Journal of Applied and Natural Science
16(4), 1719 - 1730 (2024)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Phenotypic and genotypic detection of biofilm genes for *Proteus, Morganella, Providencia* (PMP) bacterial group isolated from clinical samples of some hospitals in Mosul/Iraq

Eman M. Taher*
Department of Biology, College of Science, University of Mosul, Mosul, Iraq
Basima A. Abdullah
Department of Biology, College of Science, University of Mosul, Mosul, Iraq
Anmar A. AL Taie
Department of Biology, College of Science, University of Mosul, Mosul, Iraq

*Corresponding author. E-mail: imansbio116@uomosul.edu.iq

Article Info
https://doi.org/10.31018/jans.v16i4.5935
Received: July 10, 2024
Revised: November 29, 2024
Accepted: December 3, 2024

How to Cite
Taher, E. M. *et al.* (2024). Phenotypic and genotypic detection of biofilm genes for *Proteus, Morganella, Providencia* (PMP) bacterial group isolated from clinical samples of some hospitals in Mosul/Iraq. *Journal of Applied and Natural Science*, 16(4), 1719 - 1730. https://doi.org/10.31018/jans.v16i4.5935

Abstract
The three genera *Proteus spp., Morganella spp., Providencia spp.* (PMP) is a group of bacteria belonging to the Enterobacteriaceae family that is predominantly implicated in nosocomial infections within hospital environments. The research aimed to ascertain the frequency of PMP in various clinical samples from some hospitals by isolation and confirming the identification of bacteria from biofilms using Viteck-2 and phenotypic and genotypic determinants of biofilm genes. Out of 380 isolates from different clinical sources that were identified phenotypically (microscopic examination, culture, and biochemical tests) confirmed identification of bacteria from biofilms using Viteck-2 and genotypic determinants of biofilm genes DNA were extracted from twenty identified strains, the PCR products derived were utilized for sequencing. The Congo Red Agar and Microplate methods employed encompassed qualitative and quantitative approaches to analyze the formation of biofilms. In this approach, bacteria that develop black colonies were assessed as positive for biofilm formation.*Proteus mirabilis* showed strong producers (19.8%), *Providencia stuartii* (100%), and *Morganella morganii* produced pink colonies. The strains were assessed as non-producers and analyzed quantitatively using the microplate technique. which shows strong biofilm production in most PMP groups. Genetic detection of biofilm genes showed that the *mrpA* gene was identified in *P. mirabilis* strains utilizing the PCR technique as positive for all strains at (100%) but *M. morganii* at (25%), positive amplification for all strains (100%) for the *rsm* gene in *P. mirabilis strains*. *P. stuartii* gave positive detection for the *fim A* gene by utilizing the PCR technique as (100%). All genes were involved in biofilm formation.

Keywords: Biofilm, *fim A* gene, *Proteus Morganella Providencia* (PCR), *mrp A* gene, *rsm* gene

INTRODUCTION

Proteus spp., Morganella spp. and Providencia spp. (PMP) are a group of bacteria belonging to the family Enterobacteriaceae known, for being gram-negative, consisting of facultatively anaerobic rods capable of thriving in environments with limited nutrients (Temkin *et al*., 2014). Various diseases of Enterobacteriaceae can be induced in both plants and animals. *Proteus mirabilis* is frequently identified as the etiological factor in a variety of clinical infections that can manifest in individuals undergoing prolonged bladder catheterization. Moreover, they are responsible for opportunistic infections (Milo *et al.,* 2016). The biofilm formation process is of utmost importance in facilitating bacterial survival in adverse conditions and contributes to the persistence of infections (Hall-Stoodley and Stoodley, 2009). The occurrence of biofilm development by *Pr. mirabilis* on catheter surfaces has been extensively documented in scientific literature. The crystallized biofilms causing catheter incrustation and obstruction represent significant concerns. The biofilms contain two primary types of crystals: struvite (magnesium ammonium phosphate) and apatite (hydroxyl calcium phosphate). These crystals are typically present in urinary tract biofilms and can impede urine flow (Tabataba *et*

