Vol. 06, No. 01, pp. 21 –25 (2025)
**ISSN: 2708-1818**

JOURNAL OF LIFE AND BIO-SCIENCES RESEARCH

www.jlbsr.org

# Molecular Detection of *hpmA* Gene in *Proteus mirabilis* Isolates and its Association with Inflammatory Biomarkers among Rheumatoid Arthritis Patients

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## *Abstract*

***Proteus mirabilis* is a Gram-negative rod-shaped bacterium most noted for its swarming motility and urease activity. It is recognized as one of considerable uropathogens. It is particularly common among patients with chronic inflammatory conditions. One of these conditions is rheumatoid arthritis, an immune-mediated inflammatory disorder that primarily affects and damages joints. This study aimed to investigate the prevalence of *P. mirabilis* along with its virulence gene (*hpmA*) in urinary tract infections and determine its association with key inflammatory biomarkers among rheumatoid arthritis patients. Eighty urine samples were collected from clinics and laboratories in Zakho city from September to December 2024. Phenotypic tests were used to identify *P. mirabilis* in patients' urine, and PCR assays were performed to investigate the *hpmA* gene. In addition, Blood samples were analyzed for Erythrocyte sedimentation rate (ESR), Rheumatoid Factor (RF), and C- Reactive protein (CRP) from the same RA suspected patients to confirm Rheumatoid Arthritis and evaluate inflammation level. Cultural and biochemical tests identified *P. mirabilis* in 15 samples (18.75%). Molecular analysis revealed that all 15 isolates (100%) of *P. mirabilis* samples had *hpmA* gene. Notably, patients with *P. mirabilis*–positive UTIs, especially those carrying *hpmA*, showed elevated ESR and CRP levels. This indicates an association between hpmA-mediated pathogenicity and inflammatory responses in RA. These results suggest the importance of screening *P. mirabilis* and its virulence factors in RA patients with UTIs. The presence of *hpmA* which is responsible for hemolysin production, may be associated with increased levels of inflammation in these patients.**

***Keywords*:** *Rheumatoid Arthritis, Proteus mirabilis, UTI, PCR, hpmA gene*

Received: January 10th, 2024/ Accepted: March 21st, 2025/Online: March 31st, 2025.

## I. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic auto-immune disorder that mainly affects joints and tissues surrounding them. It leads to permanent inflammation, pain, and progressive joint destruction (Gibofsky, 2014; Jinno *et al.,* 2018). Although the precise etiology of RA is unknown, it appears that a combination of genetic susceptibility, and environmental factors lead to the onset of the illness (Ebringer and Rashid, 2014; Rashid *et al.,* 2017). The findings of several immunological and microbiological studies suggest that urinary tract infections (UTI), which are primarily caused by the *Proteus mirabilis* (*P. mirabilis*) bacteria, may be related to RA since infection is often manifested early in the course of RA and may occur ahead of clinical arthritis (Gibofsky, 2014; Geerlings, 2016).

*Proteus mirabilis* is a Gram-negative and rod-shaped bacterium that belongs to the *Enterobacteriaceae* family. This bacterium is well-known for its ability to produce urease enzymes and swarming motility on agar plates. *Proteus mirabilis* bacteriuria is usually associated with inflammation of the urothelium. *P. mirabilis* produces a toxin known as α-hemolysin which damages kidney tissue. This destructive protein is under the control of one of the two genes, *hpmA* and *hpmB* (Armbruster *et al.,* 2018). Increased *hpmA* production



*doi: 10.38094/jlbsr601144*

is associated with the ability of *P. mirabilis* strains to invade and is coordinated throughout cell growth into colony forms and infection (Schaffer and Pearson, 2017). Hemolysin has been shown to possess homologous sequences that cross-react to particular self-antigens found in synovial tissues, establishing "molecular mimicry" as the most likely mechanism in the development of RA (Coelho *et al.,* 2020). The antibodies' binding to cross-reactive self-antigens triggers an inflammatory cascade to induce cytokine release and as a result, local or systemic inflammation may appear. The passive impact of such activation on other immune cells, including T lymphocytes, may result in further amplifying the inflammatory responses with the onset of arthritis (Puntis *et al.,* 2013).

