

Molecular detection of some virulence genes of proteus mirabilis isolated from different samples in Babylon province /Iraq

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ABSTRACT

A total of (130) alkaline urine samples, as well as vaginal, wound, and burn samples, were collected from patients with urinary tract infections of both sexes who visited Al-Hilla general Teaching Hospital in Al-Hilla governorate, Iraq, between September and December of 2021. Genotyping of *P.mirabilis* isolates was carried out at the molecular level to define genotypic features using a PCR approach targeting the key virulence factors genes. Three virulence genes (*hpmA*, *rsbA*, and *pta*) were screened from the total of 25 *P.mirabilis* positive samples.

Twenty-five isolates of *P.mirabilis* were discovered. 17 (68%) isolates were obtained from patients with urinary tract infection, while 3 (12%) isolates were obtained from patients with vaginal infection, 3 (12%) isolates from patients with surgical wound infection, and 2 (8%) isolates were obtained from patients with burn infection.

For the identification of the *ureR* gene, a specific PCR primer was utilized. The *ureR* gene was discovered in 25 (100%) isolates of *P.mirabilis*, including all UTI and vaginal infections, wound infections, and burn infections. A particular PCR primer was used to determine the presence of the *hpmA* gene. The *hpmA* gene was found in 24 *P.mirabilis* bacterial isolates (96%). PCR was used on genomic DNA from each *P.mirabilis* isolate to detect the *rsbA* gene using appropriate primers. PCR amplification of the *rsbA* gene revealed that only 20 (80%) of the 25 *P.mirabilis* isolates tested positive for this gene. Using specific PCR primers, the *pta* gene was also detected in *P.mirabilis*. Out of 25 isolates of *P.mirabilis*, the *pta* gene was found in 23 (92%) of *P.mirabilis*.

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INTRODUCTION

Proteus spp. are gram-negative bacteria that belong to the Enterobacteriaceae family, which is quite common (Foris and Snowden, 2019). Gustav Hauser initially identified the genus *Proteus* in 1885, and separated it into two species, *P.mirabilis* and *P.vulgaris* (Armbruster et al., 2018). *Proteus spp.* are rods typically 1-3µm in length and 0.4-0.8µm in diameter, motile by peritrichous flagella, facultatively anaerobic, non-spore producing, and non-capsulated, with fimbriae present in the majority of isolates. Many animals, including humans, have *Proteus* species in their soil, water, and intestinal tracts (Nahar et al., 2014; Drzewiecka, 2016). *P.mirabilis* is found in the human intestine as part of the microflora, but when it comes into contact with urea in the urinary system, it can cause sickness. *P.mirabilis* is common in hospitals and care institutions, accounting for 90% of *Proteus* infections (Armbruster et al., 2017). Urease enzymes are produced by all strains of *Proteus* species, and it is also a diagnostic and distinguishing trait that distinguishes members of this genus from the rest of the intestinal family (Ali and Yousif, 2015). *UreR*, a member of the AraC family, is a positive regulator of urease activity that enhances the transcription of urease genes in the presence of urea; *UreR* has a dimerization domain and a DNA-binding domain, based on sequence homology with AraC (Zhang et al., 2013). *P.mirabilis* has the potential to lyse blood cells and induce (alpha-hemolytic) hemolysis on blood agar media, which is usually associated with pathogenic bacteria. Because it can form pores in biological membranes, it is regarded an important virulence component in their pathogenesis (Afriani et al., 2014). *RsbA* protein is a two-component protein that functions as a Histidine Kinase sensor in the membrane of *P.mirabilis*. Furthermore, proteins can receive external signals and respond to them via regulating genes. The *rsbA* gene is involved in the initiation and stability of swarming, as well as the appearance of disease bacteria in urinary tract infections; in other words, *rsbA* acts as a positive or negative swarming regulator (Naseri et al., 2018). *Proteus* toxic agglutinin (*Pta*) is a surface-associated, calcium-dependent, alkaline protease that demonstrates time

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and dose-dependent cytotoxicity in cultured epithelial cells (Alamuri and Mobley, 2008; Pearson et al., 2011).

METHODS

From September (2021) to December (2021), 130 samples were collected from patients with uncomplicated UTI (alkaline urine) and vaginal, wound, and burn infections who visited Hilla teaching hospital (2021). *P.mirabilis* genomic DNA was isolated using the Geneaid Genomic DNA Purification Kit (UK) according to the manufacturer's instructions; the bacterial culture was seeded in 10 ml brain heart infusion broth medium and cultured at 37°C overnight in a shaking incubator.

