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OCCURRENCE AND IDENTIFICATION OF FLUOROQUINOLONE RESISTANCE IN PROTEUS MIRABILIS CLINICAL ISOLATES FROM INTENSIVE CARE UNIT PATIENTS WITH URINARY TRACT INFECTIONS

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Abstract

Proteus mirabilis is a small gram-negative bacillus commensal of GIT and mainly causes urinary tract infections (UTIs). Fluoroquinolones are synthetic antimicrobial agents and are one of the most prescribed antibiotics. In this study, Proteus mirabilis isolates were obtained from urine cultures of ICU patients. Briefly, urine samples (n=100) were inoculated on CLED, MacConkey, and Blood agar. The isolates were primarily identified by using conventional microbiological tools and biochemical testing and confirmed based on ureC gene amplification using PCR. The confirmed isolates were initially screened for fluoroquinolone resistance using the Kirby-Bauer disk diffusion method. The MICs were estimated using E-Test strips. Lastly, PCR was done to screen isolates for PMQR (plasmid-mediated quinolone resistance) genes, namely qnrA, qnrB, and qnrS. A total of nine samples (9%) yielded Proteus mirabilis, while all other culture-positive plates were either non-Proteus Gram-negatives and some samples had gram-positive organisms. All the isolates were Gram-negative rods, motile, and non-spore former. All isolates were Indole, oxidase, and VP negative, while positive for urease, catalase, H_2S, and Methyl red. Further API 20E test strips also confirmed the isolates as P. mirabilis. PCR testing yielded 533bp bands of the ureC gene in all nine isolates, which confirmed them as P. mirabilis. Antibiogram analysis revealed diverse resistance patterns but all the isolates were found to be quinolone-resistant both in disk diffusion as well as in E test results with the highest MIC of ≤64 µg/ml. The qnrA band size was recorded ≈516 bp, while the qnrS band size was recorded ≈417 bp. All the isolates were screened for qnrA, qnrB, and qnrS genes. The overall frequency of qnrA was (4/9)44.44%, qnrB (0/9)0.00%, qnrS (6/9)66.66%, respectively.

Keywords: Proteus mirabilis, Urinary Tract Infections (UTIs), Gram-Negatives Organisms, Gram-Positive Organisms, Genes

1. Introduction

Proteus mirabilis belongs to the Enterobacteriaceae family and is a highly motile microorganism. It is known as a Gram-negative bacterium with ubiquitous characteristics as it can survive in various environments like polluted soils and water. Globally, UTIs are known to be the most common infections among urological disorders with indoor and outdoor cases. Its epidemic is linked with horizontal transmission; early detection and intervention are required for control(Setsukinai et al., 2003). It is estimated that approximately 150 million UTI cases occur every year worldwide. Among them, almost 70-80% of uncomplicated UTIs are caused by E. coli (Takeda et al., 2003). Ceftriaxone, cephalexin, nitrofurantoin, and cotrimoxazole are commonly recommended drugs for the acute phase of UTIs. For the chronic phase of UTIs, commonly used drugs are fluoroquinolones, which include levofloxacin and ciprofloxacin(Carson & Naber, 2004).

Proteus mirabilis is known to be the major causative agent of UTIs, more specifically in individuals having structural and functional abnormalities of the urogenital tract or dwelling catheters. This can ultimately lead to bacteremia and sepsis(Flores-Mireles et al., 2015). According to a study after E. coli, Proteus mirabilis is categorized as a second member of Enterobacteriaceae, which was repeatedly isolated in French hospitals (De Champs et al., 2000). Proteus mirabilis gets attached to any tissue or surface (living or non-living) and forms a slimy layer known as biofilm(Debiaggi et al., 2010), which exhibits a significant role in antibiotic resistance. Proteus mirabilis forms biofilms and adheres to living surfaces, which protect the microorganisms from the host's immune system and from antibacterial drugs that mostly lead to recurrent infections due to Proteus mirabilis (Jacobsen & Shirtliff, 2011). There is a positive association between biofilm formation and drug resistance, which has been reported in clinical studies (Rao et al., 2008; Yousefi et al., 2016). Biofilms are associated with increased antimicrobial resistance, and these are also linked with a horizontal transfer of genes responsible for antibiotic resistance (Subramanian et al., 2012). Proteus mirabilis causes ammonia production and increases the urine pH (>7.2), which promotes the aggregation and precipitation of Apatite crystals or struvite (Borghi et al., 2012). These crystal aggregates are directly stored in the microbial biofilms or on the surface of the catheter that leads to the blockage of the catheter and urine retention in the bladder, resulting in the development of bacteriuria (Jombo et al., 2012).

