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Molecular Characterization of Bacteria Isolated from Urinary Tract Infections and Detection of Antibiotic Resistance Genes

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ABSTRACT

The study aimed to identify the causative agents of urinary tract infections in patients with urinary tract stones.

A total of 50 urine samples were collected from patients with urinary tract stones, comprising 30 samples from females and 20 samples from males. Blood agar and MacConkey agar media were used for culturing the samples by the streaking method. Following the incubation period, 20 bacterial isolates were obtained, including 17 Gram-negative isolates and 3 Gram-positive isolates. The study was conducted from January 2018 to March 2019.

The results showed that females had a higher incidence of bacterial infections causing urinary tract infections among patients with urinary tract stones. The total number of isolates obtained from females was 16 out of 20, representing 80%, whereas 4 isolates out of 20 were obtained from males, representing 20%.

The study also demonstrated that *Escherichia coli* (*E. coli*) was the most prevalent bacterial pathogen among patients with urinary tract stones, with 9 isolates out of 20 (45%). *Proteus mirabilis* ranked second, with 4 isolates out of 20 (20%), while *Staphylococcus* spp. ranked third, with 3 isolates (10%). *Klebsiella* ranked fourth, with 2 isolates (10%), whereas *Proteus* and *Salmonella* ranked last, with one isolate each out of 20 (5%). The disc diffusion method was used to assess antimicrobial susceptibility. Ten antibiotics were used for Gram-negative bacteria, while seven antibiotics were used for Gram-positive bacteria. The diameter of the inhibition zone was measured in millimeters, and the results were compared and classified as either susceptible (S) or resistant (R) according to the international standards of the Clinical and Laboratory Standards Institute (CLSI, 2017).

The results demonstrated differences in the inhibitory activity of the tested antibiotics against the isolated bacterial species, with some antibiotics showing relatively low inhibitory effects.

Ciprofloxacin produced the highest inhibition rate, reaching approximately 75% against Gram-negative bacteria. It also showed the highest inhibitory effect against Gram-positive bacteria, with an inhibition rate of 100%.

In contrast, trimethoprim showed the lowest effect against Gram-negative bacteria, with an inhibition rate of 0%, while penicillin showed the lowest effect against Gram-positive bacteria, also with an inhibition rate of 0%. The effects of the other antibiotics ranged between these values, as presented in the tables accompanying the study.

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Introduction

The urinary system, which consists of the kidneys, urinary bladder, ureters, and urethra, is one of the important systems in the human body because of its role in filtering the blood from harmful substances and excess materials that are not required by the body and eliminating them in the form of urine. The characteristics and composition of urine provide good indicators that reflect the normal and pathological physiological state of the body, in addition to the other functions of the kidneys in maintaining the normal balance of body fluids.

Urinary tract infections (UTIs) are considered one of the major health problems affecting millions of people worldwide each year. At the same time, these infections are among the most common infections acquired in hospitals. They represent the most common bacterial infections and may affect individuals of both sexes during their lifetime, encompassing several clinical conditions ranging from bacteriuria and bladder infections to prostatitis and renal pelvic infections.

Urinary tract infections in young males are usually rare and complicated and are often attributed to the presence of abnormalities in the urinary system. In contrast, the acquisition of uncomplicated urinary tract infections may result from unprotected sexual contact with infected individuals.

Most sources indicate agreement among researchers that the causative agents of community-acquired urinary tract infections are predominantly Gram-negative bacteria, with *Escherichia coli* (*E. coli*) being the predominant species in most cases, accounting for approximately 80–90% of all urinary tract infections. The incidence of urinary tract infections ranks second after upper respiratory tract infections; however, the mortality rate associated with urinary tract infections is higher, as one-third of patients who die annually from renal failure have a previous history of UTI.

Women are more susceptible to urinary tract infections than men because of anatomical differences that increase the likelihood of ascending migration of urinary pathogens into the urinary tract and consequently the development of infection.

The widespread and inappropriate use of antibiotics has increased the problems associated with urinary tract infections, as the development of antimicrobial resistance is considered one of the major problems facing the medical community, in addition to being a serious threat to public health. This is attributed to the continued use of these antibiotics over prolonged periods, which has resulted in the development of bacterial resistance and consequently the emergence of strains with high levels of antimicrobial tolerance (Brooks et al., 2007; Fagade et al.; Pitout & Laupland, 2008).