Primer Sequences

Molecular assay in this study includes 4 genes; one for diagnosis, three for virulence. The oligonucleotide primers for all genes used in this study were obtained from previous studies and pubmlst.org, each one has specific nucleotide sequences and product size. The primer sequences and PCR conditions that used are listed in Tables (1 to 3).

RESULTS

Isolation of *Proteus mirabilis*

There are 95 positive results out of 130 clinical specimens, with 25(19.2%) isolates belonging to *Proteus mirabilis*, 70(53.8%)

isolates belonging to other bacterial genera, and 35(26.7%) samples showing no growth as shown in Tables 4 and 5.

Distribution of *P.mirabilis* Isolated from Different Clinical Samples Confirmation and Diagnosis of *P. mirabilis* by PCR using specific primer

All the isolates of *P.mirabilis* were used to produce a specific size of the 225-bp fragment of the *ureR* gene 25(100%) as shown in Figure 1, and Table 3.

Molecular detection of virulence genes in *P. mirabilis*

The genotypic characters were tested for all *P.mirabilis* isolates in this study in order for detection of some virulence factors. The specific primers were used for screening the presence of (*hpmA*, *rsbA*, and *pta*) genes as shown in Table 4.

Molecular Detection of (*hpmA* gene) in *P. mirabilis*

In this study, *hpmA* was been, and the results showed that 24 isolates in a rate of (96%) had *hpmA* gene as shown in Figure 2, and Table 5.

Molecular Detection of (*rsbA* gene) in *P. mirabilis*

The results show that 20(80%) of *Proteus mirabilis* isolates gave positive result at 467bp in PCR amplification of *rsbA* gene, as shown in Figure 3, and Table 6.

Table 1: Primer used in PCR assays for the detection of *P.mirabilis*

Genes	Primer sequence (5'-3')	Size bp	Reference
ureR F	GT GAG ATT TGT ATT AAT GG	225	(Zhang et al., 2013)
ureR R	ATA ATC TGG AAG ATG ACG AG		

Table 2. Virulence genes primers sequences with their amplicon size base pair (bp)

Genes	Primer sequence (5'-3')	Size bp	Reference
rsbA F	TTGAAGGACGCGATCAGACC	467	(Badi et al.,2014)
rsbA R	ACTCTGCTGTCTGTGGGTA		
hpmA F	GTTGAGGGCGTTATCAAGAGTC	709	(Cestari et al., 2013)
hpmA R	GATAACTGTTTTGCCCTTTTGTGC		
Pta F	AAAAGCCAGGTGTTTGATG	181	(Design this study)
Pta R	CGGGCCATAGTTGTTGCTAT		

Table 3: The PCR amplification conditions performed with a thermal cycler were specific to each single primer set depending on their reference procedure, as follows:

Gene	Initial denaturation	Denaturation	Annealing	extension	Final extension	Cycle
<i>ureR</i>	94°C / 4 m	94°C / 40 s	58°C / 60s	72°C / 20s	72°C/10 m	30
<i>rsbA</i>	94°C / 5 m	94°C / 60 s	58°C / 45s	72°C / 60s	72°C/7 m	30
<i>hpmA</i>	95°C / 5 m	95°C / 30 s	62°C / 30s	72°C / 20s	72°C/5 m	30
<i>Pta</i>	95°C / 5 m	95°C / 30 s	57°C / 30s	72°C / 20s	72°C/5 m	30

Table 4: Prevalence of *Proteus mirabilis* among other etiological agents associated with sample isolated

No. Sample	Culture			Molecular diagnosis by <i>ureR</i> gene	
	<i>Proteus mirabilis</i>	Negative results	Other Bacteria	Positive results	Negative results
130	25(19.2%)	35(26.9%)	70(53.8%)	25 (100%)	0 (0%)

Table 5: Bacterial Isolated from UTI and Vagina , Wound, Burn infection

No. of <i>P. mirabilis</i> Isolated	urinary tract infection	vagina infection	wound infection	burn infection
25	17 (68%)	3 (12%)	3 (12%)	2 (8%)

