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Article

Evaluation of Antibiotic Resistance and Prevalence Among Gram-negative Bacteria in Fecal- derived Lactose Sugar Tinctures: An in-depth Study

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Abstract: Background and Objective: Diarrheal diseases remain a significant health concern worldwide, necessitating detailed microbial analysis to guide treatment strategies. This study aimed to identify gram-negative bacteria from stool samples of affected individuals, focusing on their ability to ferment lactose and their resistance patterns to specific antibiotics.
Methodology: Conducted at Shomali General Hospital from 2023 March to 2023 December, eighty stool samples were collected and cultured on MacConkey agar to distinguish lactose fermenting from non-fermenting gram-negative bacteria. The antibiotic sensitivity of the isolates was assessed using the disk diffusion method against ten antibiotics, in accordance with the 2017 Clinical and Laboratory Standards Institute (CLSI) guidelines.
Results: Of the eighty samples analyzed, 78% showed lactose fermenting bacteria. The remaining 22% comprised non-lactose fermenters, with further analysis identifying 55% as Salmonella, 22% Pseudomonas, 11.5% Proteus, and 11.5% Shigella. Antibiotic sensitivity testing revealed Ciprofloxacin as the most effective antibiotic, inhibiting 95% of the bacteria, followed by Ceftriaxone at 85%. Ampicillin was the least effective, with a lower inhibition rate.
Conclusion: The high prevalence of lactose fermenting gram-negative bacteria among the diarrheal samples underscores the importance of lactose fermentation as a diagnostic marker. The varying degrees of antibiotic resistance highlight the critical need for ongoing surveillance of resistance patterns to inform effective treatment regimens. This study emphasizes the utility of the disk diffusion method for antibiotic sensitivity testing, providing essential data for managing diarrheal diseases.

Keywords: Antibiotic resistance, Gram-negative bacteria, Shigella dysenteriae , Proteus mirabilis , Nosocomial infections , Public health

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1. Introduction

The burgeoning crisis of antibiotic resistance among gram-negative bacteria constitutes a grave threat to public health globally, demanding rigorous and targeted research to unravel and thwart this escalating menace. The seminal discovery of the Shigella genus by Kiyoshi Shiga, a distinguished Japanese bacteriologist, has been a cornerstone in understanding bacterial pathogenesis. This genus, comprising non-motile, rod-shaped bacteria adept at surviving in both oxygenated and anaerobic conditions, includes pivotal species like Shigella dysenteriae, notorious for causing shigellosis or bacterial dysentery (Nayak, 2020).

This ailment, often characterized by severe, bloody diarrhea, results primarily from ingesting contaminated food, spotlighting the critical public health issue of foodborne in-

fectious diseases (Cornelis, 2008). Equally significant is the Proteus genus, residing in varied environments such as animal waste, soil, and the human gastrointestinal tract. This genus encompasses species like Proteus mirabilis and Proteus vulgaris, which are implicated in a broad array of infections including urinary tract infections, wound infections, and more grave conditions like bacteremia and meningitis, particularly in the context of hospital settings (Ramos, 2020). This underscores the imperative to delve deeper into the pathogenicity and antibiotic resistance patterns of these bacteria, to devise effective countermeasures (Bowen, 2016). Moreover, Morganella morganii, first delineated in 1906, has been identified as a causative agent of multiple nosocomial infections, including urinary and surgical site infections, further entangling the complex web of antibiotic resistance challenges faced in medical care settings (Davey, 2000). Although less prevalent, the Providencia species also play a significant role in opportunistic infections, especially among patients subjected to long-term urological catheterization or those recovering from severe burns. This highlights the adaptability and diversity of gram-negative pathogens in contributing to human infectious diseases (Shan, 2019).