al.,2021). *Pr. mirabilis* exhibits various virulence factors, including flagella, fimbriae, Hemolysin production, protease enzyme production, biofilm production, urease enzyme, and quorum sensing. (Armbruster *et al.*,2018). The adherence of fimbriae to renal cells to demonstrate the onset of pyelonephritis and the essential role of fimbriae in cystitis has been elucidated in previous research investigations (Scavone *et al*., 2015). Mannose-resistant Proteus-like fimbriae (MR/P) can be identified in urinary tract infections (UTIs) and play a role in the pathogenesis. The direct observations of *Pr. mirabilis* within mice's bladder, urine, and kidneys have provided evidence of MR/P fimbriation throughout the urinary tract. Variation in the orientation of the mrp promoter may aid in evading host defenses. The development of biofilm formation relies on MR/P fimbriae (Zisman, 2017). The genus *Providencia* comprises ubiquitous, facultative anaerobic microorganisms capable of motility through peritrichous flagella. At this time, nine distinct species within the genus (Wie *et al*., 2015). The ability of pathogenic bacteria to cause disease in a vulnerable host is enabled by the combined impact of multiple virulence factors and determinants throughout the infection process, which is further enhanced by their capacity to generate biofilms (El-Khatib *et al.* 2018). Biofilms are distinguished by a matrix primarily consisting of Exopolysaccharides (EPS), which play a crucial role in enhancing the conveyance of nutrients by permeating through pores and channels. It is common to observe bacteria residing in colonies or adhering to the surfaces of living or inanimate objects, including human body tissues and medical devices (Pelling *et al.,* 2019). Factors such as bacterial adhesion, flagella expression, polymers, and adhesion fimbriae are recognized as significant contributors to biofilm formation (Flemming *et al*., 2016*).*

Morganella morganii, a Gram-negative rod-shaped bacterium, is well recognized. as an opportunistic pathogen mainly associated with nosocomial infections or neonatal sepsis (Bujňáková *et al.,* 2022). This bacterium can be found in various environments and within the intestinal tracts of humans, mammals, and reptiles as part of their normal flora. The diverse range of hosts and unique symbiotic relationships established by *M. morganii* present a challenging aspect that increases threats and potentially leads to more intricate infections. Numerous virulence factors have been detected in the genome of *M. morganii*, such as the type III secretion system (T3SS), type I fimbriae, endotoxins, and toxins. The fim *cdh* genes are expected to function as virulence factors related to type I fimbriae, thereby playing a crucial role in the colonization and pathogenicity of *M. morganii* through facilitating adherence to surfaces or other cells. and enhancing their capability for biofilm formation (Minnullina *et al.,* 2019).

So, the present study aimed to determine the prevalence of PMP bacterial group from different clinical samples by isolation and confirmed identification by Viteck-2 of bacterial strains from biofilms and phenotypic and genotypic determinants of biofilm genes.

MATERIALS AND METHODS

Sample collection isolation of bacteria

The present investigation was conducted at the Laboratories of Bacteriology and the Department of Molecular Biology within the College of Sciences, University of Mosul in Iraq. From March to September 2023. Three hundred and eighty clinical specimens were obtained from patients treated at Hospitals in Mosul City/Iraq. The pathogenic PMP bacterial group was isolated by streaking bacterial colonies on MacConkey's agar and blood agar plates and incubating at 37°C for 24 hours. The colonies were identified based on phenotypic characteristics, involving microscopic examination of the cells through gram staining, and culture and biochemical tests. The process aimed at identifying the PMP group to the species level was conducted by using the kit of Vitek-2 system BioMerieux, France (Guidone *et al*.,2023).