The intensive care units (ICUs) are often considered as epicenters for the spread of antimicrobial resistance. This renders ICU patients more vulnerable and severely affects the patients and, in other wards, impacts the community through recently discharged patients(Rosenberg & Watts, 2000). ICU stay has been reported as a risk factor for colonization and an increase in infections due to extended-spectrum beta-lactamase (ESBL) generating Enterobacteriaceae that further lead to the proliferation of ESBLs in the community(Paterson & Bonomo, 2005). Moreover, another emerging issue in the ICU is often linked with the production of ESBL enzymes produced by Enterobacteriaceae members leading to multidrug resistance(Brusselaers et al., 2011). Quinolones are considered to be one of the most prescribed antibiotics among synthetic antimicrobial agents, and its invention is linked to unplanned nalidixic acid discovery, a by-product during the manufacturing of an antimicrobial agent known as chloroquine(Aldred et al., 2014).

Fluoroquinolones (FQ) have become a more frequently used antimicrobial compound globally due to their wide antibacterial spectrum and increased bioavailability (Geetha et al., 2014). Due to their discriminating restrain of bacterial DNA generation, these have been considered powerful weapons against Gram +ve and Gram -ve bacteria, and these drugs are in use for humans as well as animals. It is considered the third major class of antibiotics, and in the global market, it accounts for about a 17% share(Van Doorslaer et al., 2014). Fluoroquinolones are mainly released as unchanged particles through urine (44% to 60%) and feces (15% to 25 %) and subsequently reach into municipal sewage. The production of broad-spectrum fluoroquinolones provides various innovative treatment options for Proteus mirabilis infections. Quinolones hinder the production of DNA and split the bacterial chromosomes by changing the targets, these mainly act on topoisomerase and gyrase enzymes. The resistance against quinolones in Enterobacteriaceae mostly comes from nucleotide substitutions in DNA gyrase genes (gyrA and gyrB) and Topoisomerase IV genes (parC and parE), within the

quinolone-resistance determining-region (QRDR). It may come from the mechanism that decreases the concentration of cytoplasmic drugs through the active transportation of quinolones from the cells by efflux pumps, consequently reducing the uptake of quinolones(Yuan & Gao, 2017).

The extreme rise in antimicrobial resistance among bacterial pathogens creates an alarming situation for the recovery of critically sick patients and successful therapeutic outcomes, leading to global concern, according to the World Health Organization (WHO)(Noppon et al., 2018). WHO considers antibiotic resistance as the third most threatening public health issue of the 21st century. Several genes encoding exhibit variations in the mechanism of resistance on mobile gene elements, which reduce the quinolones or fluoroquinolones' antibiotics susceptibility. Most of these resistance mechanisms are plasmid-mediated, causing fast proliferation among microorganisms. Therefore, it is essential to manage and control the susceptibility of Enterobacteriaceae, more specifically in ICUs. Native epidemiological efforts could be helpful in diagnosis and suitable response to MDR outbursts. These observational researches expand the efforts by identifying the resistance trend(Gopalan et al., 2016).

2. MATERIALS AND METHODS

2.1 Sampling and Isolation of Proteus mirabilis

2.1.1 Sample Collection

One hundred midstream urine samples were collected from patients with urinary tract infections (UTIs) using labeled, sterile, screw-capped containers. The samples were obtained from intensive care units of various private and public hospitals in Faisalabad, Pakistan.

