Evaluation of ureC and zapA genes expression of Proteus mirabilis isolated from UTI and their role in bacterial pathogenicity in Iraqi patients

Osama Attai Razaq[1], Parsia Tahmasebi[1]*, Mahdi Saber AL-Deresawi[2]
[1]Department of Biology, Faculty of Science, Ilam University, Ilam, Iran
[2]Department of biology, Faculty of science, University of Wasit, Wasit, Iraq.
Email: p.tahmasbi@ilam.ac.ir .

Abstract

Background and objectives: This study focused on zapA, and ureC genes of Proteus mirabilis. The ure operon is responsible for the production process of the urease enzyme and ureC is a major gene, causative for urease production. The zap operon genes are important for the production of protease, especially zapA for regulating IgA protease expression during the differentiation of swimmer cells to swarmer cells. **Materials and Methods**: Clinical 150 samples of urine samples were collected from different hospitals in Iraq. Isolates were identified by API 20E and traditional biochemical tests. The Real-time-PCR technique was included in this study to detect the level of gene expression to ureC and zapA. **Results:** The results of cultural, microscopic, and biochemical tests, which were confirmed by API 20E indicated just 73 of 150 urine samples was p. mirabilis. There were non-significant differences in male (43.84%) and (56.16%) in female, also there was no significant difference between gene expression of both ureC, zapA and pH, pus cell, and epithelial and there were no significant between gene expression of ureC and zapA according to the age, sex, and bacteria. The statistical analysis showed that there was no significant difference between zapA gene expression and amorphous crystals. While there is a significant (P≤ 0.05) difference between ureC gene expression and amorphous crystals.**Conclusion**: An increase of ureC gene expression leads to an increase the pathogenicity of P. mirabilis and there is a synergistic effect of elevation of ureC and zapA gene expression.

Keywords: Proteus mirabilis, ureC, zapA, UTI.

1. Introduction

Proteus mirabilis is a member of the Enterobacteriaceae family that unlike the other members of Enterobacteriaceae, is a common pathogen causing urinary tract infections (UTIs) (1). *P. mirabilis* is well known for its ability to produce urease, which generates ammonia and elevates the pH of the urine to >7.2. Calcium and magnesium crystallization in the urine of alkaline pH blocks the catheter lumen and causes acute urinary retention and the development of bacteriuria and other ascending infections, thereby leading to pyelonephritis, bacteremia, and shock (2).

This study will be focused on *zapA*, and *ureC* genes. Since they have been identified to code most important virulent factors and have been found to be more common in previous studies.The *zap* operon genes is important for the production of protease, especially *zapA* for regulating IgA protease expression during the differentiation of swimmer cells to swarmer cells (3) Urease is the most important enzyme for kidney and bladder stone formation in *Proteus* infection. The *ure* operon are responsible for the production process of urease enzyme and *ureC* as a major gene, causative for urease production (4)

The aims of study were to detection the synergism effect of *ureC* and *zapA* genes and their role in bacterial pathogenicity and assessment of *ureC* and *zapA* genes expression of *P. mirabilis* during UTI.

Subjects In this study totally 150 UTI sample were collected but 73/150 was *Proteus mirabilis*, samples collected from the patients suffering from urinary tract infection with various age groups of both genders from different hospitals in Iraq (Baghdad medical City/ Teaching Laboratory, Al-zahraa Teaching Hospital, and Al-aziziyah hospital) from the beginning of November 2020 to March 2021. Identification was done by cultural, biochemical tests, and finally identification by API 20E system technique.

Isolation and identification of *Proteus mirabilis*

73 isolates of Proteus mirabilis were characterized by general urine examination, cultural, biochemical testes, API 20E techniques. The colony morphology of *Proteus* genus was initially identified according to their swarming phenomenon on blood agar, also they have a distinctive fish odor, while *Proteus* growth on MacConkey appeared to be pale due to incapability of this genus to ferment lactose sugar.