Animal ethical approval

Clinical specimens were obtained from patients treated at Hospitals in Mosul City/Iraq according to the animal ethics approval under administrative order number 1388 on 22/3/2023**.**

DNA extraction of bacteria strains

The genomic DNA from the strains was isolated in the form Bacterial DNA Extraction Kit (Geneaid) following the manufacturer's guidelines. The concentration of the genomic DNA was determined with a NanoDrop spectrophotometer from Thermos Fisher Scientific, USA.Assessment of both the quality and quantity of DNA extracted was conducted through the process of electrophoresis. Following extraction, the DNA of high quality underwent PCR amplification of 16SrRNA genes utilizing universal primers F AGAGTTTGATCTGGCTCAG R GGTTACCTTGTTACGACTT (Schulze-Schweifing *et al*., 2014). Subsequently, the quality of the PCR products was assessed by subjecting them to electrophoresis in a 2% agarose gel. The identity of the quality-trimmed 16S rRNA sequence was determined by comparing it with sequences in the NCBI (National Center for Biotechnology Information) nucleotide database utilizing the BLAST algorithm Basic Local Alignment Search Tool (Altschul *et al*., 1990).

Detection of biofilm formation

Two approaches were implemented to detect Biofilm formation - Congo Red method and Microtiter plate assay.

1-Congo Red Agar method

The Congo Red agar (CRA) method represents a straightforward, qualitative screening technique utilized to identify biofilm formation. The employed medium was made of brain heart infusion broth (BHI) (37 g/l), along with sucrose (50 g/l), agar No.1 (15 g/l), and Congo red (0.8 g/l). At first, a dense aqueous solution of Congo Red dye was formulated and sterilized using an autoclave (121ºC for 15 minutes), distinct from the autoclaving process of the other medium constituents. Subsequently, the solution was introduced to the autoclaved brain heart infusion agar with sucrose at 55ºC. Following this, CRA plates were subjected to inoculation with the test organisms and then incubated at 37°C for 24 hours under aerobic conditions. The results were interpreted using a predefined color scale for the indication (Tayal Ruchi *et al.*,2015)

- Black, dry, crystalline colonies as Biofilm producer
- Red and pink colonies indicate a Non-Biofilm Producer, specifically of the Red/pink variety.
-The Bordeaux Darkening of colonies is observed without dry, crystalline features, suggesting an Indeterminate producer.

2-Microtiter plate assay

The capacity of PMP group strains to generate biofilms. Was detected using a modified procedure (Wu *et al.,* 2011). PMP strains were grown overnight at 37°C in a 10mL-plane tube containing 3 mL brain heart infusion broth (BHI). 160 µl was added to the Microtiter plate wells, including BHI broth inoculation with the tested strains. The bacterial suspension corresponding to 0.5 McFarland at D600 was used as a starting concentration for all samples tested, and plates were incubated for 30h at 37°C . The negative control was prepared by using sterile BHI broth. Bacterial cultures were discarded, and wells were stained for 20 min with 200µl of 0.5% crystal violate. The surplus stain underwent three wash cycles with 200 µl of sterile distilled water, followed by the addition of 200 µl of 95% ethanol into each well. Subsequently, the optical density was assessed at 570 using a microtiter plate reader. The isolates were assessed and grouped into the following categories:

-none biofilm producers (-) (OD≤ ODc).
-weak biofilm producers (+) (ODc <OD ≤ 2*ODc)
-moderate biofilm producers (++) (2*ODc <OD≤*4*ODc)
-strong biofilm producers (+++) (4*ODc<OD) Optical density Cut-off value (*ODc)is defined as three standard deviations above the mean OD of the negative control (Gopinathan *et al.,*2021)

Genetic detection of biofilm formation genes:

Only ten *Pr. mirabilis*, eight *M. morganii*, and two *P. stuartii* strains were utilized for molecular detection through PCR. The DNA extraction from the samples was carried out using the Geneaid kit following the manufacturer's protocol. The identification of strains' biofilm genes was accomplished through PCR Thermocycler amplification and using specific primers as outlined in Table 1, with specific PCR reaction conditions detailed in Table 2. Each gene's reaction mixture included Taq PCR Premix 2012 (0.2 /20 µl rxns /96 tubes from iNtRON, Korea), forward primer (1 µl at 10 picomols/µl), reverse primer (1 µl at 10 picomols/µl), variable DNA sample concentration, variable free nuclease water, and a final reaction volume of 25µl (Table -3). Electrophoresis was conducted using a 2% agarose gel to visualize the PCR products stained with red safe stain (Korea). The sequencing of the PCR products was carried out by Macrogen DNA sequencing Korea (Jaber, 2022).

