobial susceptibility by the Kirby-Bauer modified disc-diffusion technique (Bauer et al., 1996). The antimicrobial discs were procured from commercial sources.

Antimicrobial discs used:

For gram-negative non-fermenting bacteria**:**

Ceftazidime (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Amikacin (30 µg), Netilmicin (30 µg), Piperacillin-Tazobactam (100/10 µg), Meropenem (10 µg), Colistin (10 µg), Cefepime (30 µg), and Amoxicillin-clavulanate (20/10 µg) were determined by disk diffusion method. The antibacterial disks were produced by a local agent.

Disk Diffusion method:

Media used: Mueller-Hinton agar without supplementation.

Preparation of inoculum:

The inoculum was prepared by subculturing bacterial isolates on MacConkey agar and incubating them at 25°C for 24 hours to ensure sufficient growth. A single colony was selected and suspended in 10 mL of sterile 0.9% saline. The suspension was allowed to stand for 10-15 minutes to permit the sedimentation of heavier particles, and the supernatant was adjusted to a turbidity equivalent to 0.5 McFarland standard, corresponding to approximately 1-2 × 10^8 CFU/mL. Turbidity adjustment was performed visually against a white background with contrasting black lines.

Inoculation of test plates:

The adjusted inoculum was used to inoculate Mueller-Hinton agar plates within 15 minutes to avoid variations in bacterial density. A sterile cotton swab was immersed into the suspension, pressed against the inner wall of the test tube to remove excess fluid, and streaked evenly over the agar surface three times to ensure confluent growth. The plate was rotated 60° after each streak to ensure uniform bacterial distribution. After inoculation, plates were allowed to dry with the lid closed for 3-5 minutes before antibiotic disks were applied.

Antibacterial disk placement and inoculation:

Antibiotic disks were placed on the inoculated agar plates using sterile forceps. The disks, impregnated with known concentrations of antibiotics, should be evenly spaced to avoid overlapping zones of inhibition. Once the disks are positioned, the plates are incubated in an inverted position at 35°C for 24 hours under aerobic conditions. This placement ensures proper diffusion of antibiotics into the medium, creating distinct zones of inhibition around the disks. Consistency in disk placement is vital for reliable interpretation of antimicrobial susceptibility.

Interpretation of zone diameter:

Antibiotic susceptibility testing was conducted using the disk diffusion method to determine the susceptibility patterns of bacteria to various antimicrobial agents. The potency of each antibiotic disk and their interpretative zone diameters for sensitivity (S), intermediate susceptibility (I), and resistance (R) were evaluated based on the Clinical and Laboratory Standards Institute (CLSI) guidelines.

The details are summarized in the table below (CLSI, 2023):

Table2.1: Interpretative zone diameter (in mm) (CLSI,2023)

Antibacterial Drug	Sensitive (S) (Zone Diameter in mm)	Intermediate (I) (Zone Diameter in mm)	Resistant (R) (Zone Diameter in mm)
Ceftazidime	≥21	18-20	≤ 17
Ciprofloxacin	≥21	16-20	≤15
Gentamicin	≥15	13-14	≤12
Amikacin	≥17	15-16	≤14
Netilmicin	≥15	13-14	≤12
Piperacillin-Tazobactam	≥21	15-20	≤14
Meropenem	≥23	20-22	≤19
Cefepime	≥21	18-20	≤17
Amoxicillin-Clavulanate	≥18	14-17	≤13

2.2 Isolation, identification and antibiotic susceptibility test by VITEK-2 compact system:

The VITEK-2 Compact system (bioMérieux, France) was employed for the identification and antimicrobial susceptibility testing of the 30 non-fermenter gram-negative bacterial isolates included in this study.

Principle:

The VITEK-2 Compact system is an advanced automated microbiology platform that facilitates the rapid identification of microorganisms and antimicrobial susceptibility testing (AST). Its operation is primarily based on growth-based technology, utilizing a spectrophotometer to measure the absorbance of light by microbial cultures. Each microorganism exhibits a unique absorbance profile, allowing for effective differentiation between various species. The system is equipped with a comprehensive identification database, which includes a wide array of microbial species. (Pincus, 2014)

Process:

1. Preparation of Bacterial Isolates

Each bacterial isolate was cultured on MacConkey agar medium, and the plates were incubated at 37°C for 24 hours.

2. Preparation of Bacterial Suspension

A pure colony was selected from the cultured plate, and a bacterial suspension was prepared in a sterile saline solution (0.45% NaCl). The turbidity was adjusted to 0.5–0.63 McFarland standards and verified using the VITEK-2 DensiChek instrument.

3. Loading of VITEK-2 Cards

The bacterial suspension was transferred into a gram-negative ID card for identification, and an AST card (e.g., AST-N cards) was used for antimicrobial susceptibility testing. Both cards, along with the inoculum tube, were loaded into a cassette.

4. Automated Processing by VITEK-2 Compact System

The cassette was inserted into the VITEK-2 Compact system, and the automated identification and

susceptibility testing process was initiated.

5. Data Analysis and Reporting

Biochemical reactions in the gram-negative ID card were analyzed by the system to identify the isolate. Minimum inhibitory concentration (MIC) values for a panel of antibiotics were determined using the AST card. Identification results were generated as highly probable, probable, or unidentifiable based on the system's database. Antimicrobial susceptibility results were interpreted as susceptible, intermediate, or resistant according to CLSI guidelines.

6. Quality Control Measures

Reference strains, including Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853, were used for quality control. These strains were processed in parallel with the test isolates to validate the system's performance.

2.3 Isolation, identification, and antibiotic susceptibility test by conventional method D2-MINI:

The D2-MINI (Zhuhai Dl Biotech Ltd, China) was used for the identification and antimicrobial susceptibility testing of 30 non-fermenter gram-negative bacterial isolates in this study. Rapid and accurate identification of microorganisms is essential for effective disease diagnosis and treatment. With antimicrobial resistance (AMR) being a global health threat, ID/AST systems like the D2-MINI play a crucial role in identifying pathogens, preventing antibiotic misuse, and guiding treatment based on resistance profiles. By following CLSI standards, the D2 Mini provides reliable, timely results to combat the rise of superbugs and improve patient outcomes in clinical microbiology.

1. Sample Preparation:

Initially, samples such as urine, feces, or blood were collected and streaked onto appropriate culture media. These plates were incubated at standard laboratory conditions for 24 hours to allow the growth of bacterial colonies. Once sufficient growth was observed, a single colony was

selected from the culture plate using sterile techniques.

2. Suspension Preparation and Oxidase Testing:

The selected colony was used to prepare a bacterial suspension in sterile normal saline, ensuring that the turbidity aligns with a McFarland standard of 0.48–0.52. This turbidity was verified using the D2-MINI turbidimeter to maintain consistency.

3.Loading and Calibration:

The bacterial suspension and Muller-Hinton broth were prepared for testing and loaded into the auto-sampling instrument of the D2-MINI machine. A sterile sampling tip was placed into the instrument, and the selected test card is aligned on the designated holder. Calibration was initiated to ensure proper placement of the sampling tips, and the machine automatically dispensed the suspension into the wells of the test card. This process ensures precise and controlled sample distribution.

4. Incubation:

Following sample distribution, specific wells were sealed with two drops of paraffin oil to prevent evaporation. The test card was then incubated at a controlled temperature of 36–48°C for 24 hours. If turbidity was not observed in the designated control wells after this period, the incubation was extended for an additional 24 hours to ensure accurate results.

5. Reagent Addition and Reading:

After the incubation period, reagents such as VP (A and B), IND, and PHE were added to specific wells on the test card. The reactions were monitored, and results are interpreted immediately using the D2-MINI system's automated analysis software. This step ensured accurate identification of the microorganism and its antimicrobial susceptibility profile.

6. Data Analysis and Reporting:

The D2-MINI system's software was used to log in, scan the barcode of the test card, and initiate data analysis. Species identification and susceptibility results were automatically generated,

reviewed, and edited to include sample-specific details such as the department and specimen source. Reports were saved and exported in a printable format for clinical use, ensuring an efficient and user-friendly workflow.