2. Materials and Methods

2.1. Materials

Table 1. The antibiotics used and their concentrations B m

No	Antibiotic Name	Symbol	Concentration (mg)
1	Amikacin	AK	30
2	Trimethoprim	TM	5
3	Gentamicin	GEN	10
4	Ceftriaxone	CTR	30
5	Cefepim	CFP	30
6	Ciprofloxacin	CIP	5
7	Nitrofurantoin	NIT	100
8	Ampicillin	AMP	25
9	Chloramphenicol	C	30
10	Pipracillin	PI	100

Table 2 : The media culture

No.	Media Name	Purpose
1	MacConkey Agar	Selective for gram-negative bacteria and differential between lactose fermenters and non-fermenters.
2	Triple Sugar Iron Agar	Used to distinguish between bacteria that ferment sugars and those that produce hydrogen sulfide.
3	Muller Hinton Agar	Employed for antibiotic sensitivity testing.
4	Peptone Water	Used for indole production testing.
5	S-S Agar	Utilized to differentiate between Salmonella and Shigella.
6	MR-VP Media	Used for Methyl Red and Voges-Proskauer tests.
7	Nutrient Slants	Used for preservation and collection of bacterial samples.
8	Citrate Utilization Agar	Used to detect bacteria capable of fermenting citrate.

The preparation of the culture media follows the manufacturer's guidelines provided on the packaging of each medium.

Solutions: A normal saline solution, known as phlegsalin, was sterilized using an autoclave. The process involved heating the solution to a temperature of 121°C under a pressure of 15 psi for a duration of 15 minutes.

Equipment and Instruments: The essential equipment in a microbiology laboratory includes the autoclave, a critical device for sterilization. The autoclave is a pressure chamber constructed from metal, designed to heat solutions beyond their boiling points at standard atmospheric pressure, thereby achieving sterilization. This process is accomplished by elevating the temperature to 121°C and applying a pressure of 15 psi for 15 minutes. The autoclave is primarily used for sterilizing culture media and for the safe disposal of contaminated samples.

2.2. Methods

2.2.1. Sample Collection:

Stool samples were collected from patients suffering from diarrhea at Shomali General Hospital, ensuring they were free of parasites after a general stool examination. The samples were gathered in sterile containers and immediately cultured to avoid contamination.

2.2.2. Sample Collection:

Culture of Samples: The collected samples were directly inoculated using a flame-sterilized loop in a special culturing chamber (hood) onto MacConkey agar plates. These plates were then incubated at 37°C for 24 hours.

2.2.3. Identification of Samples: The bacterial samples were identified based on:

1. Morphological Characteristics: Observing the physical characteristics of the bacteria's colonies, such as texture, smell, shape, color, transparency, colony borders, growth density, and their ability to ferment lactose in MacConkey agar.
2. Microscopic Examination: Smears from the bacterial colonies were fixed and stained with Gram stain to differentiate between gram-positive and gram-negative bacteria according to their staining properties.
    - Biochemical Tests: A series of biochemical tests were conducted to further identify unclassified bacteria, utilizing various chemical substances. These tests are fundamentally based on the bacterial enzymes secreted into their environment according to their metabolic activity. The tests include:
    - Catalase Test: This test involves transferring a portion of the colony onto a glass slide using a loop or wooden sticks, followed by the addition of hydrogen peroxide (H2O2). The presence of bubbles indicates a positive result, while their absence denotes a negative outcome.
    - Oxidase Test: This test distinguishes between bacteria that produce the enzyme oxidase and those that do not. A portion of the colonies is placed on filter paper, treated with oxidase reagent, and observed for a purple color change within 10 seconds to indicate a positive result.
    - Urease Test: This test differentiates bacteria based on the production of the enzyme urease, which breaks down urea. It involves using urea broth in tubes inoculated with bacterial colonies and incubated aerobically at 35°C for 24 hours. A positive result is indicated by a change in the medium color to purple.
    - Gas and Fermentation Production Test: Using Triple Sugar Iron (TSI) Agar, this test is conducted by stabbing the bacterial isolates into the tubes and incubating at 37°C for 24 hours. Results are read by observing color changes, with yellow indicating glucose fermentation; yellow at both top and bottom suggests fermentation of sucrose, glucose, and

lactose; black sediment and bubble formation indicate H2S production and gas formation, respectively.