RESULTS AND DISCUSSION

Forty-seven strains out of (380) isolates were identified as PMP bacterial groups using VITEK- 2 (BioMerieux, USA) were identified, *Pr. mirabilis* from urine was 21 (5.52%), burn 9(2.36), pus and wound swabs 4(1%), stool 3 (0.78%). The most common pathogens were followed by *M. morganii* from urine 8(2.1%) and *P. stuartii* from pus, wound swabs and stool 2(0.52%). The majority of the isolates were recovered from urine

Table 1. Oligonucleotide primers from various genes utilized during the investigation

Gene	*Sequence [5'-3']*		Amplicon Size (bp)	Reference
mrp A	F	ACACCTGCCCATATGGAAG ATACTGGTACA	550	Zunino et al. 2001
	R	AAGTGATGAAGCTTAGTGATGGTGATGGTGATGAGAG-TAAGTCACC		
FimA	F	GCATGTGCTGTTGATGCTAAC	215	- Guidone et al.,2023
	R	GAACCCGCAACAGCTAGAA		
Rsm	F	TAG CGA GTG TTG ACG AGT GG	562	Shi et al., 2016
	R	AGC GAG GTG AAG AAC GAG AA		
16s rRNA	F	AGAGTTTGATCTGGCTCAG	1492	Schulze-Schweifing *et al.*,2014
	R	GGTTACCTTGTTACGACTT		

F =Forward; R= Reverse

Table 2. Programs of PCR Thermocycling conditions of primers

Gene Name	Initial Denaturation C^0 /min	Denaturation C^0/sec.	Annealing C^0/sec.	Extension C^0/sec.	Final Extens C^0/ sec.	Cycles Number
mrpA	95/5	94/30	40/30	72 /30	72 /5	35
fim A	95/5	94/30	54/30	72 /30	72/ 5	35
rsm	95/5	94/30	56/30	72 /30	72/ 5	35
16rRNA	95/5	94/40	55/40	72 /30	72 /5	35

Table 3. PCR reaction volume and its components

Components	Volume of sample (µL)
Master Mix	12.5
Forward primer	1
Reverse primer	1
DNA	Variable
Nuclease Free Water	To 25
Total volume	25

(16.20 %; n=179), followed by burn (9.8%; n=91) The findings of this investigation align perfectly with the outcomes achieved by (Elhoshi *et al*.,2023), who isolated *P. mirabilis* from cases of UTIs and other clinical sources. Enteric bacteria were observed on differential MacConkey's agar as colorless or pale colonies due to bile salts and crystal violet employed to inhibit the proliferation of gram-positive bacteria-particularly Enterobacteriaceae. PMP Group, a motile Gram-negative bacterium, is a frequent etiological agent of complicated UTIs. These infections typically manifest in individuals with urinary tract functional or anatomical abnormalities and patients undergoing prolonged catheterization, significantly amplifying the bacterium's influence on public health. Previously, multidrug-resistant *Pr. mirabilis* cases were identified in human clinical samples (urine, blood, sputum, stool, pus) and livestock (Algammal *et al.,*2021).

The DNA extracted from the strains was visualized using Agarose gel electrophoresis for molecular diagnosis. Its purity was confirmed and the DNA sequences obtained were cross-referenced with the information in GenBank through utilization of the BLAST program (https://blast.ncbi.nlm.nih.gov/). The resulting 20 bacterial strains from 16SrRNA gene sequencing were 100% identical to *Pr. mirabilis* 16S rRNA gene using BLAST (Mukhtar *et al*.,2018). The 16S rRNA sequence of eight isolated strains revealed 99% of identity with strains of *M. morganii* available in the database agree with (Ferheen *et al*. (2020); Behera *et al*.,2023) and with the two strains of *P.stuartii (*Al□Gburi and Mohammed,2020) . The samples were subjected to sequencing and subsequent analysis for comparison through a National Center for Biotechnology Information (NCBI) database. The results indicated a 99% similarity in genetic identity with strain *P. stuartii* within the Enterobacteriaceae family. Regarding 16S rRNA gene sequence similarity, the family of Enterobacteriaceae demonstrated the highest resemblance, particularly with members of the genus *Providencia.*