Formula to get Percentage of identification concordance between VITEK-2 and D2-MINI or conventional method and VITEK-2 or conventional method and D2-MINI: Concordance (%): Percentage agreement between VITEK-2 and D2-MINI or conventional method and VITEK-2 or conventional method and D2-MINI; Concordance Calculation: Concordance (%) = (Number of matches between VITEK-2 and D2 MINI / Total number of organisms) × 100. (Hernández-Durán et al., 2017).

The formula to get the Percentage of susceptibility concordance between disk diffusion method and D2-mini for 30 isolates: Concordance (%) = (Number of matching susceptibility results between D2-MINI and disk diffusion method / Total number of susceptible isolates) × 100 (Hernández-Durán et al., 2017). Formula to get percentage of susceptibility concordance between disk diffusion method and VITEK-2 for 30 isolates: Concordance= percentage of susceptibly agreements between disk diffusion method and VITEK-2; Concordance (%) = (Number of matching susceptibility results between VITEK-2 and disk diffusion method / Total number of susceptible isolates) × 100 (Hernández-Durán et al., 2017)

Chapter 3

RESULTS

This study analyzed 30 known isolates of gram-negative non-fermentative bacteria to evaluate the performance of the automated D2-MINI system in comparison with conventional methods and the VITEK-2 system. In addition to bacterial identification, antibiotic susceptibility testing was conducted to further assess the efficacy of the D2-MINI system. Conventional identification involved bacterial culture followed by a series of biochemical tests, while conventionally antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method. Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) 2023 guidelines to ensure standardization and accuracy. These 30 isolates were recovered from five specimen types, including blood, urine, pus, wound swabs, and catheter tips.

Figure 3.1 illustrates the distribution of isolates according to their specimen types. Blood was identified as the predominant source of isolates, accounting for 43.3% of the total samples. Pus samples represented the second most common source, contributing 23.3% of the isolates, followed by wound swabs at 20.0%. In contrast, urine (3.3%) and catheter tips (10.0%) yielded fewer isolates.

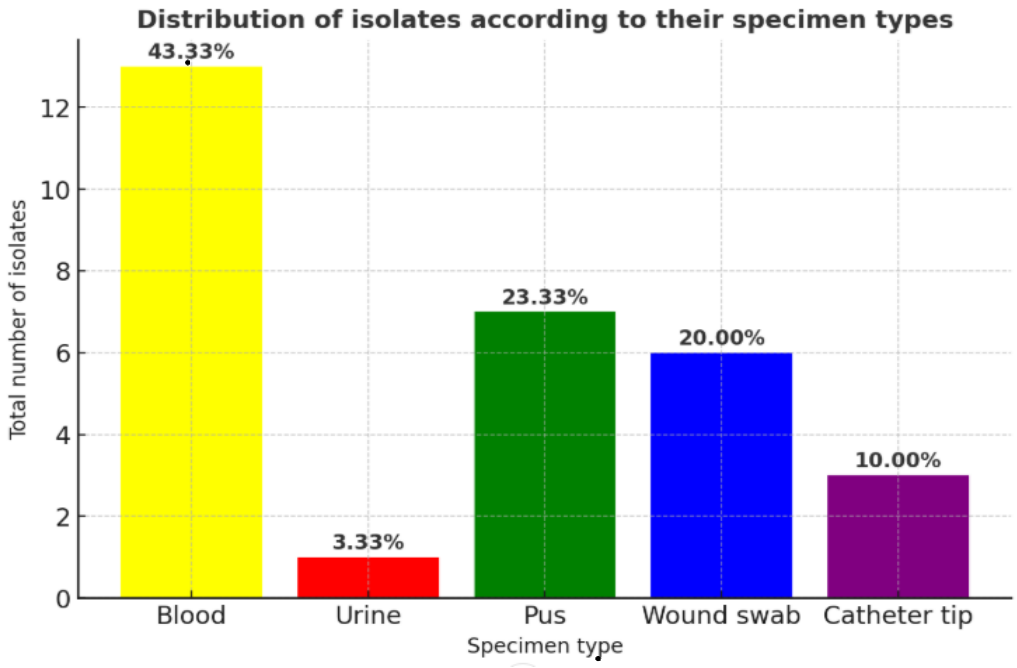

Figure 3.1: Distribution of isolates according to their specimen types.

The detailed biochemical profiles of the 30 gram-negative non-fermenting bacterial isolates are summarized in Table 3.2. The isolates were identified using microbiological tests, including gram staining, colony morphology on MacConkey Agar media, oxidase, catalase, bile esculin, citrate utilization, motility, indole, urease tests (MIU), and Triple Sugar Iron (TSI) reaction. The majority of the isolates were identified as gram-negative rods, except for *Acinetobacter baumannii complex* (n=4), which displayed a distinctive coccobacilli morphology. These isolates demonstrated consistent biochemical patterns across key diagnostic tests.

All isolates exhibited an alkaline/alkaline (K/K) reaction on Triple Sugar Iron (TSI) agar, confirming their inability to ferment carbohydrates such as glucose, lactose, or sucrose. Furthermore, none of the isolates produced hydrogen sulfide (H_2S) or gas. Motility testing revealed that most isolates were motile, except for *Acinetobacter baumannii complex* and *Stenotrophomonas maltophilia*, which were non-motile.

The oxidase test, an important biochemical marker, was positive for *Pseudomonas* (n=8), *Burkholderia* (n=12), and *Achromobacter xylosoxidans* (n=1), whereas *Acinetobacter baumannii complex* and *Stenotrophomonas maltophilia* tested negative. Catalase activity, indicative of aerobic metabolism, was universally positive across all isolate.

Table 3.2: Conventional methods for identification of gram-negative bacteria (N=30)

Prospective Bacterial isolates	**Gram staining**	**Colony characteristics a**	**TSI (Slant/ Butt/ H_2S/ Gas)**	**MIU (Motility/ Indole/ Urease)**	**Citrate test**	**Bile esculin hydrolysis test**	**Oxidase test**	**Catalase test**
Pseudomonas	Gram negative rods	Metallic sheen, large	K/K H_2S -ve Gas -ve	M +ve I -ve U -ve	+ve	-ve	+ve	+ve
Burkholderia	Gram negative rods	Pink, smooth, circular	K/K H_2S -ve Gas -ve	M +ve I -ve U -ve	+ve	-ve	+ve	+ve
Acinetobacter	Gram negative cocco bacilli	Pale, smooth,mucoid.	K/K H_2S -ve Gas -ve	M -ve I -ve U -ve	+ve	-ve	-ve	+ve
Stenotrophomonas	Gram-negative curved rod	Pale, smooth.	K/K H_2S -ve Gas -ve	M +ve I -ve U -ve	-ve	+ve	-ve	+ve
Achromobacter	Gram negative rods	Pale, smooth, circular	K/K H_2S -ve Gas -ve	M +ve I -ve U -ve	+ve	-ve	+ve	+ve
Rhizobium	Gram negative rods	Pale, small, round	K/K H_2S -ve Gas -ve	M +ve I -ve U -ve	+ve	+ve	+ve	+ve

Note: **"+"** = positive**, "-"** = negative; **MIU**: Motility/Indole/Urease; **TSI**: Triple Sugar Iron; **K/K** = alkaline/alkaline; **H_2S:** Hydrogen Sulfide **a:** Colony characteristics on MacConkey agar media; **n**= number

Table 3.3 presents the distribution of 30 gram-negative non-fermentative bacterial isolates identified from various clinical specimens, including blood, urine, pus, wound swabs, and catheter tips. Blood samples yielded the highest proportion of isolates (47.8%, n=11), Pus specimens accounted for 21.7% (n=5), while wound swabs contributed 17.4% (n=4). Catheter tips and urine samples accounted for fewer isolates, contributing 8.7% (n=2) and 4.3% (n=1), respectively.