Most causative agents of urinary tract infections are Gram-negative bacteria, such as *E. coli*, *P. mirabilis*, and other species. A study conducted by Khalaf Kadhim (2012) indicated that *P. mirabilis* was responsible for 11.85% of urinary tract infections. The study also showed that the isolation rate of this bacterium was higher among females than males.

There are also complicated urinary tract infections. Gram-negative bacterial infections of the urinary tract are often associated with complicated urinary tract infections, in addition to their association with structural abnormalities of the urinary tract, obstructions, and congenital abnormalities (Pospiech & Neuman, 1995). This type of infection is difficult to treat using antibiotics because of the presence of bacteria within the casts (Brenner et al., 2004).

Antibiotics

An antibiotic is a term used to describe organic substances produced by microorganisms that can be used systemically either to kill microorganisms or to inhibit their multiplication, regardless of their origin. Antibiotics are among the natural products known as secondary metabolites.

Antibiotics are of great importance in human life because they contribute to reducing disease incidence and, consequently, reducing mortality. When selecting an appropriate antibiotic for treatment, several factors must be taken into consideration, the most important of which are the patient's clinical condition and the required dose.

Antibiotics possess selective toxicity toward bacteria, meaning that the antibiotic is harmful to the disease-causing microorganism without affecting the host cells. Antibiotics may either be bactericidal, in which case they kill the bacteria, or bacteriostatic, in which case they inhibit bacterial growth without causing harm to the host cells.

Patients with bladder infections suffer from a range of symptoms, including dysuria, increased frequency of urination, urinary urgency, and pain above the pubic region (Brooks et al., 2010; Pecet et al., 2005).

Bactericidal antibiotics cause lysis and death of the pathogenic microorganism; that is, they directly damage one of the essential components of the bacterial cell. In contrast, bacteriostatic antibiotics inhibit bacterial multiplication by binding to structures necessary for cell division (Pitout & Laupland, 2008).

Antibiotic resistance is encoded by resistance genes, which can be transferred between bacteria through three mechanisms: conjugation, transformation, and bacteriophage-mediated transduction (Pospiech & Neuman, 1995). Many antibiotic-resistance genes are carried on plasmids, facilitating their transfer between bacterial cells (Kieser et al., 2007).

Bacteria carrying multiple antibiotic-resistance genes are generally referred to as multidrug-resistant (MDR) bacteria (Quinn et al., 2002).

Mechanisms of Action of Antibiotics

The mechanisms of action of antibiotics fall under four main categories:

- **Inhibition of cell membrane function.**
- **Inhibition of cell wall synthesis.**
- **Inhibition of protein synthesis**, such as inhibition of the translation and transcription processes of genetic material.
- **Inhibition of nucleic acid synthesis.**

Materials and Methods

The study was conducted according to the following procedures:

1. **Isolation of bacteria causing urinary tract infections in patients with urinary stones.**
2. **Identification of bacteria causing urinary tract infections using MacConkey agar, biochemical tests (Biochemical tests), and morphological and microscopic characteristics.**

3. Use of the commercially prepared diagnostic kit for identifying Gram-negative bacteria of the Enterobacteriaceae family (API 20E) to confirm the identification.

4. Use of the commercially prepared diagnostic kit for identifying Gram-positive bacteria of the Enterobacteriaceae family (Staph API) to confirm the examination.

5. Determination of the resistance of bacteria causing urinary tract infections to selected antibiotics.

Table 1. Antibiotics Used

No.	Antibiotic	Code	Concentration	S	I	R
1	Amikacin	AK	30 mcg	≤14	15–16	≥17
2	Nitrofurantoin	NIT	300 mcg	≤14	15–16	≥17
3	Pipracillin	PI	100 mcg	≤17	18–20	≥21
4	Trimethoprim	TM	5 µg	≤10	11–15	≥16
5	Gentamicin	GEN	10 mcg	≤12	13–14	≥15
6	Chloramphenicol	C	30 mcg	≤12	13–17	≥18
7	Cefepim	CFP	30 µg	≤14	15–17	≥18
8	Ceftriaxone	CTR	30 µg	≤19	20–22	≥23
9	Ciproflaxalin	CIP	5 mg	14	16–20	21
10	Pencillin	P	10 unit	—	28–29	—

Solutions

1. Normal Saline

Normal saline was prepared by dissolving 5.8 g of NaCl in a small amount of distilled water. The volume was then made up to 1 liter. The solution was subsequently sterilized by autoclaving (Autoclave) at 121°C, under a pressure of 15 lb/in², for 15 minutes.