Biofilm producers by Congo Red assay as a qualitative approach

In the qualitative assessment using Congo red agar plates, merely seven strains (18.9%) of the 37 isolates of *Pr. mirabilis*, along with two strains of *P. stuartii,* produced black colonies exhibiting a crystalline appearance at 100% is indicative of being strong biofilm formers as in Fig. 2 agrees with (Dhanalakshmi *et al*.,2018) who reported that 46% of uropathogens producing black colony indicated as strong producer, 100% and 81%, respectively for *M. morganii and Pr .mirabilis* produce pink colony indicative of nonbiofilm formers as in Table-4- and Fig. 3.This agrees with Gopinathan *et al*. (2021) who showed the ability of *Pr. mirabilis* to produce biofilm o on CRA. Biofilm producers by Congo Red agar and the same study disagree with the present result for *M. morganii* which appears as pink colony on CRA method (ALjeeliz *et al.* 2022) indicated that the Congo Red technique proved to be a swifter and more sensitive approach than other phenotypic methodolo-

Table 4. Number and percentage of biofilm formation by Congo Red Agar method (CRA)

Bacterial Strains	Biofilm producers N	Percentage %	Non-biofilm producing N	Percentage %
Proteus mirabilis	7 out of 37	18.9	30	81.1
Morganella morganii	0 out of 8	00	8	100
Providencia stuartii	2out of 2	100	0	00