General urine examination

All sample were examined by Compound light microscope, and it was noticed the crystals formation, pus cells, and also examined the PH, Oder, color, turbidity and most of sample were alkaline and contain bacteria.

doi.org/10.31838/hiv23.01.74 Received: 15.09.22, Revised: 19.10.22, Accepted: 14.12.22

Biochemical testes

A. Catalase Test

The growth bacteria were transferred by the wooden stick, and it was put on the surface of a clean slide, a drop of (3% H2O2) was added. The Formation of gas bubbles indicates appositive result (5) .

B. Urea agar slant

Heavy inoculum from bacteria that has grown on blood agar was streaked over the surface of slope of urea agar and stab into the bottom, incubated aerobically at 37°C for 24 hrs. The development of pink color indicates a positive result, while the yellow indicate a negative result (6).

c. Triple Sugar Iron Agar

Heavy inoculum was streaked over the surface of slope of TSI agar and stab into the bottom, incubated aerobically at 37°C for 24 hrs. Interpretation of the results was recovered by the change of color at surface and bottom (7).

D. Phenylalanine deaminase test

Using a loopful of inoculum from an 18–24-hour pure culture, streak the slant surface with sterile inoculating wire. Incubate the inoculated slant for 18-24 hours at 35°-37°C in ambient air with cap loose. Following incubation, add 4-5 drops of a 10% Ferric Chloride solution directly to the slant. Gently agitate the tube and observe for the development of a green color within 1-5 minutes (8).

API 20E system technique

To identify and differentiate members of family Enterobacteriaceae. The API range provides a standardized, miniaturized version of existing identification techniques, which up until now were complicated to perform and difficult to read. In the API 20E, the plastic strip holds twenty mini-test chambers containing dehydrated media having chemically defined compositions for each test. They usually detect enzymatic activity, mostly related to fermentation of carbohydrate or catabolism of proteins or amino acids by the inoculated organisms.

A bacterial suspension is used to rehydrate each of the wells and the strips are incubated. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. All positive and negative test results are compiled to obtain a profile number, which is then compared with profile numbers in a commercial codebook (or online) to determine the identification of the bacterial species (9).

RNA extraction

Total RNA of all samples was extracted using the GENEzol™ Reagent following the protocol provided by the manufacturer company, Korea.

cDNA synthesis

Total RNA was reversely transcribed to complementary DNA (cDNA) using WizScript™ RT FDmix Kit. The procedure was carried out in a reaction volume of 20µl according to the manufacturer's instructions. The total RNA volume to be reversely transcribed was (20µl).

Primer design

The design process for primers was obtained for the first time by Primer3 web version 4.1.0 (online at website http://primer3.ut.ee) for *Urec*, *ZapA* and *16sRNA* genes. Primers were used for the detection the gene expression are shown in table (1).

Table (1) Primer sequences were used in this study.

Primer names	Sequence (5'→3' direction)	Product size(bp)
UreC FUreC R	TGGCAAGGCAGGTAATCCAGCGCCAGAAACGAGACCTTCT	164
ZapA FZapA R	ACGTGCTGGGAACTTTTCTGATATCGTCTCCTTCGCCTCCA	155
16S-R F16S-R R	AGATCTGATGGGTTGTCAGGTCCTGCCCATCAAGAAACGGA	161

QRT- PCR Reactions

QRT-PCR was performed using the strata gene Real-time PCR System (Analytic Jena Technologies) with qPCR soft software. The gene expression levels and fold change were quantified by measuring the threshold cycle (Ct) employing the qPCR Master Mix Kits components. Every reaction was done in a duplicate and included a non-template control (NTC), non-amplification control (NAC) and non-primer control (NPC) were concluded as negative controls.

Before preparing qPCR reactions, the qPCR Master Mix, template DNA, and primers were thoroughly mixed. The required volume of each component was calculated according to table2.

Table (2) qPCR components used in the ureC and zapA and proteus housekeeping 16S rRNA genes expression experiment.

