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# Molecular detection of some virulence genes of Proteus mirabilis in rheumatoid Arthritis patient's urine and other clinical samples: Comparative study

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> **Abstract**---This study was aimed to detecting three different virulence genes (UreC, HmpA and ZapA) for Proteus mirabilis isolated from different clinical samples ( urine from UTI non-Rheumatoid arthritis patients, urine from UTI Rheumatoid arthritis patients, wound swaps , chronic supportive otitis media(CSOM) and cerebrospinal fluid aspirate(CSF). Three hundred (300) different clinical samples were collected and cultured on blood agar and macConky agar. 40 of samples were identified as P. mirabilis, biochemical test and VITEK 2 system used to complete the identification. Phenotypic detection appeared the present 100% of urease enzyme (on urea agar) hemolysin enzyme ( on blood agar), swarming phenomena (on agar plate) while protease enzyme detection (on skim milk agar) present in 54% of isolates. Molecular results by PCR present UreC, HmpA and ZapA genes in 100 % in 40 isolates. Antibiotic Susceptibility test of Proteus mirabilis based on Kirby-Bauer disk diffusion method on Muller-Hinton agar to (14) different antimicrobial agents appeared 100% resistance to Aminoxicillin /clav, Cefotaxime and Erythromycin while the lowest resistance was against Impenem, Ciprofloxacin, Meropenem and Gentamicin (12.5%, 15%, 20% and 47.5%), respectively. In conclusion: Proteus mirabilis can isolated from different clinical samples and have virulence factors responsible for its pathogenicity and there is no difference in PCR result for these genes (UreC, HmpA and ZapA) between RA and different clinical samples.

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*Keywords*---RA, proteus mirabilis, virulance genes, UreC, HmpA, ZapA genes.

### Introduction

Proteus mirabilis are gram negative rod shape bacteria belong to Enterobacteraceae family are opportunistic bacterial pathogens .Its capable of causing serious invasive illnesses and has been linked to a variety of human disorders including respiratory, gastrointestinal, eye, ear, skin infections urinary tract infections (UTIs), meningitis in newborns or children and rheumatoid arthritis( RA)(Jamal et al., 2018). Swarming, growth rates, fimbria, flagella, , hemolysins, in proteases, addition to the ureases, expression of lipopolysaccharide (LPS) antigens and capsular polysaccharides(CPSs), are all potential virulence factors and bacterial behaviors associated with infection processes and disease in Proteus mirabilis which explain the difficulty in achieving clinical therapy success (Seo et al., 2015 and Al-Mayahi, 2017).Infections induced by Proteus mirabilis in the urinary system can lead to bacteriuria, cystitis, kidney and bladder stones, catheter blockage owing to stone encrustation, and severe pyelonephritis (Leelakrishna and Karthik, 2018; Letica-Kriegel et al., 2019). Virulence factors played a key role in causing infection in many parts of the urinary tract (Stankowska et al ., 2008) including toxins like hemolysin and its function of pore formation, biofilm and regulation of the pathogenicity (Schaffer and Pearson, 2015). Urease enzyme which causes kidney and bladder stones(Al-Duliami et al., 2011). Proteus mirabilis has a remarkable capacity to live, persist, and cause illness after entering the host's urinary system, despite antibiotic therapy and catheter replacement, because to its ability to avoid innate and adaptive immune responses. Because of its capacity to destroy antimicrobial peptides (particularly polymyxin). The findings of many immunological and microbiological research suggest that there may be a relationship between urinary tract infections (UTI) and RA, which are mostly caused by the *P. mirabilis* bacteria (Gibofsky *et al.*, 2014). The aim of this study is to compare the virulence factors encoding genes of Proteus mirabilis between Proteus mirabilis isolated from urine of RA patient and Proteus mirabilis isolated from different clinical samples.