2. Preparation of Culture Media

The ready-to-use culture media were prepared according to the manufacturer's instructions. The media used in this study are presented in the following table.

Table 2. Culture Media Used

No	Culture Medium	Purpose of Use
1	MacConkey Agar	Used as a selective medium for Gram-negative bacteria and to differentiate lactose-fermenting colonies from non-lactose-fermenting colonies.
2	Muller-Hinton Agar	Used to perform the antibiotic susceptibility test.
3	Peptone Water	Used for detection of the indole reaction.
4	Citrate Utilization Medium	Used to detect bacteria capable of utilizing citrate as a sole carbon source.
5	Kligler Iron Agar (KIA)	Used to differentiate bacteria based on hydrogen sulfide production and sugar fermentation.
6	Methyl Red–Voges-	Used to perform the Methyl Red and Voges-Proskauer tests.

	Proskauer (MR-VP) Medium	
7	Blood Agar	Used for the cultivation of Gram-negative and Gram-positive bacteria.

Routes of Infection

There are three possible routes through which bacteria can invade the urinary tract: the ascending route, hematogenous route, and lymphatic route (Raksha et al., 2003).

Predisposing Factors for Urinary Tract Infections

Factors that interfere with the flow of urine and emptying of the urinary bladder, as well as factors that facilitate the access of pathogenic microorganisms to the bladder, are considered predisposing factors for infection. Researchers have indicated that the retention of approximately 2–3 mL of urine may constitute a predisposing factor for bacterial growth (Ranjan et al., 2010).

Other Predisposing Factors for Urinary Tract Infections

Other factors may contribute to bacterial infection and the development of urinary tract infections, including changes in osmotic pressure and urinary urea concentration. In addition, the accumulation of various urinary toxins may inhibit defensive blood cells, such as phagocytic cells, as well as other immune mechanisms (Reinthal et al., 2010).

A large proportion of urinary tract infections enter through the urethra and urinary bladder, and subsequently reach the ureters and kidneys (Bashir et al., 2000).

Sample Collection

Urine samples were collected from patients diagnosed with urinary tract infections or suspected of having urinary tract infections, according to the physicians' diagnoses at Afak General Hospital and Al-Diwaniyah Teaching Hospital, during the period extending from December 2018 to March 2019.

Patients were instructed to collect urine samples in sterile, single-use containers. The initial portion of the urine was discarded, and the samples were then transported directly to the laboratory for culture.

Sample Culturing

The urine samples collected from the patients were cultured immediately without delay to prevent contamination. Using a loop, the samples were inoculated onto Blood Agar and MacConkey Agar media. The inoculated plates were then incubated at 37°C for 24 hours.

Identification of Isolated Bacteria

The growing bacterial colonies were identified based on the following criteria:

A. Phenotypic Characteristics

The phenotypic characteristics of the growing colonies were examined, including their shape, color, colony surface, characteristic odors, texture, transparency, and lactose fermentation on MacConkey Agar (Blanco et al., 2006).

B. Microscopic Examination

The bacterial colonies were examined microscopically by preparing smears from the growing bacterial colonies. The smears were fixed and stained using the Gram staining technique according to the procedure recommended by the manufacturer. This examination was performed to determine the arrangement and shape of bacterial cells and their reaction to the stain.

C. Biochemical Tests

A series of biochemical tests was performed to identify the bacteria under investigation, as follows:

Catalase Test

A portion of a young bacterial colony was transferred using a wooden applicator stick onto a glass slide. A drop of 3% hydrogen peroxide (H_2O_2) was then added. A positive reaction was indicated by the formation of oxygen gas bubbles (Blanco et al., 1997).

Oxidase Test

A portion of a young bacterial colony was transferred using a sterile wooden applicator stick onto filter paper saturated with the oxidase reagent. The appearance of a purple color within 10 seconds was considered a positive reaction (Blanco et al., 1997).

Sugar Fermentation and Gas Production Test

This test was performed using tubes containing Kligler Iron Agar (KIA). The medium was inoculated with the bacterial isolates by stabbing the butt and streaking the slanted surface. The tubes were then incubated at 37°C for 24 hours.

Bacteria that do not ferment lactose produce a yellow color in the butt only, whereas fermentation of sucrose results in a yellow color in both the slant and butt. A black color indicates the formation of ferrous sulfide.

IMViC Tests

The IMViC test series consisted of the following tests:

1. Indole Test

Peptone water was inoculated with a portion of the pure bacterial colony under examination and incubated at 37°C for 24 hours. After incubation, several drops of Kovac's reagent were added to each tube. The appearance of a red-colored ring indicated a positive reaction.

