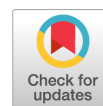


MOLECULAR INVESTIGATION OF *PROTEUS VULGARIS* VIRULENCE GENES FROM ASYMPTOMATIC BACTERIURIA IN PREGNANT WOMEN



S. Ghali Jabur

University of Thi-Qar, Thi-Qar, Iraq

Abstract. *Background.* It is a well-known bacterium *Proteus vulgaris* that causes urinary tract infections specifically bacteriuria. This study aimed to genetically search for the role of some virulence genes in this bacterium via bacteriuria in pregnant women of the Thi-Qar Governorate of Iraq. *Materials and methods.* A mid-stream urine specimen with a total of 235 specimens was gleaned from pregnant women, which were performed in a random manner from various hospitals, when 116 cases suffered from UTI (patient group). The others (119 specimens) were collected from married women, but not pregnant women, and apparently in healthy cases (control group). MacConkey agar and blood agar were used for culturing and identification of bacteria under aerobic conditions, followed by microscopic examination, appearance description, biochemical test, API 20E analysis, and PCR analysis. *Results.* Genetic analysis revealed bacteriuria in 39 (33.6%) pregnant women infected with *Proteus* sp., but it stooped to 3 (2.5%) in apparently healthy women (control). When phenotypically and genotypically with specific identification genes of *P. vulgaris* the same samples were infected with this bacterium in eight (6.8%), while it receded to 1 (0.84%) in the control. The PCR and statistical analyses demonstrated that the total existence of each study gene (*ureC*, *mrpA*, and *zapA*) in bacteriuria isolates of *P. vulgaris* was 11/9 (122.2%), 9/9 (100%) and 9/9 (100%), respectively. There was significant variation in the position of *zapA* on chromosomes 8/9 (88.8%) and on the plasmid that was dropped to 1/9 (11.1%). *Conclusion.* The role of these bacteria is serious and significant in pathogenicity and attachment to the epithelial tissues of the urinary tract, the opportunistic nature of *P. vulgaris*, biofilm production, and elevated urine pH, which lead to stone production in pregnant women. PCR is the fastest and most accurate method for diagnosing *P. vulgaris* and its virulence genes.

Key words: *Proteus vulgaris*, PCR, bacteriuria, virulence genes, asymptomatic bacteriuria, urinary tract infections.

МОЛЕКУЛЯРНОЕ ИССЛЕДОВАНИЕ ГЕНОВ ВИРУЛЕНТНОСТИ *PROTEUS VULGARIS* ПРИ БЕССИМПТОМНОЙ БАКТЕРИУРИИ У БЕРЕМЕННЫХ ЖЕНЩИН

Гали Джабур С.

Университет Ти-Кар, г. Ти-Кар, Ирак

Резюме. *Введение.* *Proteus vulgaris* является хорошо изученной бактерией, которая вызывает инфекции мочевыводящих путей (ИМП), в частности бактериурию. Целью настоящего исследования было проведение генетической оценки роли ряда генов вирулентности *Proteus vulgaris* у беременных женщин с бактериурией в провинции Ти-Кар, Ирак. *Материалы и методы.* Исследование включало изучение 235 образцов средней порции

Адрес для переписки:

Санаа Гали Джабур
Научный колледж Университета Ти-Кар, г. Ти-Кар, Ирак.
Тел./факс: +9647810510523.
E-mail: medicalresearch68@yahoo.com; sanaaghali@sci.utq.edu.iq;
sanaagali12345@gmail.com

Contacts:

Sanaa Ghali Jabur
College of Science, University of Thi-Qar, Thi-Qar, Iraq.
Phone/fax: +9647810510523.
E-mail: medicalresearch68@yahoo.com; sanaaghali@sci.utq.edu.iq;
sanaagali12345@gmail.com