Recent studies demonstrated the critical role of urinary tract pathogens in rheumatoid arthritis. For instance, a study conducted by Jin *et al*. (2023), reported a strong correlation between RA-linked urinary microbiota and systemic immune responses in hosts. While, Coelho *et al*. (2020) found that *Proteus* bacteria is one of the causes that might trigger an immunological response in RA patients, which is demonstrated by increased levels of antibodies. in urine samples of UTI patients (Coelho *et al.,* 2020). Additionally, a study by Magdy *et al*. (2024) revealed that approximately 17% of RA patients experienced UTI infections almost a month before the disease onset. Hence, it is crucial to determine the role of the *hpmA* gene in *P. mirabilis* in the severity of the disease and the inflammatory responses in RA patients with urinary tract infections. Accordingly, this study was aimed to molecularly determine *hpmA* gene of *P. mirabilis* isolates obtained from RA patients with UTIs and to evaluate its association with key inflammatory indicators.

## II. Materials And Methods

### A. Sample collection and processing

Eighty mid-stream urine of both sexes (15-60) years were collected in sterile closed tubes (Citotest/China) from suspected RA patients. Samples were collected from September to December 2024 from main private laboratories and clinics in Zakho city. Eighty Venous blood samples (5 ml) were aspirated from the same patients, some were put in the Erythrocyte Sedimentation Tube (ESR) (Sediplast/Italy) to be clot at room temperature within one hour. The remained blood was centrifuged to separate the Serum which was kept at deep freezing condition (-20º C). To confirm the RA status of suspected patients, routine tests associated with RA including Rheumatoid Factor (RF), and C- Reactive protein (CRP) were performed on blood samples. But only patients with confirmed RA were included in this study Additionally, their urine was prepared for further analysis.

### B. Identification of P. mirabilis

*Proteus mirabilis* that was detected in the urine of RA patients was identified through routine laboratory methods including colony morphology on both Blood agar and MacConkey agar, biochemical tests were also performed for further confirmation including; Motility, Oxidase, Catalase test, Urease test, IMViC test, H2S precipitation production in TSI agar (Talaiekhozani *et al.,* 2015).

### C. Bacterial DNA extraction and PCR

Genomic DNA was extracted from 15 isolates via Genomic DNA Mini extraction kit (Favorgen/Taiwan). Then NanoDrop Microvolume Spectrophotometers (Thermo Fisher Scientific/USA) were used to determine the purity of the extracted DNA. After that Polymerase Chain Reaction (PCR) (Macrogen/Korea) was used for amplification of the *hpmA* gene by using *hpmA* primer as shown in Table 1. The PCR was conducted according to the manufacturer's instructions as its conditions are clarified in Table 2. Finally, all amplicons were run on agarose gel for observation and their size was determined by comparing them with a DNA ladder (1000 bp) (GDSBio/China).

Table1. Primer of *hpmA* gene utilized in this study for amplification in the PCR reaction

| Gene | Primer (5'-3') | Size (bp) | Reference |
|---|---|---|---|
| *hpmA* | F: GTTGAGGGGCGTTATCAAGAGTC<br>R: GATAACTGTTTTGCCCTTTTGTGC | 709 | (Cestari *et al*., 2013) |

Where: F: Forward primer; R: Reverse primer; bp: base pair

Table 2. PCR program for gene amplification

| Step | Cycle | Temperature (ºC) | Time |
|---|---|---|---|
| Initial Denaturation | 1 | 95 | 5 Minutes |
| Denaturation | 35 | 95 | 30 Seconds |
| Annealing | | 62 | 30 Seconds |
| Extension | | 72 | 20 Seconds |
| Final Extension | 1 | 72 | 5 Minutes |

### D. Statistical analysis

Data was analyzed using Jamovi version 2.6.17 statistical software to describe and determine the correlation between variables. A p-value was considered statistically significant when it was less than 0.05 (P-value < 0.05).