Reagent Master Mix	Final concentration	20 µl rxn
Forward Primer (1 µL)	ureC 164 nmzapA 155nm16sRNA 161nm	1
qPCR Master (SYBR)	1 ml	10
Reverse Primer (1 µL)	UreC 164nmzapA 155nm16sRNA 161nm	1
Template DNA	(1.4 -2.8mg/ml)	2
RNA free water		6

The qPCR Reaction run the cycling protocol was programmed according to the thermal profile for ureC, zapA and 16S rRNA genes shown in Table3

Table (3) Thermal profile for ureC, zapA and 16S rRNA genes.			
Step	Temperature (C°)	Duration	Cycles
Initial Denaturation	95	5 min	Hold
Denaturation	95	30 sec	
Anneal/extend	UreC gene (58) zapA gene (55)16S rRNA gene (57)	30 sec	35
Melting tem.	1min /95 °C-	30sec /55 °C-30sec/95 °C	

Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) and T-test was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in. Estimate of correlation coefficient between Urec folding and ZapA folding in this study(10)

2. Results

General characterization of samples

In this study totally 150 UTI isolates were examined. But 73/150 was proteus mirabilis, the sex distribution was 43.84% male and 56.16% were female and there are no Significant. The urine pH was alkaline in 90.41% (P= 0.0001) and there are high Significant. The bacteria was existed many in 54.79% of UTI samples and rest of them were moderate and there are no Significant. The Pus was many in 26.03%, moderate in 73.97% of samples and there are high Significant. The amorphous crystal was 13.7%, 67.12% and 19.18% for many, moderate and few and there are high Significant, respectively. The epithelial cells were existed many in 17.81%, Moderate in 49.32% and few in 32.88% and there are high Significant. In addition, all samples were positive for Urease, TSI and Catalase test. The mean age of individuals were 31.32 years.

zapA and ureC expression in relationship with amorphous crystal of urine in patients

The relationship between amorphous crystals and virulence factor was indicated in Table.5 The Urec folding was 1.667 ±0.10, 2.427 ±0.18, and 2.651 ±0.49 for patients with many, moderate and few amorphous crystal of urine table (4-10). Our statistical analysis showed that there is a significant difference between Urec expression and amorphous crystals (p< 0.05).

ureC and zapA determination in positive UTI isolates of Proteus Mirabilis

Our data showed that just 7/73 (9.6%) of isolates were negative and 66/73 were positive for presence of ureC. As described in table (4-3) further analysis showed that the mean of ureC in all samples was 2.1. Our finding also showed that just 24/73 (32.8%) of isolates were negative and 49/73 (67.2%) were positive for presence of *zapA*. As presented in table (4) analysis showed that the mean of *zapA* in all samples was 3.49. Our data showed that just 5/73 (6.8%) of isolates were double negative for both *ureC* and *zapA*.

Table (4) descriptive analysis of *ureC* and *zapA* gene.			
Gene	N	Mean	Std. Deviation
zapA	49	3.494	3.410
urec	66	2.148	1.406

Table (5) relationship between Amorphous with ureC folding and zapA folding		
Amorphous	Mean ± SE	
	Urec folding	ZapA folding
Many	1.667 ±0.10 b	3.387 ±0.57
Moderate	2.427 ±0.18 ab	5.199 ±0.44
Few	2.651 ±0.49 a	5.888 ±1.20
LSD	0.887 *	3.071 NS
P-value	0.041	0.378
Means having with the different letters in same column differed significantly. * (P≤0.05).		

3. Discussion

Our results showed that among 150 UTI samples, seventy-three isolates of Proteus mirabilis were characterized API 20E and were confirmed by cultural, microscopic diagnosis, biochemical testes, as a confirmatory technique. The colony morphology of Proteus genus was initially identified according to their swarming phenomenon on blood agar, also they have a distinctive fish odor. While Proteus growth on MacConkey appeared to be pale due to incapability of this genus to ferment lactose sugar(11) .