### **Material and Methods**

A total of 300 different clinical samples were collected during the period from September (2021) to April (2022).These samples were (200 urine sample from non RA patient ,30 urine sample from RA patient ,25 swap from CSOM ,35 swap from wound and 10 CSF aspirate ),out-patient of both sex (234 female &66 male ). All these samples were collected from Al-Sadder Medical City, Al-Hakeem General Hospital. All *P.mirabilis* isolates present were identified by routine tests: colony morphology by culturing 37°C for 24 hrs on (blood and macckongy agar), biochemical tests including; catalase test, oxidase reaction, urease test and also VITEK 2 system used for more identification. The antibiotic susceptibility tests was carried out for all the *P. mirabilis* isolates using Mueller - Hinton method to measure the inhibition zones according to the recommendations of clinical and laboratory standards institute (CLSI 2022). The antibiotics used were: (Ak)

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Amikacin (10 µg/ml-disk): (AMC) Aminoxicillin /clav (30 µg/ml-disk): (CTX) Cefotaxime (30µg/ml-disk): (CRO) Ceftriaxone (10  $\mu g/ml$ -disk) : (CAZ) Ceftrazidime (C)Chloramphenicol(30µg/ml-disk):  $(30\mu g/ml-disk)$ : (CIP)Ciprofloxacin(10µg/ml-disk): (E)Erythromycin (10) $\mu g/ml$ -disk): (IPE)Imipenem / EDTA(10 µg/ml-disk) (CN)Gentamicin(30  $\mu$ g/ml-disk): (LEV)Levofloxacin(5: µg/ml-disk): (MEM) Meropenem(10 µg/ml-disk): (NET) Netilmicin and (30  $\mu$ g/ml-disk): (TMP) Trimethoprim (5  $\mu$ g/ml-disk). Genomic DNA was extracted from all Proteus mirabilis isolates using commercial genomic DNA purification kit (Dongsheng Biotech / China). DNA was extracted to provide a PCR template for amplification. The concentration and purity of extracted DNA were directly determined by Nano drop device, extracted DNA purity ranged between (1.6-2.0) and DNA concentration was between (18.5-812) ng/µl. Extracted DNA was confirmed by gel electrophoresis. Conventional PCR were used in this study to detect the presence of the important virulence genes (UreC, HmpA and ZapA) of the P.mirabilis bacteria using specific primers as shown table 1 and the PCR reaction mix preparation which used for detection of each gene are in table 2.

Primer type		Primer sequence (5'-3')	Product	Conditions	References
			size (bp)		
	F	GGGGGCAATTTCGGTGATGT	319	1min /95°C	
Urease	R	TGGCGCATAAGCGACCATAC		30 sec95°C	
				25sec/57°C	
				30sec/72°C	
				5min /72°C	-
Haemolysi	F	GCGGTGTAACGAAGCCAGTT	210	1min /95°C	igi
n	R	CGGCTTGGCTATCGGTTTGT		30 sec95°C	les
				25sec/57°C	r c
				30sec/72°C	ne
				5min /72°C	rin
Protease	F	GTTATTGGGCAACCCGCTGA	260	1min /95°C	Ц
	R	GCCTGAAATGTGCCATTGGG		30 sec95°C	
				25sec/57°C	
				30sec/72°C	
				5min /72°C	

Table 1: PCR primer sequence o	of UreC, HmpA d	and ZapA	genes and	their
	programs			

Table 2	The PCR	reaction	mix	preparation
1 abic 2.	THE LOR	reaction	IIIIA	preparation

Mixture Contents	Volume (µl)		
Master Mix	12.5		
Primer	3		
Template DNA	2		
Nuclease free water	7.5		
Total	25		

Once the PCR reaction volume get ready in PCR tube the mixture was spin down and then PCR tube placed in the PCR thermo cycler and the amplification reactions was started according to the program described in Table 1.

