Comparison of Automated System D2 Mini with Conventional method and VITEK-2 for Identification and Antibiotic Susceptibility Pattern of Gram-Negative Fermentative Bacteria.

By
Kaniz Fatema
Student ID: 20126019
Tasnim Rahman
Student ID: 20126021

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

Department of Mathematics and Natural Sciences

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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.

Student's Full Name & Signature:

Kaniz Fatema	**Tasnim Rahman**
Student ID: 20126019	**Student ID:20126021**

Approval

The thesis/project titled "Evaluation of D2 Mini for Identification of Gram-Negative Non-Fermenter Bacteria: Comparative Analysis with Conventional Methods and VITEK" submitted by

1. **Kaniz Fatema (20126019)**
2. **Tasnim Rahman (20126021)**

Of Spring, 2020 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on 20th February 2025.

Examining Committee:

Supervisor: _____
(Member)

Dr. Md. Shariful Alam Jilani
Professor and Head, Department of Microbiology
Ibrahim Medical College

Co-supervisor: _____
(Member)

Dr. Mahboob Hossain, PhD
Professor, Microbiology Program, Department of Mathematics and Natural Sciences
BRAC University

Program Coordinator: _____

(Member) **Nadia Deen, PhD**
Associate Professor, Microbiology Program,
Department of Mathematics and Natural Sciences
BRAC University

Department Head: _____

(Chair)

Md. Firoze H. Haque, PhD
Associate Professor and Chairperson
Microbiology Program, Department of Mathematics and Natural Sciences
BRAC University

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 fermentative bacteria. A total of 34 clinical isolates were analyzed, including *Escherichia coli* (n=9), *Klebsiella pneumoniae* (n=7), *Klebsiella oxytoca* (n=1), *Salmonella* Typhi (n=7), *Salmonella* Paratyphi (n=1), *Serratia marcescens* (n=3), *Aeromonas hydrophila* (n=2), *Morganella morganii* (n=1), *Proteus hauseri* (n=1), *Proteus mirabilis* (n=1), and *Edwardsiella hoshinae* (n=1). The isolates were recovered from various clinical specimens, including blood, urine, pus, wound swabs, catheter tips, and bronchial wash. Identification was performed using conventional methods, including Gram staining, biochemical tests, and antibiotic susceptibility testing (AST) by the disk diffusion method, followed by 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 most isolates, except for *Klebsiella pneumoniae* (85.7%) and *Serratia marcescens* (66.7%) at the species level. The D2 Mini system failed to identify *Salmonella* species at the species level. Antibiotic susceptibility testing revealed that both automated systems (VITEK-2 and D2 Mini) exhibited high concordance with the disk diffusion method for several antibiotics, including amoxicillin-clavulanate, ceftazidime, ciprofloxacin, gentamicin, and amikacin. However, discrepancies were observed for antibiotics such as netilmicin and colistin, with low concordance values. The D2 Mini demonstrated a restricted antibiotic panel, lacking profiles for antibiotics such as ceftriaxone colistin, and TZP, while VITEK-2 showed higher concordance but also displayed limitations for certain antibiotics. This study highlights the strengths and limitations of automated systems in microbial diagnostics, emphasizing the need for further improvements in their antibiotic testing capabilities, particularly for last-resort antibiotics and non-fermenting bacteria.

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1.Introduction

Gram-negative bacteria represent a diverse and significant group of microorganisms characterized by a unique cellular architecture. Their cell wall structure comprises a thin peptidoglycan layer surrounded by an outer membrane enriched with lipopolysaccharides (LPS). This distinct composition plays a pivotal role in conferring resistance to external stressors, including antimicrobial agents, detergents, and digestive enzymes. The outer membrane not only serves as a permeability barrier, limiting the influx of hydrophobic compounds but also facilitates the active efflux of toxic substances through specialized efflux pumps. During Gram staining, the structural features of gram-negative bacteria prevent the retention of the crystal violet dye. Consequently, the counterstain is absorbed, yielding a characteristic red or pink coloration under microscopic observation (NCBI, 2023).

Among gram-negative bacteria, fermenting species are a distinct subgroup. They are characterized by their ability to metabolize carbohydrates, such as lactose, into acids or gases through fermentation. This metabolic activity is a key distinguishing feature that sets them apart from non-fermentative bacteria (Cheesbrough, 2017). Selective and differential media, such as MacConkey agar, are frequently employed to identify and differentiate these groups. On MacConkey agar, fermentative gram-negative bacteria ferment lactose, leading to acid production that lowers the pH of the medium. This pH change activates the neutral red dye, resulting in the formation of pink-colored colonies (Forbes et al., 2016). In contrast, non-fermentative bacteria cannot ferment lactose and therefore form colorless or pale colonies (Koneman et al., 2017). When these bacteria possess the metabolic capability to ferment carbohydrates through anaerobic pathways, it provides them with a distinct advantage for thriving in low-oxygen environments, such as the gastrointestinal tract, where oxygen is limited. Prominent examples of these bacteria include *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter spp.*, and *Serratia marcescens*. They are commonly found in soil, water, and on the surfaces of plants. (Madigan et al., 2015) In the human body, gram-negative bacteria are a significant component of the gut microbiota. While many of these bacteria are harmless and even beneficial, some can become pathogenic under certain conditions, leading to infections. Notable examples include *Escherichia coli and Klebsiella pneumoniae*, which can cause gastrointestinal and urinary tract infections, respectively (Bäumler & Sperandio, 2016) While many strains are commensals, contributing to the host's microbiota, others possess significant pathogenic potential, leading to infections such as urinary tract

infections, septicemia, and neonatal meningitis (Kaper et al., 2004). Infections caused by fermentative Gram-negative bacteria present a critical challenge in clinical settings.

In Bangladesh, these fermentative gram-negative pathogens are frequently associated with hospital-acquired infections, including urinary tract infections, ventilator-associated pneumonia, and neonatal sepsis (Shama et al., 2024). For instance, *Salmonella* infections can cause gastroenteritis, typhoid fever, and septicemia. Along with *Klebsiella pneumoniae* causes pneumonia, UTIs, and infections of the bloodstream. In addition, *E. coli* is a significant cause of nosocomial and community-associated infections in Bangladesh, including respiratory tract infections, UTIs, and enteric infections. (Shama et al., 2024). Furthermore, *Serratia marcescens* is recognized as a significant pathogen in neonatal units, causing septicemia and pneumonia with rapid and severe disease progression (Paterson, 2006; Aghapour et al., 2019). A study highlighted that *M. morganii* infection has been reported in several countries, including Bangladesh (Wei et al., 2023). As a formidable opportunistic pathogen, *M. morganii* poses significant health risks, with a high mortality rate among infected patients (Bandy, 2020). This bacterium can lead to a range of conditions, including urological infections, pneumonia, diarrhea, liver abscess, osteomyelitis, peritonitis, bacteremia, meningitis, and complications from invasive surgery or immune dysfunction (Zaric et al., 2021; Lee and Liu, 2006; McDermott and Mylotte, 1984). Additionally, *M. morganii* is recognized as a potential cause of food poisoning (Ryser et al., 2021). However, these fermentative gram-negative pathogens are frequently involved in infections observed in intensive care units (ICUs) and neonatal intensive care units (NICUs). In addition**,** they are commonly associated with severe clinical outcomes in these settings.

However, the escalating prevalence of antimicrobial resistance among fermentative gram-negative bacteria has compounded the complexity of treating these infections. (Saxena et al., 2023). Resistance mechanisms such as the production of extended-spectrum beta-lactamases, carbapenems activity, and the overexpression of efflux pumps have rendered many first-line antibiotics ineffective. In Bangladesh, these multidrug-resistant strains are major contributors to hospital-acquired infections, resulting in increased morbidity, mortality, and healthcare costs **(**Shamsuzzaman et al.2017**).** The emergence of these resistant pathogens underscores the critical need for accurate and timely diagnostic methodologies to guide therapeutic strategies and implement effective infection control measures (Saxena et al., 2023). Moreover, the increasing

prevalence of infections caused by fermentative gram-negative bacteria in Bangladesh highlights the urgent need for enhanced monitoring systems, improved infection control practices, and continuous research to better understand their resistance mechanisms. Such efforts will not only help improve patient outcomes but also alleviate the strain these infections place on healthcare facilities.

In our study, we gathered 34 clinical samples from a trusted laboratory to identify fermenting gram-negative bacteria. The samples were processed using standard microbiological diagnostic techniques, beginning with culturing on MacConkey agar and incubation at 37°C for 18–24 hours. Upon the development of bacterial colonies, we carried out traditional biochemical tests, including oxidase, catalase, Gram staining, and metabolic assays using Triple Sugar Iron (TSI) agar, Simmons Citrate Agar, Bile Esculin Agar, and MIU (Motility, Indole, Urease) tests. This systematic procedure enabled the precise identification of bacterial species. Additionally, we conducted antimicrobial susceptibility testing (AST) using the Kirby-Bauer disc diffusion method, in line with the Clinical and Laboratory Standards Institute (CLSI) guidelines, to determine the most effective treatment strategies (NCBI, 2023).

In recent years, microbiology has witnessed transformative advancements, particularly in diagnosing and identifying fermentative Gram-negative bacteria. 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 48 to 72 hours or more for results, delaying clinical decision-making. They also struggle with detecting emerging drug resistance and also less convenient in differentiating between rare or closely related bacterial species. With the rise in multidrug-resistant infections, the need for faster and more precise diagnostic tools has become critical (Rajkumari and Roy, 2023).

To overcome diagnostic challenges in microbiology, advanced technologies such as VITEK-2 and D2 Mini have been introduced. VITEK-2 is an automated system widely employed for bacterial

identification and antimicrobial susceptibility testing (AST). It uses colorimetric biochemical analysis to deliver rapid, high-throughput results, making it especially valuable in clinical microbiology laboratories. Its compact design is suitable for smaller facilities, while its automation reduces manual workload, minimizes human error, and provides detailed bacterial resistance profiles within 10 hours. However, VITEK-2 has limitations, including the high cost of testing cards, occasional misidentifications due to incomplete reference databases, a primary focus on bacteria and yeast, and reduced effectiveness in detecting low-level resistance patterns (Gupta and Sood, 2021).

The D2 Mini offers another cutting-edge solution, providing fast and accurate identification of a wide range of microorganisms, including rare pathogens. It requires minimal sample preparation, has a lower cost per test compared to VITEK 2, and supports the identification of bacteria and fungi. While the D2 Mini excels in rapid identification, it cannot directly detect antimicrobial resistance. For comprehensive diagnostics, it can be paired with other tools, such as molecular diagnostic systems or automated AST platforms like VITEK 2. This integrated approach enhances the detection of resistance mechanisms and provides a complete diagnostic profile, enabling more effective patient management (Ingle, 2024).

However, the D2 Mini also has challenges, including high initial setup and maintenance costs, the need for specialized training, and reliance on an extensive reference database for accurate identification. Overcoming these hurdles requires thorough training programs for laboratory personnel, focusing on technical operation, troubleshooting, and result interpretation. Collaboration with manufacturers or academic institutions to organize workshops and certifications can help standardize the system's use across laboratories, ensuring its effective implementation (Petersen and Becker, 2022).

