



Investigating virulence gene and sulfonamide antibiotic resistance in *Proteus mirabilis* isolated from urinary tract infections in Iran

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Abstract

Background: *Proteus mirabilis* is one of the most important causes of urinary tract infection (UTI) and an opportunistic pathogen that can cause infection, especially in the urinary tract and bladder, pyelonephritis or kidney stones, especially in people with catheters or people with abnormalities of the urinary system. The pathogenicity of this bacterium causes by several genes, and sulfonamides prevent the growth of these bacteria by inhibiting DNA synthesis. The study aimed to investigate the pathogenic genes that cause sulfonamide antibiotic resistance in *Proteus mirabilis* isolated from urinary infections in Iran.

Methods: In this study, biochemical tests were performed to confirm the identity of *Proteus mirabilis* bacteria and DNA extraction, and then multiplex PCR tests were performed to identify pathogenicity genes Fal and Urea and antibiotic resistance genes Sul1 and Sul2 using specific primers.

Results: In this research, biochemical tests confirmed the identity of the bacteria and the target strain, and multiple resistance of the *Proteus mirabilis* strain was observed to several different types of antibiotics. In addition, Multiplex PCR verified that more than 90% of the isolated strains had pathogenic genes (91.7% Fal and 95% Urea gene), and more than 50% of the strains had antibiotic resistance genes (51.7% Sul1 and 56.7% Sul2).

Conclusion: Considering the excessive use of antibiotics worldwide and the increasing spread of antibiotic resistance, it is better to check the pathogenic genes and their resistance to the antibiotics during the outbreak of the disease, consequently, the use of the antibiotics and their side effects must be reduced and to prevent the spread of antibiotic resistance.

Keywords: *Proteus mirabilis*, Multiplex PCR, Sul1, Fal, Urea, Sul2

Background

The *Proteus* genus is included in the Enterobacteriaceae family and the Proteae subfamily along with the *Morganella* and *Providencia* genera. The distinguishing feature of *Proteus* species from most other species is the swarming ability on the agar surface. They are constantly found in rotten meat, sewage, and often in human and animal feces, and they can usually be isolated from soil and vegetables. In addition to being saprophytic, *Proteus* strains are also the cause of infection in humans and animals (1). Morphology is gram-negative and facultatively anaerobic and has peri-trich flagella. On the solid culture medium, most of the cells are rod-shaped and have a length of 1-3 μm and a diameter of 0.4-0.6 μm . In liquid cultures,

the cells are short rods and about 0.6 μm in diameter and 1.2 μm in length (2). The most important feature that distinguishes *Proteus* from other members of Enterobacteriaceae is the ability to deaminate certain amino acids and convert them into ketoacids and ammonia (3). All *Proteus* strains produce glucose, acid, and mostly a small amount of gas. Unlike *Morganella* and *Providencia* strains, none of the *Proteus* strains produce acid from mannose, mannitol, adenitol, and inositol. Lactose fermentation is rare and when present it is acquired and coded for by an exogenous plasmid (4). *Proteus* strains produce several types of colonies on McConkey agar. Belyavin identified three types of these colonies, he introduced these colonies as phases. Phase A colonies are smooth colonies and have a diameter of 3-5 mm and look like hammered copper.