The test is based on the ability of bacteria to metabolize the amino acid tryptophan and produce indole (MacFaddin, 2000).

2. Methyl Red Test

The test was performed by inoculating tubes containing VP-MR Medium with the bacterial culture, followed by incubation at 37°C for 24–48 hours. After incubation, several drops of Methyl Red reagent were added. The development of a red color after 15 minutes indicated complete glucose fermentation and therefore a positive reaction (Collee et al., 1996).

3. Voges–Proskauer Test

The test was performed by inoculating tubes containing VP-MR Medium with the bacterial culture and incubating them at 37°C for 24–48 hours. Subsequently, 0.6 mL of alpha-naphthol reagent and 0.2 mL of potassium hydroxide solution were added to each tube.

The appearance of a pink color within 2–5 minutes indicated a positive reaction, suggesting partial sugar degradation and the production of acetyl methyl-carbonyl (Collee et al., 1996).

4. Citrate Utilization Test

Simmons citrate medium, prepared in a slanted form (slant), was inoculated with a portion of the colony to be examined and incubated at 37°C for 24 hours. A positive reaction was indicated by a change in the color of the medium from green to blue, indicating bacterial utilization of citrate as an energy source (Collee et al., 1996).

Identification Using the API 20E System

After obtaining the results of the biochemical tests, the results were confirmed using the API 20E system. This strip contains 20 microtubes/wells designated for biochemical tests. The procedure was performed as follows:

A. Preparation of Bacterial Suspension

5 mL of Normal Saline was inoculated with 1–3 colonies to prepare the bacterial suspension.

B. Inoculation of the API 20E Strip

Using a clean, dry pipette, the wells of the API 20E strip were inoculated by filling them with the bacterial suspension according to the manufacturer's instructions. Each well was filled with 0.12 mL of the suspension, whereas 0.28 mL of inoculum was used for the Gel, VP, and Citrate tubes.

Sterile oil was added to the tubes indicated by underlining. These tests included:

URE, ADH, LDC, ODC, and H₂S.

C. Incubation of the API 20E Strip

The strip was placed inside the special incubation tray supplied with the kit and incubated at 37°C for 24 hours.

D. Addition of Reagents

- One drop of TDA reagent was added to the Tryptophan deaminase test. A change in color to brown indicated a positive reaction.

- One drop of James reagent was added to the Indole test. The appearance of a red ring indicated a positive reaction.

- One drop each of 1VP and 2VP reagents was added successively to the Voges–Proskauer test. A change in color to pink indicated a positive reaction.

- Drops of 1NIT and 2NIT reagents were added to the Glucose test.

- Glucose: A change in color to blue indicated a positive result.

E. Reading the Results

The bacteria were identified using the numerical profile generated by assigning + or – according to the positive or negative results of the biochemical tests included in the API 20E strip. The bacterial identification was then determined using the API 20E identification guide.

Antibiotic Sensitivity Test

The antibiotic susceptibility of the bacterial isolates was tested using the disc diffusion method, based on the method described by Bauer et al. (1966). The procedure involved transferring 2–4 bacterial colonies into a sterile test tube containing 5 mL of Tryptone Soy Broth, followed by incubation at 37°C for 8 hours.

The resulting bacterial growth was diluted using normal saline, and the turbidity was compared with the standard 0.5 McFarland reference tube. A sterile cotton swab was then immersed in the suspension and used to inoculate solid Mueller–Hinton agar plates by streaking the bacteria repeatedly in different directions to ensure uniform distribution of the bacterial inoculum.

A total of 10 antibiotic discs were placed on each 150-mm plate. The distance between adjacent discs was 20 mm, measured from the center of one disc to the center of the next.

The plates were incubated at 37°C for 18 hours. The diameters of the inhibition zones were then measured using a Vernier caliper and compared with the values specified by CLSI (2017).