This study aims to evaluate the diagnostic efficacy of the D2 Mini system for the identification of fermentative Gram-negative bacteria, comparing its performance to conventional diagnostic methods. By emphasizing its applicability in resource-limited settings such as Bangladesh, this research seeks to address diagnostic bottlenecks and improve clinical outcomes in managing multidrug-resistant infections. The integration of advanced diagnostic tools like the D2 Mini with

traditional methods holds the promise of enhancing infection control measures and promoting effective antimicrobial stewardship.

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 fermentative bacteria. For this purpose, a total of 34 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 Tetramethyle-p-phenylenediaminedihydrochloride) 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 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 fermenting bacteria**:**

Ceftazidime (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Amikacin (30 µg), Netilmicin (30 µg), Cefixime (5 µg), Piperacillin-Tazobactam (100/10 µg), Aztreonam (30 µg), Meropenem (10 µg), Colistin (10 µg), Cefepime (30 µg), Ceftriaxone (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% NaCl. 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)

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
Cefixime	≥19	16-18	≤15
Piperacillin-Tazobactam	≥21	15-20	≤14
Aztreonam	≥21	18-20	≤17
Meropenem	≥23	20-22	≤19
Cefepime	≥21	18-20	≤17
Ceftriaxone	≥23	20-22	≤19
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 34 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:

2.2.1 Preparation of Bacterial Isolates

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

2.2.2 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.

2.2.3 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.

2.2.4 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.

2.2.5 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.

2.2.6 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 34 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.

2.3.1 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.

2.3.2 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.

2.3.3 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 was 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.

2.3.4 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.

2.3.5 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 were interpreted immediately using the D2 Mini system's automated analysis software. This step ensured accurate identification of the microorganism and its antimicrobial susceptibility profile.

2.3.6 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.

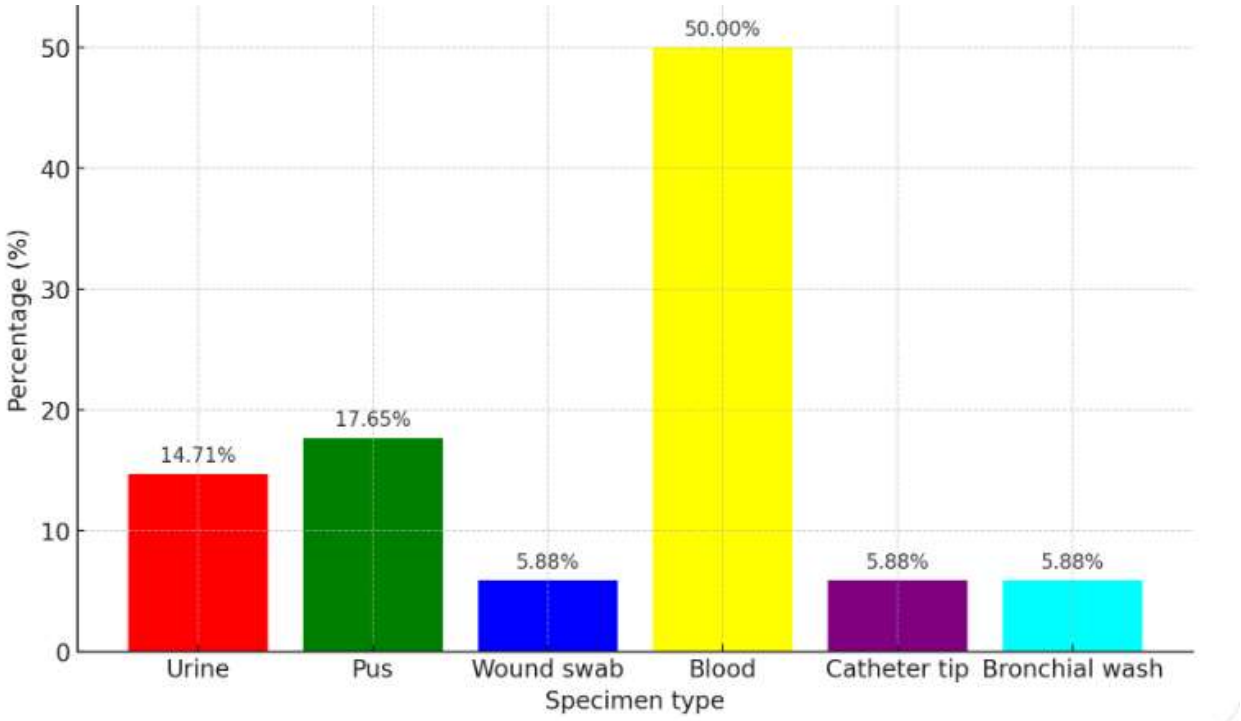
Formula to get Percentage of identification concordance between VITEK-2 and D2-MINI or conventional method and VITEK-2 or conventional method and D2-MINI: Concordance (%): Percentage agreement between VITEK-2 and D2 MINI or conventional method and VITEK-2 or conventional method and D2-MINI; Concordance 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 34 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 34 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)**;**

3. RESULTS

This study analyzed 34 known isolates of gram-negative 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) guidelines to ensure standardization and accuracy. These 34 isolates were recovered from six specimen types, including blood, urine, pus, wound swabs, catheter tips, and bronchial wash.

Figure 3.1 illustrates the distribution of isolates according to their specimen types. Blood (50.00%) accounted for the highest proportion of isolates among the specimen types analyzed. This was followed by pus (17.65%) and urine (14.71%). Wound swabs, catheter tips, and bronchial wash each contributed 5.88% to the total isolates, representing the lowest proportions among the specimen types.



Note: Y-axis percentages (%) indicate the total number of isolates in each specimen type.

Figure 3.1: Distribution of isolates according to their specimen types (N=34)

Table 3.2 outlines the microbiological characteristics of 34 isolates of gram-negative fermentative bacteria recovered in this study. The isolates were identified using a combination of microbiological tests, including gram staining, colony morphology on MacConkey agar, oxidase, catalase, bile esculin, citrate utilization, motility, indole, and urease tests (MIU), along with Triple Sugar Iron (TSI) reactions.

Among the isolates, *Escherichia coli* (n = 9) exhibited characteristic pink, circular, and smooth colonies on MacConkey agar. These were Gram-negative rods, catalase-positive, and negative for oxidase, citrate, and bile esculin. In the MIU test, *E. coli* displayed positive motility and indole production but was urease-negative. On TSI agar, it produced an acid/acid (A/A) result with no H_2S production (-H_2S) and gas production (+Gas).

Klebsiella pneumoniae (n = 7) and *Klebsiella oxytoca* (n = 1) shared similar colony characteristics, appearing large, pink, and mucoid. Both were Gram-negative rods, catalase-positive, bile esculin-positive, citrate-positive, and oxidase-negative. However, the two species differed in their indole test results, with *Klebsiella oxytoca* being indole-positive and *Klebsiella pneumoniae* indole-negative.

The isolates of *Salmonella* species, including *Salmonella* Typhi (n = 7) and *Salmonella* Paratyphi (n = 1), exhibited pale, colorless, smooth colonies on MacConkey agar. Both species were Gram-negative rods and displayed similar results for oxidase, catalase, citrate, bile esculin, and MIU tests. However, differences were evident in their TSI results. *Salmonella* Typhi produced H_2S but no gas, yielding alkaline/acid (K/A), +H_2S, -Gas profile, while *Salmonella* Paratyphi did not produce H_2S but was gas-positive, resulting in a K/A, -H_2S, +Gas profile.

Serratia marcescens (n = 3) formed distinctive red, circular, and smooth colonies and was positive for catalase, citrate, and bile esculin. On TSI agar, it showed a K/A result with no H_2S or gas production. *Aeromonas hydrophila* (n = 2), which formed pale, circular, and smooth colonies, was positive for catalase, citrate, bile esculin, and oxidase. On TSI agar, it produced a K/A result with H_2S production but no gas. In the MIU test, both species demonstrated positive motility and were urease-negative. However, *Aeromonas hydrophila* was indole-positive, while *Serretia marcescens* was indole-negative.

Morganella morganii (n = 1) and *Proteus hauseri* (n = 1) exhibited similar results for most of the microbiological tests. However, they differed in their TSI reactions. *Morganella morganii* was unable to produce gas or H2S (K/A, -H2S, -Gas), while *Proteus hauseri* could produce H_2S but not gas (K/A, +H_2S, -Gas).

Proteus mirabilis (n = 1) formed pale, circular colonies and exhibited positive motility and urease activity. It was indole-negative, oxidase-negative, catalase-positive, citrate-positive, and bile esculin-negative. On TSI agar, it demonstrated a K/A result with H2S production but no gas (K/A, +H_2S, -Gas).

Lastly, *Edwardsiella hoshinae* (n = 1) and *Escherichia coli* (n = 9) displayed overlapping microbiological characteristics. Both were Gram-negative rods, catalase-positive, and bile esculin-negative, with identical motility, indole, and urease patterns. On TSI agar, both exhibited an A/A result with no H_2S production and gas production (A/A, -H_2S, +Gas). However, differences were evident in their colony morphology on MacConkey agar media. *E. coli*, a lactose fermenter, formed pink colonies, whereas *Edwardsiella hoshinae*, unable to ferment lactose, produced pale colonies.

Table 3.2: Conventional methods for identification of gram-negative bacteria (N=34)

Isolates	Gram Staining	Colony[a] Characteristics	Oxidase (+/-)	Catalase (+/-)	Bile Esculine (+/-)	Citrate (+/-)	MIU (Motility/Indole/Urease) (+/-)	TSI (Slant/Butt/H_2S/Gas)
Escherichia coli (n=9)	Gram-negative rods	Pink, circular, smooth	-	+	-	-	+ / + / -	**A/A, - H_2S, +Gas**
Klebsiella pneumoniae (n=7)	Gram-negative rods	Large, pink, mucoid, dome shaped	-	+	+	+	- / - / +	**A/A, - H_2S, +Gas**
Klebsiella oxytoca (n=1)	Gram-negative rods	Large, pink, mucoid,smooth	-	+	+	+	- / + / +	**A/A, - H_2S, +Gas**
Salmonella Typhi *(n=7)*	Gram-negative rods	Pale, colorless, smooth	-	+	-	-	+ / - / -	**K/A, + H_2S, -Gas**
Salmonella Paratyphi *(n=1)*	Gram-negative rods	Pale, colorless, smooth	-	+	-	-	+ / - / -	**K/A, - H_2S, +Gas**
Serratia marcescens (n=3)	Gram-negative rods	Red, circular, smooth	-	+	+	+	+ / - / -	**K/A, - H_2S, -Gas**
Aeromonas hydrophila (n=2)	Gram-negative rods	Pale, circular, smooth	+	+	+	+	+ / + / -	**K/A, + H_2S, +Gas**
Morganella morganii (n=1)	Gram-negative rods	Pale, circular, smooth	-	+	-	-	+ / + / +	**K/A, - H_2S, -Gas**
Proteus hauseri (n=1)	Gram-negative rods	Pale, circular, smooth	-	+	-	-	+ / + / +	**K/A, + H_2S, -Gas**
Proteus mirabilis (n=1)	Gram-negative rods	Pale, circular, smooth	-	+	-	+	+ / - / +	**K/A, + H_2S, -Gas**
Edwardsiella hoshinae (n=1)	Gram-negative rods	Pale, small colonies	-	+	-	-	+ / + / -	**A/A, - H_2S, +Gas**

Note: **N**= total number of isolates; **"+"** = positive**, "-"** = negative; **MIU**: Motility/Indole/Urease; **TSI**: Triple Sugar Iron; **K/A**: Alkaline/Acid; **A/A**: Acid/Acid; **H_2S:** "+" = positive, "-" = negative; **Gas:** "+" = positive, "-" = negative; **a:** Colony characteristics on MacConkey agar media.