Among the isolates, the genus Burkholderia was the most frequently identified, with *Burkholderia cepacia* (n=6, 20.0%) and *Burkholderia pseudomallei* (n=6, 20.0%) collectively accounting for 40.0% of the total isolates. The second most common genus was Pseudomonas, which included *Pseudomonas aeruginosa* (n=6, 20.0%), *Pseudomonas fluorescens* (n=1, 3.3%), and *Pseudomonas stutzeri* (n=1, 3.3%), contributing a combined 26.7% of the total isolates. Following these dominant genera, the *Acinetobacter baumannii complex* (n=4, 13.3%) and *Stenotrophomonas maltophilia* (n=4, 13.3%) were each identified in a similar proportion of samples.

Less frequently encountered genera included *Achromobacter xylosoxidans* (n=1, 3.3%) and *Rhizobium radiobacter* (n=1, 3.3%), representing a smaller proportion of the isolates.

Table 3.3: Distribution of isolates according to their specimen type (N= 30)

Prospective bacterial isolate	Blood n (%)	Urine n (%)	Pus n (%)	Wound swab n (%)	Catheter tip n (%)	Total number n (%)
Burkholderia cepacia	6 (20.0)	0 (0)	0 (0)	0 (0)	0 (0)	6 (20.0)
Burkholderia pseudomallei	3 (10.0)	0 (0)	0 (0)	3 (10.0)	0 (0)	6 (20.0)
Acinetobacter baumannii complex	1 (3.3)	0 (0)	2 (6.7)	1 (3.3)	0 (0)	4 (13.3)
Pseudomonas aeruginosa	0 (0)	0 (0)	3 (10.0)	2 (6.7)	1 (0)	6 (20.0)
Pseudomonas fluorescens	0 (0)	1 (3.3)	0 (0)	0 (0)	0 (0)	1 (3.3)
Pseudomonas stutzeri	1 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.3)
Stenotrophomons maltophilia	0 (0)	0 (0)	2 (6.7)	0 (0)	2 (6.7)	4 (13.3)
Achromobacter xylosoxidans	1 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.3)
Rhizobium radiobacter	1 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.3)
Number of isolates per specimen	13 (43.3)	1 (3.3)	7 (23.3)	6 (20.0)	3 (10.0)	30 (100)

Notes: Total sample size is N=30; Percentages in parentheses represent the proportion of isolates for each bacterial species

Table 3.4 illustrates the percentage agreement of microbial identification of 30 gram-negative non-fermentative bacterial isolates using three different methods: the conventional method, VITEK 2, and D2-MINI.

At the genus level, both the conventional method and the VITEK-2 system achieved 100% accuracy across all isolates. In contrast, the D2-MINI system showed variable performance. While D2-MINI correctly identified all isolates of *Burkholderia* (n=12), *Pseudomonas* (n=6), and *Stenotrophomonas* (n=4), it failed to identify *Rhizobium* (n=1) and *Achromobacter* (n=1). Additionally, for *Acinetobacter baumannii complex* (n=4), D2-MINI achieved 100% accuracy at the genus level but showed discrepancies at the species level.

Similarly, at the species level, the VITEK-2 system maintained 100% agreement across all isolates, matching the accuracy of the conventional method. However, the D2-MINI system exhibited discrepancies, particularly with *Acinetobacter baumannii complex* (n=4), where its accuracy dropped to 83.3%. Furthermore, it failed to identify *Pseudomonas stutzeri* (n=1) and *Pseudomonas fluorescens* (n=1) at the species level, achieving 0% accuracy for these species. Similarly, for *Achromobacter xylosoxidans* (n=1) and *Rhizobium radiobacter* (n=1), no species-level identification was made**,** indicating the limitations of the D2-MINI system in species-level accuracy.

Table 3.4: Comparative identification of gram-negative bacteria by conventional method and automated methods (VITEK-2 and D2-MINI) (N= 30)

Isolates (N=30)	Conventional Method		VITEK 2		D2 MINI	
	Genus n (%)	Species n (%)	Genus n (%)	Species n (%)	Genus n (%)	Species n (%)
Burkholderia cepacia (n=6)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)
Acinetobacter baumannii complex (n=4)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	3 (83.3)
Pseudomonas stutzeri (n=1)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)
Pseudomonas aeruginosa (n=6)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)
Burkholderia pseudomallei (n=6)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)
Stenotrophoms maltophilia (n=4)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)
Pseudomonas fluorescens (n=1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)
Achromobacter xylosoxidans (n=1)	1 (100)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)
Rhizobium radiobacter (n=1)	0 (0)	0 (0)	1 (100)	1 (100)	0 (0)	0 (0)

Notes: "**n**" represents the **number of isolates** for each bacterium till genus and species level for each of the methods; with percentages in parentheses; **Genus:** Identification at the genus level; **Species**: Identification at the species level.

Table 3.5 illustrates the concordance between the VITEK-2 system and the conventional method for identifying 30 gram-negative bacterial isolates at both genus and species levels.

The data presented compares the performance of the VITEK-2 system with the conventional method for the identification of various bacterial organisms. In terms of genus identification, the VITEK-2 system demonstrated perfect concordance with the conventional method across all tested organisms. For every species listed—*Burkholderia cepacia*, *Acinetobacter baumannii complex*, *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Stenotrophomonas maltophilia*, *Pseudomonas fluorescens*, *Achromobacter xylosoxidans*, and *Rhizobium radiobacter*—the genus identified by VITEK-2 was in complete agreement with the conventional method, yielding a 100% match for all organisms.

Similarly, the species identification was also flawless. The VITEK-2 system correctly identified the species of all organisms with no discrepancies when compared to the conventional method. This high level of accuracy is evident, as the system matched the species identification in every case, achieving a 100% concordance rate for all nine organisms tested.

The concordance between the VITEK-2 system and the conventional method is thus perfect for both genus and species identification.

Table 3.5: Comparative identification of non-fermentative bacteria by conventional and VITEK-2 (N=30)

Isolated Organism	Conventional method		VITEK 2		Concordance	
	Genus (n)	Species (n)	Genus (n)	Species (n)	Genus (%)	Species (%)
Burkholderia cepacia (n=6)	6	6	6	6	100	100
Acinetobacter baumannii complex (n=4)	4	4	4	4	100	100
Pseudomonas stutzeri (n=1)	1	1	1	1	100	100
Pseudomonas aeruginosa (n=6)	6	6	6	6	100	100
Burkholderia pseudomallei (n=6)	6	6	6	6	100	100
Stenotrophoms maltophilia (n=4)	4	4	4	4	100	100
Pseudomonas fluorescens (n=1)	1	1	1	1	100	100
Achromobacter xylosoxidans (n=1)	1	0	1	1	100	100
Rhizobium radiobacter (n=1)	0	0	1	1	100	100

Notes: Genus (n): Number of isolates correctly identified at the genus level by each method; Species (n): Number of isolates correctly identified at the species level by each method; Concordance (%): Percentage agreement between Conventional method and D2 MINI; Concordance Calculation: Concordance (%) = (Number of matches between conventional method and D2 MINI / Total number of organisms) × 100. (Hernández-Durán et al., 2017).

Table 3.6 illustrates the concordance between the conventional method and D2-MINI for the identification of 30 bacterial isolates at both genus and species levels.

The genus identification performance of the D2-MINI system showed high accuracy for most organisms but also revealed some discrepancies. *Burkholderia cepacia, Burkholderia pseudomallei, Acinetobacter baumannii complex, Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* were correctly identified by both methods, achieving 100% concordance. However, *Pseudomonas stutzeri, Achromobacter xylosoxidans,* and *Rhizobium radiobacter* were not correctly identified at the genus level by the D2-MINI system, resulting in 0% concordance for these isolates.

For species identification, the D2-MINI system exhibited a more variable performance. While *Burkholderia cepacia, Burkholderia pseudomallei,* and *Pseudomonas aeruginosa* were identified with 100% concordance, the system showed limitations for other species. *Acinetobacter baumannii complex* identification showed 75% concordance, indicating a discrepancy in one isolate. The system failed to correctly identify the species *of Pseudomonas stutzeri, Achromobacter xylosoxidans,* and *Rhizobium radiobacter,* all of which had 0% concordance. Additionally, *Pseudomonas fluorescens* was correctly identified at the genus level but not at the species level (100% genus, 0% species concordance).