In terms of morphology, they include bacilli with dimensions of 0.5 micrometers x 5-6 micrometers, and they create continuous swarming in other environments (5). *Proteus* species are naturally resistant to polycationic cyclic antibiotics, namely polymyxins. Due to having positive charges in their structure, these antibiotics bind to negatively charged surfaces on the bacterial cell cover, for example, LPS, capsular antigens, and phospholipids. This problem disrupts the order of external and internal membranes of gram-negative bacteria. Polymyxin B inhibits the synthesis of α -TNT and the release of IL-1 from human macrophages, which are induced by LPS (6). In addition to UTT, *Proteus mirabilis*, and *Proteus vulgaris* can play a role as opportunistic agents in infections of the respiratory tract, wounds, burns, skin, eyes, ears, nose, and throat. In addition, they can cause gastroenteritis caused by consuming meat or other contaminated foods. The possible role of *Proteus* in causing diarrhea was investigated in the 1950s. The epidemiological and serological investigation confirmed the presence of *Proteus* or *Providencia-Morganella* strains in the feces of patients (7). Bacterial resistance is generally not considered a problem in disease pathology. But it is a limiting option in the treatment of the disease, so the strategies to solve this case should be considered according to the control of the disease and the treatment plan, as well as observing the behavior of resistance over some time and space. The amount of bacterial resistance to antibiotics is different in different regions, that's why it is necessary to have information about the pattern of antibiotic resistance of bacteria in each region and associated risk factors to increase the probability of resistance for appropriate experimental treatment. For example, in various studies, things such as age, gender, race, history of hospitalization, history of UTI, use of antibiotics to treat previous UTI prophylaxis, complicated UTI, having a urinary catheter, community-acquired urinary infection compared to the type of Hospitalization, history of taking antibiotics outside the hospital, underlying chronic disease, use of immunosuppressive drugs and history of recent surgery are considered as factors related to increasing the risk of resistance to common experimental antibiotics (8).

2. Method and materials

2.1 Types of study

This research was designed and implemented using a cross-sectional descriptive method. In this descriptive-cross-sectional study, urine samples were collected after the initial diagnosis of infection from outpatients to laboratories and patients admitted to hospitals, keeping safety points and covering the plates with parafilm.

2.2 Definition of the study population

In total, 160 non-repetitive samples were collected completely randomly from different clinical samples of people referring to Tehran hospitals. After transferring all the samples, additional biochemical tests were performed on them. These samples were cultured on Mueller Hinton agar culture media and transferred to the laboratory and cultured on Blood agar, McConkey, and Stremid agar media (Merck, Germany) and after being kept in a greenhouse at a temperature of 37 degrees Celsius for 24 Hours were examined for colony formation and bacterial growth. Cochran's statistical formula was used to

calculate the sample size. The expected number of samples was 60 *Proteus mirabilis* isolates. Because sampling is not done directly from the patient and the samples are related to microbial plates and the name of the patient will not be mentioned, therefore there was no interference in the process of diagnosis and treatment of the disease. Internet resources and authentic articles were used to order the required materials and primers. Also, the samples were collected in the laboratory and coded using culture and molecular techniques. Consumable and non-consumable materials have been used for this thesis. The isolates examined in this study were previously identified as *Proteus mirabilis* in the microbiology laboratories of the hospital, but to ensure them, cultures were prepared, and routine diagnostic tests were performed on them.

2.3 Culture media

All culture media were available in ready form and powder form. First, according to the manufacturer's instructions written on the box, a certain amount of powder was weighed and mixed with a certain amount of distilled water and completely dissolved by heat. Then the culture medium was autoclaved for 15 minutes at a temperature of 121 °C and a pressure of 15 pounds per square inch and spread in a sterile plate near the flame.

This medium was prepared by dissolving 45.3 g of cetramide agar in 1000 ml of distilled water and adding 10 ml of glycerol to it and then sterilizing it with an autoclave at 121 °C for 15 minutes. After cooling to 45 °C, it was poured into containers. This medium is used for the isolation and diagnosis of *Proteus mirabilis*.

2.4 SIM culture medium (Sulfide-Indol-Motility)

Three tests of H₂S production, indole production from tryptophan, and bacterial movement were investigated in this culture medium. Positive indole was concluded by pouring 5 drops of cox reagent on the culture for 24 hours in this environment and creating a purple color. In the case of indole-negative bacteria, no color change was obtained after pouring the reagent. The last component, i.e. positive movement, was determined by creating uniform turbidity in the cultivation environment. *Proteus* strains are motile and bacterial growth was observed as a ring around the tube.

2.5 Culture medium OF (Oxidative Fermentative)

By culturing bacteria in this environment, it can be determined that the bacteria used carbohydrates through oxidation or fermentation. Two samples were prepared from this medium, one with paraffin and the other without paraffin, and the samples were cultured in both mediums.