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мочи, собранных от 116 беременных женщин с ИМП (группа пациентов) случайным образом из разных больниц. В группу контроля (119 образцов) вошли замужние, не беременные, практически здоровые женщины. Агар Макконки и кровяной агар использовались для культивирования и идентификации бактерий в аэробных условиях с последующим микроскопическим исследованием, описанием внешнего вида, проведением биохимического теста, анализа на стрипе API 20E и ПЦР. **Результаты.** Генетический анализ выявил бактериурию у 39 (33,6%) беременных женщин, инфицированных *Proteus* sp., которая была обнаружена в 3 (2,5%) случаях у практически здоровых женщин (контроль). При фенотипическом и генотипическом исследовании с использованием специфических идентификационных генов *P. vulgaris* она была выявлена в тех же образцах в восьми (6,8%) и одном (0,84%) образце в группе пациентов и в контроле соответственно. ПЦР и статистический анализ показали, что общее наличие каждого изучаемого гена (*ureC*, *mrpA* и *zapA*) в изолятах бактериурии *P. vulgaris* составило 11/9 (122,2%), 9/9 (100%) и 9/9 (100%) соответственно. Отмечена достоверная вариация в положении *zapA* на хромосомах 8/9 (88,8%) и плазмиде, которая снизилась до 1/9 (11,1%). **Заключение.** *P. vulgaris* играет важную роль в патогенности и прикреплении к эпителиальным тканям мочевыводящих путей, условно-патогенной активности, образовании биопленки и повышенном pH мочи, что приводит к образованию камней у беременных женщин. ПЦР является самым быстрым и точным методом диагностики *P. vulgaris* и его генов вирулентности.

Ключевые слова: *Proteus vulgaris*, ПЦР, бактериурия, гены вирулентности, бессимптомная бактериурия, инфекции мочевыводящих путей.

Introduction

In general, women are more susceptible to different diseases than men, specifically in developed countries; among the most important is asymptomatic bacteriuria (ASB), which is a common finding clinically without symptoms or signs of infection of a urinary route [34]. It occurs in approximately 20% of women throughout lifetime [13]. The average incidence of ASB infection in females that being associated with sexual vitality and pregnancy. Most women are at a risk of ASB related to the urethral stumpy nature (which causes bacteria to be released from the urethral stream and perineum), and some social agents that contain hygiene of person and sexual vitality [13]. Most women are infected with transient bacteriuria, when a small number of women develop infections of symptomatic bacteriuria for the body's mechanisms of ordinary defense block infection of symptomatic bacteriuria in more status [2]. However, there are specific conditions via pregnancy, the main adjustment event in the mother's immune regulation to secure the mother and her future infant from infection by pathogens while averting harmful immune restraint versus allogeneic fetal irritation, and how definite physiological, endocrinological, and immunological agents raise the risk of infection demands careful consideration. For example, urinary route infections are more common [1].

Bacteria are the fundamental causative factors of bacteriuria. Bacteria of the family *Enterobacteriaceae* are common in UTIs, and the essential pathogenic isolation is uropathogenic (UPEC) [8, 9, 22, 23]. *Proteus* spp. are regarded as the main *Enterobacteriaceae* and uropathogenic bacteria that cause urinary tract infections (UTIs), specifically (ASB) after *Escherichia coli* and *Staphylococcus* spp. [22, 37]. It occurs in 18 percent of patients with bacteriuria. The maximum number of isolates were

obtained from patients in the hospital. *P. vulgaris* is famous for its behaviorism to cause urinary tract infections, and mainly causes infections in people with a weak immunity system, and its plurality can cause complications in the urinary tract, wounds, and nosocomial infections [24, 25]. *Proteus vulgaris* is a member of the *Enterobacteriaceae* family and is gram-negative, rod-shaped, and motile. It is more widespread and ordinary; therefore, it gives a Latin name meaning [31]. It is a popular opportunistic pathogen in humans that can be present in fecal matter, soil, and water [12, 36].

The pathogenesis of *Proteus* sp. bacteria occurs because of their capacity to produce a variety of virulence factors, such as biofilm formation, adhesion molecules, enzymes such as proteases and urease, and siderophore factors and agents of toxins [10, 20, 25]. The genetic agent that controls urease enzyme production is the ure operon, where the major gene of urease production is *ureC*, but operon genes of protease production are zap genes, specifically, *zapA*, which acts to organize the expression of IgA protease via swimmer cell differentiation to swarmer cells [33].