Our data showed that all positive isolates of *Proteus Mirabilis* which was extracted from UTI infection were examined to detection of UreC and *zapA* using real time PCR. Our data showed that just 90.4% of isolates were positive for presence of UreC. In addition, the present study showed 67.2% of isolate

were positive for presence of ZapA. Moreover, our data showed that just 6.8% of isolates were double negative for both UreC and ZapA. Similarly, Pathirana and colleagues 2017, investigate the prevalence of *ureC*, *rsbA*, *zapA* and *mrpA* virulence genes using polymerase chain reaction (PCR) in Proteus spp. isolated from 5 commercially popular species of pet turtles and comparison of the mrpA gene sequences of Proteus mirabilis isolates with human clinical isolates. A total of 24 isolates in pet turtles were identified, comprised of *P. mirabilis* (15), *Proteus vulgaris* (7) and *Proteus hauseri* (2). The prevalence of *ureC, rsbA, zapA and mrpA* genes among all identified Proteus spp. isolates were 91.7%, 50%, 45.8% and 45.8%, respectively. The average percentage similarities of mrpA gene sequence of pet turtle *P. mirabilis* isolates to human urinary and respiratory isolates were 96.35% and 94.85%, respectively. The prevalence of virulence genes and high similarity of mrpA gene sequences between pet turtles and human *P. mirabilis* isolates revealed that though pet turtles are healthy, these animals may pose a potential risk of urinary and respiratory infections to humans(12).

Proteus mirabilis have many virulence factors that are vital for inflicting UTI, several of these factors appear to be more important for establishing infection in different areas of the urinary tract (13). For instance, recently it has been shown that several potential virulence factors may be responsible for the pathogenicity of P. mirabilis. Among them, flagella, necessary for motility, Proteus mirabilis expresses several virulence factor involved in infection like adhesins, flagella, toxins, quorum-sensing, enzymes and immune invasion are involved in establishing infection(14) . Urease is important enzyme in pathogenesis of Proteus mirabilis. Urease enzyme has ability of the formation of kidney and bladder stones and block urinary tract. The cluster of urease gene (ureRDABCEFG) that breakdown the urea into ammonia and carbon dioxide and increase the pH of urine. The change in pH can facilitate P. mirabilis adherence, colonization, and biofilm formation (15).

The urease of P. mirabilis is unambiguously associated with the development of infection induced stone formation urolithiasis(16) .

The present study also showed that there is no significant relationship between zapA and ureC expression and Urine pH and other microscopic parameters. To date no relationship between virulence factor and biochemical parameters have been elucidated yet, however potential virulence factors and bacterial behaviors associated with the infection processes and disease, including swarming, growth rates, fimbria expression, flagella, and the production of hemolysins, ureases, proteases, amino acid deaminases, in addition to the expression of lipopolysaccharide (LPS) antigens and capsular polysaccharides (CPSs), have been described in many studies (17) .

4. Conclusion

The pus, amorphous and epithelial cell formation are coinciding with UTI. Increase of *ureC* gene expression leading to increase the pathogenicity of *Proteus mirabilis* and there is a synergistic effect of elevation of *ureC* and *zapA* gene expression.

Authors' Contributions

Study design: PT, MSD; Data analyzing and draft manuscript preparation: OAR, PT, and MSD; Perform Experiment: OAR, MSD; Data Critical revision of the paper: PT, MSD; Supervision of the research: PT, MSD; Final approved of the version to be published: OAR, PT, and MSD.