2.6 Oxidase test

If the bacterium has an oxidase enzyme, it converts tetramethyl-p-phenylenediamine dihydrochloride to indophenol, which is seen in purple color. 1% tetramethyl-p-paraphenylenediamine dihydrochloride solution was used. Usually, for daily consumption, 50 milligrams of powder can be poured into a small plastic container and 5 milliliters of

of distilled water can be added to it, and one vial can be used every day.

2.7 Maintenance of approved strains

After confirming the collected strains by various biochemical tests, these strains should be kept safely for the next stages of the project, for this purpose, two methods were used (A- short-term method and B- Long-term method).

2.8 Antibiotic discs and MIC test

Antibiotic susceptibility profile on Mueller Hinton Agar medium (Merck, Germany) and using disk diffusion method for antibiotics including piperacillin (PRL-100 µg), piperacillin-tazobactam (TPZ-10-100 µg), ticarcillin-clavulanate (TIM 10.75 micrograms), ceftazidime (CAZ-30 micrograms), cefepime (CPM-30 micrograms), aztronam (ATM-30 micrograms), imipenem (IPM-10 micrograms), meropenem (MEN-10 micrograms), gentamicin (GM-10 micrograms), and ciprofloxacin (CIP-5 micrograms) was obtained from Mast, England. Based on the provided guideline, *Proteus mirabilis* strains are insensitive to at least one agent in three or more antibiotic categories; the test was performed according to standard laboratory and clinical guidelines.

McFarland standard is used to check the effect of antimicrobial substances from antimicrobial suspension with appropriate density. For example, a 0.5 McFarland tube contains approximately 1.5×10^8 cfu/ml of bacteria.

Minimum inhibitory concentration (MIC) bacteria were grown in a broth medium.

Above antibiotics were used to evaluate the antibacterial effects on *Proteus mirabilis*. Concentrations were determined by broth micro-dilution technique in a sterile 96-well plate. A volume of 100 µL of nanoparticles synthesized at a concentration of 20 mg/mL was placed into the first well of the microtiter plate that contained 100 µL NB medium to obtain a concentration of 10 mg/mL. Serial dilution was performed by diluting the contents of the first well and removing 100 µL and adding to the second well.

Gram staining was performed according to Hucker's method. Also, to confirm the staining result, a 3% KOH test was performed. The microscopic image was detected using a 100-light microscope lens.

2.9 PCR tests

To prepare the necessary biomass for genomic DNA extraction, fresh bacterial culture was used on the plate. Fresh bacterial colonies were collected from the surface of the plate with a sterile loop and dissolved in the primary buffer. At first, Wilson's modified method was used to extract the genomic DNA of the strains. Due to the high toxicity of phenol and the difficulty of working with it in existing laboratory conditions, this method was modified in such a way that there is no need to use phenol. PCR test stands for polymerase chain reaction (PCR). This means that in laboratory conditions, millions of copies of one or more genes are made from DNA. The used primers and PCR program are listed in Tables 5 and 6, respectively.

3. Results

3.1 Isolation examples of *Proteus mirabilis*

As a result of screening 160 clinical samples in the laboratory, a total of 60 suspected *Proteus* colonies were isolated, which were identified as *Proteus mirabilis* based on morphological, microscopic, and biochemical tests (fig1). This means that in laboratory conditions, millions of copies of one or more genes are made from DNA. The used primers and PCR program are listed in Tables 5 and 6, respectively.



Figure 1. *Proteus mirabilis* colony

3.2 Identification based on morphological and microscopic characteristics

The colony of isolates suspected to be *Proteus mirabilis* was compared with the standard colonies taken from the microbial bank and also examined under the microscope, and the results indicated that the isolates were *Proteus mirabilis*.

3.3 Identification based on biochemical tests

The biochemical tests mentioned in the previous chapter were carried out for the biochemical confirmation of the genus *Proteus mirabilis*. The results are given in Table 1.