Among other virulence agents, various fimbriae have been identified in *Proteus* sp. Four types of genetic fimbria sites are more interest and accountable for the pathogenicity and the cohesion of *P. vulgaris* to urinary epithelium tract (*mrp*, *mrkA*, *uca* and *atf*) [21]. The vigor type is MR/P fimbria encrypted by *mrp* (*A*, *B*, *C*, *D*, *E*, *F*, *G*, and *I*) genes, but the *mrpA* gene is regarded as significantly remarkable in terms of pathogenicity because it inputs copious virulent agents such as the attachment of bacteria to the epithelium tissue, biofilm figuration, and swarming [26, 33].

All virulence factors are encoded by specific genes on the chromosomes or plasmids of bacteria or other genetic elements that are acquired by bacteria. Plasmids are universally present in bacteria and play key roles in the dissemination of genes such as antibi-

otic resistance and virulence factors [14]. Horizontal gene delivery of all virulence genes takes place by a plasmid from one bacterium to another, which is related to different species residing in the same niche, such as in the gastrointestinal tract [17, 29].

Audience education with immediate monitoring of (asymptomatic bacteriuria) is important for decreasing the risk of future complications of reproductive health. In addition, there is little information about *P. vulgaris* in UTI infection in general, and regarding pregnant women, particularly in Iraq, especially in the Thi-Qar province where infections in pregnancy cases should be given special attention, because there is a risk of vertical transmission to the fetus, and adverse events can occur (preterm birth, and others). Polymerase chain reaction (PCR) is the most rapid and sensitive technique [26]. Based on what was mentioned above, the investigation aimed to determine the percentage of *P. vulgaris* percentage including its virulence gene location, in pregnant women infected with asymptomatic bacteriuria in the Thi-Qar province of Iraq by molecular method.

Materials and methods

Ethical approval. The Medical Ethical Committee of the Pathological analysis department, Collage of Science, University of Thi-Qar approved this study (No. 015 on 12/2/2022). All the women involved in this study signed informed consent forms.

Collection of Samples. A mid-stream urine specimen with a total of 235 specimens was gleaned from women in case of pregnancy, which were performed in a random way from various hospitals of Thi-Qar or called (Nasirya city) in Iraq, when 116 cases had UTI (patient group). The others (119 specimens) were collected from married women, but not pregnant women, and apparently in healthy cases (control group). Specimens were collected at the AL-Habboby Hospital and Al-Hussein Teaching Hospital, from beginning January 2023 until the beginning of April 2024. The specimens were immediately transferred to the microbial identification laboratory-pathological analysis department/science faculty by cary blair (transport media). MacConkey agar and blood agar were used for culturing and identification of bacteria under aerobic conditions and temperature 37°C for 24 h. Microscopic examination, appearance description, biochemical tests, and API 20E analysis were performed by PCR analysis [11].

Isolation and identification of bacteria. *Proteus* spp. diagnosis was conducted in [7, 33].

Phenotypic identification of virulence criteria

Urease production identification test. Christensen's Urea Agar was employed to identify the ability of bacteria to produce the enzyme of urease, which takes place by a heavy inoculum (of pure culture of bacteria about 18- to 24-hour incubation) was used to streak on the entire slant surface of this agar without stab-

bing the butt, which was regarded as a color control. The tubes were Incubated at 35°C in loosened caps. The slant was observed to indicate the change in color at 6 h, 24 h, and every day for six days, the Diagnosis of urease production is by fuchsia color or called (bright pink) formation on a slant that may protracted until the bottom; in addition, any grade of pink was set as a positive reaction where the protracted incubation period may lead to a forged positive product because protein hydrolysis occurred in the culture medium, and the same medium without urea was used as the control medium to avoid protein hydrolysis that caused a positive test [3, 11].

Protease production detection test. Protease production was achieved according to the methods of [4]. The skimmed milk agar was used to examine the ability of the study [8] isolate to produce protease, where each isolate was inoculated on the superficies of plate of this medium, at 37°C for about 24–48 h, and the pates were incubated with inverted it. A specific zone surrounding the line of growth was observed, indicating successful results.