Results and Discussion

The results of the biochemical tests obtained in this study are presented in the following table:

Table 3. Results of Biochemical Tests

N	Diagnosis	Gram stain	M-Indol	Urease	Kliglar	Oxidase	Catalase	Citrate	VP
1	E. coli	K/A	-ve	+ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve
2	E. coli	K/A	-ve	+ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve
3	Salmonella	K/A	-ve	+ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve
4	Prot. M	K/A	-ve	+ve	-ve Gas & H ₂ S	+ve	-ve	+ve	-ve
5	E. coli	K/A	-ve	+ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve
6	Staph	K/A	-ve	+ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve
7	E. coli	K/A	+ve	+ve	-ve Gas & no H ₂ S	+ve	-ve	-ve	-ve
8	Prot. M	K/A	+ve	+ve	-ve Gas & no H ₂ S	+ve	-ve	-ve	-ve
9	—	K/A	-ve	+ve	-ve Gas & H ₂ S	+ve	-ve	+ve	-ve
10	E. coli	K/A	-ve	+ve	-ve No Gas & no H ₂ S	-ve	+ve	+ve	-ve
11	Staph	K/A	-ve	+ve	-ve Gas & H ₂ S	+ve	-ve	+ve	-ve
12	Prot. M	K/A	-ve	-ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve
13	E. coli	K/A	-ve	+ve	-ve Gas & H ₂ S	+ve	-ve	+ve	-ve
14	Staph	K/A	-ve	+ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve

15	E. coli	K/A	-ve	+ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve
16	E. coli	K/A	-ve	+ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve
17	E. coli	K/A	+ve	+ve	-ve Gas & no H ₂ S	+ve	-ve	-ve	-ve
18	Klebsiella	K/A	+ve	+ve	-ve Gas & no H ₂ S	+ve	-ve	-ve	-ve
19	Prot. M	K/A	-ve	+ve	-ve Gas & H ₂ S	-ve	-ve	+ve	+ve
20	Klebsiella	K/A	+ve	+ve	-ve Gas & no H ₂ S	+ve	-ve	-ve	-ve

Results of API 20E

The identification of the bacterial isolates in this study was confirmed using the API 20E system, following their preliminary identification based on the results of biochemical tests and their morphological and microscopic characteristics.

E. coli was the most frequently isolated bacterium in this study. This may be attributed to its natural habitat in the gastrointestinal tract, as well as the close proximity of the urinary tract to the anus, particularly in females, which facilitates the transmission of this bacterium to the urinary tract and may result in infections among patients with kidney stones.

Kidney stones may also cause abrasion of the walls of the kidneys and urinary tract, resulting in bleeding. The blood may contribute to increased bacterial growth because it provides a nutrient-rich medium for bacterial proliferation.

The study found that Ticarcillin was the antibiotic to which the Gram-negative bacteria showed the highest resistance, whereas Penicillin was the antibiotic to which the Gram-positive bacteria showed the highest resistance. Ciproflaxalin showed the lowest level of resistance among both groups of bacteria.

The results of the antibiotic susceptibility test for the Gram-negative bacteria are presented in the following table:

Table 4. Isolation and Identification of Bacteria Causing Urinary Tract Infection in Patients with Kidney Stones and Their Resistance to Selected Antibiotics

Table: Antibiotic Susceptibility of Bacterial Isolates

No	Antibiotic	E. coli (N=9)	%	Proteus mirabilis (N=4)	%	Proteus vulgaris (N=1)	%	Klebsiella sp. (N=2)	%	Salmonella sp. (N=1)	%
1	AK	5	55.5	1	25	1	100	1	50	1	100
2	C	3	33.3	1	25	0	0	2	100	1	100
3	PI	4	44.4	2	50	1	100	2	100	0	0
4	TM	6	66.6	2	50	1	100	2	100	0	0
5	TI	2	22.2	1	25	1	100	1	50	1	100

							0				
6	CIP	4	33	1	25	1	10 0	1	50	0	0
7	NIT	9	100	4	10 0	1	10 0	2	10 0	2	10 0
8	GFN	4	44.4	1	25	1	10 0	1	50	1	10 0
9	CEF	2	22.2	1	25	0	0	1	50	1	10 0
10	Ceftriaxone	—	—	—	—	—					

Likewise, the results of the antibiotic susceptibility test and the resistance values for the **Gram-positive bacteria** are presented in the following table:

Table 5. Antibiotic Susceptibility Test Results for Gram-Positive Bacteria

No	Antibiotic Name	Number N=3	Percentage (%)
1	AK	2	66.6%
2	GEN	2	66.6%
3	TM	1	33.3%
4	C	1	33.3%
5	NIT	1	33.3%
6	CIP	0	0%
7	Pen	3	100%

Table 6. Infection Rate According to Age Group

Age Group (years)	Number of Cases	Percentage (%)
1–10	1	5%
11–30	6	30%
31–50	12	60%
>50	1	5%
Total	20	100%

Table 7. Infection Rate According to Sex

Sex	Number of Cases	Percentage (%)
Male	16	80%
Female	4	20%
Total	20	100%

Discussion

A total of 50 urine samples were collected from patients with kidney stones in Al-Qadisiyah Governorate. The samples were divided into 30 samples from females and 20 samples from males during the period from ... to Following bacterial culture, 20 bacterial isolates were obtained.