- Gelatin Liquefaction Test: This test assesses the ability of microorganisms to hydrolyze gelatin using the enzyme gelatinase or protease. Tubes containing nutrient gelatin medium are inoculated with bacteria, incubated at 25°C for 48-72 hours, and then observed for gelatin liquefaction after placing in ice for 15 minutes. A liquid state indicates a positive result, while a solid state denotes a negative outcome.
- IMViC Tests: A set of tests including the Indole test, Methyl Red test, Voges-Proskauer test, and Citrate utilization test, each designed to examine different metabolic capabilities of the bacteria.

3. Antibiotic Sensitivity Test: Sensitivity testing was performed on the bacterial isolates grown on appropriate media by transferring two colonies using a loop to a tube containing 3-5 ml of normal saline or distilled water. The turbidity was adjusted, and a cotton swab was dipped into the solution and streaked onto a Muller Hinton agar plate. Antibiotic discs were placed on the surface of the medium using sterile forceps, ensuring proper spacing. The plates were left to stand for half an hour to allow antibiotic diffusion before incubating at 37°C for 18 hours. The inhibition zones for each antibiotic were measured with a ruler and compared to the 2017 CLSI standards.

3. Results

In the process of this study which was carried out at Shomali General Hospital between the months of March and December in 2023, a total of eighty stool samples were collected from patients who were experiencing diarrhoea. Eighteen of the eighty isolates were found to be non-lactose fermenting after being subjected to stringent biochemical, morphological, and microscopic studies, which were reinforced by confirmation using E20 API. Among them, the distribution was as described in the following: In accordance with the findings of the antibiotic sensitivity test (Table 2), there were ten isolates of Salmonella, four isolates of Pseudomonas, two isolates of Proteus, and two isolates of Shigella. The inhibition rate of ciprofloxacin was the greatest, coming in at 95%, while the rate of inhibition for ampicillin was the lowest, just 10%. It is important to note that Pseudomonas isolates exhibited a large level of antibiotic resistance, which highlights the enormous issue that is faced by the introduction and evolution of antibiotic resistance mechanisms among these bacteria.

The results of this research indicate the predominance of gram-negative bacteria that do not digest lactose in diarrheal illnesses. Variations in the frequency of these bacteria may be impacted by variables such as age, seasonality, personal cleanliness, the health state of the patient, and dietary choices. The investigation into the phenotypic attributes of bacterial colonies revealed distinct appearances for each type of bacteria cultured from the samples. Salmonella colonies were identified by their large size, ranging from 2 to 3 mm in diameter, with a circular, slightly convex shape, and a transparent center, indicating their inability to ferment lactose sugar present in MacConkey agar. Proteus species produced medium-sized, pale colonies with a slight convexity, characterized by their moist, transparent appearance and a unique spreading across the entire dish due to swarming motility, also showing no lactose fermentation. Shigella colonies were observed as circular, convex, colorless, semi-transparent with a smooth surface and flat edges, not fermenting lactose sugar. In contrast, Pseudomonas species formed large, transparent, convex colonies that did not ferment lactose sugar. Microscopic analysis of the bacterial isolates revealed that all were Gram-negative and motile, with the exception of the Shigella genus, which displayed no motility. This differentiation highlights the

diversity in mobility among the studied gram-negative bacteria, further contributing to our understanding of their distinct characteristics.