Figure 3.3 shows the percentage distribution of the 34 gram-negative fermentative bacterial isolates identified in this study. Among the isolates, *Escherichia coli* was the most prevalent species, accounting for 26.5% (n=9) of the total. This was followed by *Klebsiella pneumoniae* and *Salmonella* Typhi, each contributing 20.6% (n=7). While *Aeromonas hydrophila/*punctata accounted for (5.9%, n=2) and *Serratia marcescens* represented 8.8% (n=3).

Other less frequent isolates included *Salmonella* Paratyphi, *Klebsiella oxytoca, Morganella morganii, Edwardsiella hoshinae, Proteus hauseri,* and *Proteus mirabilis*, each contributed 2.9% (n = 1).

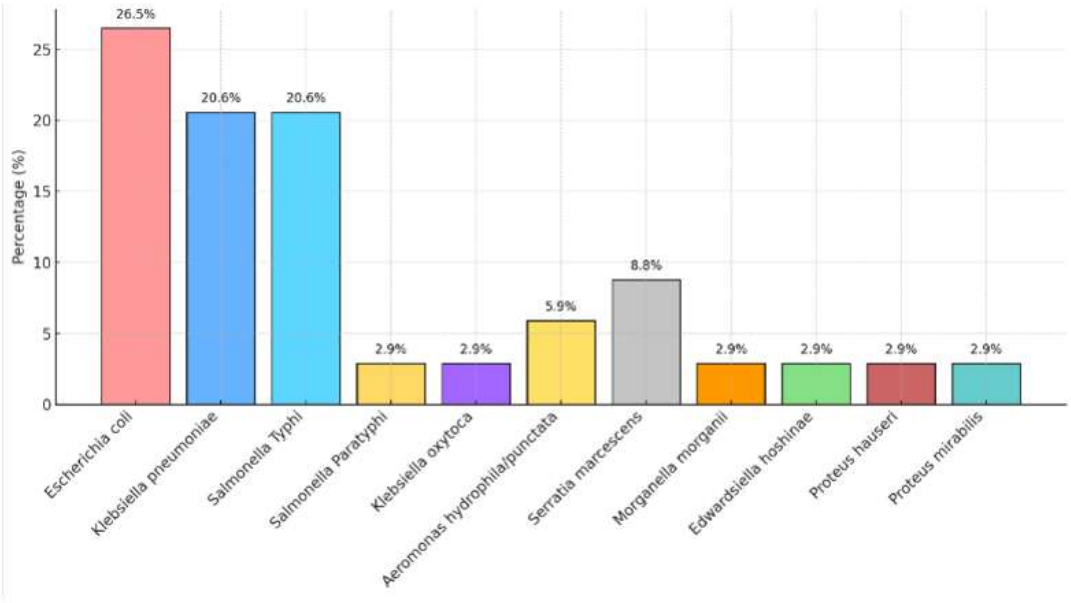


Figure 3.3: Percentage distribution of gram-negative organisms (N=34)

The distribution of 34 gram-negative fermentative bacterial isolates across various specimen types is summarized in Table 3.4. *Escherichia coli* (n = 9) was predominantly isolated from urine (55.56%), with smaller proportions recovered from blood and catheter tip specimens (22.22%) each, *Klebsiella pneumoniae* (n = 7) was most frequently recovered from blood (42.86%) and equally from pus and wound swabs (28.57%) each, while *Klebsiella oxytoca* (n = 1) was exclusively found in blood (100%).

Salmonella species, including *Salmonella* Typhi (n = 7) and *Salmonella* Paratyphi (n = 1), were isolated only from blood (100%). *Serratia marcescens* (n = 3) was primarily recovered from bronchial wash specimens (66.67%), with the remainder isolated from blood (33.33%). Similarly, *Aeromonas hydrophila* (n = 2) was exclusively recovered from blood (100%).

Less frequent isolates, including *Morganella morganii*, *Edwardsiella hoshinae*, *Proteus hauseri*, and *Proteus mirabilis* (n = 1 each), were all recovered from pus specimens (100%).

Table 3.4: Distribution of isolates according to their specimen type (N=34)

Isolates	Urine no. (%)	Pus no. (%)	Wound swab no. (%)	Blood no. (%)	Catheter tip no. (%)	Bronchial wash no. (%)
Escherichia coli (n=9)	5 (55.56)	0 (0.00)	0 (0.00)	2 (22.22)	2 (22.22)	0 (0.00)
Klebsiella pneumoniae (n=7)	0 (0.00)	2 (28.57)	2 (28.57)	3 (42.86)	0 (0.00)	0 (0.00)
Klebsiella oxytoca (n=1)	0 (0.00)	0 (0.00)	0 (0.00)	1 (100)	0 (0.00)	0 (0.00)
Salmonella Typhi (n=7)	0 (0.00)	0 (0.00)	0 (0.00)	7 (100)	0 (0.00)	0 (0.00)
Salmonella Paratyphi (n=1)	0 (0.00)	0 (0.00)	0 (0.00)	1 (100)	0 (0.00)	0 (0.00)
Serratia marcescens (n=3)	0 (0.00)	0 (0.00)	0 (0.00)	1 (33.33)	0 (0.00)	2 (66.67)
Aeromonas hydrophila (n=2)	0 (0.00)	0 (0.00)	0 (0.00)	2 (100)	0 (0.00)	0 (0.00)
Morganella morganii (n=1)	0 (0.00)	1 (100)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Edwardsiella hoshinae (n=1)	0 (0.00)	1 (100)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Proteus hauseri (n=1)	0 (0.00)	1 (100)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Proteus mirabilis (n=1)	0 (0.00)	1 (100)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)

Note: N= number of isolates; n= number of isolates for each bacterial species; no.=number of isolates belonging to each specimen types; percentage in parenthesis represent the proportion of isolates for each bacterial species.

The comparative identification of 34 gram-negative fermentative bacterial isolates using the conventional method, VITEK-2, and D2 MINI. Both the conventional method and VITEK-2 achieved 100% accuracy in identifying all isolates at the genus and species levels.

The D2 MINI system successfully identified *Escherichia coli* (n=9), *Klebsiella oxytoca* (n=1), *Morganella morganii* (n=1), *Proteus hauseri* (n=1), *Proteus mirabilis* (n=1), and *Edwardsiella hoshinae* (n=1) with 100% accuracy. However, its performance was lower for *Klebsiella pneumoniae* (85.7%, n=6) in both genus and species level and *Serratia marcescens* (66.7%, n=2) in species level only. Notably, it failed to identify any isolates of *Salmonella* Typhi (n=7) and *Salmonella* Paratyphi (n=1) at the species level. These findings are summarized in Table 3.5.

Table 3.5: Comparative identification of gram-negative bacteria by conventional method and automated methods (VITEK-2 and D2 MINI) (N=34)

Isolates	Conventional Method		VITEK-2		D2-MINI	
	Genus n (%)	Species n (%)	Genus n (%)	Species n (%)	Genus n (%)	Species n (%)
Escherichia coli (n=9)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)
Klebsiella pneumoniae (n=7)	7 (100)	7 (100)	7 (100)	7 (100)	6 (85.7)	6 (85.7)
Klebsiella oxytoca (n=1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Salmonella Typhi *(n=7)*	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	0 (0.00)
Salmonella Paratyphi *(n=1)*	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0.00)
Serratia marcescens (n=3)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	2 (66.7)
Aeromonas hydrophila (n=2)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
Morganella morganii (n=1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Proteus hauseri (n=1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Proteus mirabilis (n=1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Edwardsiella hoshinae (n=1)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)

Notes**: N=** total number of isolates; "**n"** represents the number of isolates for each bacterium; **Genus (n):** Number of isolates correctly identified at the genus level by each method with percentages in parentheses; **Species (n):** Number of isolates correctly identified at the species level by each method with percentages in parentheses;

Table 3.6 illustrates the concordance between the VITEK-2 system and the conventional method for identifying 34 gram-negative bacterial isolates at both genus and species levels.

All 34 isolates demonstrated 100% concordance between the VITEK-2 system and the conventional method at the genus and species level. This includes *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Salmonella* Typhi, *Salmonella* Paratyphi, *Serratia marcescens*, *Aeromonas hydrophila*, *Morganella morganii*, *Proteus hauseri*, *Proteus mirabilis* and *Edwardsiella hoshinae*.

Table 3.6: Percentage of identification concordance between the conventional method and VITEK-2 (N=34)

Isolates	Conventional Method		VITEK-2		Concordance	
	Genus (n)	Species (n)	Genus (n)	Species (n)	Genus (%)	Species (%)
Escherichia coli (n=9)	9	9	9	9	100	100
Klebsiella pneumoniae (n=7)	7	7	7	7	100	100
Klebsiella oxytoca (n=1)	1	1	1	1	100	100
Salmonella Typhi *(n=7)*	7	7	7	7	100	100
Salmonella Paratyphi *(n=1)*	1	1	1	7	100	100
Serratia marcescens (n=3)	3	3	3	3	100	100
Aeromonas hydrophila (n=2)	2	2	2	2	100	100
Morganella morganii (n=1)	1	1	1	1	100	100
Proteus hauseri (n=1)	1	1	1	1	100	100
Proteus mirabilis (n=1)	1	1	1	1	100	100
Edwardsiella hoshinae (n=1)	1	1	1	1	100	100

Notes: **N=** total number of isolates; "**n"** represents the number of isolates for each bacterium; **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 VITEK-2; **Concordance Calculation**: Concordance (%) = (Number of matches between conventional method and VITEK-2 / Total number of organisms) × 100. (Hernández-Durán et al., 2017).

The concordance between the conventional method and D2 MINI for both genus and species-level identification of 34 gram-negative bacterial isolates is presented in Table 3.7.

The genus-level concordance was 100% for most isolates, including *Escherichia coli*, *Klebsiella oxytoca*, *Salmonella* Typhi, *Salmonella* Paratyphi, *Aeromonas hydrophila*, *Morganella morganii*, *Proteus hauseri*, *Proteus mirabilis*, and *Edwardsiella hoshinae* and *Serretia marcescens*. However, for *Klebsiella pneumoniae*, the concordance at the genus level was reduced to 85.7% because D2 MINI correctly identified 6 out of 7 isolates.

At the species level, the performance of D2 MINI was slightly lower compared to the genus level. 100% concordance was observed for *Escherichia coli*, *Klebsiella oxytoca*, *Aeromonas hydrophila*, *Morganella morganii*, *Proteus hauseri*, *Proteus mirabilis*, and *Edwardsiella hoshinae*. Again, for *Klebsiella pneumoniae*, the concordance at the species level was also 85.7% because D2 MINI identified 6 out of 7 isolates correctly. For *Serratia marcescens*, the species-level concordance was 66.7% as D2 MINI identified only 2 out of 3 isolates correctly at the species level. Notably, for *Salmonella* Typhi and *Salmonella* Paratyphi, the concordance at the species level was 0%, as D2 MINI failed to detect these isolates at the species level.