Table 1. The result of biochemical tests to identify <i>Proteus mirabilis</i>	
Test Done	Test Result
Beta-galactosidase	-
Cytochrome Oxidase	-
Gelatinase	+
Lysine Decarboxylase	-
Ornithine Decarboxylase	+
Tryptophan Deaminase	+
Urease	+

3.4 Antibigram and MIC test results of *Proteus* strains

The results of the antibiogram of *Proteus* strains isolated from patients with urinary tract infections showed that the highest level of resistance in these strains to ampicillin was 100%, and the lowest level of resistance to amikacin was 16% (Table 2).

Table 2. Examining the sensitivity of bacteria to antibiotics										
Antibiotics	AMP	AMX	CAZ	CTX	TE	CIP	AK	GN	TOB	TS
Proteus strains	98	80	48	40	80	32	100	52	48	56

AMP= Ampicillin, AMX= Amoxicillin, CAZ= Ceftazidime, CTX = Cephataxime, TE = Tetracycline, CIP = Ciprofloxacin AK= Amikacin, GN= Gentamycin, TOB= Tobramycin, TS= Co-trimoxazole

To investigate the antibiotic resistance of the strains, the antibiogram was performed by disc diffusion method and the percentage of sensitivity and resistance of all the strains to the antibiotics in question was determined. The results can be seen in the corresponding Table 2. As inferred from the table and graphs; the highest bacterial sensitivity was observed in amikacin (100%) and ampicillin (98%) antibiotics.

Table 3. Examining the minimum grain resistance of bacteria to antibiotics										
Antibiotics	AMP	AMX	CAZ	CTX	TE	CIP	AK	GN	TOB	TS
Proteus	>64	>64	>32	32	>64	32	64	>32	32	>32

AMP= Ampicillin, AMX= Amoxicillin, CAZ= Ceftazidime, CTX = Cephataxime, TE = Tetracycline, CIP = Ciprofloxacin AK= Amikacin, GN= Gentamycin, TOB= Tobramycin, TS= Co-trimoxazole

3.5 Multiplex PCR test results

The results of the multiplex PCR test for the presence of pathogenicity genes: *Fal* and *Urea* and antibiotic resistance genes: *SulI* and *SulII* of the isolated *Proteus mirabilis* isolate can be seen in Figures 2- 3 and table 4. Also, the frequency of genes can be seen in the table and graph below. Distilled water was used as a negative control and the tested samples were used as a positive control to check the contamination in this PCR reaction research.

Table 4. Frequency of genes examined in this study		
Gene	Number	Percentage
Fal	55	91.7
Urea	57	95
SulI	31	51.7
SulII	34	56.7