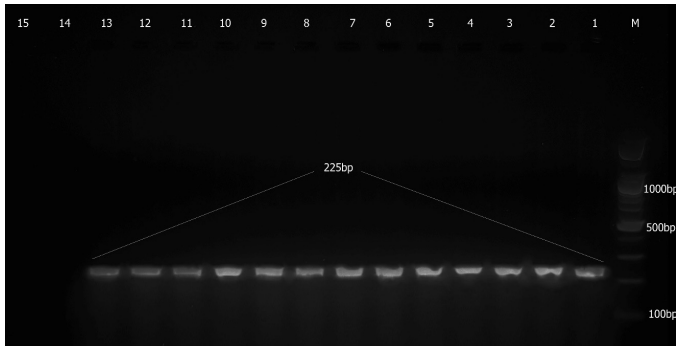


Fig. 1: Agarose gel electrophoresis of PCR products obtained by using ureR-specific primer. lanes 1-25 represent the identified ureR gene products with 225bp, Lane M represent 100bp DNA ladder

Table 4: Distribution of Virulence factors (hpmA, rsbA and Pta) among *P. mirabilis* isolates

genes	No.	(%)
<i>hpmA</i>	24	96
<i>rsbA</i>	20	80
<i>Pta</i>	23	92

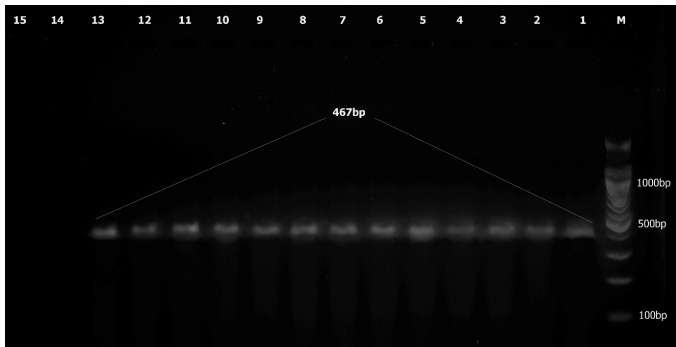


Fig. 3: Agarose gel electrophoresis of PCR products obtained by using rsbA-specific primer. lanes 1-25 represent the identified rsbA gene products with 467bp, Lane M represent 100bp DNA ladder

Table 6: Identification of *rsbA* gene of *P. mirabilis* in patients with different sample sources

Results	<i>rsbA</i> N (%)Positive	<i>rsbA</i> N(%)Negative
Urine	15 (88)	2(12%)
Vagina	2 (67)	1(33%)
Wound	2(67)	1(33%)
Burn	1 (50)	1(50%)
Total	20/25	5/25

Molecular Detection of (pta gene) in *P. mirabilis*

The result of the current study was shown that *pta* gene was present in 23 isolates out 25 isolates of *P.mirabilis* at rate (92%) from different samples sources with long length in (181bp) as shown in Figure 4 and Table 7.

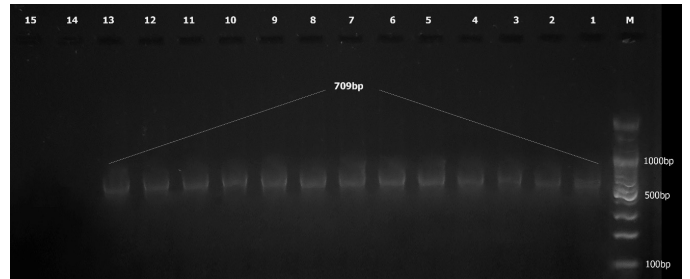


Fig. 2: Agarose gel electrophoresis of PCR products obtained by using hpmA -specific primer. lanes 1-25 represent the identified hpmA gene products with 709bp, Lane M represent 100bp DNA ladder.

Table 5: Identification of ureR gene gene of *P. mirabilis* in patients with different sample sources

Results	<i>ureR</i> N (%) Positive	<i>ureR</i> N(%) Negative
Urine	17 (100)	0(0%)
Vagina	3 (100)	0(0%)
Wound	3 (100)	0(0%)
Burn	2 (100)	0(0%)
Total	25/25	0/25

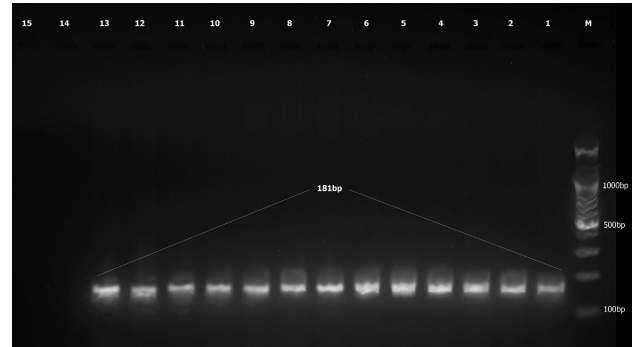


Fig. 4: Agarose gel electrophoresis of PCR products obtained by using Pta-specific primer. lanes 1-25 represent the identified Pta gene products with 181bp, Lane M represent 100bp DNA ladder

Table (3-7): Identification of Pta gene gene of *P. mirabilis* in patients with different sample sources.