Table 3.7: Percentage of identification concordance between conventional method and D2-MINI (N =34)

Isolates	Conventional Method		D2-MINI		Concordance	
	Genus (n)	Species (n)	Genus (n)	Species (n)	Genus (%)	Species (%)
Escherichia coli (n=9)	9	9	9	9	100	100
Klebsiella pneumoniae (n=7)	7	7	6	6	85.7	85.7
Klebsiella oxytoca (n=1)	1	1	1	1	100	100
Salmonella Typhi (n=7)	7	7	7	0	100	0
Salmonella Paratyphi (n=1)	1	1	1	0	100	0
Serratia marcescens (n=3)	3	3	3	2	100	66.7
Aeromonas hydrophila (n=2)	2	2	2	2	100	100
Morganella morganii (n=1)	1	1	1	1	100	100
Proteus hauseri (n=1)	1	1	1	1	100	100
Proteus mirabilis (n=1)	1	1	1	1	100	100
Edwardsiella hoshinae (n=1)	1	1	1	1	100	100

Notes: : N= total number of isolates; **"n"** represents the number of isolates for each bacterium; **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)

The concordance between VITEK-2 and D2 MINI for genus-level identification of 34 gram-negative fermentative bacterial isolates. VITEK-2 achieved 100% accuracy at the genus level for all isolates, including *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Salmonella* Typhi, *Salmonella* Paratyphi, *Serratia marcescens*, *Aeromonas hydrophila*, *Morganella morganii*, *Proteus hauseri*, *Proteus mirabilis*, and *Edwardsiella hoshinae*.

The D2 MINI system also demonstrated high concordance at the genus level, achieving 100% for most isolates. However, for *Klebsiella pneumoniae*, D2 MINI correctly identified 6 isolates, resulting in 85.7% genus-level concordance.

At the species level, VITEK-2 continued to show 100% concordance across all isolates, confirming its reliability in species identification.

Conversely, the D2 MINI system showed variability. For *Klebsiella pneumoniae*, the species-level concordance was also 85.7%. For *Serratia marcescens*, the species-level concordance was reduced to 66.7% as D2 MINI identified only 2 out of 3 isolates correctly. Notably, for *Salmonella* Typhi and *Salmonella* Paratyphi, the species-level concordance for D2 MINI was 0%, as the system failed to detect these isolates at the species level. Other isolates, including *Escherichia coli*, *Klebsiella oxytoca*, *Aeromonas hydrophila*, *Morganella morganii*, *Proteus hauseri*, *Proteus mirabilis*, and *Edwardsiella hoshinae*, achieved 100% concordance at the species level. These findings are illustrated in Table 3.8.

Table 3.8: Percentage of identification concordance between VITEK-2 and D2-MINI (N=34)

Isolates	VITEK-2		D2-MINI		Concordance	
	Genus (n)	Species (n)	Genus (n)	Species (n)	Genus (%)	Species (%)
Escherichia coli (n=9)	9	9	9	9	100	100
Klebsiella pneumoniae (n=7)	7	7	6	6	85.7	85.7
Klebsiella oxytoca (n=1)	1	1	1	1	100	100
Salmonella Typhi *(n=7)*	7	7	7	0	100	0
Salmonella Paratyphi *(n=1)*	1	1	1	0	100	0
Serratia marcescens (n=3)	3	3	3	2	100	66.7
Aeromonas hydrophila (n=2)	2	2	2	2	100	100
Morganella morganii (n=1)	1	1	1	1	100	100
Proteus hauseri (n=1)	1	1	1	1	100	100
Proteus mirabilis (n=1)	1	1	1	1	100	100
Edwardsiella hoshinae (n=1)	1	1	1	1	100	100

Notes: N= total number of isolates; **"n"** represents the number of isolates for each bacterium; **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 VITEK-2 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).

Table 3.9 illustrates the percentage of susceptibility concordance between the Kirby-Bauer disk diffusion method and VITEK-2 for 19 lactose fermenter isolates, which included *Escherichia* spp. (n=9), *Klebsiella* spp. (n=7), and *Serratia* spp. (n=3). The susceptibility profiles were compared for 12 antibiotics to assess the agreement between the disk diffusion method and VITEK-2.

Among the antibiotics tested, high concordance rates (100%) were observed for amoxicillin-clavulanate**,** ceftriaxone**,** ciprofloxacin**,** amikacin**,** and gentamicin, indicating perfect agreement between the two methods for these antibiotics. Moderate concordance levels were seen for piperacillin-tazobactam (76.92%), cefepime (61.54%), meropenem (56.25%), cefixime (44.44%) and ceftazidime (41.67) suggesting variability in susceptibility results. Whereas netilmicin **s**howed lower concordance at 7.14%, highlighting substantial discrepancies between the two methods. For colistin, no concordance was observed, with a 0% agreement, indicating a complete lack of correlation between the two methods for this antibiotic.

Table 3.9: Percentage of susceptibility concordance between disk diffusion method and VITEK-2 for lactose fermenting isolates (N=19)

Lactose fermenting isolates	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by VITEK-2 (n)	Concordance (%)
***Escherichia* spp., *Klebsiella* spp., *Serratia* spp.**	Amoxiclav	4	4	100
	Ceftazidime	12	5	41.67
	Ceftriaxone	11	11	100
	Cefepime	13	8	61.54
	Cefixime	9	4	44.44
	Ciprofloxacin	11	11	100
	Amikacin	14	14	100
	Netilmicin	14	1	7.14
	Gentamicin	14	14	100
	Meropenem	16	9	56.25
	Colistin	7	0	57.14
	TZP	13	10	76.92

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.

The percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and the D2-MINI system for 19 lactose-fermenting isolates is presented in Table 3.10. Meropenem demonstrated the highest concordance (100%), indicating complete agreement between the two methods. Other antibiotics, such as amikacin (92.86%), gentamicin (92.86%), cefepime (92.31%), and ceftazidime (91.67%) also showed higher concordance, highlighting the strong reliability of the D2-MINI system for these antibiotics. In contrast, lower concordance was observed for amoxiclav (25%) and netilmicin (7.14%), indicating significant discrepancies between the methods for these antibiotics.

It is worth noting that the D2-MINI system did not provide susceptibility profiles for certain antibiotics, including ceftriaxone, cefixime, ciprofloxacin, colistin, and piperacillin-tazobactam (TZP).

Table 3.10: Percentage of susceptibility concordance between disk diffusion method and D2-mini for lactose fermenting isolates (N=19)

Lactose fermenting isolates	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by D2-MINI (n)	Concordance (%)
***Escherichia* spp., *Klebsiella* spp., *Serratia* spp.**	Amoxiclav	4	1	25
	Ceftazidime	12	11	91.67
	Ceftriaxone	11	-	-
	Cefepime	13	12	92.31
	Cefixime	9	-	-
	Ciprofloxacin	11	-	-
	Amikacin	14	13	92.86
	Netilmicin	14	1	7.14
	Gentamicin	14	13	92.86
	Meropenem	16	16	100
	Colistin	7	-	-
	TZP	13	-	-

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.

Table 3.11 compares the percentage of susceptibility concordance between VITEK-2 and D2-MINI systems with the Kirby-Bauer (KB) disk diffusion method for 19 lactose-fermenting isolates. VITEK-2 exhibited the highest concordance (100%) for amoxicillin-clavulanate, amikacin and gentamicin. Similarly, D2-MINI also showed much higher concordance for amikacin (92.86%) and gentamicin (86.67%), but significantly lower concordance for amoxicillin-clavulanate (25%).

VITEK-2 also achieved the highest concordance (100%) for ceftriaxone and ciprofloxacin; however, a comparison with D2-MINI was not possible as it did not provide susceptibility profiles for these antibiotics.

Moderate concordance values for VITEK-2 were observed with cefepime (61.54%), meropenem (56.25%), and ceftazidime (41.67%). D2-MINI, in contrast, showed the highest concordance for meropenem (100%) and higher concordance levels for cefepime (92.32%) and ceftazidime (91.67%).

For tazobactam-piperacillin (76.92%), and cefixime (44.44%), VITEK-2 demonstrated moderate concordance. However, these antibiotics could not be compared with D2-MINI as it did not provide susceptibility profiles for them.

Moreover, both systems demonstrated lower concordance for netilmicin, with each achieving only 7.14%.

For colistin, VITEK-2 showed the lowest concordance (0%) with the KB disk diffusion method, while D2-MINI did not provide any susceptibility profile for this antibiotic, which is why concordance could not be observed.

Table 3.11: Percentage of susceptibility concordance between VITEK-2 and D2-mini with disk diffusion method for lactose fermenting isolates (N=19)

Lactose fermenting isolates	Antibiotics	Percentage of concordance with disk diffusion method	
		VITEK 2 (%)	**D2 MINI (%)**
***Escherichia* spp., *Klebsiella* spp., *Serratia* spp.**	Amoxiclav	100.0	25.0
	Ceftazidime	41.67	91.67
	Ceftriaxone	100.0	-
	Cefepime	61.54	92.31
	Cefixime	44.44	-
	Ciprofloxacin	100.0	-
	Amikacin	100.0	92.86
	Netilmicin	7.14	7.14
	Gentamicin	100.0	92.86
	Meropenem	56.25	100.0
	Colistin	0	-
	TZP	76.92	-

Note: "-" =D2-MINI 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 3.12 presents the percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and VITEK-2 for six lactose non-fermenting isolates, including *Aeromonas spp.* (n=2), *Edwardsiella* spp. (n=1), *Morganella* spp. (n=1), and *Proteus* spp. (n=2). The susceptibility profiles for 12 antibiotics were compared to evaluate the agreement between the Kirby-Bauer (KB) disk diffusion method and VITEK-2.

The highest concordance (100%) was observed for amoxiclav, ciprofloxacin, amikacin and gentamicin indicating complete agreement between the two methods for these antibiotics. In contrast, cefixime and colistin **s**howed the lowest concordance at 0%, highlighting substantial discrepancies between the two methods. Moderate concordance was noted for ceftazidime (80%), piperacillin-tazobactam (66.67%), ceftriaxone (60%), cefepime (50%) reflecting variability between the two methods for these antibiotics. It is worth noting that VITEK-2 did not provide a susceptibility profile for netilmicin.

Table 3.12: Percentage of susceptibility concordance between disk diffusion method and VITEK-2 for lactose non-fermenting isolates (N=6)

Lactose non-fermenting isolates	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by VITEK-2 (n)	Concordance (%)
***Aeromonas* spp., *Edwardsiella* spp., *Proteus* spp., *Morganella* spp.**	Amoxiclav	2	2	100
	Ceftazidime	5	4	80
	Ceftriaxone	5	3	60
	Cefepime	6	3	50
	Cefixime	5	0	0
	Ciprofloxacin	5	5	100
	Amikacin	6	6	100
	Netilmicin	6	-	-
	Gentamicin	6	6	100
	Meropenem	6	3	50
	Colistin	1	0	0
	TZP	6	4	66.67

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

The percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and the D2-MINI system for six lactose non-fermenting isolates is described in this analysis. Among the antibiotics tested, the highest concordance (100%) was observed for ceftazidime indicating strong agreement between the two methods. Moderate concordance values were also observed for amikacin (83.33%), cefepime (83.33%), gentamicin (83.33%), meropenem (83.33%), ciprofloxacin (60%), and amoxiclav (50%). However, piperacillin-tazobactam (TZP) and cefixime displayed the lowest concordance, with values of 33.33% and 20%, respectively. Furthermore, the D2-MINI system did not provide susceptibility profiles for ceftriaxone, netilmicin, and colistin. These findings have been illustrated in Table 3.13.