The fimbriae production test. This test is called resistant to mannose-hemagglutination analysis (MRHA). It was made by a conglomerate of red cells with fimbriae produced in bacteria and D-mannose occurring by hemagglutination in bacteria in direct tests, which include the slide method and resistance in mannose hemagglutination tests. Bacterial isolates were inoculated into nutrient broth at approximately 1%, followed by incubation at 37°C for 48 h for full-fimbriation. Erythrocytes were chosen by obtaining the blood of humans specifically ("O" group), which was rinsed thrice with normal saline, and then fresh saline was used to make a 3% suspension. They were employed immediately and within a week, when storage accrued within (3–5°C). Approximately one drop of blood erythrocyte suspension was added to an acollyrium of the broth culture, and then a slide was shaken at room temperature for approximately 5 min. The hemagglutination of sensitive to mannose was identified by the hemagglutination was absence in a parallel set of analysis in that an acollyrium of two percent D-mannose was supplemented to an acollyrium of broth culture and red cells when hemagglutination resistant Mannose was diagnosed by the carried out of hemagglutination in three percent RBC specifically group "O" of human and in 2% mannose presence. Tannic acid bacterial agglutination of treated human erythrocytes (agglutination of MR/K) was performed using a previously described method to reveal the manifestation of type 3 fimbriae [26].

Molecular identification. The total isolates of *Proteus* sp. that appeared from the biochemical investigation were the target of molecular identification by PCR with primers specific for the 16S rRNA gene of *Proteus* sp. and *ureC* gene of *P. vulgaris* [6] (Table 1) for confirmed identification of *P. vulgaris* where extraction and purification of bacterial DNA were per-

Table 1. The primers that used in study

Proteus Isolates gene	Primer	Primers sequence 5'→3'	Product size bp	T(°C) of annealing	Reference
<i>ureC</i> specific identification gene of <i>P. vulgaris</i>	F	GTTATTCGTGATGGTATGGG	317	56.2	34
	R	ATAAAGGTGGTTACGCCAGA			
16SrRNA specific identification gene of <i>Proteus</i> sp.	F	GGAAACGGTGGCTAATACCGCATAAT	101	60	22
	R	GCAGCGCTAGGTGAGCCTAATGGG			
<i>mrpA</i>	F	ACACCTGCCCATATGGAAGATACTGGTACA	550	40	34
	R	AAGTGATGAAGCTTAGTGATGGTGTGGTGTGATGAGAGTAAGTCACC			
<i>zapA</i>	F	ACCGCAGGAAAACATATAGCCC	540	59	5 and 34
	R	GCGACTATCTCCGCATAATCA			

formed using Genomic DNA (Trans/Korea), and the total nine isolates of *P. vulgaris* were extracted and purified using plasmid mini kit (Trans/Korea) [19, 32] and chromosomal DNA mini kit (Trans/Korea) to the DNA of the chromosome and plasmid of each isolate, followed by PCR for identification of virulence genes using the primers listed in Table 1.

These genes were multiplied by PCR operation in an aggregate volume (50 µl) with the mode demonstrated in the study of [28]. The PCR conditions for all genes in the study are listed in Table 1 and 2, taking into account that the conditions are the same for all genes except the annealing temperature. The multiplying samples were analyzed on an agarose gel containing red stain in electrophoreses to clarify the PCR outputs.

The PCR steps for PCR the 16 srRNA diagnostic gene specific to *Proteus* sp. Were as followed: the initial denaturation at 94°C for 30 s in one cycle. The samples were then denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s for 30 cycles. The final extension 72°C for 10 min for one cycle. Then hold at 4°C forever.

Statistical analysis. The data were processed and analyzed using SPSS (Statistical Package for Social Sciences) version 23 for Windows (SPSS Inc., Chicago, IL, USA). The qualitative results are presented as frequencies and comparative percentages. The (χ^2) Chi square test was employed to account for the difference between two or more groups, and a P value < 0.05 was considered significant.

Results

Phenotyping and genotyping identification of *P. vulgaris*. The molecular analysis of the study indicated that the number and rate of bacteriuria samples infected with *Proteus* sp. in pregnant women were 39 (33.6%) when that in the control (apparently healthy women) was dropped to 3 (2.5%). However, the phenotypically and genotypically with specific identification (*ureC*) of *P. vulgaris* showed that the number and percentage of samples infected with *P. vulgaris* in the pregnant women were 8 (6.8)%, but it decreased to 1 (0.84)% in the control (apparently healthy women) (Table 2).