The culture results showed that Gram-negative bacteria accounted for 17 of the 20 bacterial isolates, whereas Gram-positive bacteria accounted for 3 isolates out of 20.

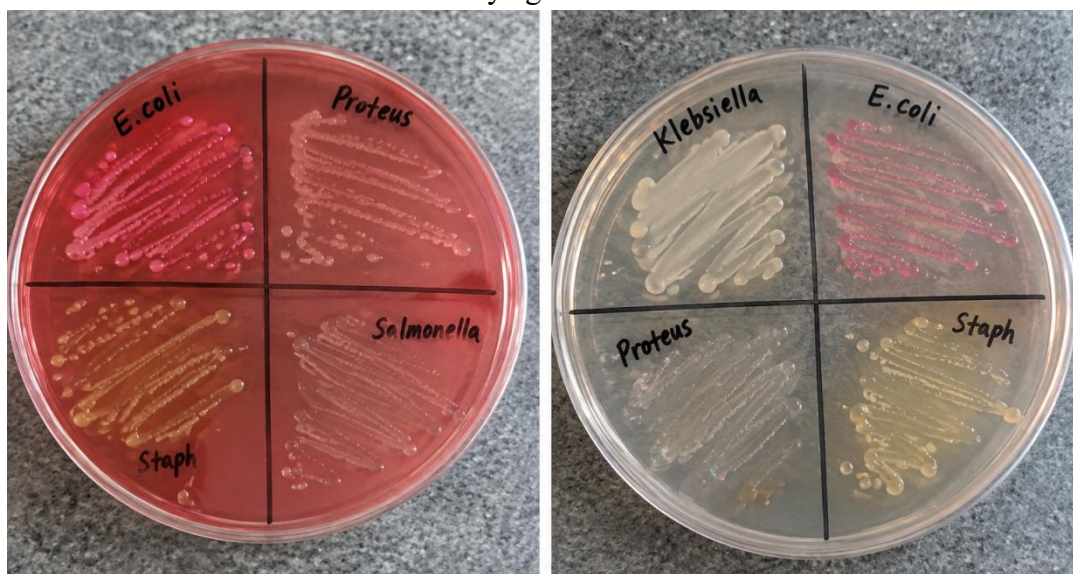
The study showed that *E. coli* had the highest rate of infection, with 9 isolates out of 20, reported as 20%. *Proteus mirabilis* ranked second, with 4 isolates (20%), while *Staphylococcus* spp. ranked third, with 3 isolates (15%). *Salmonella* / *Proteus vulgaris* were reported at the lowest frequency, with one isolate out of 20 (5%).

The antibiotic Ciproflaxalin produced the highest inhibition rate, approximately 75%, against Gram-negative bacteria. The same antibiotic also showed the highest inhibitory effect against Gram-positive bacteria, with a rate of 100%.

In contrast, Trimethoprim showed the lowest inhibitory effect against Gram-negative bacteria, with a rate of 0%, whereas Penicillin showed the lowest inhibitory effect against Gram-positive bacteria, also with a rate of 0%. The effects of the other antibiotics ranged between these values, as shown in the tables included in the study.

Cultural Characteristics

The colonies of the Gram-negative bacterium *E. coli* appeared pink on MacConkey agar, indicating lactose fermentation. In contrast, *Proteus* and *Salmonella* produced pale-colored colonies and did not ferment lactose on MacConkey agar.



Conclusions

1. The study showed that Gram-negative bacteria, particularly *E. coli*, were responsible for a high proportion of urinary tract infections among patients with kidney stones.
2. *Staphylococcus* spp. were also responsible for a proportion of urinary tract infections among patients with kidney stones.

Recommendations

1. Avoid the indiscriminate use of antibiotics without medical consultation.
2. Conduct further studies on these bacterial species because of their high capacity for antibiotic resistance, particularly since these bacteria can cause various infections in humans in addition to urinary tract infections.
3. Conduct periodic surveillance in hospitals and diagnostic centers to determine and monitor the levels of bacterial resistance to antibiotics.
4. Avoid the indiscriminate use of medications and consult a specialist physician in cases of kidney stones and urinary tract infections.

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