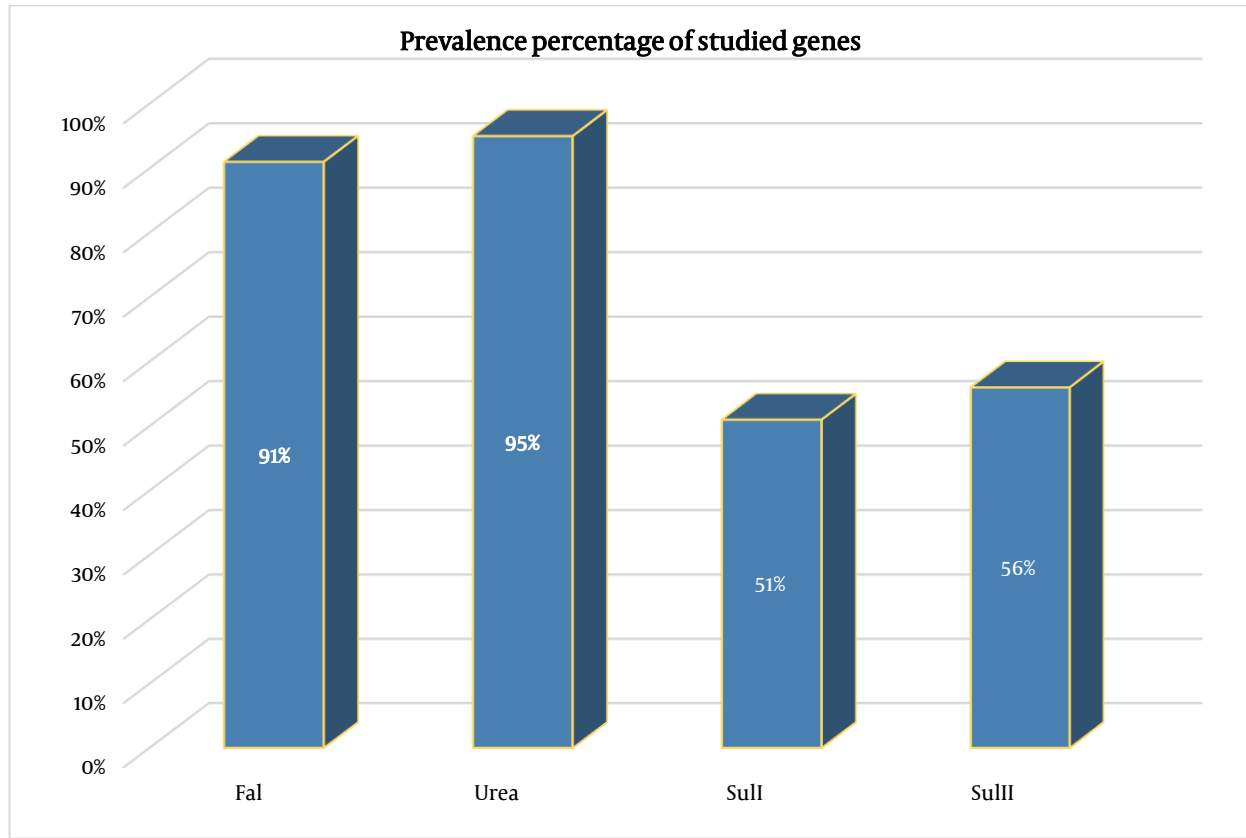


Figure 2. Prevalence percentage of Fla, Urea, Sul1 and Sul2 gene

Table 5. Primers used to detect pathogenicity and antibiotic resistance in *proteus mirabilis*

Primer length (bp)	Oligonucleotide sequence (' 3 ' → 5)	Gene name
362	F:5'- GATCTGGCGACATAATCGT - 3'	Urea
	R:5'- TCACCGGGGATCATGTTATT-3'	
417	R:5'- TCACCGGGGATCATGTTATT-3'	flaA
	R:5'- CGGCATTGTTAATCGCTTTT-3'	
Primer length (bp)	Oligonucleotide sequence (' 3 ' → 5)	Gene name
433	F - CGGCGTGGGCTACCTGAACG	SULI
	R - CGGCGTGGGCTACCTGAACG	
293	F - GCGCTCAAGGCAGATGGCATT	SULII
	R- GCGTTTGATACCGGCACCCGT	

Table 6. The schedule of temperature and the number of cycles in PCR

Stage	Time (minutes)	Temperature(°C)	The Number of Cycling
Pre-denaturation	3	95	1
Denaturation	0.5	95	35
Annealing	0.5	54	
Extension	1	72	
Final extension	5	72	1