Table 3.13: Percentage of susceptibility concordance between disk diffusion method and D2-mini for lactose non-fermenting isolates (N=6)

Lactose non-fermenting isolates	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by D2-MINI (n)	Concordance (%)
***Aeromonas* spp., *Edwardsiella* spp., *Proteus* spp., *Morganella* spp**	Amoxiclav	2	1	50
	Ceftazidime	5	5	100
	Ceftriaxone	5	-	-
	Cefepime	6	5	83.33
	Cefixime	5	1	20
	Ciprofloxacin	5	3	60
	Amikacin	6	5	83.33
	Netilmicin	6	-	-
	Gentamicin	6	5	83.33
	Meropenem	6	5	83.33
	Colistin	1	-	-
	TZP	6	2	33.33

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;

Table 3.14 compares the percentage of susceptibility concordance between VITEK-2 and D2-MINI systems with the Kirby-Bauer (KB) disk diffusion method for six lactose non-fermenting isolates.

VITEK-2 exhibited the highest concordance (100%) for amoxicillin-clavulanate, ciprofloxacin, amikacin, and gentamicin indicating strong agreement with the KB disk diffusion method. Similarly, D2-MINI showed higher concordance for amikacin (83.33%) and gentamicin (83.33%), while displaying moderate concordance for ciprofloxacin (60.00%) and amoxicillin-clavulanate (50.00%).

Moderate concordance values for VITEK-2 were observed with ceftazidime (80.00%), TZP (66.67%), cefepime (50.00%), and meropenem (50.00%). D2-MINI, in contrast, demonstrated the highest concordance for ceftazidime (100%) and higher concordance for cefepime (83.33%) and meropenem (83.33%). However, D2-MINI showed lower concordance for piperacillin-tazobactam (33.33%).

For ceftriaxone (60.00%) VITEK-2 also demonstrated moderate concordance. However, this antibiotic could not be compared with D2-MINI as it did not provide a susceptibility profile for ceftriaxone.

For cefixime and colistin, VITEK-2 showed the lowest concordance which is 0%, indicating no agreement with the KB disk diffusion method, while D2-MINI exhibited slightly better concordance at 20% for cefixime and did not provide a susceptibility profile for colistin.

Furthermore, neither D2-MINI nor VITEK-2 demonstrated any susceptibility concordance for netilmicin.

Table 3.14: Percentage of susceptibility concordance between VITEK-2 and D2-mini with disk diffusion method for lactose non-fermenting isolates (N=6)

Lactose non-fermenting isolates	Antibiotics	Percentage of concordance with disk diffusion method	
		VITEK-2 (%)	**D2-MINI (%)**
***Aeromonas* spp., *Edwardsiella* spp., *Proteus* spp., *Morganella* spp.**	Amoxiclav	100.00	50.00
	Ceftazidime	80.00	100.00
	Ceftriaxone	60.00	-
	Cefepime	50.00	83.33
	Cefixime	0.00	20.00
	Ciprofloxacin	100.00	60.00
	Amikacin	100.00	83.33
	Netilmicin	-	-
	Gentamicin	100.00	83.33
	Meropenem	50.00	83.33
	Colistin	0.00	-
	TZP	66.67	33.33

Note: "-" = VITEK-2/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 VITEK-2 or 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 3.15 presents the percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and VITEK-2 for eight isolates of *Salmonella* spp. The susceptibility profiles for 11 antibiotics were compared to evaluate the agreement between the Kirby-Bauer (KB) disk diffusion method and VITEK-2.

Among the antibiotics tested, the highest concordance (100%) was observed for amoxiclav, ceftriaxone, ciprofloxacin, meropenem, and piperacillin-tazobactam (TZP), reflecting the strong agreement between the two methods for these antibiotics. Moderate concordance was observed for cefepime (87.5%). However, lower concordance values were seen for cefixime (33.33%) and aztreonam (16.67%), indicating inconsistencies in susceptibility results. Ceftazidime and colistin showed the lowest concordance at 12.5% and 0%, suggesting significant discrepancies between the methods for this antibiotic. For amikacin, VITEK-2 did not demonstrate the susceptibility profile.

Table 3.15: Percentage of susceptibility concordance between disk diffusion method and VITEK-2 for *Salmonella* spp., (N=8)

Isolates of *Salmonella* spp.	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by VITEK-2 (n)	Concordance (%)
***Salmonella* spp.**	Amoxiclav	8	8	100
	Ceftazidime	8	1	12.5
	Ceftriaxone	8	8	100
	Cefepime	8	7	87.5
	Cefixime	6	2	33.33
	Aztreonam	6	1	16.67
	Ciprofloxacin	3	3	100
	Amikacin	8	-	-
	Meropenem	7	7	100
	Colistin	5	0	0
	TZP	8	8	100

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

The percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and the D2-MINI system for eight isolates of *Salmonella* spp is illustrated in Table 3.16. Here, the highest concordance (100%) was observed for amoxiclav, ceftazidime, cefepime, amikacin, and meropenem, demonstrating strong agreement between the KB disk diffusion method and the D2-MINI system for these antibiotics. However, moderate concordance exhibited by ciprofloxacin (33.33%) indicates limited agreement between the two methods for this antibiotic. Notably, D2-MINI did not demonstrate the susceptibility profile for ceftriaxone, cefixime, aztreonam, colistin, and piperacillin-tazobactam (TZP).

Table 3.16: Percentage of susceptibility concordance between disk diffusion method and D2-mini for *Salmonella* spp. (N=8)

Isolates	Antibiotics	No. of sensitive isolates by disk diffusion method (n)	No. of sensitive isolates by D2-MINI (n)	Concordance (%)
***Salmonella* spp.**	Amoxiclav	8	8	100
	Ceftazidime	8	8	100
	Ceftriaxone	8	-	-
	Cefepime	8	8	100
	Cefixime	6	-	-
	Aztreonam	6	-	-
	Ciprofloxacin	3	1	33.33
	Amikacin	8	8	100
	Meropenem	7	7	100
	Colistin	5	-	-
	TZP	8	-	-

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 KB disk diffusion method / Total number of susceptible isolates) × 100; (Hernández-Durán et al., 2017).; Amoxiclav= Amoxicillin-Clavulanic acid; TZP= Piperacillin-Tazobactam;

Table 3.17 compares the percentage of susceptibility concordance between VITEK-2 and D2-MINI systems with the Kirby-Bauer (KB) disk diffusion method for eight *Salmonella* spp.

VITEK-2 demonstrated the highest concordance (100%) for amoxicillin-clavulanate, ciprofloxacin, and meropenem, reflecting strong agreement with the KB disk diffusion method for these antibiotics. Similarly, D2-MINI achieved 100% concordance for amoxicillin-clavulanate and meropenem, indicating reliable performance. However, D2-MINI showed lower concordance for ciprofloxacin (33.33%).

VITEK-2 also achieved the highest concordance (100%) for ceftriaxone and TZP; however, a comparison with D2-MINI was not possible as it did not provide susceptibility profiles for these antibiotics.

D2-MINI also demonstrated the highest concordance (100%) for ceftazidime and cefepime. In comparison, VITEK-2 showed slightly lower concordance for cefepime (87.50%) but exhibited significantly lower concordance for ceftazidime (12.50%).

D2-MINI also achieved highest concordance (100%) for amikacin; however, a comparison with VITEK-2 was not possible as it did not provide susceptibility profiles for this antibiotic.

For colistin, cefixime, and aztreonam, VITEK-2 showed a range of concordance values, from moderate (33.33% for cefixime) to lower (16.67% for aztreonam and 0% for colistin). However, D2-MINI did not provide susceptibility concordance for any of these antibiotics.

Table 3.17: Percentage of susceptibility concordance between VITEK-2 and D2-MINI with disk diffusion method for *Salmonella* spp. (N=8)

Isolates of *Salmonella* spp.	Antibiotics	Percentage of concordance with disk diffusion method	
		VITEK-2 (%)	D2-MINI (%)
***Salmonella* spp.**	Amoxiclav	100.00	100.00
	Ceftazidime	12.50	100.00
	Ceftriaxone	100.00	-
	Cefepime	87.50	100.00
	Cefixime	33.33	-
	Aztreonam	16.67	-
	Ciprofloxacin	100.00	33.33
	Amikacin	-	100.00
	Meropenem	100.00	100.00
	Colistin	0	-
	TZP	100.00	-

Note: "-" = VITEK-2/D2-MINI 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.

5. DISCUSSION

The identification of gram-negative fermentative bacteria has traditionally relied on conventional microbiological methods, including Gram staining, colony morphology from culture followed by 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 (Basak & Shetty, 2021). However, despite their benefits, traditional approaches present several limitations that hinder their efficiency and reliability. Accurate microbial identification through conventional methods often demands substantial expertise, as the subjective interpretation of results can lead to inconsistencies and potential misidentifications (Monteiro et al., 2016). Additionally, the preparation of culture media is labor-intensive and time-consuming and requires quality control to ensure the quality. Antimicrobial susceptibility testing (AST) further adds complexity, as the manual preparation of McFarland standards to standardize inoculum density introduces variability and potential errors (Basak & Shetty, 2021). The overall reliance on human expertise and labor increases the likelihood of errors and significantly extends turnaround times, which can delay critical clinical decisions and negatively impact patient outcomes.

To overcome these challenges, 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 fermentative species, with high precision. For example, Monteiro et al. (2016) reported that the VITEK 2 system correctly identified 94.7% of isolates in a clinical setting. The VITEK 2 system offers numerous advantages over conventional methods. Its automation reduced the reliance on individual expertise and minimized 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. 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 (Monteiro et al., 2016).

A similar automated system developed by Zhuhai DL Biotech Co., Ltd., the D2mini, serves as an alternative to conventional methods and the VITEK 2 system for the identification and antimicrobial susceptibility testing (AST) of gram-negative fermentative bacteria. The D2mini system integrates advanced colorimetric technology for bacterial identification and turbidimetric analysis for AST, ensuring reliable and rapid results (Zhuhai DL Biotech Co., Ltd., 2023). It features a semi-automated workflow with combo test cards capable of detecting *Enterobacteriaceae* and non-fermentative bacteria, among others. The system's compatibility with multiple antibiotic panels, updated according to the latest CLSI guidelines, offers accurate minimum inhibitory concentration (MIC) values, which is crucial for guiding effective antimicrobial therapy. The D2mini system presents several advantages over conventional methods and the VITEK 2 system. Unlike conventional approaches, which are labor-intensive and prone to subjective interpretation errors, the D2mini system minimizes human error and reduces variability in results through automation. In contrast to the VITEK 2, the D2mini system offers unique features that make it an attractive option for clinical microbiology laboratories. The D2mini utilizes a combo test card with unlimited throughput, providing a more flexible and scalable solution compared to the VITEK 2's fixed antibiotic combination and limited capacity of 15, 30, or 60 test cards per run. Notably, the D2mini system delivers true minimum inhibitory concentration (MIC) values with more antibiotic concentration levels, ensuring higher accuracy in antimicrobial susceptibility testing. Additionally, it supports free and timely updates of CLSI guidelines, which are essential for maintaining current standards in microbial diagnostics (Zhuhai DL Biotech Co., Ltd., 2023). Unlike the VITEK 2, the D2mini system does not require air conditioning (AC) or an uninterrupted power supply (UPS) for operation, making it more cost-effective and suitable for resource-limited environments. Given these distinct advantages, this study aims to analyze the efficacy of the D2mini system in comparison to the VITEK-2 system and conventional methods for identifying and determining the antibiotic susceptibility patterns of gram-negative fermentative bacteria (Zhuhai DL Biotech Co., Ltd., 2023).