2.1.2 Transportation of Samples

The urine samples were transported under optimal conditions (4°C temperature and sterile environment) to the Medical Microbiology Laboratory at Government College University, Faisalabad.

2.1.3 Isolation of Proteus mirabilis

Primary isolation was carried out by culturing urine samples on MacConkey agar, Cystine Lactose-Electrolyte-Deficient (CLED) agar, and Blood agar. After inoculation, labeled culture plates were incubated at 37°C for 24 hours, followed by observation of bacterial growth.

2.1.4 Pure Culture of Isolated Bacterial Colonies

Colonies resembling Proteus mirabilis based on morphology were subcultured on MacConkey agar, CLED agar, and Blood agar to obtain pure cultures.

2.2 Identification of Bacterial Isolates

2.2.1 Swarming Motility

To assess swarming motility, isolated P. mirabilis colonies were cultivated on Blood agar.

2.2.2 Lactose Fermentation

Pure colonies were cultured on MacConkey agar to determine whether the organism was lactose fermenting (LF) or non-lactose fermenting (NLF).

2.2.3 Colony Morphology

The colonies were observed for shape, size, texture, elevation, margins, and color (Tanvir et al., 2012).

2.2.4 Gram Staining

1. Gram staining was performed a standard protocol (Maelegheer & Nulens, 2017) to determine cell morphology, arrangement, and Gram reaction.

2.2.5 Biochemical Identification of Proteus mirabilis

Biochemical tests were performed for identification (Poirel et al., 2016):

- **Catalase Test:** To detect catalase enzyme production using hydrogen peroxide.
- **Oxidase Test:** To determine the presence of cytochrome c oxidase using tetramethyl-p-phenylenediamine reagent.
- **Urease Test:** To assess urease activity using Christensen’s urea agar.
- **Indole Test:** To detect tryptophanase enzyme activity using Kovac’s reagent.

- **Triple Sugar Iron (TSI) Test:** To test fermentation of lactose, glucose, and sucrose, and H2S production.
- **Methyl Red (MR) Test:** To evaluate acid production during glucose fermentation.
- **Voges-Proskauer (VP) Test:** To detect acetoin production using alpha-naphthol and potassium hydroxide (KOH).
- **API 20E Test System:** A biochemical strip method for confirmation.

2.3 Molecular Confirmation of Proteus mirabilis

2.3.1 Genomic DNA Extraction

Genomic DNA was extracted using FavorPrep Blood Genomic DNA Extraction Kit following the manufacturer's protocol.

2.3.2 Quantification of Extracted DNA

DNA purity and concentration were evaluated using a Calibri micro-volume Spectrophotometer.

2.3.3 Gel Electrophoresis of Extracted DNA

Agarose gel electrophoresis was performed to visualize DNA bands under UV light.

2.3.4 Storage of Extracted DNA

Extracted DNA was stored at -20°C for further processing.

2.3.5 Confirmation of P. mirabilis by Amplification of ureC Gene

PCR amplification of ureC gene was performed using species-specific primers (Zhang et al., 2013).

2.4 Antibiotic Susceptibility Testing

2.4.1 0.5 McFarland Standard Preparation

A 0.5 McFarland turbidity standard (1.5 x 10^8 CFU/ml) was prepared using barium chloride and sulfuric acid.

2.4.2 Preparation of Inoculum

Bacterial suspension was prepared in PBS and adjusted to 0.5 McFarland standard.

2.4.3 Antibiotic Discs

According to CLSI (2019) guidelines, the following antibiotics were tested: Amikacin, Tobramycin, Gentamicin, Meropenem, Imipenem, Piperacillin, Cefuroxime, Cefotaxime, Ceftazidime, Ceftriaxone, Cefepime, Sulbactam-Ampicillin, Piperacillin-Tazobactam, Amoxicillin-Clavulanic Acid, Cefoperazone-Sulbactam, Ciprofloxacin, Levofloxacin, Trimethoprim-Sulfamethoxazole, Doxycycline, and Aztreonam.