## III. Results

Among 80 urine samples collected from RA patients, *P. mirabilis* was isolated in 15 cases (18.75%). Among these isolates, 11 were from female patients (73.33%) and four from male patients (26,67%), indicating a higher prevalence among female patients. This disparity between men and women may reflect different susceptibility or physiological factors, suggesting further research.

In culture, *P. mirabilis* had unique characteristics that were helpful for its identification. On blood agar, it had a typical fish-like odour and clear swarming motility, which are hallmark diagnostic characteristics of this bacterium. On

MacConkey agar, the isolates grew as pale, non-lactose fermenting colonies and low convex colonies confirming their identification as *P. mirabilis* as presented in Figure 1.

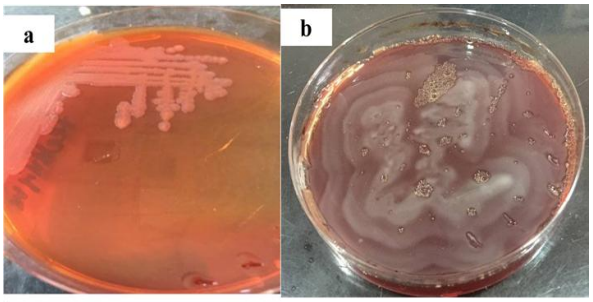


Figure 1. Cultural Characteristics of *P. mirabilis* (a): Isolates of *P. mirabilis* on MacConkey agar, (b): swarming of *P. mirabilis* on Blood agar.

Furthermore, as we mentioned before to confirm that the patients were infected by this bacterium some biochemical tests were conducted including the motility test, Oxidase test, Catalase test, and Urease test. These tests were chosen based on their reliability to accurately identify *P. mirabilis* and differentiate it from other bacteria. The results showed that all 15 isolates were found to have similar results, which match the biochemical features that are already known to be found in *P. mirabilis*. The results of these tests are summarized in table 3, and Figures 2 and 3. However, *Streptococcus pneumoniae* was used as a negative control for the Motility test, Catalase test, and Urease test. Whilst in the Oxidase test *Pseudomonas aeruginosa* was used as a positive control.

Table 3. Results of the biochemical test of *P. mirabilis*

| No. | Biochemical test | Result |
|---|---|---|
| 1 | Motility | Positive |
| 2 | Oxidase | Negative |
| 3 | Catalase test | Positive |
| 4 | Urease test | Positive |
| 5 | H2S Production | Positive |

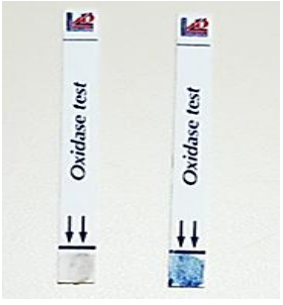


Figure 2. Oxidase test sticks: the left-handed stick was negative (no color changes) and it was *P. mirabilis*. Whereas the right-handed stick that was wiped off by *Pseudomonas aeruginosa* is positive (blue-purple), it was used as a control.

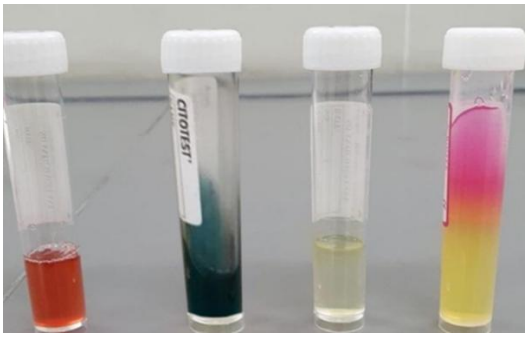

Figure 3. Results of biochemical tests of *P. mirabilis*. From left 1. Methyl Red test, 2. Citrate utilization test, 3. Indole test, 4. Urease.

RA related tests were also done for all 15 blood samples that had UTI infection due to *P. mirabilis* for detecting logical and scientific evidence of correlation between RA and UTI infections caused by *P. mirabilis*. All (CRP, RF, and ESR) tests showed elevated rates of inflammation among RA patients infected with *P. mirabilis*. The results revealed that both CRP and ESR rates are distributed with low variability as shown in Table 4.