Urease production. The phenotypic analysis of the study expounded that the urease production ratio in the total investigation isolates of *P. vulgaris* was 9/9 (100%).

Protease production. All the study isolates in 9/9 (100%) showed phenotypic ability to produce protease enzymes (Table 3).

Fimbriae production. Analysis of the virulence characteristics of the bacteria in the study isolates proved that all of these isolates were capable of producing fimbriae 9/9 (100%), with no significant differences between the isolates of pregnant women infected with bacteriuria disease 8/8 (100%) and the control isolate (apparently healthy women) 1/1 (100%) (Table 4).

Genotyping identification of virulence gene. The PCR technique used in the study showed that the total percentage of each virulence gene in the study (*ureC*, *mrpA*,

Table 2. Number and percentage of *P. vulgaris* isolated from pregnancy women

Patient sample	Number of sample	Number and percentage of sample infected with <i>Proteus</i> sp.	Number and percentage of sample infected with <i>P. vulgaris</i>
Pregnancy women	116	39 (33.6)	8 (6.8)
Apparently healthy women (control)	119	3 (2.5)	1 (0.84)
Total	235	42 (17.8)	9 (21.4)
P. value = 95 Df = 1	CalX ² = 0.035 Tab.X ² = 3.84	CalX ² = 30.82 Tab.X ² = 3.84	CalX ² = 5.4 Tab.X ² = 3.84

Table 3. Phenotyping identification of virulence factors in *P. vulgaris* isolated from bacteriuria sample in pregnancy women

Sample source	Total number of isolates	fimbriae production	Protease production	Urease production
Pregnancy women	8	8/8 (100)	8/8 (100)	8/8 (100)
Apparently healthy women (control)	1	1/1 (100)	1/1 (100)	1/1 (100)
Total	9	9/9 (100)	9/9 (100)	9/9 (100)
P. Value = 95 Df = 1		CalX ² = 3.6 Tab.X ² = 3.59		

Table 4. Comparison number and percentage of virulence gene in chromosome and plasmid of *P. vulgaris* isolated from bacteriuria samples from pregnancy women

Genetic site	Total number of isolates	Number and percentage of isolates appeared gene		
		<i>ureC</i>	<i>mrpA</i>	<i>zapA</i>
Chromosome	9	9/9 (100)	6/9 (66.6)	8/9 (88.8)
Plasmid		2/9 (22.2)	4/9 (44.4)	1/9 (11.1)
Total	9	11/9 (122.2)	9/9 (100)	9/9 (100)
P. Value = 95 Df = 1		CalX ² = 3.6 Tab.X ² = 3.84	CalX ² = 0.5 Tab.X ² = 3.84	CalX ² = 4.5 Tab.X ² = 3.84

and *zapA*) was 11/8 (122.2), 8/8 (100), and 8/8 (100)%, respectively, while *ureC* was located in chromosome 9/9 (100%) and in plasmid 2/9 (22.2%) without differences between these two locations, and the percentage of *mrpA* showed that there was no significant difference in the gene on the chromosome than on the plasmid if it was on chromosome 6/9 (66.6%), while on the plasmid it was 4/9 (44.4%). The position of *zapA* was manifested on the chromosome at a rate of 8/9 (88.8%), which decreased significantly on the plasmid to 1/9 (11.1) (Table 4).

Discussion

The molecular identification of *Proteus* sp. in pregnant women with 16 srRNA genes perhaps regarded as an accurate identification and classification center of *Proteus* sp. [6]; therefore, the identification of bacteriuria in pregnant women of Thi-Qar province may be a perfect diagnosis. In addition, *P. vulgaris* was found in eight (6.8%) pregnant women in the same governorate of Iraq, because the primers of the Urease C gene were used to rapidly and precisely identify *Proteus vulgaris* [6].

The complete phenotypic percentage of urease enzyme was confirmed through molecular diagnosis of the genes encoding the urease enzyme under investigation (genotyping identification of virulence gene). This return assures the finding of [30], which states that enzyme urease production is a diagnostic characteristic of *Proteus* sp., especially the two types *P. vulgaris* and *P. mirabilis*. The overall rate of protease production was also confirmed in a subsequent test (genotyping identification of virulence genes), which showed a complete percentage. These results are similar to those of other studies in Iraq, including that all *P. vulgaris* isolated from a variety of clinical sources can produce extracellular proteases [8].