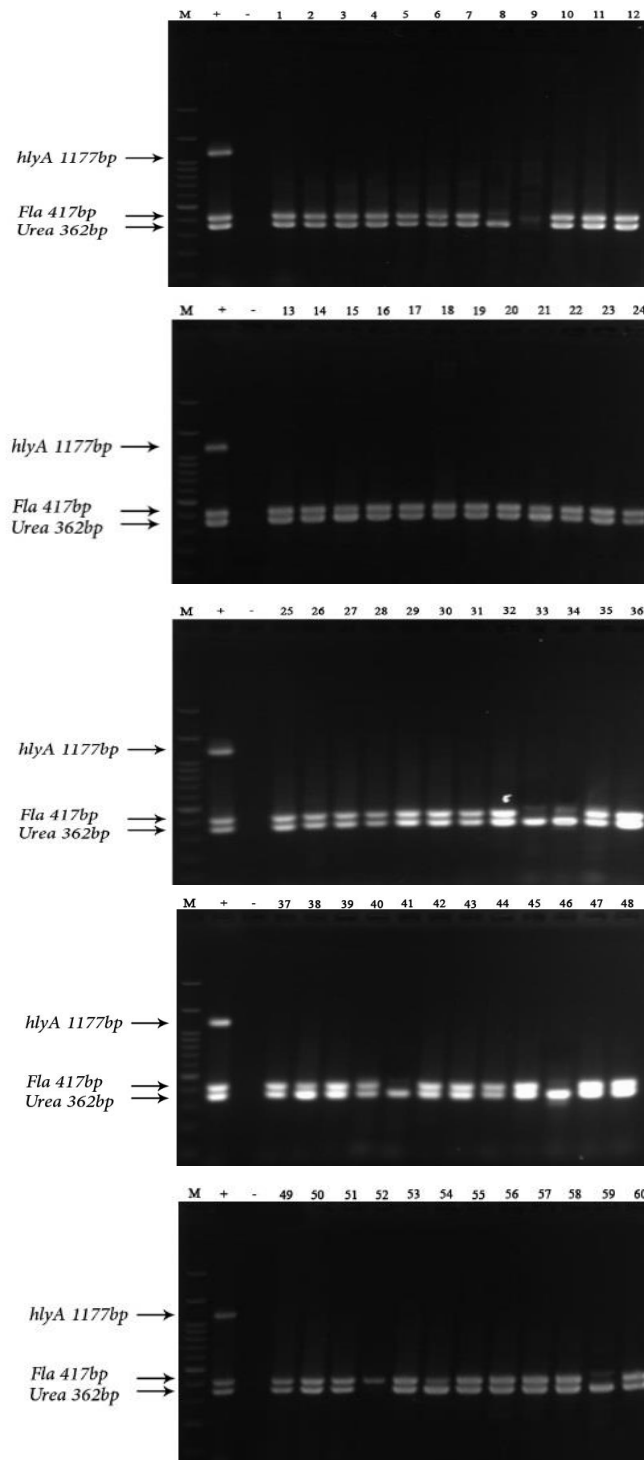


Figure 3. The result of checking the frequency of pathogenic genes by PCR test using Fla, Urea primers. Columns from left to right: M marker, positive control, negative control samples 1 to 60.