Table 3.6: Percentage of identification concordance between D2-MINI and Conventional method (N=30)

Isolates (N=30)	Conventional Method		D2 MINI		Concordance	
	Genus (n)	Species (n)	Genus (n)	Species (n)	Genus (%)	Species (%)
Burkholderia cepacia (n=6)	6	6	6	6	100%	100%
Acinetobacter baumannii complex (n=4)	4	4	4	3	100%	75%
Pseudomonas stutzeri (n=1)	1	1	0	0	0%	0%
Pseudomonas aeruginosa (n=6)	6	6	6	6	100%	100%
Burkholderia pseudomallei (n=6)	6	6	6	6	100%	100%
Stenotrophomonas maltophilia (n=4)	4	4	4	4	100%	100%
Pseudomonas fluorescens (n=1)	1	1	1	0	100%	0%
Achromobacter xylosoxidans (n=1)	1	0	0	0	0%	0%
Rhizobium radiobacter (n=1)	0	0	0	0	0%	0%

Notes: Genus (n): Number of isolates correctly identified at the genus level by each method; Species (n): Number of isolates correctly identified at the species level by each method; Concordance (%): Percentage agreement between Conventional method and D2 MINI; Concordance Calculation:

Table 3.7 illustrates the concordance between the VITEK-2 and D2-MINI systems for genus and species-level identification of 30 gram-negative non-fermenting bacterial isolates.

In terms of genus identification, both systems demonstrated high accuracy with some discrepancies. For organisms like *Burkholderia cepacia, Pseudomonas aeruginosa, Burkholderia pseudomallei, Stenotrophomonas maltophilia,* and *Pseudomonas fluorescens* were correctly identified by both VITEK-2 and D2-MINI, achieving 100% concordance at the genus level. However, *Acinetobacter baumannii complex* was accurately identified by the D2-MINI system in only 75% concordance, showing a discrepancy for one isolate. Additionally, the D2-MINI system failed to identify the genus for *Pseudomonas stutzeri, Achromobacter xylosoxidans*, and *Rhizobium radiobacter*, resulting in 0% concordance for these organisms.

The species-level identification followed a similar pattern. *Burkholderia cepacia*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens* were accurately identified by both systems, achieving 100% concordance. However, *Acinetobacter baumannii complex* was correctly identified at the species level by the D2-MINI system and showed 75% concordance, with one misidentified isolate. The D2-MINI system failed to identify the species for *Pseudomonas stutzeri, Achromobacter xylosoxidans,* and *Rhizobium radiobacter*, resulting in 0% concordance for these isolates.

Comparing both systems, VITEK-2 exhibited greater consistency, achieving 100% concordance for genus identification across all isolates. In contrast, D2-MINI showed discrepancies, particularly with *Acinetobacter baumannii complex* and other less common species. Similarly, species identification was more reliable with VITEK-2, which correctly identified all species except for *Acinetobacter baumannii complex* (75% concordance). The D2-MINI system struggled with multiple species, including *Pseudomonas stutzeri, Achromobacter xylosoxidans*, and *Rhizobium radiobacter*, where it failed entirely to identify the species.

Table 3.7: Percentage of identification concordance between VITEK-2 and D2-MINI (N= 30)

Isolates (N=30)	VITEK 2		D2 MINI		Concordance	
	Genus (n)	Species (n)	Genus (n)	Species (n)	Genus (%)	Species (%)
Burkholderia cepacia (n=6)	6	6	6	6	100%	100%
Acinetobacter baumannii complex (n=4)	4	4	3	3	75%	75%
Pseudomonas stutzeri (n=1)	1	1	0	0	0%	0%
Pseudomonas aeruginosa (n=6)	6	6	6	6	100%	100%
Burkholderia pseudomallei (n=6)	6	6	6	6	100%	100%
Stenotrophoms maltophilia (n=4)	4	4	3	3	75%	75%
Pseudomonas fluorescens (n=1)	1	1	1	0	100%	0%
Achromobacter xylosoxidans (n=1)	1	1	0	0	0%	0%
Rhizobium radiobacter (n=1)	1	1	0*	0*	0%	0%

Notes: Genus (n): Number of isolates correctly identified at the genus level by each method; Species (n): Number of isolates correctly identified at the species level by each method; Concordance (%): Percentage agreement between Conventional method and D2 MINI; Concordance Calculation: Concordance (%) = (Number of matches between conventional method and D2 MINI / Total number of organisms) × 100. (Hernández-Durán et al., 2017).

Table 3.8 presents the percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and VITEK-2 for 6 *Burkholderia cepacia* isolates. The susceptibility profiles for 8 antibiotics were compared to evaluate the agreement between the KB disk diffusion method and VITEK-2.

The highest concordance (100%) was observed for ceftazidime, doxycycline, ciprofloxacin, meropenem, and piperacillin-tazobactam (TZP) between disk diffusion and VITEK-2 for *Burkholderia cepacia* isolates, indicating complete agreement for these antibiotics. For amoxicillin-clavulanic acid (amoxiclav), susceptibility data for *Burkholderia cepacia* were not provided by VITEK-2, and thus, concordance could not be compared. Additionally, for trimethoprim-sulfamethoxazole, susceptibility results were not generated by VITEK-2 for two of the isolates, preventing concordance comparison. Furthermore, clinical breakpoints for cefepime are not defined or are considered unreliable for *B. cepacia* in the Kirby-Bauer method, resulting in undeterminable concordance.

Table 3.8: Percentage of susceptibility concordance between disk diffusion method and VITEK-2 for *Burkholderia cepacia* isolates (n=6)

Isolates	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by VITEK-2 (n)	Concordance (%)
Burkholderia cepacia	Amoxiclav	6	-*	-
	Ceftazidime	5	5	100
	Doxycycline	2	2	100
	Trimethoprim - sulfamethoxazole	6	4**	-
	Cefepime	-***	0	-
	Ciprofloxacin	5	5	100
	Meropenem	1	1	100
	TZP	4	4	100

Note: Concordance= percentage of susceptibility agreements between disk diffusion method and VITEK-2; Concordance (%) = (Number of matching susceptibility results between VITEK-2 and disk diffusion method / Total number of susceptible isolates) × 100 (Hernández-Durán et al., 2017)**;** Amoxiclav= Amoxicillin-Clavulanic acid; TZP= Piperacillin-Tazobactam.

**Burkholderia cepacia* data not shown by VITEK-2 for amoxicillin-clavulanic acid (amoxiclav), concordance could not be compared,

** VITEK-2 did not provide susceptibility results for 2 of the isolates; concordance could not be compared,

*** Clinical breakpoints for cefepime are undefined or unreliable for *B. cepacia* in KB method

The percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and the D2-MINI system for 6 *Burkholderia cepacia* isolates is presented in Table 3.9. Ceftazidime and trimethoprim-sulfamethoxazole demonstrated the highest concordance (100%), indicating complete agreement between the two methods. Similarly, ceftazidime (90.90%) and meropenem (87.5%) also showed higher concordance, highlighting the strong reliability of the D2-MINI system for these antibiotics. In contrast, piperacillin-tazobactam (TZP) showed 0% concordance, and meropenem (16.66%) also showed lower concordance, indicating significant discrepancies between the methods for this antibiotic.

It is worth noting that the D2-MINI system did not provide susceptibility profiles for amoxiclav, doxycycline, and ciprofloxacin; all isolates were resistant, resulting in 0% concordance for these antibiotics.

Table 3.9: Percentage of susceptibility concordance between disk diffusion method and D2-MINI for *Burkholderia cepacia* isolates (n=6)

Isolates	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by D2-MINI (n)	Concordance (%)
Burkholderia cepacia	Amoxiclav	6	-*	-
	Ceftazidime	5	5	100
	Doxycycline	2	-*	-
	Trimethoprim - sulfamethoxazole	6	6	100
	Cefepime	-	0	-
	Ciprofloxacin	5	-*	-
	Meropenem	1	6	16.66
	TZP	4	0	0

Note: "-" = did not demonstrate the susceptibility profile for those specific antibiotics; Concordance= percentage of susceptibility agreements between disk diffusion method and D2 MINI; Concordance (%) = (Number of matching susceptibility results between D2-MINI and disk diffusion method / Total number of susceptible isolates) × 100 (Hernández-Durán et al., 2017); Amoxiclav= Amoxicillin-Clavulanic acid; TZP= Piperacillin-Tazobactam.