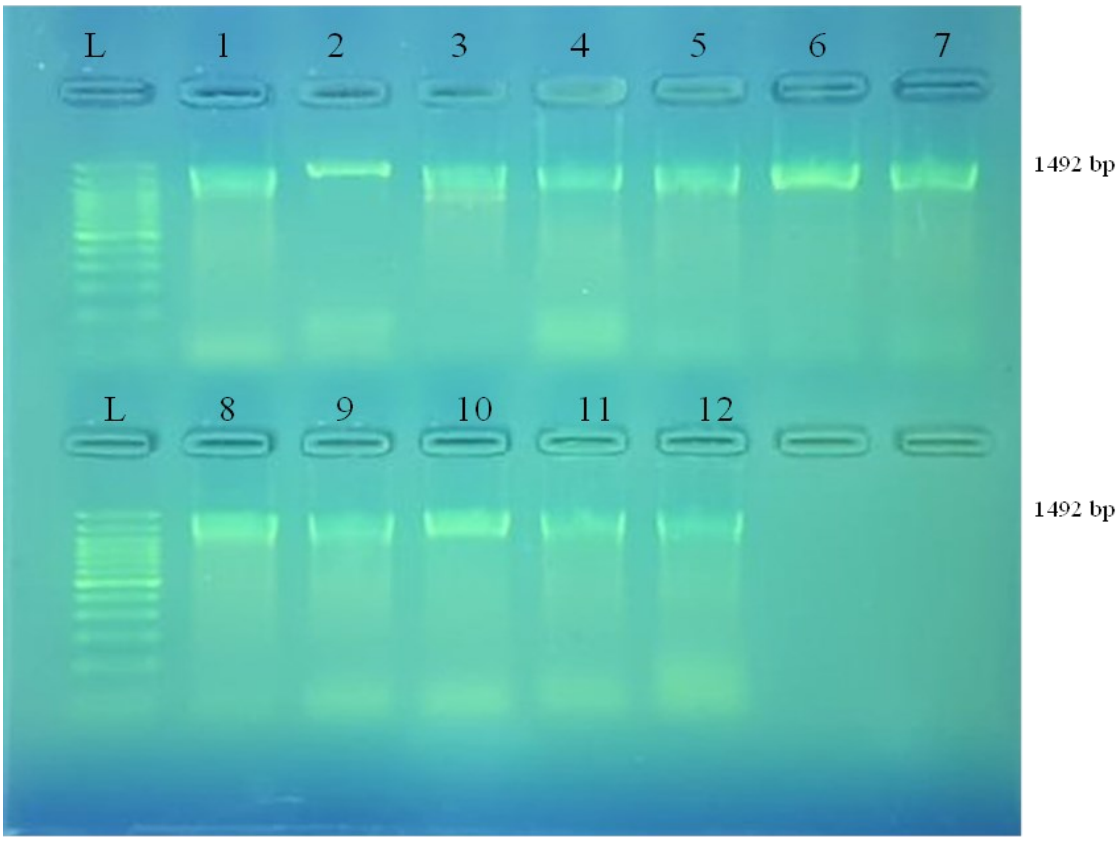

Fig.1a. *Visualization of bacterial DNA isolated in gel electrophoresis. (Detection of 16SrRNA gene (1492 bp). Line (L) marker (100bp). Lines (1-10) represent positive results for Proteus mirabilis and11,12 line for Providencia stuartii*

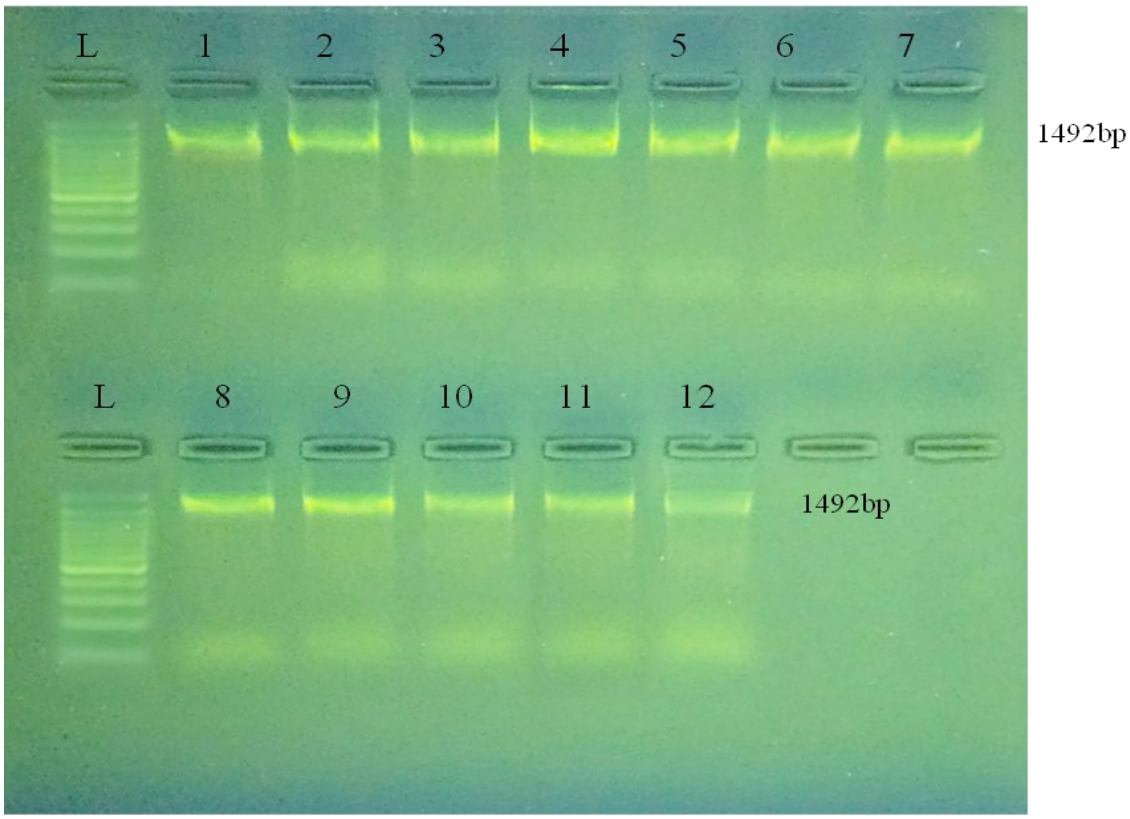

Fig.1.b. *Visualization of bacterial DNA isolated in gel electrophoresis. (Detection of 16SrRNA gene (1492 bp). Line (L) marker (100bp). Lines (1-8) represent positive results for Morganell morganii*