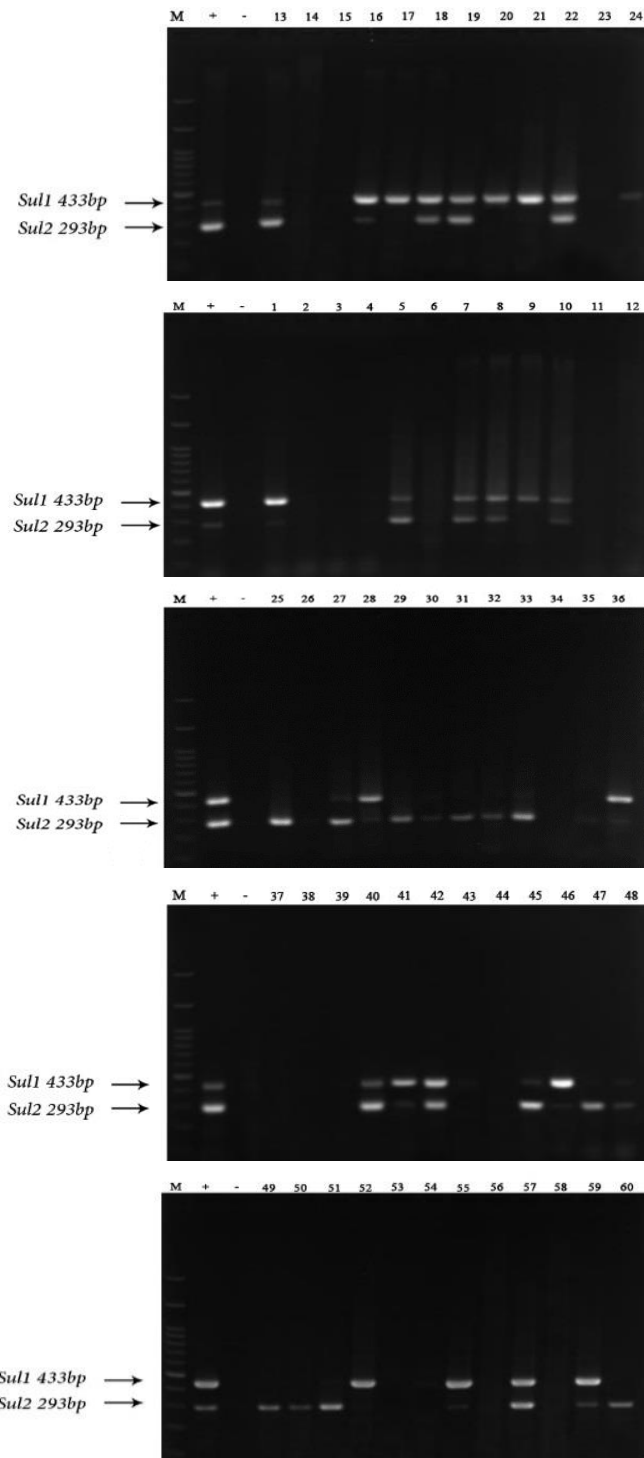


Figure 4. The result of checking the frequency of antibiotic resistance genes by PCR test using *SulI* and *SulII* primers. Columns from left to right: M marker, positive control, negative control samples 1 to 60.

4. Discussion

Proteus mirabilis is a gram-negative, aerobic, and facultative anaerobic bacillus with flagella. And they are not able to ferment lactose, the production of sugar fermentation gas is similar to salmonella, but unlike salmonella, they can break down Urea (18).

This bacterium has an active movement and can move, due to peritrichous flagella. One of the prominent characteristics of *Proteus* is the swarming movement on the solid culture medium. When a strain of *Proteus* is cultured in two spots on a plate, the bacteria had mixed growth. If two strains of the same species of *Proteus* are cultured on the surface of a plate, the propagation of *Proteus* is not mixed and a narrow space that is easily visible separates the two *Proteus*. The occurrence of this phenomenon is called Dines, which is used to identify similar and non-similar strains in epidemiology studies. This bacterium is very resistant and therefore can cause bladder, and kidney infections and even penetrate the bloodstream.

Proteus mirabilis can be found in different environments including soil, water sources, and sewage, but it is mainly found in the digestive system of humans and animals.

Urinary tract infections (UTIs) are recurrent bacterial infections in humans and include about 20% of infections outside the hospital. Approximately 90% of urinary tract infections are progressive, which means that the bacteria have reached the upper parts of the urinary tract through the bladder (19).

Many urinary infections are caused by an underlying disease in the urinary system. The susceptibility of the host, the presence of structural abnormalities in the urinary system, and the pathogenicity of microorganisms are among the most important primary factors in the occurrence and recurrence of UTI (20). *Proteus mirabilis* is the second most common pathogen after *Escherichia coli* which causes urinary tract infections. Sulfonamides are synthetic antibiotics that are widely used in the treatment of microbial infections. By inhibiting DNA synthesis, sulfonamides stop the growth of bacteria (bacteriostatic) (21).

But today, due to the indiscriminate use of antibiotics, inappropriate prescription, insufficient dosage, and non-observance of the treatment period, the strains of *Proteus mirabilis* that are involved in hospital infections have multi-drug resistance to different antibiotics all over the world which increases day by day.

The results obtained in this study are consistent with the results of the study by Shikh-Bardsiri et al. To investigate the antibiotic resistance of the strains, an antibiogram was performed by disk diffusion method and the percentage of sensitivity and resistance of all the strains to the antibiotics in question was determined. The highest rate of sensitivity is to amikacin (100%) and ampicillin (98%) (22). Also, 10% of the strains were resistant to ciprofloxacin. In the study of Shaibani et al., among 88 hospital isolates of *Proteus*, 67% had the highest resistance to ceftriaxone and the least resistance to chloramphenicol (46.5%). Moreover, the isolated *Proteus* had lower MIC to ciprofloxacin and cefotaxime antibiotics. These researchers stated that the reason for the lower resistance of the isolates to chloramphenicol may be related to the lack of routine use of this drug in the hospital. As a result, microbes have less contact with this drug and less resistance occurs (23). Antibiotic resistance is both intrinsic and acquired. In natural resistance, a natural or wild cell is capable of inhibiting antibiotics and has a chromosomal origin; while acquired resistance is caused by the exposure of sensitive and natural

populations to various factors and the transformation of sensitive strains into resistant ones (24).