* The D2-MINI system did not provide susceptibility profiles for amoxiclav, doxycycline, and ciprofloxacin

Table 3.10 compares the percentage of susceptibility concordance between VITEK-2 and D2-MINI systems with the Kirby-Bauer (KB) disk diffusion method for 6 *Burkholderia cepacia* isolates. VITEK-2 exhibited the highest concordance (100%) for ceftazidime, doxycycline, ciprofloxacin, meropenem, and TZP, indicating complete agreement between the two methods for these antibiotics. Meanwhile, D2-MINI showed the highest concordance (100%) for ceftazidime and trimethoprim-sulfamethoxazole only. However, D2-MINI demonstrated significantly lower concordance for meropenem (16.67%) and 0% concordance for piperacillin-tazobactam (TZP), highlighting substantial discrepancies between the two methods.

It is worth noting that neither automated systems provide susceptibility profiles for amoxiclav. Whereas only the D2-MINI system did not provide susceptibility profiles for doxycycline, cefepime, and ciprofloxacin, thus, concordance could not be determined for these antibiotics. Additionally, VITEK-2 did not provide susceptibility profiles for amoxicillin-clavulanic acid, cefepime, and Trimethoprim-sulfamethoxazole which is why concordance could not be observed for these antibiotics.

Table 3.10: Percentage of susceptibility concordance between VITEK-2 and D2-mini with disk diffusion method for *Burkholderia cepacia* isolates (n=6)

Isolates	Antibiotics	Percentage of concordance with disk diffusion method	
		VITEK-2 (%)	**D2 MINI (%)**
Burkholderia cepacia	Amoxiclav	-	-
	Ceftazidime	100	100
	Doxycycline	100	-
	Trimethoprim-sulfamethoxazole	-	100
	Cefepime	-	-
	Ciprofloxacin	100	-
	Meropenem	100	16.66
	TZP	100	0

Note: "-" =D2-MINI and VITEK-2 did not demonstrate the susceptibility Profile for those specific antibiotics; Concordance= percentage of susceptibly agreements between disk diffusion method and VITEK-2 or D2-MINI; Concordance (%) = (Number of matching susceptibility results between VITEK-2/D2-MINI and disk diffusion method / Total number of susceptible isolates) × 100 (Hernández-Durán et al., 2017); Amoxiclav= Amoxicillin-Clavulanic acid; TZP= Piperacillin-Tazobactam.

Table 3.11 illustrates the percentage of susceptibility concordance between the Kirby-Bauer disk diffusion method and VITEK-2 for 8 *Pseudomonas* spp. and 4 *Acinetobacter baumannii complex* isolates. The susceptibility profiles were compared for 9 antibiotics to assess the agreement between the disk diffusion method and VITEK-2.

Among the antibiotics tested, high concordance rates (100%) were observed for trimethoprim-sulfamethoxazole, piperacillin-tazobactam (TZP), meropenem and amikacin indicating perfect agreement between the two methods for this antibiotic. Moderate concordance levels were seen for cefepime (85.7%), ciprofloxacin (80%), and gentamicin (75%), suggesting variability in susceptibility results. For doxycycline, no VITEK-2 results were available, and for ceftazidime, results were only available for one *P. aeruginosa* isolate; thus, concordance could not be determined for either antibiotic. However, All *Acinetobacter baumannii complex* isolates were resistant to Ciprofloxacin, Amikacin, Gentamicin, Meropenem, and TZP; numbers shown in the table for these antibiotics are results of *Pseudomonas* spp.

Table 3.11: Percentage of susceptibility concordance between disk diffusion method and VITEK-2 for *Pseudomonas* spp. and *Acinetobacter baumannii complex* isolates (n=12)

Isolates	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by VITEK-2 (n)	Concordance (%)
Pseudomonas* spp. , *Acinetobacter baumannii complex	Ceftazidime	5	1*	-
	Doxycycline	2	-**	-
	Trimethoprim-sulfamethoxazole	1	1	100
	Cefepime	6	7	85.7
	Ciprofloxacin***	4	5	80
	Amikacin***	6	6	100
	Gentamicin***	4	3	75
	Meropenem***	6	6	100
	TZP***	5	5	100

Note: Concordance= percentage of susceptibly agreements between disk diffusion method and VITEK-2; Concordance (%) = (Number of matching susceptibility results between VITEK-2 and disk diffusion method / Total number of susceptible isolates) × 100 (Hernández-Durán et al., 2017)**;** Amoxiclav= Amoxicillin-Clavulanic acid; TZP= Piperacillin-Tazobactam.

*VITEK-2 did not provide ceftazidime results for most Pseudomonas species and *Acinetobacter baumannii complex;* thus, concordance could not be compared.

**VITEK-2 did not provide doxycycline results for both organisms; thus, concordance could not be compared

***All *Acinetobacter baumannii complex* isolates were resistant to Ciprofloxacin, Amikacin, Gentamicin, Meropenem, and TZP; numbers shown in the table for these antibiotics are results of *Pseudomonas* spp.

The percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and the D2-MINI system for 8 *Pseudomonas* spp. and 4 *Acinetobacter baumannii complex* isolates is presented in Table 3.12. Ceftazidime, doxycycline, TZP, trimethoprim-sulfamethoxazole, and amikacin demonstrated the highest concordance (100%), indicating complete agreement between the two methods. Moderate concordance levels were seen for cefepime (85.7%), meropenem (83.3%), and ciprofloxacin (80%), highlighting the strong reliability of the D2-MINI system for these antibiotics.

It is worth noting that the D2-MINI system did not provide susceptibility profiles of gentamicin for *Pseudomonas* spp.; thus, concordance could not be determined for this antibiotic.

Table 3.12: Percentage of susceptibility concordance between disk diffusion method and D2-MINI for *Pseudomonas* spp. and *Acinetobacter baumannii complex* isolates (n=12)

Isolates	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by D2-MINI (n)	Concordance (%)
Pseudomonas* spp. , *Acinetobacter baumannii complex	Ceftazidime	5	4	100
	Doxycycline	2	2	100
	Trimethoprim-sulfamethoxazole	1	1	100
	Cefepime	6	7	85.7
	Ciprofloxacin	4	5	80
	Amikacin	6	6	100
	Gentamicin	4	0*	-
	Meropenem	6	5	83.3
	TZP	5	5	100

Note: "-" = D2-MINI did not demonstrate the susceptibility Profile for those specific antibiotics; Concordance= percentage of susceptibly agreements between disk diffusion method and D2 MINI; Concordance (%) = (Number of matching susceptibility results between D2-MINI and disk diffusion method / Total number of susceptible isolates) × 100 (Hernández-Durán et al., 2017); Amoxiclav= Amoxicillin-Clavulanic acid; TZP= Piperacillin-Tazobactam.

*D2 MINI did not provide susceptibility profiles of gentamicin for *Pseudomonas* spp. thus, concordance could not be compared

Table 3.13 compares the percentage of susceptibility concordance between VITEK-2 and D2-MINI systems with the Kirby-Bauer (KB) disk diffusion method for 8 *Pseudomonas* spp. and 4 *Acinetobacter baumannii complex* isolates. VITEK-2 exhibited the highest concordance (100%) for trimethoprim-sulfamethoxazole, amikacin, meropenem and piperacillin-tazobactam. In contrast, D2-MINI also showed the highest concordance (100%) for ceftazidime, doxycycline, trimethoprim-sulfamethoxazole, amikacin, and piperacillin-tazobactam.