Results	<i>Pta</i> N (%)Positive	<i>Pta</i> N(%)Negative
Urine	15 (88)	2(12%)
Vagina	3 (100)	0(0%)
Wound	3 (100)	0(0%)
Burn	2(100)	0(0%)
Total	23/25	2/25

DISCUSSION

95 of 130 clinical specimens were found to be positive, with 25(19.2%) belonging to *Proteus mirabilis* and the remaining 70(53.8%) to various bacterial species, with 35(26.7%) samples showing no growth.

According to Abbas *et al.*, (2015), 17(12.6%) of 135 isolates were identified as *P.mirabilis* from various sites of infection, including urine, vaginal, wounds, and burns swabs, which is similar to our findings. Also, according to AL-Ta'ee (2002), 50(16.6%) of 302 isolates from various infection sites were identified as *P.mirabilis*. However, AL-Jumaa (2011) identified just 7% of *P.mirabilis* from a total of 100 samples acquired from various clinical samples including urine, vaginal, wound, and burn.

Variations in *Proteus* isolation between studies can be attributed to a variety of factors, including sanitary practices in hospitals and staff, environmental conditions, isolation and identification techniques, social and cultural level of patients, and use of multidrug (antibiotics) that may lead to bacterial resistance development, or differences in sample size; all of these factors may combine and play an important role in inhibiting or stimulating bacterial resistance development.

Distribution of *P.mirabilis* Isolated from Different Clinical Samples

17(68%) isolates from patients with urinary tract infection, 3(12%) isolates from patients with vaginal infection, 3(12%) isolates from patients with surgical wound infection, and 2(8%) isolates from patients with burn infection were obtained from the total of 25 isolates of bacteria *P. mirabilis*.

Proteus mirabilis, after *Escherichia coli* and *Klebsiella pneumoniae*, is the third most common cause of urinary tract infection (UTI) (Peng *et al.*, 2016; Armbruster *et al.*, 2018). In addition to UTIs, this pathogen causes infections of the respiratory tract and infections of the skin and soft tissue (such as postoperative wounds and burns) (Fernández-Delgado *et al.*, 2015).

The results of bacterial isolation reveal that 17 isolates of *P.mirabilis* (68%) were found in urine samples. These findings are similar to those of Abbas *et al.*, (2015). *P.mirabilis* was isolated from urine specimens in 11 cases (64.7%). While Lamees A.Abdul-Lateef *et al.*, (2018) found 9 *P.mirabilis* isolates (39.2%) in urine of UTI patients. However, this result resembles that of Al-Bassam and Al-Kazaz (2013), who found 20 *P.mirabilis* isolates from the urinary tract, accounting for 40% of the total *P. mirabilis* isolates. The discrepancies in isolation percentages could be related to variances in the size and number of hospitals examined, as well as the season of sample collection and medicine taken prior to sampling. Several virulence factors in the bacteria are important for generating UTI, and some of these factors appear to be more significant for establishing infection in particular parts of the urinary tract. Adherence ability, urease synthesis, and flagella production are examples of virulence factors (Burall *et al.*, 2004).

These bacteria are present in the pus of patients with post-surgical wounds, and wounds can lead to bacteremia or meningitis (Smith *et al.*, 2000). Three isolates of *Proteus mirabilis* were isolated from wound infections, according to the findings of this investigation. However, the findings of Abbas *et al.*, (2015), who recovered *Proteus mirabilis* from patients with wound infections in 1(5.9%) of the total *Proteus mirabilis* isolates, are similar to those of this investigation. The results of the current study differ with those of Al-Bassam and Al-Kazaz (2013), who found *Proteus mirabilis* isolates in (24%) of total *Proteus mirabilis* isolates from wounds.