In this study, a total of 34 known isolates were analyzed, including *Escherichia coli* (n=9), *Klebsiella pneumoniae* (n=7), *Klebsiella oxytoca* (n=1), *Salmonella Typhi* (n=7), *Salmonella*

Paratyphi (n=1), *Morganella morganii* (n=1), *Aeromonas hydrophila* (n=2), *Serratia marcescens* (n=3), *Edwardsiella hoshinae* (n=1), *Proteus hauseri* (n=1), and *Proteus mirabilis* (n=1). In this study the majority of isolates were obtained from blood specimens (50%), followed by pus (17.65%), urine (14.71%), wound swabs (5.88%), catheter tips (5.88%), and bronchial washings (5.88%). This distribution is consistent with previous studies highlighting the prevalence of gram-negative bacterial infections in blood samples. For instance, a study analyzing 51,189 non-repetitive strains of gram-negative bacilli found that *Escherichia coli* (31.4%) and *Klebsiella spp.* (21.2%) were the most frequently isolated pathogens, with a significant proportion originating from blood cultures (Zhang et al., 2023). Specifically, *Escherichia coli* was predominantly isolated from urine specimens (55.56%), aligning with its well-documented role as the leading cause of urinary tract infections (UTIs). A study conducted in Dhaka, Bangladesh, reported that out of 120 UTI patients, *E. coli* was the most prevalent pathogen, emphasizing its dominance in urinary infections (Sarker et al., 2023). *Klebsiella pneumoniae* was most frequently recovered from blood samples (42.86%), with additional isolates from pus and wound swabs (28.57% each). This is in line with findings from El-Mahallawy et al. (2009), who reported that *K. pneumoniae* is commonly associated with bloodstream infections, particularly in immunocompromised patients. All Salmonella species isolates, including *Salmonella Typhi* and *Salmonella Paratyphi*, were found in blood samples (100%), highlighting their role as the cause of enteric fevers, which are systemic infections diagnosed through blood cultures. This observation is supported by the established clinical presentation of enteric fever, where blood culture remains the gold standard for diagnosis (Parry et al., 2002). Other organisms like *Serratia marcescens* were primarily isolated from the bronchial wash (66.67%) and blood (33.33%), reflecting its role in respiratory infections. *Aeromonas hydrophila* was exclusively recovered from blood (100%), consistent with its association with bacteremia. Rare isolates, including *Morganella morganii*, *Edwardsiella hoshinae*, *Proteus hauseri*, and *Proteus mirabilis* (n=1 each), were all from pus specimens (100%), aligning with their role in wound infections.

In this study, we observed significant challenges in differentiating between *Escherichia coli* and *Edwardsiella hoshinae* using traditional microbiological methods. Conventional techniques, such as the Motility-Indole-Urease (MIU) test, citrate utilization, oxidase, catalase, and bile esculin hydrolysis, proved insufficient for distinguishing these species. Even Triple Sugar Iron (TSI) agar,

a commonly used differential medium, failed to provide clear differentiation, as both species exhibited an Acid/Acid (A/A) reaction with gas production. While other *Edwardsiella* species typically produce an Alkaline/Acid (K/A) reaction with hydrogen sulfide (H_2S) production on TSI agar, *E. hoshinae* exhibits a unique profile that closely resembles *E. coli* (Reichley et al., 2017). This phenotypic similarity has historically led to the misidentification of *E. hoshinae* as a non-lactose-fermenting variant of *E. coli* (Grimont et al., 1980). In this study, differentiation was only possible through careful observation of colony characteristics. Unlike *E. coli*, *E. hoshinae* is incapable of fermenting lactose, which is evident from its pale-colored colonies on MacConkey agar, in contrast to the pink colonies of lactose-fermenting *E. coli*. However, such differentiation is contingent on the availability of known strains for comparative purposes, underscoring the limitations of conventional methods in identifying atypical isolates. The advent of automated systems like VITEK-2 and D2-MINI has significantly improved the accuracy of bacterial identification. These systems utilize a broader range of biochemical tests, including the fermentation of sugars such as arabinose, melibiose, rhamnose, and raffinose (bioMérieux, 2017). Furthermore, the Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) test, which assesses β-galactosidase activity, serves as a critical discriminator between these organisms, with *E. coli* showing positive results and *E. hoshinae* testing negative. Such advanced biochemical profiling was unavailable in traditional methods, leading to the frequent misclassification of these isolates (Pincus, 2006).

In this study, we also identified challenges associated with detecting *Proteus hauseri* using different methods. Conventionally, *P. hauseri* can be differentiated from other closely related species by observing its positive indole test and negative bile esculin hydrolysis. These biochemical markers provide a reliable means of identifying *P. hauseri*, which was previously misclassified under *Proteus vulgaris* biogroup 3 before being reclassified as a separate species based on genotypic and phenotypic characteristics (O'Hara et al., 2000). Automated systems, such as VITEK-2, successfully identified *P. hauseri* in this study. This success can be attributed to the extensive and regularly updated database of VITEK-2, which includes distinct biochemical profiles for *P. hauseri* (Monteiro et al., 2016). In contrast, the D2-MINI system faced limitations in detecting *P. hauseri* due to its database containing only profiles for *P. vulgaris*. The absence of *P. hauseri* in the D2-MINI database highlights a critical drawback that may lead to

misidentification or the inability to detect this organism altogether (Zhuhai DL Biotech Co., Ltd, 2023). The findings underscore the importance of comprehensive and up-to-date databases in automated systems for accurate microbial identification. The inability of D2-MINI to detect *P. hauseri* highlights the risks posed by incomplete databases, particularly when dealing with clinically significant species. This limitation emphasizes the need for ongoing updates and the inclusion of newly recognized or reclassified species, such as *P. hauseri*, to enhance the diagnostic utility of such systems.

A critical aspect of this study involved differentiating *Salmonella* species, particularly *Salmonella* Typhi and *Salmonella* Paratyphi, using conventional microbiological methods and automated systems. In traditional diagnostics, biochemical tests such as the Triple Sugar Iron (TSI) test play a pivotal role in distinguishing these serovars. Both *S.* Typhi and *S.* Paratyphi exhibit an alkaline slant and acidic butt (K/A) on TSI agar. However, *S.* Typhi produces hydrogen sulfide (H_2S), evident as a black precipitate, while *Salmonella* Paratyphi does not. Additionally, gas production is observed in *Salmonella* Paratyphi but is absent in *S.* Typhi (Jiang et al., 2024). These distinct biochemical characteristics provide a reliable means of differentiation in conventional microbiology. The advent of automated systems such as VITEK-2 demonstrated its ability to identify *Salmonella* isolates at both genus and species levels, including differentiation between *Salmonella* Typhi and *Salmonella* Paratyphi. *Salmonella Typhi* and *Salmonella Paratyphi* can be distinguished based on several key biochemical characteristics detected by the VITEK-2 system. First, *S. Typhi* typically does not ferment lactose, whereas some strains of *S. Paratyphi* (especially *S. Paratyphi A*) may show delayed lactose fermentation (Latif et al., 2014), which can be detected over time by the VITEK-2 system. Additionally, *S. Typhi* does not produce gas during glucose fermentation, while *S. Paratyphi* produces gas, a key differentiating feature. In terms of H_2S production, *S. Typhi* produces a moderate amount of H_2S, which is detectable in biochemical tests, whereas *S. Paratyphi* does not produce H_2S, further aiding in their differentiation. These biochemical differences, which are captured by the VITEK-2 system, highlight its ability to accurately distinguish between *S. Typhi* and *S. Paratyphi* without requiring antisera-based testing (Pincus, 2006). In contrast, the D2-MINI system identifies *Salmonella* isolates only at the genus level, necessitating additional serological testing for specific serovar identification. This reliance on antisera testing by D2-MINI underscores a limitation in its database, which lacks the necessary

biochemical profiles to differentiate between *S. Typhi* and *S. Paratyphi* (Zhuhai DL Biotech Co., Ltd, 2023).

In this study, an important finding was the misidentification of a *Klebsiella pneumoniae* isolate by the D2-MINI system. Of the seven *Klebsiella pneumoniae* isolates, one sample was incorrectly identified as *Serratia odorifera* Biogroup 1 by the D2-MINI system, while both conventional methods and the VITEK-2 system correctly identified it as *Klebsiella pneumoniae*. The organism's identity was confirmed through Gram staining and colony morphology on MacConkey agar. On MacConkey agar, *K. pneumoniae* typically forms large, mucoid, and pink colonies, distinguishing it from slow-lactose fermenters such as *Serratia odorifera* Biogroup 1 (Rafii, 2014). Gram staining revealed gram-negative rods with a characteristic capsule, confirming its identity as *Klebsiella pneumoniae* (Podschun & Ullmann, 1998). Biochemical tests conducted using both VITEK-2 and conventional methods yielded consistent results, including citrate positive, bile esculin positive, and a Triple Sugar Iron (TSI) reaction of yellow slant and yellow butt (A/A) with gas production, consistent with glucose, lactose, and sucrose fermentation. The sample also demonstrated a negative motility result, negative indole, and slow positive urease activity—typical traits of *Klebsiella pneumoniae* (Podschun & Ullmann, 1998). Furthermore, VITEK-2 confirmed a positive result for lysine decarboxylase and negative results for arginine and ornithine decarboxylase, matching the expected biochemical profile for *Klebsiella pneumoniae* (Podschun & Ullmann, 1998). In contrast, the D2-MINI system misclassified the MIU test results as motility negative, indole negative, and urease negative, partially matching the expected profile for *Klebsiella pneumoniae*, but failing to differentiate it from *Serratia odorifera*. *Serratia odorifera*, which is motility-positive, indole-positive, and urease-negative, differs significantly from *Klebsiella pneumoniae* in these traits (Farmer et al., 1985). The D2-MINI system also showed a negative result for ornithine decarboxylase, further contributing to the misclassification, which led to a probability of *Serratia odorifera* Biogroup 1 identification at 77.15%, rather than a higher, more definitive probability. These discrepancies in biochemical profiles underscore the limitations of the D2-MINI system in distinguishing closely related species and highlight the need for further refinement in its database and testing capabilities to ensure more accurate species identification.

