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Molecular Study of Hemolysin genes in *Proteus mirabilis* isolated from patients with urinary tract infections.

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ABSTRACT

Proteus mirabilis is associated with Urinary tract infections (UTIs) mainly in patients with urinary tract abnormality and the formation of stones in the kidney and bladder. One of the important virulence factors in *P. mirabilis* is the production of hemolysin hpmA, which cause damage in the kidney tissues. A total of 110 (26.5%) uropathogenic *P. mirabilis* isolates were obtained from 415 urine samples and analyzed to detect the presence of the *hpm*A and *hpm*B genes by polymerase chain reaction (PCR) and sequencing. The presence of the *hpm*A and *hpm*B genes was confirmed by PCR in 96 (87.3 %) of the 110 isolates. The sequencing confirmed the presence of the *hpm*A and *hpm*B genes in the isolates. The two genes were detected by multiplex PCR. Five strains with the highest hemolytic activity (PMK1-PMK5) were chosen to study the effect of pH on the gene expression by RT-PCR. It was found that the highest value of gene expression fold was recorded at the pH 8 for the gene *hpm*A (7.8) in the local isolate PMK3 in contrast with the samples in the neutral pH, while the highest value of fold for the *hpm*B gene was 5.57 at the pH 8. It was obvious there was a high decreasing of the folds of gene expression of hemolysin genes at low pH, therefore the increase of pH in the growth medium led to increasing of gene expression.

Keywords: *Proteus mirabilis*, Hemolysin genes, UTI.

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INTRODUCTION

Urinary tract infections (UTIs) are among the most frequently occurring human bacterial infections, accounting for about 20% of all infections acquired outside the hospital. *Proteus mirabilis* is one of the most common causes of UTI in individuals with long-term indwelling catheters and complicated UTIs [1, 2]. The main pathogenic features of *P. mirabilis* rods are their ureolytic, proteolytic, and hemolytic abilities, swarming motility, and lipopolysaccharide (LPS) presence [3]. Calcium independent hemolysin system is one of the main virulence factors in *P. mirabilis* and consisting of two proteins hpmA and hpmB. HpmB is responsible for the activation and extracellular secretion of the hemolysin HpmA. Activated HpmA once released into the external environment forms pores in and lyses the red blood cells [4]. The importance of *P. mirabilis* as a nosocomial pathogen due to its risk factors with dangerous virulence attributes that is difficult to eradicate and as a microorganism with multiple drug resistance because of the beta-lactamase activity [4, 5].

The aim of this study was investigation the prevalence of hemolysin genes (*hpm*A and *hpm*B) in *P. mirabilis* isolated from UTIs in Baghdad city and studies the effect of pH on the gene expression of these genes.

MATERIALS AND METHODS

Isolation and identification of *Proteus mirabilis*

This study was performed at Al-Kindy Teaching Hospital in Baghdad, Iraq, between February and July 2018. A total 415 Urine sample were collected from patients with Urinary Tract Infections. Blood agar and McConkey agar were used for isolation uropathogenic *P. mirabilis*. These isolates were identified using traditional bacteriological methods and biochemical testing, with VITEK 2 system (bioMerieux, France).

DNA extraction and identification of hemolysin genes by PCR

Bacterial DNA was extracted from all *P. mirabilis* isolates using ready kit (Promega, USA). Purity of the isolated DNA was monitored by NanoDropper 2000 (Thermo Scientific, USA). The PCR reactions for detection *hpmA* and *hpmB* genes were done within a total volume of 25 µL. The mixture of reaction contained 1x buffer (10 mM Tris-HCl, 50 mM KCl), 0.2µM of each deoxynucleoside triphosphate, 1 mM MgCl2, 0.5 µM of forward and reverse primers. The Primer sequences, which were used for detection of hemolysin genes in this study, were as in Table 1. PCR conditions for amplification was according to the reference of Table 1.

Table 1. Primer sequences for PCR detection of *hpmA* and *hpmB* genes in *P. mirabilis.*

Target gene	Oligonucleotide primer sequence 5' to 3'	Amplicon size (bp)	Reference
***hmpA*-F** ***hmpA*-R**	**GTTGAGGGGCGTTATCAAGAGTC** **GATAACTGTTTTGCCCTTTTGTGC**	**709**	**[6]**
***hmpB*-F** ***hmpB*-R**	**CAGTGGATTAAGCGCAAATG** **CCTTCAATACGTTCAACAAACC**	**422**	**[6]**

For multiplex PCR, PCR conditions for amplification of the two gene was carried out by the thermocycler (Applied Biosystems, Malaysia) as follows: initial denaturation at 94°C for 5 min, denaturation at 95°C for 1 min, annealing at 55 °C for 30 sec, and extension at 72°C for 1 min, was repeated for 30 cycles; a final extension at 72°C for 5 min. Agarose gel electrophoresis was done a 1.2 % agarose gel at 80V for 2 hours. After electrophoresis fragments were stained by ethidium bromide, and then visualized with ultraviolet light.