According to the findings of this investigation, two isolates of *Proteus mirabilis* (8%) are isolated from burn diseases. However, the findings of Zafar *et al.*, (2019) who identified *Proteus mirabilis* from patients with burn infections (3%) and surgical wound infections (5%) of the total *Proteus mirabilis* isolates are similar to those of this investigation. *P.mirabilis* was more commonly isolated from surgical wounds 19(47.4%) than burn 19(21%) in Nigeria, according to Mohammed *et al.*, (2013)

Finally, vaginitis is a vaginal inflammation that can cause discharge, irritation, and discomfort. A disturbance in the normal balance of vaginal flora or an infection are the most common causes. *E.coli* and *Proteus* are the most common bacteria found in postmenopausal women (Alwash *et al.*, 2008). Iraq has a high infection load, and genito-urinary infections are on the rise; this could be related to poor personal/community hygiene among some of the world's poorest people (Abass *et al.*, 2014).

In this investigation, *P.mirabilis* was found to be the causal agent of vaginitis in 3(12%) of the participants. This finding contradicts Pal *et al.*, (2014) and Alwash *et al.*, (2008) they isolated *P.mirabilis* from vaginal tissue at percentages of (3.1%) and (4%), respectively. While Abbas *et al.*,(2015) isolated (5.9%) *P.mirabilis* from vaginal swabs, the current study found 3 *P.mirabilis* in vaginal swabs.

Changes in vaginal microflora that show a critical role in promising vaginal colonization (Aiyegoro *et al.*, 2007), and my hypothesis is that the reason intestinal bacteria are associated with urinary tract and vaginal infections is due to the close proximity of the anal opening to the vagina and urethra, so contamination from the anus can lead to bacteria being found in the vagina area, though this is a much less common occurrence. Gram-negative bacteria have a higher resistance to the inhibitory impact of vaginal fluids, as well as their ability to cling to the epithelial cells of the urinary tract via their pili (Inabo and Obanibi, 2006).

In this investigation, species specific primers were utilized to amplify the urease gene (*ureR*) from *P.mirabilis* isolates, which is responsible for the production of urease enzyme and is recognized as a diagnostic trait of these species when employed with PCR. To create a precise size of the 225-bp segment of the *ureR* gene 25, all *P.mirabilis* isolates were used (100%).

Poore and Mobley (2003), Zhang *et al.*, (2013) developed species specific primers based on the conserved *ureR* sequence of *P.mirabilis* to identify this species using PCR, a 225-bp DNA product was amplified from this species and detected on an agarose gel. Adnan *et al.* (2014) showed that *ureR* has a strong discriminatory power for PCR-based identification of *P.mirabilis*. All *P.mirabilis* isolates from patients with urinary tract infections were able to manufacture urease and possess *ureR*, which encodes for urease, according to Alatrash and Al-Yasseen (2017). When electrophoresed on a 1 percent agarose gel, an amplicon with a molecular weight of 359 bp appeared.

Furthermore, according to Latif *et al.*, (2017), Kamil and Jarjes (2021), PCR amplification to particular *ureR* primers revealed that (100%) of *P.mirabilis* isolates produced positive results at 225bp. The results of this analysis were consistent with their findings, as (100%) of the *P.mirabilis* isolates produced *ureR* amplicon products of 225 bp.

Urease enzyme produced by *P.mirabilis* is considered to be more active than urease enzyme produced by other types of bacteria, with the exception of *H. pylori*. It works by changing the pH of urine to basic, causing the formation of crystal in biofilm by depositing calcium and magnesium phosphate in the biofilm and becoming a more complex type for it closes the urinary catheter and protects the bacteria from antibiotics, causing cure failure (Ali and Yousif, 2015).

The single polymerase chain reaction technique was used to investigate the genes responsible for virulence factor in *P.mirabilis* by using pieces of DNA with a limited number of nucleotides (oligonucleotide), which act as primers specialized for virulence genes in *P.mirabilis*, including *hpmA*. The *hpmA* gene is responsible for producing hemolysin in *P.mirabilis*. Unlike other *Proteus* spp., *P.mirabilis* α -hemolysin is organized by (*hpmA*), which encodes for the *hpmA* protein (Lazm et al. 2018). *hpmA* was tested in this investigation, and the results revealed that 24 isolates (96%) had the *hpmA* gene. Cestari et al., (2013) discovered that 96.24 percent of *P.mirabilis* isolates showed amplification for the *hpmA* gene by PCR. And this finding is in line with Filimon and Iacob, (2007) finding that the rate of this gene in *P.mirabilis* isolates was (97.15%). Also, Lazm et al., (2018) discovered that all of the isolates (100%) could manufacture hemolysin. *P.mirabilis* accounts for the majority of *Proteus* urinary tract isolates (97%); this shows that *hpmA* was the most prevalent *Proteus* hemolysin and may have a role in *Proteus* spp. extraintestinal infections Lazm et al., (2018).