### **Results and Discussion**

### Isolation of Proteus mirabilis and their Antibiotics resistance

During the period from September 2021 to April 2022; 40 (13%) *Proteus mirabilis* bacteria were isolated from a (200 urine sample from non RA patient ,30 urine sample from RA patient ,25 swap from CSOM ,35 swap from wound and 10 CSF aspirate ),out-patient of both sex (234 female &66 male ) from Al-Sadder Medical City and Al-Hakeem General Hospital in AL-Najaf province. *P. mirabilis* isolated in 66% , 18% ,8% ,5% and 3% from urine non RA, urine RA ,wound ,CSOM and CSF, respectively as in figure (1)



Figure 1: The distribution of Proteus mirabilis isolates in the clinical samples

All these culture sterile isolates were identified on the basis of microscope examination, colony morphology and comparison of the biochemical characteristics with standard description in McFadden (2000). In microscopic examination (gram stain ) the organism was appeared as gram negative bacilli . The colony which appeared colorless or bale colony on Mac-Concky agar because non lactose ferment and  $\beta$ -hemolysis on blood agar with fishy odor. The identification of P. mirabilis also involved biochemical testing with production of urease, citrate utilization and catalase positive. Moreover, the biochemical test with VITIK 2 system was confirmed that all these isolates as *P. mirabilis*. In this study ,the isolation rate of Proteus mirabilis from all urine sample ( non RA and RA) (84%) and agree with studies of Yassen and Khelkal ,(2015) and Mohson et al .,(2020) who isolated P. mirabilis from urine sample in 77%,80% ,respectively and incompatible with Al-Bassam and Al-Kazaz, (2013). These differences might be due to the collection condition, type of sample, age of patient, the technique used for culture and diagnosis tools.

*P. mirabilis* has many virulence factors make it more important and commonest pathogen in different infection especially UTI. The main virulence factors associated with different stages of infection, including flagella, pili or adhesins, urease, hemolysin, metal intake, and immune escape (Yuan *et al.*, 2021).Different immunological and microbiological evidence results support that there could be a

link between urinary tract infections (UTI) and RA, this has mainly caused by *P. mirabilis* bacterium. Gibofsky *et al.*, 2014 discovered As a result of repeated infections with *P. mirabilis*, significant titers of bacterial antibodies are produced. The binding of these antibodies to cross reactive self-antigens will initiate a cascade of inflammatory reactions that will result in the creation of numerous chemokines and cytokines, resulting in local inflammation with or without systemic consequences.

The current study recorded that all 40(100%) isolates produced  $\beta$ -hemolysis and this result in agreement with AL- Jumaa *et al.* (2011) , Jabur *et al.*, (2013) , Mohammed *et al.* (2014) and Bunyan and Albakery , (2021) demonstrated that all isolates (100%) of *Proteus mirabilis* isolated from different clinical sources exhibit  $\beta$ - hemolysis on blood agar plates. Armbruster *et al.*, (2018) illustrated *P. mirabilis* hemolysin is thought to be a major virulence factor in most UTI. Hemolysin is a protein that forms holes in target host cells. It's been suggested that *P. mirabilis'* hemolytic activity aids infection progress into the kidneys. Also this study presented that the production of extracellular protease enzyme by *Proteus mirabilis* isolates was found in (54%) and this result agree with result by Hussain, (2016) who recorded that (56.6%) of isolates produce protease and its effect on skim milk agar appear as a zone around the colony as in the figure (2)



Figure 2: Proteus mirabilis isolates on skim milk agar

The present study founded that all isolates 40 ( 100%) produced strong extracellular urease when cultured on urea agar by change its color to pink .Urease is one of the most critical variables in the pathogenesis of *P. mirabilis*. Urease hydrolyzes urea to alkaline ammonia and carbon dioxide in vitro (on basic urea agar), raising the pH and altering the color of the phenol red indicator to pink (Friedrich *et al.*, 2005). Bunyan and Albakery, (2021) revealed that all *P. mirabilis* isolated produce urease enzyme as a virulence factor in bacteria isolated from urine infection. In this study all *Proteus mirabilis* isolates (100%) showed swarming phenomena when grown on agar plates Yuan *et al.*, (2021) reported swarming is likely an important aspect of initial bladder colonization in catheterized individuals .