2.4.4 Disc Diffusion Assay

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method (Périllaud-Dubois et al., 2019).

3. RESULTS AND DISCUSSION

3.1 Culture Characteristics of Proteus mirabilis

Urine samples from male and female ICU patients were analyzed for bacterial growth. Proteus mirabilis was isolated using the streak-plate method, and non-Proteus isolates were excluded. The isolates exhibited distinct colony morphologies on different culture media. On CLED agar, colonies appeared shiny blue-green without swarming due to electrolyte deficiency. Blood agar cultures showed characteristic swarming motility, confirming Proteus mirabilis. MacConkey agar cultures produced radiant creamy-white colonies, consistent with non-lactose fermenting characteristics. Incubation at 37°C for 24 hours was required for optimal growth. As a facultative anaerobe, Proteus mirabilis also grew in aerobic conditions.

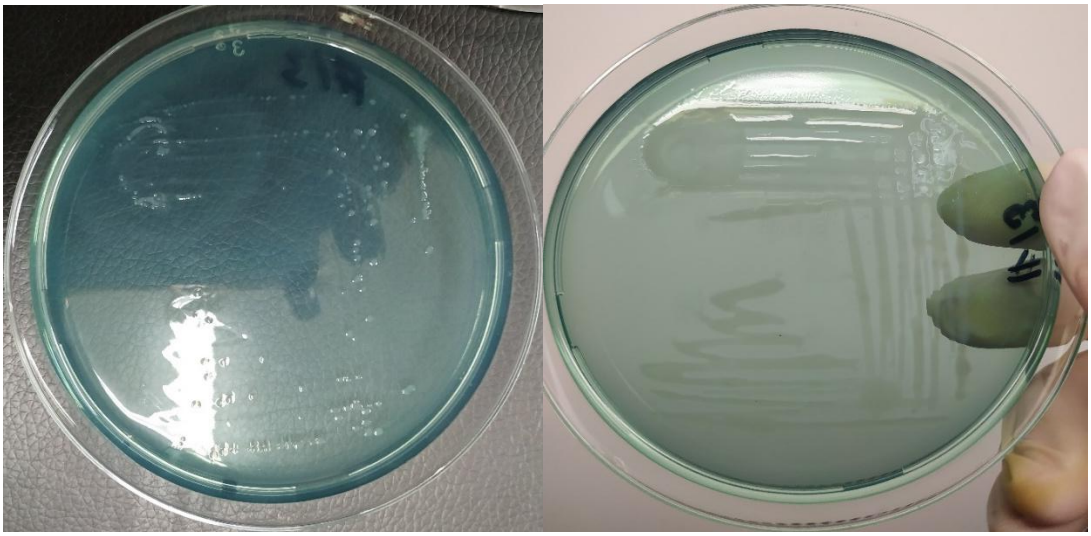

Figure 3. 1: Proteus mirabilis translucent blue-green colonies on CLED agar