In the present study, a *Serratia marcescens* isolate was misidentified as *Serratia liquefaciens* by the D2-MINI system, while both conventional methods and the VITEK-2 system correctly

identified it as *S. marcescens*. The conventional identification relied on Gram staining, colony morphology on MacConkey agar, and a series of biochemical tests, including Triple Sugar Iron (TSI) agar. The TSI test showed an alkaline slant/acidic butt with no gas or hydrogen sulfide (H_2S) production, which is characteristic of *S. marcescens*. Additional tests, such as the Motility-Indole-Urease (MIU) test, showed motility-positive, indole-negative, and urease-negative results, while citrate was positive and bile esculin was negative. These findings are consistent with the well-established biochemical profile of *S. marcescens*, known for its red-pigmented colonies due to the production of prodigiosin, which distinguishes it from *Serratia liquefaciens*, a species that does not produce this pigment (Rafii, 2014; Grimont & Grimont, 2006). The VITEK-2 system, with its extensive database and advanced algorithms, corroborated the conventional identification by accurately identifying the isolate as *S. marcescens*. However, the D2-MINI system misidentified the species, likely due to its inability to accurately interpret key biochemical distinctions between *S. marcescens* and *S. liquefaciens*. Notably, *S. marcescens* is typically positive for adonitol fermentation and negative for raffinose fermentation, while *S. liquefaciens* is positive for raffinose fermentation and negative for adonitol fermentation (Rafii, 2014; Grimont & Grimont, 2006; Janda & Abbott, 2021). Despite the D2-MINI showing positive for adonitol fermentation and negative for raffinose fermentation, it misclassified the sample as *S. liquefaciens*, with a 73.79% probability, whereas the other two samples of S. *marcescens* was supported with a 99.99% probability in D2-MINI. This misidentification highlights the limitations of the D2-MINI system in distinguishing phenotypically similar species and emphasizes the need for further refinement in its database and testing capabilities, particularly in cases where species are closely related in their biochemical profiles. These results align with previous studies, which have noted the occasional misidentification of phenotypically similar organisms by automated systems (Janda & Abbott, 2021).

In this study, the 34 gram-negative isolates were categorized into three groups: lactose-fermenting isolates, non-lactose-fermenting isolates, and *Salmonella* species. The lactose-fermenting group included *Escherichia* species, *Klebsiella* species, and *Serratia* species, while the non-lactose-fermenting group consisted of *Proteus*, *Aeromonas*, *Edwardsiella*, and *Morganella* species.

Firstly, we observe the percentage of susceptibility concordance between the Kirby-Bauer disk diffusion method and VITEK-2 for 19 lactose fermenter isolates, which included *Escherichia* spp.

(n=9), *Klebsiella* spp. (n=7), and *Serratia* spp. (n=3). The susceptibility profiles were compared for 12 antibiotics, including amoxicillin-clavulanate, ceftriaxone, ciprofloxacin, amikacin, gentamicin, piperacillin-tazobactam, cefepime, meropenem, cefixime, netilmicin, ceftazidime, and colistin, to assess the agreement between the disk diffusion method and VITEK-2. The concordance is calculated only by comparing the number of sensitive isolates determined by the disk diffusion method and VITEK-2. Here, high concordance rates (100%) were observed for amoxicillin-clavulanate, ceftriaxone, ciprofloxacin, amikacin, and gentamicin, indicating perfect agreement between the two methods, which aligns with previous studies reporting similar findings (Patel et al., 2018). Moderate concordance (76.92%) was observed for piperacillin-tazobactam, mainly due to discrepancies in *Serratia* species, where all three isolates were susceptible in the disk diffusion method, but no susceptibility profile was generated by VITEK-2. For cefepime (61.54%), the disk diffusion method identified 5 sensitive and 4 resistant *Escherichia* spp. Isolates, whereas VITEK-2 generated profiles for only 3 *Escherichia* spp. Isolates, of which 2 were resistant and 1 was sensitive, resulting in reduced concordance. *Klebsiella* spp. Showed similar discrepancies, with the disk diffusion method identifying 5 sensitive and 2 resistant isolates, while VITEK-2 provided profiles for only 6 isolates, with 4 sensitive and 2 resistant isolates, leading to a 38.46% reduction in concordance. For meropenem (56.25%), the disk diffusion method identified 7 sensitive and 2 resistant isolates in *Escherichia* spp., but VITEK-2 provided profiles for only 3 of these, with 2 resistant and 1 sensitive. The concordance was further reduced for *Klebsiella* spp., where the disk diffusion method identified 6 sensitive and 1 resistant isolate, but VITEK-2 showed profiles for only 6 isolates, with 5 sensitive and 1 resistant. A decrease in concordance (44.44%) was observed for cefixime, where VITEK-2 showed susceptibility profiles for only 1 sensitive *Klebsiella* spp. Isolate, while the disk diffusion method identified 4 sensitive and 3 resistant isolates. No susceptibility profiles were generated by VITEK-2 for *Serratia* spp. For ceftazidime (41.67%), the disk diffusion method identified 5 sensitive and 4 resistant *Escherichia* spp. Isolates, whereas VITEK-2 provided profiles for only 6 isolates, with 4 sensitive and 2 resistant isolates. *Klebsiella* spp. Showed 6 sensitive and 1 resistant isolate in the disk diffusion method, while VITEK-2 provided profiles for only 1 sensitive isolate. No concordance was observed for *Serratia* spp. in VITEK-2. Netilmicin exhibited particularly low concordance (7.14%) due to the VITEK-2 method showing susceptibility profiles for only one sensitive sample of *Escherichia* spp. Finally, for colistin, there was no concordance (0%), as most of the samples

were reported as intermediate by VITEK-2, with only one *Serratia* spp. Sample showing resistance. These results align with previous studies reporting reduced concordance for certain antibiotics, likely due to inherent limitations in the automated testing systems like VITEK-2, which may not generate susceptibility profiles for all isolates, particularly those with atypical or borderline resistance patterns (Lee et al., 2020).

Subsequently, we can observe the percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and the D2-MINI system was assessed for 19 lactose-fermenting isolates. The concordance was determined by comparing the number of sensitive isolates identified by the two methods. Meropenem demonstrated the highest concordance (100%), indicating complete agreement between the two methods. This finding is consistent with studies showing excellent agreement for meropenem in both disk diffusion and automated systems, which is commonly used for accurate susceptibility testing (Patel et al., 2018). Other antibiotics, such as amikacin (92.86%), gentamicin (92.86%), cefepime (92.31%), and ceftazidime (91.67%), also showed high concordance, further highlighting the reliability of the D2-MINI system. These results are in line with previous reports indicating that aminoglycosides (amikacin, gentamicin) and cephalosporins (cefepime, ceftazidime) generally demonstrate good agreement between traditional and automated susceptibility testing methods (Lee et al., 2020). In contrast, lower concordance was observed for amoxiclav (25%) and netilmicin (7.14%), reflecting significant discrepancies between the methods for these antibiotics. The low concordance for amoxiclav (25%) can be attributed to the resistance profiles of non-lactose fermenter isolates, which were all resistant in D2-MINI except one isolate of *Escherichia* spp., leading to a reduced number of sensitive isolates. This highlights the limitation of using sensitive isolate comparison for calculating concordance, particularly when the majority of isolates exhibit resistance, as seen in previous studies (Zong et al., 2021). Similarly, netilmicin showed the lowest concordance (7.14%), with the D2-MINI system generating susceptibility profiles for only one sensitive isolate, which aligns with studies reporting variability in the performance of automated systems, particularly with aminoglycosides (Zong et al., 2021). It is also worth noting that the D2-MINI system did not provide susceptibility profiles for certain antibiotics, including ceftriaxone, cefixime, ciprofloxacin, colistin, and piperacillin-tazobactam (TZP). This is consistent with previous reports on the limitations of automated systems like D2-MINI in generating susceptibility profiles for

certain antibiotics, especially when testing more complex resistance mechanisms or certain classes of antibiotics (Patel et al., 2018).

To further analyze the performance of the D2-MINI system, we now compare the percentage of susceptibility concordance between the VITEK-2 and D2-MINI systems with the Kirby-Bauer (KB) disk diffusion method for the 19 lactose-fermenting isolates. VITEK-2 exhibited perfect concordance (100%) for amoxicillin-clavulanate, amikacin, and gentamicin, reflecting complete agreement with the KB disk diffusion method. In comparison, the D2-MINI system also showed high concordance for amikacin (92.86%) and gentamicin (86.67%), but it displayed significantly lower concordance for amoxicillin-clavulanate (25%). This lower concordance for amoxicillin-clavulanate was primarily due to the resistance profiles of lactose fermenter isolates, where all isolates were resistant to the antibiotic in the D2-MINI system except one isolate of *Escherichia* spp., resulting in a reduced number of sensitive isolates. In contrast, VITEK-2 identified sensitivity in 4 isolates, which led to the higher concordance observed for this antibiotic. For ceftriaxone and ciprofloxacin, VITEK-2 again showed perfect concordance (100%) with the KB disk diffusion method, while the D2-MINI system was unable to generate susceptibility profiles for these antibiotics due to its more limited antimicrobial testing panel. VITEK-2 demonstrated moderate concordance for cefepime (61.54%), meropenem (56.25%), and ceftazidime (41.67%). In contrast, the D2-MINI system exhibited much higher concordance for meropenem (100%), and cefepime (92.32%), and ceftazidime (91.67%). This suggests that the D2-MINI system is more reliable in detecting susceptibility to these antibiotics, providing profiles for most of the isolates compared to VITEK-2, which displayed discrepancies for some of the isolates. For piperacillin-tazobactam (76.92%) and cefixime (44.44%), VITEK-2 showed moderate concordance, while these antibiotics could not be compared with D2-MINI due to the system's inability to generate susceptibility profiles. This further emphasizes the need for D2-MINI to expand its antimicrobial testing capabilities to include a broader range of antibiotics, as these drugs are commonly used in clinical treatment regimens. Both systems demonstrated particularly low concordance for netilmicin, with each achieving only 7.14%. This suggests that netilmicin is a challenging antibiotic for both automated systems, Finally, for colistin, VITEK-2 exhibited no concordance (0%) with the KB disk diffusion method, as most of the samples were reported as intermediate, with only one *Serratia* isolate showing resistance. In contrast, the D2-MINI system did not provide any

susceptibility profiles for colistin, preventing any concordance analysis. This highlights the ongoing challenge in accurately testing colistin susceptibility, as this last-resort antibiotic often involves complex resistance mechanisms that are difficult to detect using automated systems.