References

1. Gupta K, Grigoryan L, Trautner B. Urinary tract infection. Ann Intern Med. 2017;167(7):ITC49–64. https://doi.org/10.7326/AITC201710030
2. Jarvis TR, Chan L, Gottlieb T. Assessment and management of lower urinary tract infection in adults. Aust Prescr. 2014;37(1):7–9. DOI: 10.18773/austprescr.2014.002
3. Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Am J Med. 2002;113(1):5–13. DOI: 10.1016/s0002-9343(02)01054-9
4. Weber DJ, Sickbert-Bennett EE, Gould C V, Brown VM, Huslage K, Rutala WA. Incidence of catheter-associated and non-catheter-associated urinary tract infections in a healthcare system. Infect Control Hosp Epidemiol. 2011;32(8):822–3. DOI: 10.1086/661107
5. Wang J, Muhammad N, Li T, Wang H, Liu Y, Liu B, et al. Hyaluronic acid-coated camptothecin nanocrystals for targeted drug delivery to enhance anticancer efficacy. Mol Pharm. 2020;17(7):2411–25. https://doi.org/10.1021/acs.molpharmaceut.0c00161
6. Stuart CA, Van Stratum E, Rustigian R. Further studies on urease production by Proteus and related organisms. J Bacteriol. 1945;49(5):437–44. DOI: 10.1128/jb.49.5.437-444.1945
7. Ahmed DA. Prevalence of Proteus spp. in some hospitals in Baghdad City. Iraqi J Sci. 2015;56(1):665–72. https://www.iasj.net/iasj/article/102911
8. Falkinham 3rd JO, Hoffman PS. Unique developmental characteristics of the swarm and short cells of Proteus vulgaris and Proteus mirabilis. J Bacteriol. 1984;158(3):1037–40. DOI: 10.1128/jb.158.3.1037-1040.1984
9. Thaochan N, Drew RAI, Hughes JM, Vijaysegaran S, Chinajariyawong A. Alimentary tract bacteria isolated and identified with API-20E and molecular cloning techniques from Australian tropical fruit flies, Bactrocera cacuminata and B. tryoni. J Insect Sci. 2010;10(1):131. DOI: 10.1673/031.010.13101
10. Carley KM, Columbus D, Azoulay A. Automap user's guide 2012. CARNEGIE-MELLON UNIV PITTSBURGH PA INST OF SOFTWARE RESEARCH INTERNAT; 2012. http://www.casos.cs.cmu.edu/publications/papers/CMU-ISR-12-106.pdf

11. O'Hara CM, Brenner FW, Miller JM. Classification, identification, and clinical significance of Proteus, Providencia, and Morganella. Clin Microbiol Rev. 2000;13(4):534–46. DOI: 10.1128/CMR.13.4.534
12. Pathirana H, De Silva BCJ, Wimalasena S, Hossain S, Heo G-J. Comparison of virulence genes in Proteus species isolated from human and pet turtle. Iran J Vet Res. 2018;19(1):48. DOI:10.22099/IJVR.2018.4768
13. Al-Bassam WW. Bacteriological and genetic study on swarming in Proteus mirabilis isolated from clinical samples. M. Sc. Thesis/College of science/Baghdad University; 2010.
14. Baldo C, Rocha SPD. Virulence factors of Uropathogenic Proteus Mirabilis-a mini review. Int J Technol Enhanc Emerg Eng Res. 2014;3:24–7. https://www.semanticscholar.org/paper/Virulence-Factors-Of-Uropathogenic-Proteus-A-Mini-Baldo-Rocha/83ca1c803c54827b32e15919c8efbc837f966bfc
15. Nicholson EB, Concaugh EA, Mobley HL. Proteus mirabilis urease: use of a ureA-lacZ fusion demonstrates that induction is highly specific for urea. Infect Immun. 1991;59(10):3360. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC258891/
16. Mobley HL, Warren JW. Urease-positive bacteriuria and obstruction of long-term urinary catheters. J Clin Microbiol. 1987;25(11):2216. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC269446/
17. Jacobsen SM, Lane MC, Harro JM, Shirtliff ME, Mobley HLT. The high-affinity phosphate transporter Pst is a virulence factor for Proteus mirabilis during complicated urinary tract infection. FEMS Immunol Med Microbiol. 2008;52(2):180–93. DOI: 10.1111/j.1574-695X.2007.00358.x.