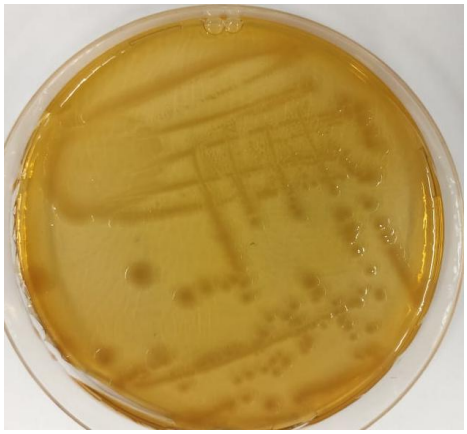

Figure 3. 2: Colorless-pale colonies of NLF P. mirabilis on MacConkey agar

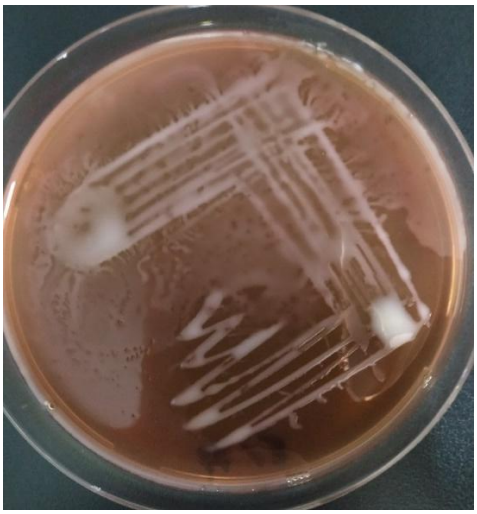

Figure 3. 3: Proteus mirabilis exhibited swarming motility and γ Hemolysis on Blood agar

3.2 Gram Reaction of Proteus mirabilis Isolates

Microscopic examination of Gram-stained isolates revealed pink-colored, Gram-negative straight rods under an oil immersion lens, confirming Proteus mirabilis morphology.

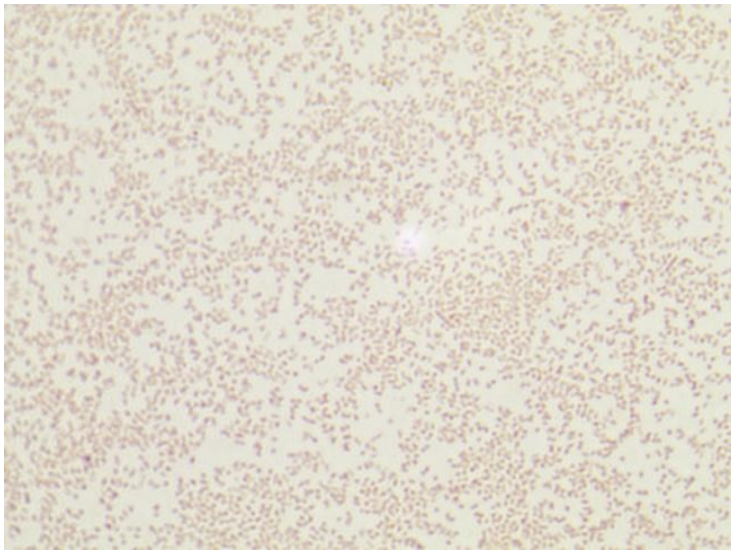

Figure 3. 4: Gram-negative rods of P. mirabilis under a microscope

3.3 Biochemical Tests

3.3.1 Catalase Test

All Proteus mirabilis isolates were catalase-positive, capable of breaking down hydrogen peroxide (H2O2) into water and oxygen.

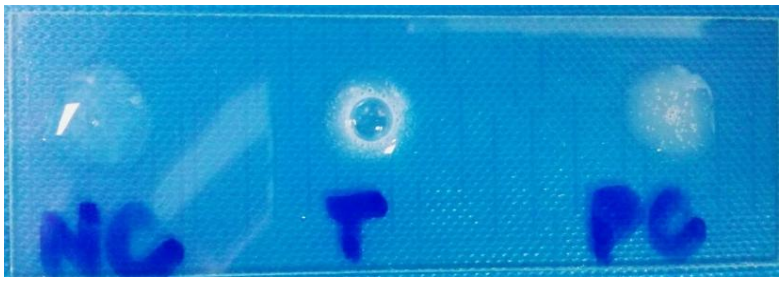

Figure 3. 5: Proteus mirabilis was catalase-positive and metabolized H_2O_2

3.3.2 Oxidase Test

Proteus mirabilis isolates tested negative for oxidase, as indicated by the absence of color change on the filter paper test.