Both VITEK-2 and D2-MINI showed similar concordance levels for cefepime (85.7%), and ciprofloxacin (80%). Vitek-2 showed moderate concordance for gentamicin (75%). In contrast, D2-MINI showed moderate concordance for meropenem (83.3%).

It is worth noting that the D2-MINI system did not provide susceptibility profiles for gentamicin, and thus concordance could not be determined for this antibiotic. Additionally, VITEK-2 did not provide susceptibility results for ceftazidime and doxycycline thus concordance could not be determined.

Table 3.13: Percentage of susceptibility concordance between VITEK-2 and D2-mini with disk diffusion method for *Pseudomonas* spp. and *Acinetobacter baumannii complex* isolates (n=12)

Isolates	Antibiotics	Percentage of concordance with disk diffusion method	
		VITEK-2 (%)	**D2-MINI (%)**
Pseudomonas* spp. , *Acinetobacter baumannii complex	Ceftazidime	-	100
	Doxycycline	-	100
	Trimethoprim-sulfamethoxazole	100	100
	Cefepime	85.7	85.7
	Ciprofloxacin	80	80
	Amikacin	100	100
	Gentamicin	75	-
	Meropenem	100	83.3
	TZP	100	100

Note: "-" =D2-MINI and VITEK-2 did not demonstrate the susceptibility Profile for those specific antibiotics;

It is worth noting that VITEK-2 did not generate susceptibility results for *Burkholderia pseudomallei* and showed only one susceptibility profile for *Stenotrophomonas maltophilia*; consequently, concordance between the Kirby-Bauer method and VITEK-2 could not be assessed for these two species. Moreover, D2 MINI misidentified *Achromobacter xylosoxidans* as *Brucella maltose* and was unable to determine *Rhizobium radiobacter*, for which concordance of susceptibility could not be compared for these two species. These findings highlight the variability in performance and incomplete reporting of susceptibility profiles by the D2-MINI and VITEK-2 system for gram-negative non-fermentative isolates.

Chapter 4

DISCUSSION

The identification of gram-negative non-fermentative bacteria has traditionally relied on conventional microbiological methods, including gram staining, colony morphology assessment, and a series of biochemical tests such as oxidase, catalase, triple sugar iron (TSI), motility-indole-urease (MIU), bile esculin, and citrate utilization. These methods are advantageous due to their cost-effectiveness and minimal reliance on sophisticated equipment, making them widely accessible in various laboratory settings. (Mishra et al., 2019). Traditional microbiological methods for identifying bacteria, while widely used, have notable limitations. These techniques are often time-consuming and labor-intensive, requiring extended periods for microbial cultures to grow and for subsequent biochemical testing. Such demands can delay diagnosis and treatment decisions in clinical settings. Additionally, the reliance on phenotypic characteristics can lead to misidentification. (Smith et al., 2004). Additionally, Traditional antimicrobial susceptibility testing (AST) methods, such as the disk diffusion test, are widely used in clinical laboratories due to their simplicity and flexibility in antibiotic selection. However, these methods have notable limitations, including the inability to determine the minimum inhibitory concentration (MIC) and delays in obtaining results, which can impede timely treatment decisions, especially for critically ill patients. (Smith et al., 2021). The reliance on manual processes in laboratory testing not only increases the potential for human error but also extends turnaround times, which can delay critical clinical decisions and adversely affect patient outcomes. For instance, errors in the pre-analytical phase, such as incorrect sample collection or handling, can significantly impact the accuracy of test results, leading to misdiagnosis or delayed treatment. The reliance on manual processes in laboratory testing not only increases the potential for human error but also extends turnaround times, which can delay critical clinical decisions and adversely affect patient outcomes. For instance, errors in the pre-analytical phase, such as incorrect sample collection or handling, can significantly impact the accuracy of test results, leading to misdiagnosis or delayed treatment. Additionally, delays in laboratory turnaround times have been associated with prolonged hospital stays and increased healthcare costs, further highlighting the need for efficient and accurate laboratory processes. (Hernandez et al., 2020).

Automated identification systems have been developed to enhance the speed and accuracy of microbial diagnostics. Among these, the VITEK 2 system has emerged as one of the most widely adopted solutions. The VITEK 2 system employs advanced colorimetric technology to efficiently identify microorganisms and determine their antimicrobial susceptibility profiles. Studies have

shown that the VITEK 2 system can accurately identify a wide range of bacteria, including Gram-negative non-fermentative species, with high precision. For example, a study reported that the VITEK 2 system correctly identified 94.7% of isolates in a clinical setting. (Funke et al., 1998). The VITEK 2 system offers numerous advantages over conventional methods. Its automation eliminates the need for extensive human intervention, reducing the reliance on individual expertise and minimizing human error. The system is capable of processing multiple samples simultaneously, significantly improving laboratory efficiency. Moreover, standardized results provided by the VITEK 2 system ensure reproducibility across different laboratories. (World Health Organization, 2016). The adoption of automated systems such as VITEK 2 in clinical microbiology laboratories has revolutionized microbial diagnostics, providing rapid and reliable results that facilitate timely and targeted therapeutic interventions, ultimately improving patient care outcomes. (Funke et al., 1998).

The D2-MINI, an automated system developed by Zhuhai DL Biotech Co., Ltd., offers an alternative to traditional methods and the VITEK 2 system for identifying gram-negative non-fermentative bacteria and conducting antimicrobial susceptibility testing (AST). D2-MINI system utilizes advanced colorimetric technology for bacterial identification and turbidimetric analysis for AST, ensuring reliable and rapid results. A study evaluating the BD Phoenix system reported that it successfully identified 92% of gram-negative non-fermentative isolates, highlighting its efficacy in clinical microbiology settings (Zhang et al., 2019). The D2mini system, developed by Zhuhai DL Biotech Co., Ltd., offers a semi-automated workflow utilizing combo test cards capable of detecting Enterobacteriaceae and non-fermentative bacteria. It supports multiple antibiotic panels that are regularly updated in line with the latest Clinical and Laboratory Standards Institute (CLSI) guidelines, providing accurate minimum inhibitory concentration (MIC) values essential for effective antimicrobial therapy. Compared to traditional methods, which are labor-intensive and susceptible to subjective interpretation errors, the D2-MINI system enhances automation to reduce human error and variability in results. In contrast to the VITEK 2 system, the D2mini offers unique features advantageous for clinical microbiology laboratories. It employs a combo test card with unlimited throughput, offering a more flexible and scalable solution compared to the VITEK 2's fixed antibiotic combinations and a limited capacity of 15, 30, or 60 test cards per run. Notably, the D2-MINI system provides true MIC values with a broader range of antibiotic concentration levels, ensuring higher accuracy in antimicrobial susceptibility testing. Additionally, it offers free

and timely updates of CLSI guidelines, which are crucial for maintaining current standards in microbial diagnostics (Monteiro et al., 2016). The D2-MINI system is more affordable and appropriate for settings with limited resources because it doesn't require air conditioning (AC) or an uninterrupted power supply (UPS) to function, unlike the VITEK 2. This study intends to examine the effectiveness of the D2mini system for identifying and determining the antibiotic susceptibility patterns of gram-negative non-fermentative bacteria in light of these clear benefits.

The detailed biochemical profiles and distribution of the 30 gram-negative non-fermenting bacterial isolates, as summarized in the study, provide critical insights into their identification, clinical significance, and prevalence in various clinical specimens. The isolates were identified using a series of standard microbiological tests, including Gram staining, colony morphology, oxidase, catalase, bile esculin, citrate utilization, motility, indole, urease tests (MIU), and Triple Sugar Iron (TSI) reaction. These tests are widely regarded as the gold standard for microbial identification due to their reliability (MacFaddin, 2000). The majority of the isolates, which include *Pseudomonas, Burkholderia, Achromobacter,* and *Rhizobium,* were identified as gram-negative rods, except for *Acinetobacter baumannii complex*, which displayed a distinctive coccobacilli morphology. The oxidase test, catalase test, and bile esculin hydrolysis test, which are crucial biochemical markers, were positive for *Pseudomonas*, *Burkholderia*, and *Achromobacter*. Catalase activity, indicative of aerobic metabolism, was universally positive across all isolates, highlighting their ability to break down hydrogen peroxide and thrive in oxygenated environments. This is a common trait among non-fermenting gram-negative bacteria, which often colonize environments with high oxygen availability, such as hospital settings (Gales et al., 2019). All isolates exhibited an alkaline/alkaline (K/K) reaction on TSI agar, indicating their inability to ferment carbohydrates such as glucose, lactose, or sucrose. This is a hallmark characteristic of non-fermenting bacteria, as they lack the metabolic pathways to break down these sugars (Janda & Abbott, 2007). Additionally, none of the isolates produced hydrogen sulfide (H_2S) or gas, further supporting their classification as non-fermenters. Motility testing revealed that most isolates were motile, except for *Acinetobacter baumannii complex* and *Stenotrophomonas maltophilia*, which were non-motile. This aligns with previous studies that have documented the non-motile nature of these species (Peleg et al., 2008).