Table 1. Biochemical Test Results for Salmonella, Proteus, Pseudomonas, and Shigella

No	Test Name	Salmonella	Proteus	Pseudomonas	Shigella
1	Gram Stain	-ve	-ve	-ve	-ve
2	Catalase	+ve	+ve	+ve	+ve
3	Oxidase	-ve	+ve	-ve	-ve
4	PV	-ve	-ve	-ve	-ve
5	Citrate	-ve/+ve	+ve	-ve/+ve	-ve/+ve
6	Indole	-ve/+ve	-ve	-ve/+ve	-ve
7	MR	+ve	-ve	+ve	+ve
8	Urease	-ve	-ve	+ve	-ve
9	TSI	K/A, H2S, CO2 gas	A/A, K/A, H2S, CO2 gas	K/K, No gas	K/A, No gas

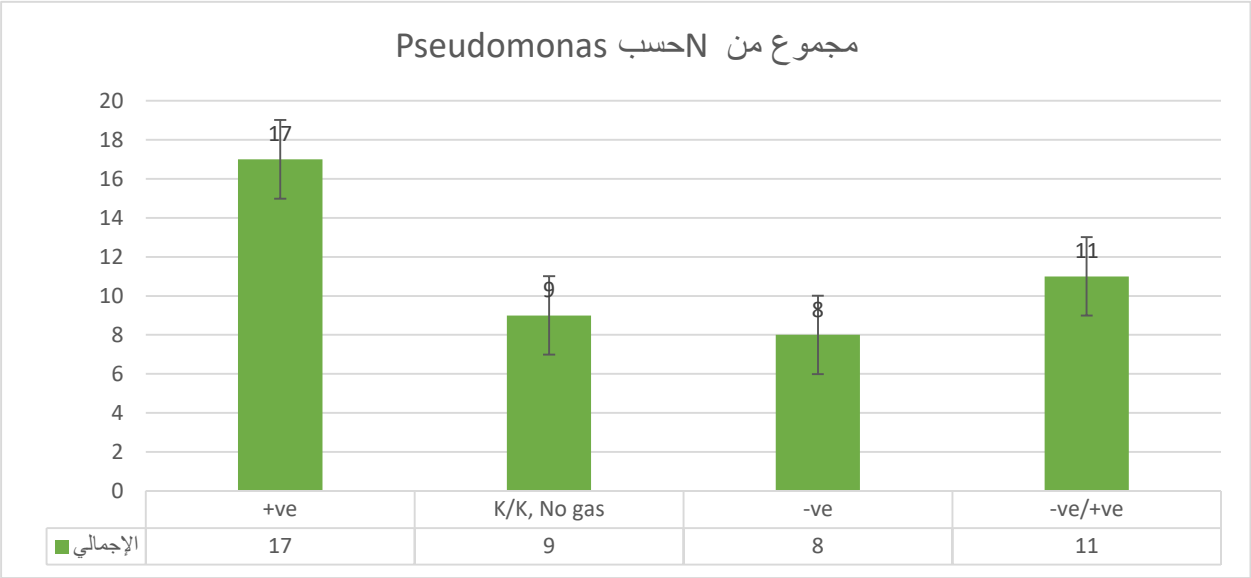

Figure 1. Biochemical Test Results for Salmonella, Proteus, Pseudomonas, and Shigella

Table 2. Antibiotic Sensitivity Test Results for Different Bacterial Species"

No	Antibiotic	Salmonella sp Resistance % (n=10)	Proteus sp Resistance % (n=2)	Pseudomonas sp Resistance % (n=4)	Shigella sp Resistance % (n=2)
1	AK	0% (0)	75% (3)	50% (1)	30% (3)
2	TM	50% (1)	50% (2)	0% (0)	10% (1)
3	GEN	50% (1)	50% (2)	50% (1)	10% (1)

No	Antibiotic	Salmonella sp Resistance % (n=10)	Proteus sp Resistance % (n=2)	Pseudomonas sp Resistance % (n=4)	Shigella sp Resistance % (n=2)
4	CTR	0% (0)	25% (1)	0% (0)	20% (2)
5	CFP	50% (1)	50% (2)	50% (1)	30% (3)
6	CIP	0% (0)	25% (1)	0% (0)	0% (0)
7	NIT	50% (1)	75% (3)	50% (1)	20% (2)
8	AMP	100% (2)	100% (4)	50% (1)	90% (9)
9	C	50% (1)	50% (2)	0% (0)	40% (4)
10	Pi	100% (2)	75% (3)	100% (2)	70% (7)