Table 4. Results of blood tests among 15 RA patients with *P. mirabilis* bacteriuria.

| Sample No. | Gender | Age (Years) | CRP (mg/L) | RF | ESR (mm/hr) |
|---|---|---|---|---|---|
| 1 | F | 35 | 82 | + | 73 |
| 2 | M | 37 | 61 | + | 55 |
| 3 | F | 40 | 77 | + | 60 |
| 4 | F | 33 | 60 | + | 67 |
| 5 | F | 50 | 80 | + | 84 |
| 6 | M | 55 | 56 | + | 45 |
| 7 | F | 46 | 71 | + | 62 |
| 8 | F | 48 | 68 | + | 70 |
| 9 | F | 56 | 85 | + | 71 |
| 10 | F | 49 | 69 | + | 49 |
| 11 | F | 40 | 77 | + | 61 |
| 12 | F | 50 | 60 | + | 69 |
| 13 | M | 36 | 80 | + | 77 |
| 14 | F | 55 | 76 | + | 66 |
| 15 | M | 57 | 53 | + | 62 |
| Normal range | | | <0.3 mg/L) | 0-15 mm/h men 0-20 mm/h women | |

Where; F: Female; M: Male; CRP: C-reactive protein; RF: Rheumatoid factor; +: Positive; ESR: Erythrocyte sedimentation rate.

The correlation between ESR and CRP levels in RA patients infected with *P. mirabilis* is demonstrated in Table (5), showing that a Pearson correlation coefficient (Pearson's r = 0.547) suggests a moderate positive association, indicating that as CRP levels are elevated, ESR levels increase. The association between both of these biomarkers is statistically significant (p-value=0.035). This finding confirms the impact

of *P. mirabilis* infection on increasing systemic inflammation in RA patients.

Table 5. Correlation between ESR & CRP among RA patients infected with *P. mirabilis*.

| Variables | Pearson's r | Degrees of Freedom | p-value |
|---|---|---|---|
| ESR & CRP | 0.547 | 13 | 0.035 |

According to the results of agarose gel electrophoresis, all 15 amplicons were processed through the PCR amplification procedure and run on the gel. The electrophoresis results demonstrated that each of the tested isolates showed a positive band corresponding to the *hpmA* primer, with expected amplicon size of (709 bps). The presence of these bands indicates that all isolates amplify the target gene, confirming that their *hpmA* gene is positive, as is obvious in Figure 4.

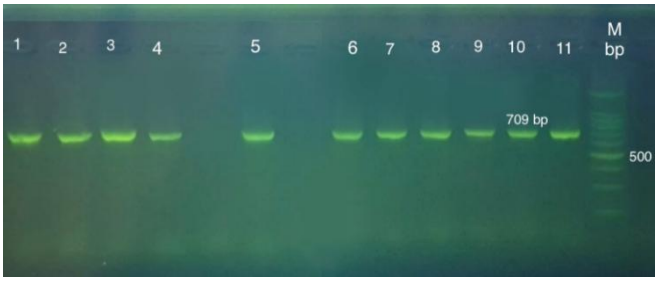


Figure 4. Amplicons of *P. mirabilis* isolates showing positive results for the *hpmA* gene (709bp). Samples from 1-11 were run on (1.2) Agarose gel for one hour at 45 volts. DNA ladder was (1000bp).

## IV. Discussion

Rheumatoid arthritis (RA) is a common autoimmune condition. Despite the mysterious causative agent of RA, various experimental evidence supports the microbial origin of it (Jin *et al.,* 2023). In this study, we investigated the prevalence of *P. mirabilis* and its virulence gene (*hpmA*) in urinary tract infections (UTIs) among RA patients, as well as its association with key inflammatory biomarkers.