While comparing the identification results of 30 gram-negative isolates between the VITEK-2 system and the conventional method, the study observed that, at the genus level, the VITEK-2 system showed complete concordance with conventional methods in identifying all tested non-fermenting gram-negative bacilli. This includes accurate genus-level identification of *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Acinetobacter baumannii complex*, *Pseudomonas fluorescens*, *Rhizobium radiobacter*, and *Achromobacter xylosoxidans*. These results demonstrate that VITEK-2 effectively identifies both common and less common genera when compared to traditional culture and biochemical approaches.

At the species level, the VITEK-2 system also aligned with conventional methods in identifying *Burkholderia cepacia*, *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Rhizobium radiobacter*, and *Achromobacter xylosoxidans*. Notably, VITEK-2 correctly identified *Rhizobium radiobacter*, which the D2-MINI system failed to identify. Identification was supported by colony morphology on MacConkey agar—smooth, translucent colonies with no lactose fermentation—and Gram staining results that revealed numerous oxidase-positive, urease-positive, gram-negative bacilli (Nakai, Kim, & Aoki, 2016). However, identifying *Rhizobium radiobacter* and *Achromobacter xylosoxidans* using conventional biochemical methods, such as TSI, MIU, citrate utilization, bile esculin hydrolysis, oxidase, and catalase tests, is often challenging due to their limited and overlapping biochemical characteristics. *Rhizobium radiobacter*, previously known as *Agrobacterium tumefaciens*, shares many phenotypic traits with closely related genera, making it difficult to distinguish accurately at both the genus and species levels using conventional methods. This challenge arises because the organism exhibits few stable and distinctive biochemical markers that can separate it from similar taxa (Coenye et al., 2001). Similarly, *Achromobacter xylosoxidans*, a non-fermenting gram-negative bacillus, also presents identification difficulties in conventional systems due to its overlapping biochemical profile with other non-fermenters. Traditional methods often cannot resolve the subtle metabolic differences necessary for species-level identification, as the phenotypic characteristics of *Achromobacter* species are not only variable but also insufficient for reliable classification (Spilker et al., 2014). In contrast, automated systems like VITEK-2 utilize extensive databases and pattern-recognition algorithms capable of analyzing a broader range of biochemical reactions, thereby overcoming the limitations of conventional tests. These systems provide higher specificity and sensitivity, enabling accurate identification of less common

or biochemically ambiguous organisms by recognizing patterns and profiles beyond the scope of standard manual tests (Coenye et al., 2001; Spilker et al., 2014).

The performance of the D2-MINI system in identifying gram-negative non-fermenting bacterial isolates, as highlighted in the study, reveals both its strengths and limitations. The system demonstrated 100% accuracy in identifying *Burkholderia cepacia, Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Burkholderia pseudomallei*, at both genus and species levels, which aligns with its ability to reliably identify common and well-characterized pathogens. However, its performance was inconsistent for less common or more complex species. For instance, the D2-MINI system accurately identified *Acinetobacter baumannii complex* at the genus level but showed reduced accuracy (83.3%) at the species level, indicating potential challenges in differentiating closely related species within the *Acinetobacter* genus (Peleg et al., 2008).

An identification discrepancy was noted for *Pseudomonas fluorescens*, which was identified by both the VITEK-2 system and conventional methods, whereas the D2-MINI system identified it as *Pseudomonas putida*, which can be attributed to the biochemical and genetic similarities between these species. Both *Pseudomonas fluorescens* and *Pseudomonas putida* belong to the *Pseudomonas* genus and share several phenotypic and metabolic traits, which can lead to misidentification in automated systems. These species are both non-fermenting, gram-negative rods, and they exhibit similar biochemical profiles, including positive oxidase and catalase reactions, as well as the ability to utilize citrate. Additionally, both species are motile and produce similar colony morphologies, which can further complicate differentiation (Peix et al., 2009). Automated systems like D2-MINI rely on pre-programmed databases and biochemical algorithms to identify bacteria, and the overlapping characteristics of these species can sometimes result in incorrect species-level identification. This is particularly true for less common or closely related species, where subtle differences in metabolic pathways may not be adequately captured by the system (Janda & Abbott, 2007). The misidentification of *Pseudomonas fluorescens* as *Pseudomonas putida* by the D2-MINI system underscores the challenges in distinguishing between closely related species, even with advanced automated methods.

Another significant finding was the inability of the D2-MINI system to correctly identify *Rhizobium radiobacter* and *Achromobacter xylosoxidans* at both genus and species level. For *Rhizobium radiobacter*, the system failed to identify the isolate at both the genus and species levels, resulting in 0% concordance. The D2-MINI system may struggle to detect *Rhizobium*

radiobacter due to several factors, including the specificity of its test panels, which may not include *Rhizobium radiobacter* as a target organism. As well as D2-MINI cannot detect this organism due to the system lacking a dedicated identification card. Additionally, the biochemical characteristics of *Rhizobium radiobacter* might not react or produce detectable results in the tests used by the D2-MINI , leading to false negatives. Furthermore, the system has specific detection limits that, if not met by the concentration of *Rhizobium radiobacter* in a sample, could result in non-detection. Lastly, the type of sample being tested may also influence detection, as *Rhizobium radiobacter* is more commonly found in specific environments, like soil or plant tissues, rather than in clinical samples, making it less likely to be identified in a clinical setting (Pérez, Sanchis, & Ramos, 2015). Automated systems often struggle with less common or atypical isolates, as their biochemical profiles may not align with the pre-programmed algorithms used for identification (Murray et al., 2015).

Similarly, *Achromobacter xylosoxidans* was misidentified as *Brucella maltose* by the D2-MINI system, which failed to recognize it at both the genus and species levels. This could be attributed to the unique biochemical characteristics of *Achromobacter xylosoxidans*, which may not be adequately captured by the D2-MINI system. The system's database may lack sufficient data on this species, leading to misidentification or failure to identify (Brooke, 2012). These findings underscore the limitations of automated systems in handling rare or less common isolates, which require more specialized or manual methods for accurate identification. Finally, the system's reliance on biochemical tests alone, without incorporating molecular methods such as 16S rRNA sequencing, may limit its ability to accurately identify species with overlapping phenotypic characteristics (Murray et al., 2015).

In this study, the 30 gram-negative isolates were categorized into three groups: *Burkholderia* spp. Isolates, *Pseudomonas* spp. and *Acinetobacter* spp. isolates and others species(*Achromobacter xylosoxidans, Rhizobium radiobacter, and Stenotrophomonas maltophilia*). The *Burkholderia* species group included *Burkholderia pseudomallei* and *Burkholderia cepacia*, and the *Pseudomonas* species group consisted of *Pseudomonas aeruginosa, Pseudomonas stutzeri* and *Pseudomonas fluorescens.*

In the comparison between the VITEK-2 system and the conventional Kirby-Bauer (KB) disk diffusion method for antibiotic susceptibility testing of non-fermenting gram-negative bacteria, *Burkholderia pseudomallei* presented significant challenges. VITEK-2 was unable to generate susceptibility profiles for this organism, likely due to its intrinsic resistance mechanisms, such as chromosomally encoded efflux pumps (AmrAB-OprA) and inducible β-lactamases (PenA), which complicate phenotypic expression and fall outside the system's standardized interpretation algorithms (Lim et al., 2019; Dance, 2020). Also, *B. pseudomallei* is a slow-growing organism, often requiring longer incubation times (typically over 48 hours) to display sufficient growth. In contrast, VITEK-2 operates on a relatively short incubation cycle (usually 8–24 hours). This mismatch can lead to inadequate bacterial growth in the test card, resulting in failure to generate AST data (Dance, 2020). Additionally, the system's database lacks comprehensive breakpoint criteria for rare organisms like *B. pseudomallei*, particularly for antibiotics such as cefepime and amoxicillin-clavulanate, which have undefined clinical breakpoints for *Burkholderia* species (Peleg et al., 2008; Hantrakun et al., 2016).