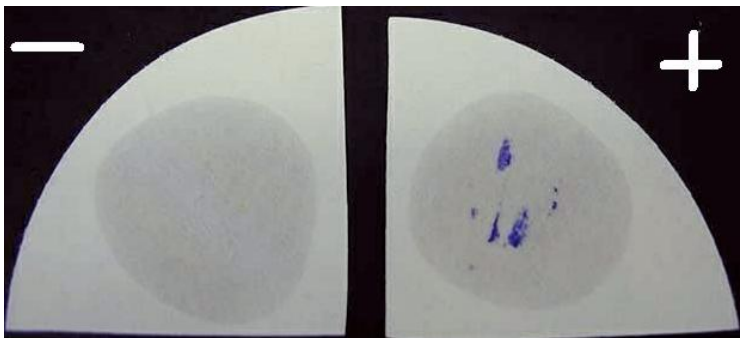

Figure 3. 6: Proteus mirabilis was oxidase negative and produced no color

3.3.3 Urease Hydrolysis

All isolates were urease-positive, converting urea into ammonia (NH3) and carbon dioxide (CO2). This reaction turned the phenol red indicator bright pink due to alkaline pH.

Figure 3. 7: Proteus mirabilis was urease positive and it converted urease agar pink from yellow

3.3.4 Tryptophan Hydrolysis

The isolates were indole-negative, differentiating Proteus mirabilis from Proteus vulgaris.

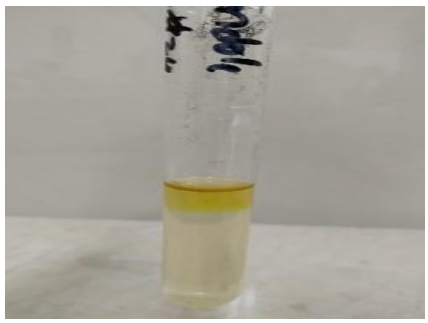

Figure 3. 8: Proteus mirabilis was indole negative

3.3.5 Triple Sugar-Iron (TSI) Agar Test Proteus mirabilis isolates tested positive for TSI, indicated by H2S gas production that blackened the medium. The slant turned red while the agar butt appeared yellow, confirming glucose fermentation and gas production.

3.3.6 Methyl Red (MR) Test All isolates were MR-positive, with a red color formation indicating stable acid production.

3.3.7 Voges-Proskauer (VP) Test Isolates tested negative for VP, as indicated by the absence of color change upon adding alpha-naphthol and KOH.

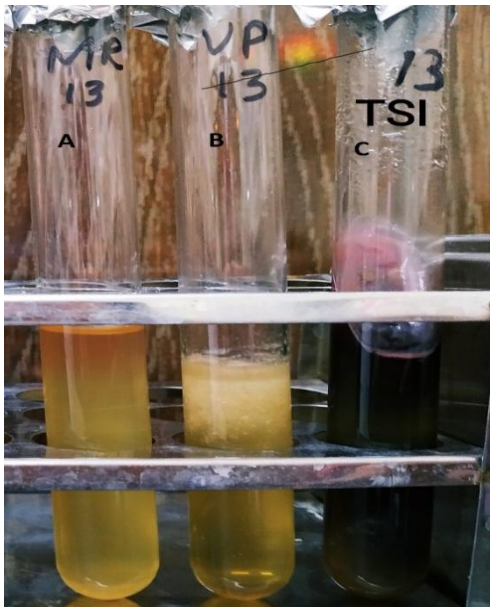

Figure 3. 9: Proteus mirabilis was MR positive (A), VP negative (B) and produce red color in TSI slant and black color exhibited production of H_2S gas (C)

Table 3.1: Proteus mirabilis Identification tests based on culture, microscopic and biochemical characteristics

Identification Tests			Proteus mirabilis
Culture Characteristics	Lactose Fermenter		-
	Swarming Motility		+
	Blood Hemolysis		γ Hemolysis
Microscopic Tests	Gram Reaction		-
	Cell Shape		Rods
Biochemical Tests	Catalase		+
	Oxidase		-
	Urease		+
	Indole		-
	TSI	Slant color	Red
		Butt color	Yellow
		H_2S Production	+
	Methyl Red		+
	Voges-Proskauer		-

3.3.8 API-20E Test

The API-20E test further confirmed Proteus mirabilis species identification. The API code was interpreted using the API web database.