Real Time PCR (qRT–PCR)

One step Quantitative Real-time PCR Assay (QRT-PCR) By using GoTaq® 1-Step RT-qPCR System (Promega, USA), Amplification of fragment of mRNA was preformed with the following master amplification reaction with the program of One-Step RT-PCR list in Table (2) and the program in Table (3). Several experiments were done for more appropriate synthesis of cDNA and annealing temperature**.**

Table 2. Component of quantitative real-time PCR used in hemolysin and *16S*rRNA genes expression experiment

Component	Volume per 20µl Reaction
qPCR Master Mix, 2X	10 µl
Forward Primer, 10X	1 µl
Reverse Primer, 10X	1 µl
cDNA Template (100 ng)	4 µl
Nuclease-Free Water	to 20 µl

Housekeeping Gene Amplification

*16S*rRNA housekeeping gene was used as an internal control to be used in calculating the ΔCT value.

The qPCR Reaction run

The cycling protocol was programmed according to the thermal profile Shown in the Table 3, for *16S*rRNA and hemolysin genes.

Table 3. Thermal profile of *16S*rRNA and hemolysin gene expression

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	5 minutes	1
Denature Anneal	95 °C 58 °C	15 seconds 60 seconds	40
Melting curve Analysis	65-95 °C	2-5 seconds/step	1

Delta delta Ct (ΔΔCt) method

This method is the simplest one, as it is a direct comparison of Ct values between the target gene and the reference gene. Relative quantification involves the choice of a calibrator sample.

Firstly, the ΔCt between the target gene and the reference gene is calculated for each sample (for the unknown samples and also for the calibrator sample).

ΔCt = Ct target – Ct reference gene

Then the difference between the ΔCt of the unknown and the ΔCt of the calibrator is calculated, giving the ΔΔCt value:

ΔΔCt = (Ct target – Ct reference) sample – (Ct target – Ct reference) calibrator.

The normalized target amount in the sample is then equal to $2^{-\Delta\Delta Ct}$ and this value can be used to compare expression levels in samples [7].

The samples were analyzed in triplicates and standardized against *16S*rRNA gene expression. The relative changes in mRNA expression levels were determined using comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$) between the samples with different pH values.

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RESULTS AND DISCUSSION

Out of the 415 urine samples, 230 (55.4 %) of the patients had significant bacterial growth. Due to the selective agar media for bacterial colonies of uropathogens from urine samples, *P. mirabilis* were identified by the biochemical tests and the confirmation of the identification of *P. mirabilis* was completed by using VITEK 2 system. One hundred and ten (26.5%) of the isolated bacteria identified as *P. mirabilis* from all collected bacterial cultures.

One of the studies revealed that Gram-negative bacteria were the most frequent and included *Escherichia coli* (48.2%), *Klebsiella pneumoniae* (9.5%), *Pseudomonas aeruginosa* (4.9%), and *Proteus mirabilis* (4.6%), while *Enterococcus* spp. (14.4%) [8]. In healthy women, *Proteus* accounts for 1% to 2% of all UTIs while in hospital-acquired UTIs, *Proteus* accounts for 5%. Complicated UTIs have an even higher association with *Proteus* infection at 20% to 45% [9]. Our results not agree with the local study conducted at a teaching hospital, Iraq from April 2012 to October 2012 which revealed the low frequency of *Proteus* spp. (1%) which isolated from Community- Acquired Urinary Tract Infection in a Baghdad Hospital [10]. *P. mirabilis* is capable of causing symptomatic infections of the urinary tract infections (UTIs) such as cystitis and pyelonephritis and is present in cases of asymptomatic bacteriuria, particularly in the elderly and patients with type 2 diabetes. These infections can also cause bacteremia and progress to potentially life-threatening urosepsis. Additionally, *P. mirabilis* infections can cause the formation of urinary stones [11]. Patients with UTI caused by *P. mirabilis* usually have alkaline pH urine due to the presence of ammonia resulting in calcium and magnesium crystallization which could in turn lead to obstruction of the lumen of indwelling catheters [12].
In order to detect the presence of hemolysin genes (*hpmA* and *hpmB*) and determination the prevalence of each gene among *P. mirabilis* clinical isolates, uniplex and multiplex polymerase chain reaction (PCR) for each DNA extracted sample have been used. The PCR reaction included 110 isolates for detection the genes. The PCR products have been confirmed by analysis of the bands on gel electrophoresis and by comparing their molecular weight with 100 bp DNA Ladder. The results of uniplex PCR reaction for hemolysin genes showed in Figures (1) and (2), and these genes also detected by multiplex PCR reaction (Figure 3).