Despite these limitations, VITEK-2 demonstrated 100% concordance with KB for *Burkholderia cepacia* when testing antibiotics like ceftazidime, doxycycline, ciprofloxacin, meropenem, and piperacillin-tazobactam. However, it failed to generate susceptibility data for amoxicillin-clavulanic acid and provided incomplete results for trimethoprim-sulfamethoxazole, missing two isolates and thus preventing a full concordance assessment.

The D2-MINI system showed high concordance with the KB method for certain antibiotics, achieving 100% agreement for ceftazidime and trimethoprim-sulfamethoxazole. In contrast, this system showed concerningly low meropenem concordance(16.66%). Nonetheless, it exhibited significant limitations, including 0% concordance for piperacillin-tazobactam and failure to provide results for amoxicillin-clavulanic acid, doxycycline, and ciprofloxacin. These shortcomings are attributed to the system's reliance on predefined databases optimized for common pathogens, which often lack comprehensive susceptibility profiles for niche organisms like *Burkholderia* species (Peleg et al., 2008). The absence of reliable susceptibility data for antibiotics such as cefepime and piperacillin-tazobactam in automated systems aligns with CLSI/EUCAST guidelines, which exclude or deem these drugs unreliable for *Burkholderia* due to undefined breakpoints (Limmathurotsakul et al., 2016). However, D2-MINI's meropenem performance issues warrant further validation studies.

Comparatively, while both VITEK-2 and D2-MINI systems offer advantages in throughput and standardization for common pathogens, their application to *Burkholderia* species remains problematic. Automated systems such as VITEK-2 and D2-MINI rely on predefined databases optimized for common pathogens, and *Burkholderia* species, being niche pathogens, often lack comprehensive susceptibility profiles in these systems (Peleg et al., 2008). For *Burkholderia cepacia,* VITEK-2 performed well for key antibiotics but showed limitations in testing amoxicillin-clavulanic acid and provided inconsistent results for trimethoprim-sulfamethoxazole. For *B. pseudomallei*, the lack of susceptibility profiles in VITEK-2 and limited data in D2-MINI (only 4 antibiotics) reinforce the necessity of manual testing in melioidosis diagnosis and treatment (Limmathurotsakul et al., 2016). Current guidelines recommend using manual methods like disk diffusion or E-tests as the gold standard for *B. pseudomallei* AST (Hernández-Durán et al., 2017)., particularly for key therapeutic agents where automated systems show poor performance (Cheng & Currie, 2005; Limmathurotsakul et al., 2016). While automated systems offer advantages in throughput and standardization for common pathogens, their application to *B. pseudomallei* remains problematic, highlighting the need for either system modifications or continued reliance on traditional methods for this important pathogen.

The comparative evaluation of antimicrobial susceptibility testing methods revealed important patterns in the performance of automated systems versus disk diffusion for *Pseudomonas* spp. and *Acinetobacter baumannii complex*. The perfect concordance (100%) between VITEK-2 and Kirby-Bauer for trimethoprim-sulfamethoxazole, piperacillin-tazobactam (TZP), meropenem and amikacin aligns with existing literature showing reliable detection of susceptibility to these first-line agents in *Pseudomonas aeruginosa* (Sader et al., 2021). This high agreement likely reflects both the clinical importance of these antibiotics and their well-established breakpoints in automated system databases. However, the moderate concordance for cefepime (85.7%) and ciprofloxacin (80%) with VITEK-2 suggests potential limitations in detecting emerging resistance mechanisms. The ciprofloxacin discrepancies could reflect the known challenges in detecting first-step quinolone resistance mutations with automated systems (Juan et al., 2019).

The complete lack of VITEK-2 results for doxycycline and ceftazidime (except one *P. aeruginosa* isolate) highlights significant database limitations. Doxycycline exclusion is unsurprising given its limited utility against *Pseudomonas*, but the ceftazidime gap is more concerning as this remains a

key anti-pseudomonal agent (EUCAST, 2023). This likely reflects either technical limitations in detecting ceftazidime resistance or database omissions for certain species-ceftazidime combinations.

The D2-MINI system showed superior performance for some antibiotics (100% concordance for ceftazidime, doxycycline, TZP, trimethoprim-sulfamethoxazole, and amikacin) but moderate meropenem concordance (83.3%). The system's failure to test gentamicin represents another notable gap given aminoglycosides' importance in combination therapy.

For *Acinetobacter baumannii complex*, the universal resistance to ciprofloxacin, amikacin, gentamicin, meropenem and TZP among tested isolates reflects the global rise of extensively drug-resistant (XDR) strains (Howard et al., 2022). The high concordance for remaining testable antibiotics suggests automated systems can reliably detect this resistance when present, though may struggle with more subtle resistance patterns.

The significant limitations observed in both VITEK-2 and D2-MINI systems for testing *Stenotrophomonas maltophilia, Achromobacter xylosoxidans, and Rhizobium radiobacter* highlight critical challenges in automated susceptibility testing for uncommon non-fermentative gram-negative pathogens. The inability of VITEK-2 to generate reliable susceptibility profiles for *S. maltophilia* (providing only one antibiotic result) aligns with multiple studies demonstrating poor performance of automated systems for this intrinsically resistant organism (Brooke, 2021). This limitation stems from several factors: *S. maltophilia's* inherent resistance to numerous antibiotic classes (including most β-lactams and aminoglycosides), its slow growth characteristics, and the lack of standardized breakpoints for many drugs in automated system databases (Chang et al., 2022).

The D2-MINI system's misidentification of *A. xylosoxidans* as *Brucella maltose* represents a concerning error with potential clinical consequences. This misidentification likely occurs due to biochemical similarities between these organisms and limitations in the system's identification database for rare non-fermenters (Tran et al., 2023). Similar identification challenges have been reported with other automated systems, particularly for glucose-nonfermenting gram-negative rods that require specialized testing for accurate speciation (Garcia et al., 2021). The system's complete failure to identify *R. radiobacter* further emphasizes these database limitations, as this

environmental organism is increasingly recognized as an opportunistic human pathogen (Ryan et al., 2022).

Conclusion

In conclusion, this study underscores the effectiveness of the D2-MINI system for identification of bacteria and antimicrobial susceptibility testing (AST) when compared to the conventional method and VITEK-2. The D2-MINI demonstrated high accuracy for common pathogens such as *Burkholderia cepacia*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia*, achieving complete agreement at both genus and species levels. However, its limitations became evident with rare or complex species like *Achromobacter xylosoxidans* and *Rhizobium radiobacter*, where it either misidentified or failed to detect these organisms entirely. For AST, the D2-MINI system showed strong agreement with the Kirby-Bauer (KB) disk diffusion method for certain antibiotics, such as ceftazidime and trimethoprim-sulfamethoxazole. However, the D2-MINI system lacked susceptibility profiles for key antibiotics like amoxicillin-clavulanate, doxycycline, and ciprofloxacin, restricting its utility in comprehensive resistance profiling. The inherent differences between the KB disk diffusion method, which employs zone diameters, and automated systems like VITEK-2 and D2-MINI, which utilize minimum inhibitory concentration (MIC), contribute to variability in susceptibility results. However, if MIC measurement were incorporated into conventional methods like the KB disk diffusion, it could lead to more accurate results and better comparability across different testing systems.

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