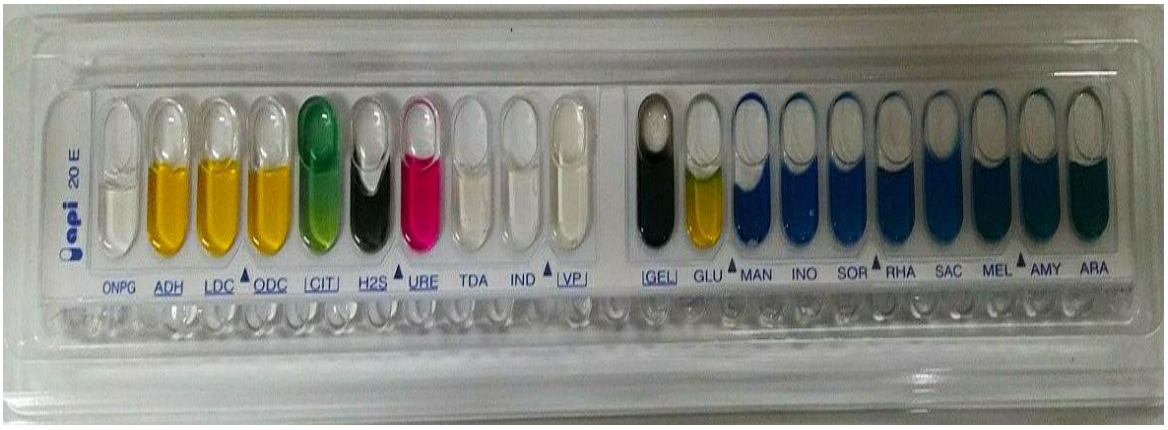

Figure 3. 10: API 20E strip confirmed the Proteus mirabilis species.

3.4 Molecular Confirmation of Proteus mirabilis

3.4.1 DNA Extraction and Quality Assessment

DNA extraction was performed using a commercial kit. The extracted DNA was evaluated using a Nanodrop spectrophotometer and agarose gel electrophoresis. Visualization under UV transillumination confirmed high-quality DNA with dense and distinct bands.

3.4.2 ureC Gene Amplification

PCR amplification targeting the ureC gene produced an expected band of approximately 533 bp, confirming Proteus mirabilis identity. All nine isolates previously identified phenotypically and biochemically were verified by PCR.

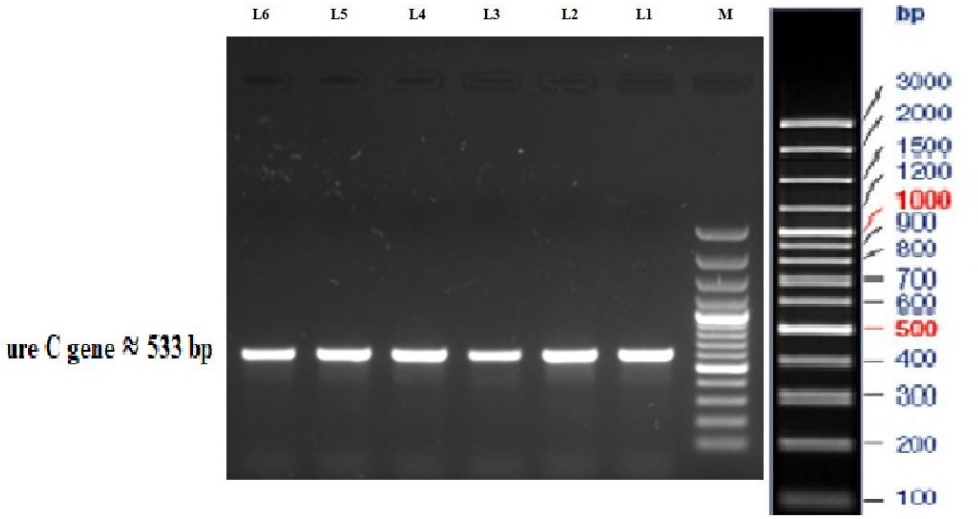

Figure 3. 11: Ethidium bromide-stained agarose gel showing ureC gene amplification.