Again, in this study, we further assessed the percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and VITEK-2 for six lactose non-lactose fermenting isolates, including *Aeromonas* spp. (n=2), *Edwardsiella* spp. (n=1), *Morganella* spp. (n=1), and *Proteus* spp. (n=2), for 12 antibiotics. The concordance is also calculated here only by comparing the number of sensitive isolates determined by the disk diffusion method and VITEK-2. High concordance (100%) was observed for amoxiclav, ciprofloxacin, amikacin, and gentamicin, indicating complete agreement between the two methods. These results are in line with previous studies where both automated and disk diffusion methods showed excellent agreement for these commonly tested antibiotics (Patel et al., 2018). However, the low concordance observed for cefixime and colistin (0%) highlights substantial discrepancies between the two methods. The low concordance for cefixime can be attributed to the fact that VITEK-2 only provided a susceptibility profile for one resistant *Morganella* spp., while the disk diffusion method detected susceptibility in other species. Similarly, for colistin, VITEK-2 reported most isolates as intermediate, with only two showing resistance, leading to no concordance with the disk diffusion method. These findings are consistent with the challenges described in other studies, where automated systems like VITEK-2 sometimes struggle to detect colistin resistance accurately due to complex mechanisms involved (Lee et al., 2020). Moderate concordance was observed for ceftazidime (80%), piperacillin-tazobactam (66.67%), ceftriaxone (60%), and cefepime (50%), reflecting variability between the methods for these antibiotics. For ceftazidime, VITEK-2 did not provide susceptibility profiles for *Proteus* and *Morganella* isolates, leading to a moderate concordance, while the disk diffusion method identified both species as susceptible. This is similar to findings in other studies, where discrepancies in automated susceptibility testing arise when isolates exhibit atypical resistance profiles that may not be detected by the system (Zong et al., 2021). For piperacillin-tazobactam, VITEK-2 generated susceptibility profiles for only one resistant isolate from *Aeromonas* spp., while the KB method identified both isolates as sensitive. Similarly, for ceftriaxone, moderate concordance (60%) was observed because VITEK-2 did not provide any susceptibility profiles for *Aeromonas* isolates, despite both showing susceptibility in the disk

diffusion method. For cefepime, moderate concordance (50%) was observed, which can be explained by the fact that VITEK-2 generated susceptibility profiles for only one isolate of *Aeromonas* spp., and did not provide profiles for *Morganella* spp. and *Proteus* spp. Additionally, it is noteworthy that VITEK-2 did not provide a susceptibility profile for netilmicin.

Building on the previous analysis, we now examine the percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and the D2-MINI system for six lactose non-fermenting isolates. A notable observation in this study was the inability of the D2-MINI system to provide susceptibility profiles for *Edwardsiella* species. This limitation can be attributed to gaps in the D2-MINI database, which lacks coverage for certain antibiotic agents specific to these organisms. Such database restrictions underscore a critical shortcoming of the system in accurately performing antimicrobial susceptibility testing (AST) for a broader range of less common pathogens. As a result, conventional methods and VITEK-2 remain indispensable in these cases to ensure comprehensive susceptibility reporting and accurate clinical decision-making.

The findings demonstrate that the D2-MINI system showed high concordance (100%) for ceftazidime, reflecting a strong agreement between the two methods. This result underscores the reliability of the D2-MINI system in detecting susceptibility for cephalosporins, as similar findings have been observed in other studies where automated systems provided excellent concordance with disk diffusion for this antibiotic (Coudray et al., 2019). Slightly moderate concordance values were observed for amikacin (83.33%), cefepime (83.33%), gentamicin (83.33%), and meropenem (83.33%), indicating a reasonable level of agreement between the two methods. However, slight discrepancies were noted, particularly with *Edwardsiella* spp., for which D2-MINI did not generate susceptibility profiles. For ciprofloxacin (60%), moderate concordance was observed, primarily due to the fact that D2-MINI did not provide susceptibility profiles for *Proteus* spp., just like *Edwardsiella* spp for this antibiotic. For amoxiclav (50%), the low concordance was noted because, aside from *Edwardsiella* spp., D2-MINI failed to provide susceptibility profiles for *Aeromonas* spp., despite both *Aeromonas* isolates showing susceptibility in the disk diffusion method. These moderate concordance values highlight the inherent challenges in automated susceptibility testing, particularly for non-lactose fermenting bacteria, as other studies have also reported similar issues when testing for specific species in automated systems (Rupp and Fey, 2020). In contrast, the D2-MINI system showed lower concordance for cefixime (20%) and

piperacillin-tazobactam (33.33%). For cefixime, the low concordance can be attributed to D2-MINI only providing susceptibility profiles for one sensitive *Proteus* isolate, whereas the disk diffusion method identified susceptibility in more isolates. Similarly, for piperacillin-tazobactam, the D2-MINI system showed low concordance because it generated profiles for only two of the *Aeromonas* spp. isolates, whereas the disk diffusion method detected susceptibility in both isolates. Additionally, it is important to note that the D2-MINI system did not provide susceptibility profiles for ceftriaxone, netilmicin, and colistin. These findings suggest that the D2-MINI system may have limitations in testing certain antibiotics, particularly those that require more complex resistance mechanisms to be detected. As reported in previous studies, automated systems often struggle to generate profiles for last-resort antibiotics like colistin, which has become increasingly critical in the treatment of multidrug-resistant infections (Miller et al., 2020).

Continuing from the previous analysis, we compared the susceptibility concordance between VITEK-2 and D2-MINI systems with the Kirby-Bauer (KB) disk diffusion method for six lactose non-fermenting isolates. VITEK-2 demonstrated the highest concordance (100%) for amoxicillin-clavulanate, ciprofloxacin, amikacin, and gentamicin, with D2-MINI showing similar results for amikacin (83.33%) and gentamicin (83.33%) but moderate concordance for ciprofloxacin (60%) and amoxicillin-clavulanate (50%). These results highlight the D2-MINI system's reliability for commonly tested antibiotics, though discrepancies were noted for *Edwardsiella* spp., where the system failed to provide profiles. For ceftazidime, D2-MINI showed 100% concordance, outperforming VITEK-2 (80%). The D2-MINI system also showed higher concordance for cefepime (83.33%) and meropenem (83.33%), but lower concordance for piperacillin-tazobactam (33.33%) due to limited profiling for *Aeromonas* isolates. D2-MINI did not provide profiles for ceftriaxone, limiting its comparability with VITEK-2, which showed moderate concordance (60%). For cefixime and colistin, VITEK-2 showed the lowest concordance (0%), while D2-MINI exhibited slight improvement for cefixime (20%) but did not provide profiles for colistin, reflecting its limitations for last-resort antibiotics. Neither system showed concordance for netilmicin. These findings suggest that while D2-MINI is effective for many antibiotics, its performance is limited by gaps in its antimicrobial panel, particularly for non-lactose fermenting isolates with complex resistance patterns. Further refinement of the D2-MINI system is needed to enhance its reliability and expand its testing capabilities for broader clinical use.

In this study, we further assessed the percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and VITEK-2 for eight isolates of *Salmonella* spp., examining 11 antibiotics for example amoxiclav, ceftriaxone, ciprofloxacin, meropenem, piperacillin-tazobactam (TZP), cefepime, cefixime, aztreonam, ceftazidime, colistin, and amikacin. High concordance (100%) was observed for amoxiclav, ceftriaxone, ciprofloxacin, meropenem, and piperacillin-tazobactam (TZP), demonstrating strong agreement between the two methods. This result aligns with previous studies, which found excellent concordance between automated and disk diffusion methods for these commonly tested antibiotics (Shen et al., 2021). However, discrepancies were observed for several antibiotics, including cefixime (33.33%) and aztreonam (16.67%), with VITEK-2 providing susceptibility profiles for 1 or 2 isolates compared to the KB disk diffusion method. Such discrepancies have been previously reported, where automated systems, like VITEK-2, struggle to detect susceptibility accurately in specific bacterial isolates, especially for antibiotics like cefixime (Moll et al., 2020). Moderate concordance (87.5%) was observed for cefepime, with VITEK-2 failing to provide susceptibility profiles for one *Salmonella* isolate. The lowest concordance was observed for ceftazidime (12.5%) and colistin (0%), indicating significant discrepancies between VITEK-2 and the KB disk diffusion method. For ceftazidime, VITEK-2 provided susceptibility profiles for only one isolate, whereas the disk diffusion method identified susceptibility in more isolates. For colistin, VITEK-2 reported most isolates as intermediate, with only two showing resistance, leading to no concordance with the disk diffusion method. This is in line with previous reports that automated systems often struggle to detect colistin resistance accurately due to the complex resistance mechanisms in multidrug-resistant organisms like *Salmonella* (Shen et al., 2021).

Continuing from the previous analysis, the percentage of susceptibility concordance between the Kirby-Bauer (KB) disk diffusion method and the D2-MINI system for eight *Salmonella* spp. Isolates were assessed. High concordance (100%) was observed for amoxiclav, ceftazidime, cefepime, amikacin, and meropenem, demonstrating strong agreement between the KB disk diffusion method and the D2-MINI system for these antibiotics. These findings are consistent with previous studies where automated systems like D2-MINI showed high reliability in detecting susceptibility for commonly tested antibiotics, including cephalosporins and aminoglycosides (Hao et al., 2021). However, moderate concordance was noted for ciprofloxacin (33.33%),

indicating limited agreement between the two methods. The moderate concordance for ciprofloxacin can be explained by D2-MINI providing susceptibility profiles for only one *Salmonella* isolate, highlighting potential limitations in automated systems when dealing with certain isolates or resistance patterns (Singh et al., 2020). Additionally, it is noteworthy that D2-MINI did not provide susceptibility profiles for ceftriaxone, cefixime, aztreonam, colistin, and piperacillin-tazobactam (TZP). This limitation aligns with findings from other studies, where automated systems often fail to generate profiles for certain antibiotics due to having a limited number of antibiotics in their testing panels. This is particularly problematic for antibiotics used to treat multidrug-resistant pathogens like *Salmonella*, as these systems may not include essential last-resort antibiotics such as colistin or others critical in managing complex resistance profiles (Chen et al., 2020).

In this study, the comparison of susceptibility concordance between VITEK-2 and D2-MINI systems with the Kirby-Bauer (KB) disk diffusion method for *Salmonella* spp. Isolates revealed notable findings. VITEK-2 showed perfect concordance (100%) for amoxicillin-clavulanate, ciprofloxacin, and meropenem, with D2-MINI performing well for amoxicillin-clavulanate and meropenem. However, D2-MINI exhibited lower concordance for ciprofloxacin (33.33%) due to its inability to generate susceptibility profiles for some isolates. While VITEK-2 achieved 100% concordance for ceftriaxone and piperacillin-tazobactam, D2-MINI did not provide profiles for these antibiotics, highlighting its restricted panel. D2-MINI also showed 100% concordance for ceftazidime and cefepime, outperforming VITEK-2, which showed lower concordance, particularly for ceftazidime (12.5%). Additionally, D2-MINI demonstrated 100% concordance for amikacin, whereas VITEK-2 did not provide profiles for this antibiotic, indicating D2-MINI's broader testing capability. However, for colistin, cefixime, and aztreonam, VITEK-2 exhibited moderate to low concordance, with significant discrepancies for colistin (0%), while D2-MINI failed to provide profiles for any of these antibiotics, showing limitations in testing critical agents. These findings underscore D2-MINI's strengths in detecting susceptibility to cephalosporins, aminoglycosides, and meropenem but also highlight the need for further refinement to expand its antibiotic panel and improve its performance in testing last-resort antibiotics and multidrug-resistant pathogens.

In conclusion, this study underscores the effectiveness of the D2-MINI system in antimicrobial susceptibility testing (AST) when compared to the Kirby-Bauer (KB) disk diffusion method and VITEK-2. The D2-MINI system, however, has a limited antibiotic panel, primarily covering amoxicillin-clavulanate, meropenem, ceftazidime, cefepime, amikacin, and gentamicin. This restricted panel limits its capacity to assess susceptibility for a broader array of antibiotics, such as ciprofloxacin and piperacillin-tazobactam, as well as critical last-resort antibiotics like colistin and ceftriaxone, which are essential for the treatment of multidrug-resistant infections. Furthermore, D2-MINI failed to provide susceptibility profiles for *Edwardsiella* species, highlighting a significant gap in its database and limited coverage for less common pathogens. 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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