The results demonstrate that in PCR amplification of the *rsbA* gene, 20(80%) of *Proteus mirabilis* isolates provided a positive result at 467bp. The findings are in line with prior research (Rather, 2005; Abbas et al., 2015). However, the findings of this study nearly contradict those of Dehnavi and Zarif, (2017), who discovered that (63%) *Proteus mirabilis* isolates. Also, the findings of this study were substantially same to those of Badi et al., (2014); Ahmadi et al., (2014), they found that (70%) of *Proteus mirabilis* isolates have the *rsbA* genes band, indicating that the foundation is widespread in *P.mirabilis*. Hussein et al., (2020), who discovered that 63(100%) *Proteus mirabilis* isolates.

The bull's-eye ring, a phenotypic hallmark of swarming, was observed in all isolates in the current study. Swarming regulated genes are not essential for swarming, however, because to the large number of genes and operons involved in the process (Pearson et al., 2010). The physiological and biochemical differences between swarm cells and vegetative cells have been established. Increased production of proteins such as flagellin, urease, hemolysin, and protease occurs during the differentiation of vegetative cells into swarm cells, which may be linked to organism persistence and correlated with *Proteus*' ability to enter cells (Ali and Yousif, 2015).

Pta is recognized as virulence factor in *P.mirabilis*. The PCR technique was used investigation of the *pta* gene through the use of pieces of DNA with a limited number of oligonucleotide which act as primer specialized a virulence gene of *P.mirabilis*. The result of the current study was shown that *pta* gene was present in 23 isolates out 25 isolates of *P.mirabilis* at rate (92%) from different samples sources with long length in (181bp).

This finding is in line with Lamees A.Abdul-Lateef, (2017), who discovered that the *pta* gene is present in practically all isolates of *Proteus mirabilis* (93.75%). Armbruster and Mobley, (2012) discovered that *pta* expression could be detected in urine

isolates. Pathology was reduced in *Proteus mirabilis* negative *pta* gene mice, as well as a deficiency in colonization of the kidneys and bladder. Because genetic diversity is possible, *Proteus mirabilis* may not be as homogeneous as previously thought, especially when pathogen specific factors are present (e.g. auto transporter).

The only described classical autotransporter from *Proteus mirabilis* is *Proteus toxic agglutinin (Pta)*. *Pta* functions as both a bacterial autoagglutinin and a poison to host cells, as its name suggests. Despite remaining linked to the bacterial cell, the *Pta*-domain is a serine protease that can cause cytotoxicity in host cells (Schaffer and Pearson, 2015). Pathology was reduced in *P.mirabilis* negative *pta* gene mice, as well as a severe colonization deficiency in the bladder, kidneys, and spleen. Although the specific mechanism of *Pta* cytotoxicity is unknown, *Pta* intoxication causes membrane damage, actin depolymerization, and final lysis in host cells. *Pta* expression is increased when cells are cultured at 37°C and an alkaline pH, which are both conditions that occur during infection (Schaffer and Pearson, 2015).

The function associated with *Pta* was critical for bacterial resistance in the host. However, the cytopathic effect of *P.mirabilis* in infected people has been a major clinical concern. *P.mirabilis* produces *pta*, which causes tissue damage and kidney dissemination, resulting in a cute pyelonephritis (Cestari et al., 2013). *Pta* punctures the host cell membrane, causing cytosol outflow, osmotic stress, and depolymerization of simple protein filaments, compromising the host cell's structural integrity and causing bladder and kidney damage. Auto-aggregation of *Pta* causes protease cell-cell interaction (Flores-Mirele et al., 2015).

CONCLUSIONS

This study reached the following conclusion:

1. Using of (*UreR*) gene for earlier identification of *P. mirabilis* infections.
2. It was found that the presences of some virulence factor genes in *P. mirabilis* as (*hpmA* and *Pta*) at high percentage increase pathogenicity of this pathogen.
3. The *rsbA* showed the lower percentage among *P. mirabilis* isolates.

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