This study illustrated that *P. mirabilis* was identified in 15 out of 85 urine samples collected from RA patients. This prevalence is consistent with the findings of Magdy *et al*. (2024), who reported 51% of *P. mirabilis* in a study of 50 urine samples of RA patients with UTIs in Najaf, Iraq. However, our results are slightly lower than those reported by Al Kady *et al*. (2019) in Egypt, where *P. mirabilis* was detected in 25% of RA patients, which is much lower than the 41% prevalence reported among 100 RA patients with UTIs by Saleem *et al*. (2021) in Pakistan.

The variability in the prevalence of *P. mirabilis* infection reported across these studies could be attributed to several factors, including differences in demographic characteristics, sampling methods, diagnostic criteria, and research design. For instance, the study by Magdy *et al*. (2024) focused on a specific population of RA patients who admitted to only a single hospital in Najaf, Iraq. With a relatively small sample size (n=50) and a higher proportion of female participants. In contrast, Saleem *et al*. (2021) recruited a larger cohort of 100 RA patients in Pakistan, with a mean age of 61.22 ± 10.18 years and a similar gender distribution (67% female, 33% male). The observed prevalence may be influenced by these demographic differences, as well as the differences in lifestyle characteristics (e.g., inactive or active lifestyle), which may influence the susceptibility to *P. mirabilis*.

Additionally, differences in sampling methods and diagnostic criteria could also account for the variability. For example, using different types of culture media, biochemical tests, and molecular techniques (e.g., PCR) may affect the sensitivity and specificity of detection. In the current study, a combination of cultural, microscopical, and biochemical tests was used for the detection of bacteria, followed by using PCR for confirmation of the presence of the *hpmA* gene in the isolated *P. mirabilis*. In a study by Magdy *et al*. (2024) despite using conventional microbiological methods and the VITEK system used for the identification of the bacteria, the PCR was then used for the gene detection. Whereas in the study conducted by Saleem *et al*. (2021), the serotypes of *P. mirabilis* were determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting to confirm the presence or absence of *P. mirabilis*.

Most infection were detected among females which was 11 out of 15 RA cases. In accordance to Al-obaidi and Al-Hashimy (2023) gender differences are seen due to biological differences and behavioural differences such as smoking are crucial factors that may influence susceptibility and phenotype of RA. Based on our findings of this investigation on *Proteus*, there is a potential association between this bacteria and RA, beginning with recurrent symptomatic *Proteus* bacteriuria and possibly contributing to the development of RA. However, it is important to note that RA is a multifactorial disease, and while *P. mirabilis* may play a role as one of those factors, no conclusive evidence of a causative relationship has been established in the broader scientific literature.

Using fragments of DNA with a limited number of nucleotides (oligonucleotides), which function as primers specifically for hemolysin-producing gene in *P. mirabilis* (*hpmA*), the single polymerase chain reaction technique was employed for investigation. Unlike other *Proteus spp., P. mirabilis* α-hemolysin is arranged according to two genes, *hpmA* and *hpmB*, which encode the HpmA and HpmB proteins, respectively (Ebringer and Rashid, 2014). In our study, out of 15 amplicons 15 (100%) isolates were positive for the *hpmA* gene with molecular size of (709 bp). The results of this study were in agreement with a study done by Khalid *et al*. (2018) who illustrated that 100% of *P. mirabilis* showed positive results for the *hpmA* gene. Furthermore, almost the same results were detected by Al-Hamdani and Al-Hashimy (2020) in Baghdad city who investigated that *hpmA*

gene prevalence was (90%) among RA patients. As a result, the confirmed rate of (100%) of the *hpmA* gene among RA patients gives reasonable evidence that this gene might play a vital role in RA linked UTI infections caused by *Proteus spp*. According to study results, the PCR technique provides affordable detection of the *hpmA* gene by PCR was sensitive enough to be used for the discovery of these virulence factors produced by *P. mirabilis* among RA patients.

## V. Conclusion

In conclusion, this study presents molecular, and clinical evidence that *P. mirabilis* plays a role in elevation of inflammation in RA. The significant correlation between CRP and ESR, in combination with the identification of virulence genes, highlights the need for further study into pathogenesis and clinical impacts of this bacteria in UTI among RA patients.

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