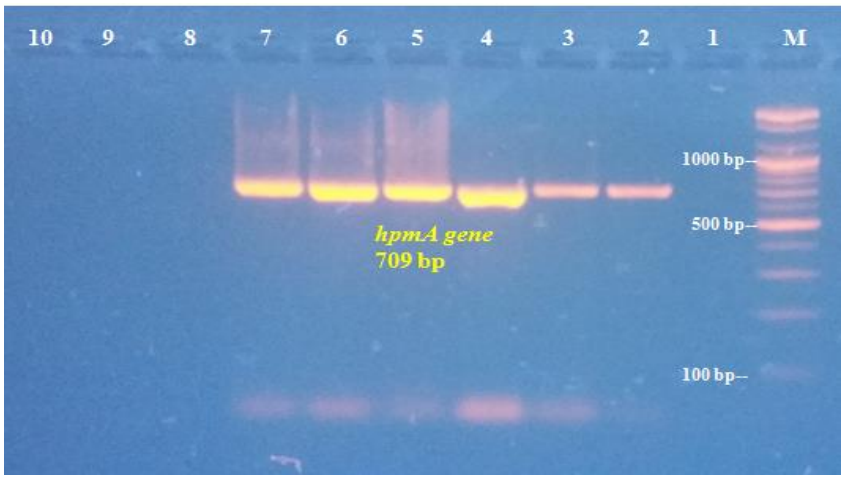

Figure 1. Agarose gel electrophoresis of PCR products for *hpmA gene* (709 bp). Lane M: 100bp DNA ladder; lanes 2-10: *P. mirabilis* PMK1-PMK9 isolates; lane 1: Negative control. (80V for 2hr).

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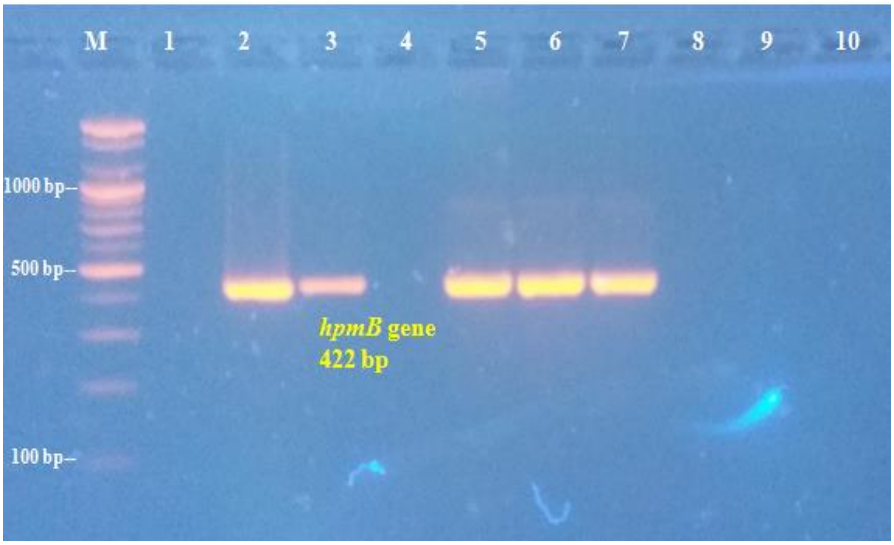

Figure 2. Agarose gel electrophoresis of PCR products for *hpmB* gene (422 bp). Lane M: 100bp DNA ladder; lanes 2-10: *P. mirabilis* PMK1-PMK9 isolates; lane 1: Negative control. (80V for 2hr).