Fimbriae are a means of bacterial attachment and adhesion. adhesion is substantial when infection occurs in an anatomically ordinary urinary tract [26]. Therefore, the presence of fimbriae in all study isolates and even in the control isolate in urinary tract samples explains the capacity of these isolates to cohesion in epithelial tissue, which can colonize the urinary tract and increase the opportunistic susceptibility of the organism.

The results of a study on Fimbriae foundation and clot blood red cells in all isolates are similar to the results of other studies in Iraq, such as the study of [16], which showed that all *P. mirabilis* and *P. vulgaris* isolates from different clinical specimens were at a rate (100%) and were able to clot human red blood cells of types A and O in the presence of mannose or tannic acid, which indicates the presence of fimbriae of type (CFA I and CFA III).

The high rate of *ureC* gene in the study *P. vulgaris* isolates may lead to stone pointing and a serious role of this bacterium in UTI infection because *ureC* is an administrator of the altitude of urine pH and causes gravel production [27].

The complete percentage of *ureC* resembled the results of [33] while mentioned, *ureC* was the only gene diagnosed in all bacteria *P. vulgaris* isolated from urinary and respiratory samples of humans, and did not agree with the same study about *zapA* and *mrpA* genes where the *zapA* gene and *mrpA* gene were not identified in *P. vulgaris* strains. This variation may be related to differences in the isolation location [18]. In addition, 100% *ureC* in *P. vulgaris* isolated from milk and meat samples [35].

The high *ureC* gene data were in agreement with [5] study, which found that *ureC* was detected in approximately 96.6% of UTI isolates.

The fimbrial genes found in all isolates of study similar to the investigation of [26] were detected

in 100% of isolates from patients with urinary tract infections in four hospitals of Babylon province in Iraq at 2013.

The existence of each virulence factor gene of study in chromosome and plasmid may be related to recurrence of the same gene by recombination process of the gene from chromosome or genetic transformation process or by conjugation of the *P. vulgaris* isolates with other pathogenic bacteria in the same clinical environment as *P. mirabilis* which is known to be present in urinary tract infections [15], then transition the gene from a conjugative plasmid of this bacterium to a conjugative plasmid of *P. vulgaris* isolates of study, which is called horizontal gene transportation [17, 29].

Based on the results of the current study, it can be concluded that the virulence factors of isolates of *P. vulgaris* are capable of producing and releasing both urease and protease enzymes, as well as producing fimbria, which are more serious and administrators of the pathogenicity and cohesion of *P. vulgaris* at a very high rate to the epithelial tissue of the urinary system and cause infections in the urinary tracts of pregnant women in Thi-Qar. The propagation of fimbria genes may be associated with the opportunistic capacity of *P. vulgaris*, which can also use

the product of the *zapA* gene that arranges the expression of IgA protease during differentiation of swimmer cells to swarmer cells. It is also possible to conclude that these bacteria are capable of reproducing and spreading from UTI samples to different clinical models because of their possession of these pathogenic genes, which makes them bacteria that threaten the public health of the community in the Thi-qar Governorate. The complete rate of *ureC* gene in *P. vulgaris* isolates may lead to stone pointing and a serious role of this bacterium in UTI infection because *ureC* is an administrator of the altitude of urine pH and originated gravel production [27]. Polymerase chain reaction (PCR) is the fastest and most accurate method for diagnosing bacteria and their virulence genes.

Additional information

Authors' contributions. Conceptualization; Data Curation; Investigation; Methodology; Project administration; Resources; Software; Writing — original draft; and writing — review and editing.

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Availability of data. On request.

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Автор:

Гали Джабур С., доктор философии, преподаватель и ассистент кафедры патологического анализа научного колледжа Университета Ти-Кар, г. Ти-Кар, Ирак.

Author:

Ghali Jabur S., PhD, Assistant Professor, Lecturer, Pathological Analysis Department, College of Science, University of Thi-Qar, Thi-Qar, Iraq.

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