# Antibiotics Susceptibility test of Proteus mirabilis

All isolates appeared 100% resistance to Aminoxicillin /clav, Cefotaxime and Erythromycin and high resistance to Amikacin, Ceftriaxone, Ceftrazidime,Chloramphenicol and trimethoprime ,while moderate resistance to Levofloxacin and Netilmicin and low resistance to Ciprofloxacin ,Gentamicin , Impenem and Meropenem as in figure (3). All isolates in this study concerned Multi-drug resistance due to their resistance to three antibiotics as reported by CLSI (2022).



Figure 3: Antibiotic Susceptibility test for Proteus mirabilis to 14 Antibiotics

Salama *et al.*, (2021) and the Iraqi study of Hussain, (2016) revealed that *P.mirabilis* recovered from different clinical specimens are characterized by multidrug resistant. According to Hussain *et al.*,(2021), bacterial isolates can develop chromosomally  $\beta$ -lactamase enzyme, which hydrolyzes chemical compounds containing a  $\beta$  -lactam ring, causing resistance to  $\beta$  -lactam antibiotics. In the same way, horizontal gene transfer (HGT) is vital in -lactam resistance. Changes in the location of penicillin binding proteins (PBPs) can lower the attraction of  $\beta$  -lactam antibiotics while increasing resistance to them. Changes in the outer membrane protein porins, which generates water-filled channels that allow the transport of chemicals into bacterial cells, may improve resistance to Cephalosporins and other –lactam antibiotics (Costello *et al.*, 2019).

### Molecular detection of Proteus mirabilis virulence gens

The current study used PCR for genotypic detection of genes that encode virulence factors, including hemolysin (*HpmA*), urease(*UreC*) and protease (*ZapA*) genes by using specific primer sequence to give a product size 210 bp ,319 bp and 260 bp respectively. The result were shown in the figures (4),(5) and (6)



Figure 4: Gel electrophoresis of PCR amplified product of *HpmA* gene primers with product 210 bp of *P. mirabilis* isolates. Lane (M), DNA molecular size marker (1KB ladder), All isolates (1-21) were positive results for *HpmA* gene.



Figure 5: Gel electrophoresis of PCR amplified product of UreC gene primers with product 319 bp of P. mirabilis isolates. Lane (M), DNA molecular size marker (1KB ladder), All isolates (1-23) were positive results for UreC gene.



Figure 6: Gel electrophoresis of PCR amplified product of *ZapA* gene primers with product 260 bp of *P. mirabilis* isolates. Lane (M), DNA molecular size marker (1KB ladder), All isolates (1-15) were positive results for *ZapA* gene.

All 40 isolates present hemolysin (*HpmA*), urease(*UreC*) and protease (*ZapA*) genes. This result is similar with Bunyan and Albakery , 2021. Chalmeau *et al*., (2011) illustrated the hemolysin enzyme destroys the leukocyte membrane by creating tiny holes in the membrane of the white blood cell and epithelial cell, and its presence is a key role in delivering iron to the bacteria; however, because it is cytotoxic, it destroys the host's kidney tissue (Dzutsev and Trinchieri, 2015; Grahl *et al*., 2021). *ZapA* is a metalloprotease that can degrade a wide variety of host proteins in vitro. IgA, IgG, secretory component, and antimicrobial peptides can be cleaved by protease enzymes, reducing their antibacterial efficacy. During *P. mirabilis* infection, *ZapA* may also help to evade the innate immune response. As a result of the existence of these genes, the organism becomes more pathogenic and resistant to antibiotic treatment (Schaffer and Pearson, 2015).

#### Conclusion

*Proteus mirabilis* can isolated from different clinical samples and have virulence factors responsible for its pathogenicity and there is no difference in PCR result for these genes (*UreC*, *HmpA* and *ZapA*) between RA and different clinical samples.

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