Resistance genes in bacteria are carried out by elements called integrons. Integrons are mobile genetic elements that by being placed in plasmids, chromosomes, and transposons, carry and move resistance genes while they are inside gene cassettes. The horizontal transfer of integrons is proposed as the most successful way of spreading resistance genes and the emergence of multiple resistance species (25); therefore, the importance of identifying this type of resistance gene is very necessary.

By considering the increase in resistance to sulfonamides and integron-dependent drug resistance in *Proteus*, and its pathogenic significance, our aim in this study is to simultaneously investigate sulfonamide and integron resistance genes in *Proteus mirabilis* isolated from urine samples. By multiplex PCR method and their antibiotic resistance pattern. Here, all the examined samples were examined using biochemical tests and confirmed the *Proteus mirabilis* according to the diagnostic standards. After biochemical tests, DNA extraction and then Multiplex PCR tests were performed to identify pathogenic genes to investigate the presence of Fal, Urea, and antibiotic resistance genes as well as Sul1 and Sul2 genes in *Proteus mirabilis*. For this purpose, the specific primers of the genes were recruited and the presence of these four genes was confirmed. In addition, the pathogenicity and antibiotic resistance genes in *Proteus mirabilis* were determined. All in all, the existence of Fal and Urea genes and their role in the expression of other pathogenic factors in the P19 wild strain of *Proteus mirabilis* and other mutants were studied (26).

In 2014, Molazadeh et al. investigated the antibiotic pattern of Gram-negative bacteria, including *Proteus*, isolated from the urine samples of patients hospitalized in different departments of Hazrat-e Vali-asr Hospital, and showed that there is a significant relationship between bacterial frequency and gender ($P=0.006$). In this study, the antibiotics cefotaxime and amikacin were recognized as the most effective drugs for the treatment of the majority of patients with urinary tract infections (27).

In this study, the sensitivity and resistance of *Proteus mirabilis* bacteria to antibiotics were investigated. The results of this study were based on the fact that more than 90% of the isolated strains had pathogenic genes associated with Fal (91.7%) and Urea (95%) genes, and more than 50% of the strains had antibiotic resistance genes related to Sul1(51.7%) and Sul2 (56.7%).

According to the results of the multiple resistance of the *Proteus mirabilis* strain to several different types of antibiotics observed in this research, the overuse of antibiotics is confirmed, worldwide. It is better to avoid the overuse of antibiotics to prevent the spread of drug resistance.

Genes for resistance to sulfonamides are usually transmitted by a plasmid, the first Sul gene is coded by three gene fragments Sul1, Sul2, and Sul3, and the previous studies showed that the prevalence of Sul2 is higher than Sul1 in human cases (28,29,31). In this study, it was also determined that the most abundant sulfonamide resistance gene in these strains is the Sul2 gene.

The results of the present study showed high resistance of *Proteus mirabilis* to antibiotics, therefore careful medical care and correct and timely use of appropriate antibiotics are necessary to prevent the spread of resistant strains.

5. Conclusion

Given the high per capita consumption of antibiotics around the world and the significant increase in multiple antibiotic resistances, it is better to avoid the use of antibiotics as much as possible, and then considering the hospital infections and common infections, first check on the genes of the disease must be undertaken. The generation and resistance of that strain should be confirmed and then, the sensitivity and resistance of the bacteria to antibiotics must be determined. It should be kept in mind that a course of treatment must be done with the most effective antibiotics and fewer side effects; because the genes can be transferred horizontally from the resistance strain to other strains prevent. To prevent the wide spread of the resistant gene in an environment this point should be always considered in healthcare systems.

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