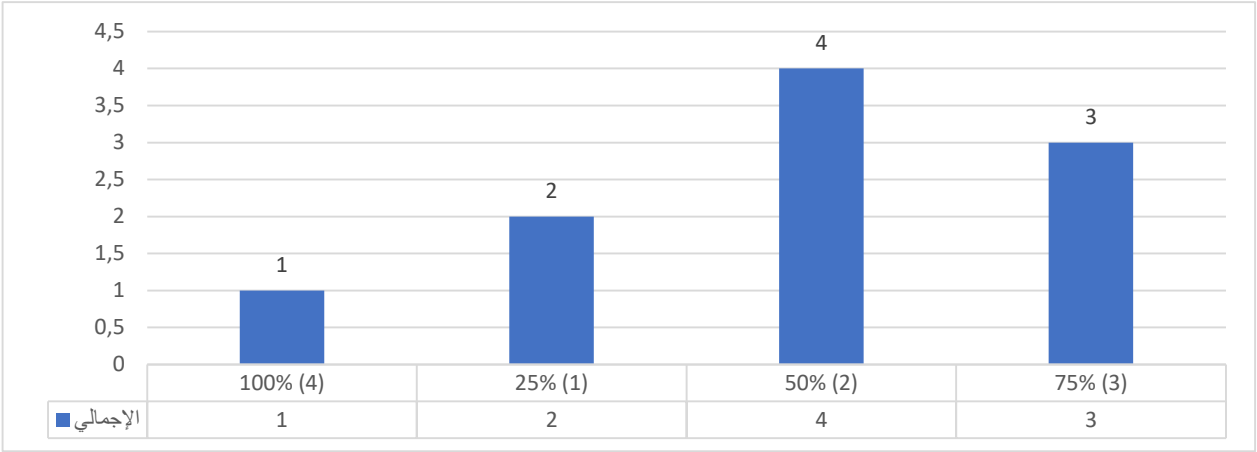

Figure 2. Antibiotic Sensitivity Test Results for Different Bacterial Species"

4. Conclusion

The findings from the study at Shomali General Hospital, focusing on the analysis of eighty stool samples from diarrheal patients, are a stark reminder of the formidable challenge posed by antibiotic resistance among non-lactose fermenting gram-negative bacteria. The identification of eighteen isolates, including Salmonella, Pseudomonas, Proteus, and Shigella, not only underscores the diversity of pathogens involved in diarrheal diseases but also highlights the critical issue of antibiotic resistance, particularly the alarming resilience displayed by Pseudomonas species. The study's revelation that ciprofloxacin retains a high rate of effectiveness, while ampicillin's efficacy plummets, serves as a clarion call for the urgent reassessment of current antibiotic prescribing

practices. The high level of resistance observed demands immediate action to steward existing antibiotics more judiciously and to accelerate the development of new antimicrobial agents. Moreover, the detailed phenotypic analysis of the bacterial isolates offers crucial insights into their identification and behavior, emphasizing the importance of advanced diagnostic tools in the effective management of infectious diseases. The variations in colony morphology and motility among the isolates not only enrich our understanding of bacterial pathogenicity but also underscore the complexity of diagnosing and treating diarrheal illnesses. The influence of external factors such as age, seasonality, hygiene, health status, and diet on the prevalence of these bacteria further indicates that combating these infections requires a holistic approach. This approach should not only involve advanced medical and pharmacological strategies but also encompass public health measures aimed at improving sanitation, hygiene practices, and awareness. In conclusion, this study is a critical reminder of the ongoing battle against antibiotic resistance and the complexity of managing diarrheal diseases. It underscores the necessity for a multifaceted strategy that includes robust surveillance, innovative research for new antibiotics, and global public health initiatives to mitigate the spread of resistant bacteria. The fight against these persistent pathogens will require concerted efforts from the medical community, researchers, policymakers, and the public at large.

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