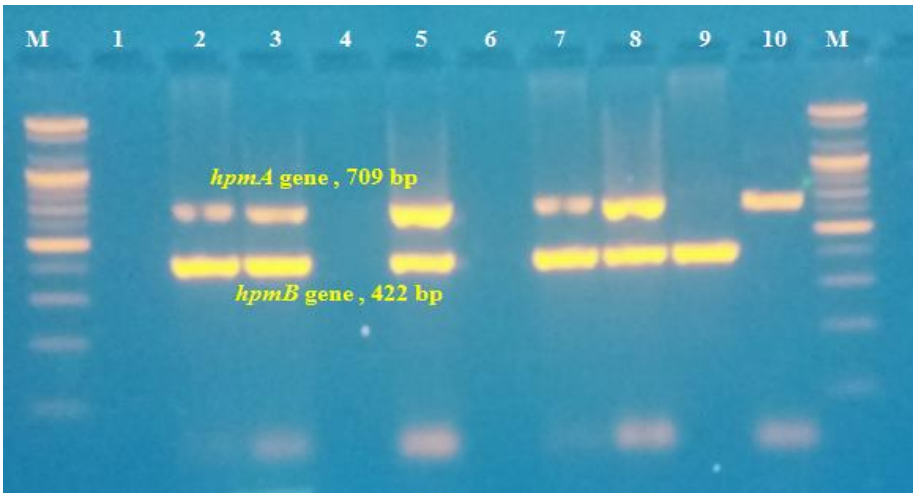

Figure 3. Agarose gel electrophoresis for detection the genes *hpmA* and *hpmB genes* by multiplex PCR. Lane M: 100bp DNA ladder; lanes 2-10: *P. mirabilis* PMK21-PMK29 isolates; lane 1: Negative control. (80V for 2hr).

The present study indicated that the hemolysin genes (*hpm*A and *hpm*B) were found in 96 (87.3 %) of the 110 *Proteus mirabilis* isolates. The results of Cestari *et al.* (2013) [6] demonstrated the presence of the *hpm*A and *hpm*B genes was confirmed by PCR in 205 (97.15 %) of the 211 isolates. There is evidence that hemolysin increases the virulence of infections by *P. mirabilis* because production of this protein has been correlated with cytotoxicity in Vero cells, also it was found that observed that *P. mirabilis* that produced hemolysin associated to the cell produced a lethal dose 50 % greater than the non-hemolytic isolates when injected transurethrally in mice [13].

The results of gene expression of hemolysin genes at different pH values (from 4 to 9) was showed in figure 4.

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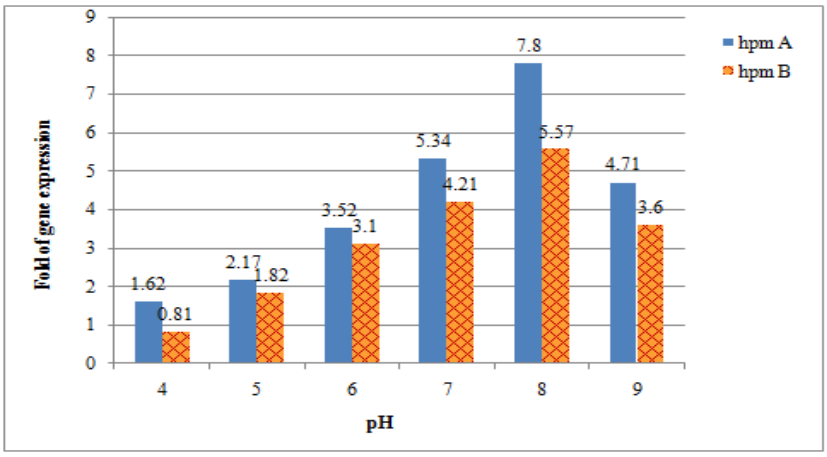

Figure 4. Fold of gene expression of hemolysin genes at different pH values depending on ΔΔCt method.

The current study revealed that the highest value of fold of gene expression was 7.8 at pH 8 for *hpm*A gene in contrast with the *hpm*B gene which recorded fold of gene expression 5.57. Also, the results demonstrated that the fold of gene expression for the 2 genes was decreased with the lowering of pH. *Proteus* rods produce three types of cytolysins, hpmA and hlyA hemolysins and Pta cytotoxic agglutinin. HpmA is the 166 kDa protein, calcium–independent cell-bound hemolysin secreted by the bacteria and activated in the process mediated by *hpm*B [14]. There are some causes play a great role to control the expression of hemolysin genes by *E.coli* isolates where some strains will produce less hemolysin production in the presence of streptomycin, sodium cyanide, rifampin, and nalidixic acid. Also, it shows maximum expression at pH (7-8) [15]. The fold of gene expression of *hpm*A was more than *hpm*B at the same conditions may be confirmed the role of *hpm*A gen as one of the important virulence factors during the urinary tract infections.

CONCLUSION

The present study showed the high frequency of hemolysin genes (*hpm*A and *hpm*B) in *Proteus mirabilis* bacteria isolated from UTIs and these genes have an obvious role in the infection, also it was found that the gene expression of these genes was highly affected by acidic pH and the optimum pH was near the alkaline.

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