3.5 Antibiotic Susceptibility Testing

3.5.1 Antibiograms of Proteus mirabilis Isolates

The antibiotic susceptibility pattern was determined using the Kirby-Bauer Disc Diffusion Assay. Antibiotics were selected and evaluated per CLSI (2019) guidelines. The resistance patterns of all nine isolates were analyzed and recorded.

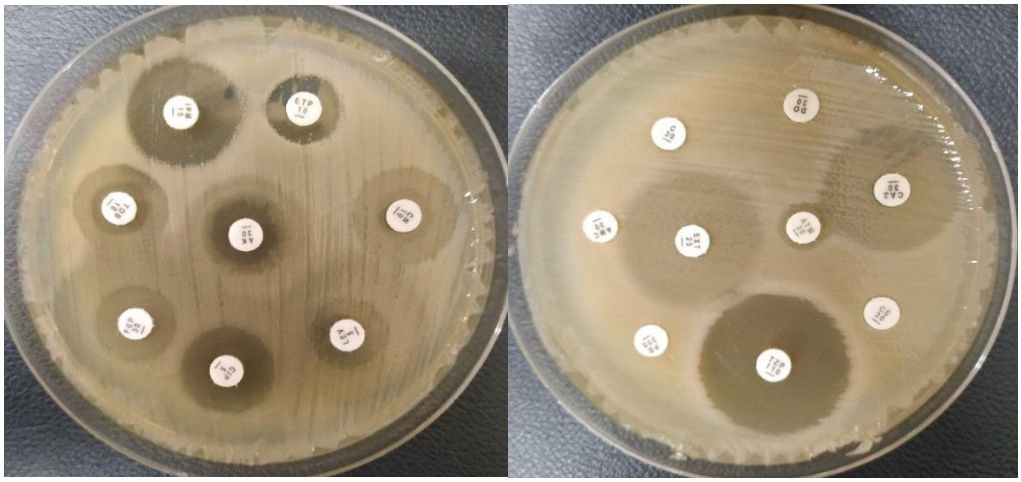

Figure 3. 12: Kirby-Bauer disc diffusion assay exhibited an antibiotic-susceptibility pattern of Proteus mirabilis.

Figure 3. 13: Inhibition zones of antibiotics were measured according to the guidelines of CLSI, 2019.

Table 3. 2: Different antibiotics and their susceptibility against Proteus mirabilis.

Isolate	Age	Gender	Specimen	AMC	SAM	FEP	CTX	CXM	CAZ	CRO	IMP	MEM	ATM	AK	CN	TOB	DO	CIP	LEV	SXT	PIP	SCF	TZP
1	65	Female	Urine	R	R	R	R	R	R	R	S	S	R	S	R	R	R	R	R	R	R	S	S
2	78	Female	Urine	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	S	S
3	45	Female	Urine	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	S	S
4	60	Female	Urine	R	R	R	R	R	R	R	S	S	R	S	R	R	R	R	R	R	R	S	S
5	49	Female	Urine	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	S	S
6	67	Female	Urine	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	S	S
7	50	Female	Urine	R	R	R	R	R	R	R	S	S	R	S	R	R	R	R	R	R	R	S	S
8	53	Male	Urine	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R	S	S
9	72	Female	Urine	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	R	R	S	S

The study successfully isolated and characterized Proteus mirabilis from ICU patient urine samples using a combination of culture, microscopic, biochemical, and molecular methods. The characteristic swarming motility on blood agar and non-lactose fermenting colonies on MacConkey agar confirmed Proteus mirabilis. Biochemical tests, including catalase positivity, urease activity, and the TSI test, further verified the identification.

Molecular confirmation using PCR amplification of the ureC gene reinforced the findings from phenotypic methods. The antibiogram results provided crucial insights into antibiotic resistance patterns, aiding in effective treatment strategies. The findings contribute to the growing body of knowledge regarding Proteus mirabilis in ICU patients, emphasizing the need for continued surveillance and antibiotic stewardship.

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