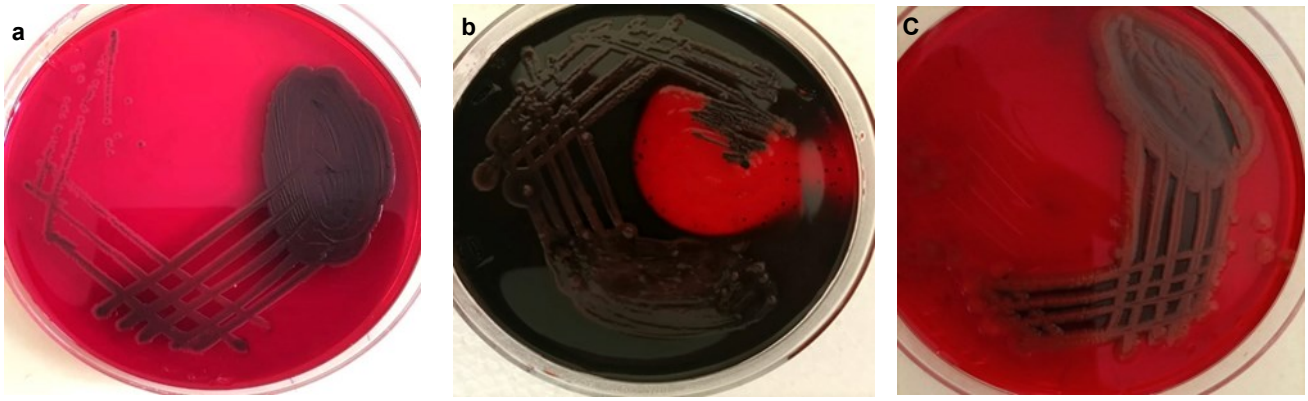

Fig. 2. *Congo Red agar plate, denoted as (a,b) exhibiting characteristics related toblack, dry, crystalline biofilm producing colonies of Providencia stuartii © Proteus mirabilis*

gies, offering the added benefit of maintaining colony viability on the culture medium. This method also circumvents issues related to variation in media batches and observer bias.

The CRA method and the Microtiter plate assay, phenotypic techniques that exhibit rapidity, reproducibility, and the preservation of cell viability, enable further examination of these microbial colonies. Utilizing spot inoculation enhances visualization and facilitates interpretations, particularly in the case of strains that produce biofilms. The interpretation of results hinges on the color spectrum of the colonies exhibiting a spectrum of colors, varying from red to black, with red indicating strains that do not produce biofilm and black indicating strains that do produce biofilm. Strains are straightforward to implement as the methodology, and the outcomes are typically contingent upon the coloration of the colony exhibited, which varies from red for strains that do not produce biofilms to black for those that do. A colorimetric scale has been established that spans from a vivid red to an intense black, very red, red, Bordeaux, nearly black, very black, and black—for the classification of biofilm production as outlined by Kaiser *et al.* (2013). Strong black colonies form due to the interaction between potent slime and the components of the growth medium, along with the Congo red dye. The intensity of colony coloration correlates directly with the degree of biofilm formation. While the reliability of this method is questionable due to possible false-positive outcomes, as noted by (Oliveira *et al.,* 2010), confirming results through quantitative assays like the Microtiter plate biofilm assay is imperative.

Quantification of pathogens producing biofilm using the Microtiter plate biofilm assay

The quantitative assay revealed a significant disparity compared to the qualitative assay, wherein 72% (n=27) were identified as strong biofilm producers, while 13.5% (n=5) were classified as moderate formers, 13.5% (n=5) were weak producers for *Pr. mirabilis,* 62.5% (n=5) as strong biofilm formers,37.5%(n=3) as moderate for *M. morganii* and 50%(n=1) strong producers,50%(n=1) moderate producer for *P. stuartii* as in Table -5- and Fig-4-.*T*he biofilm formation by MTP is considered a Gold standard method for biofilm detection . In the present study, *Pr. mirabilis* was the strong producer of this similar finding(ALjeeliz *et al.,*2022) that was isolated from urine, and in another study by(Mishu *et al*. (2022) biofilm producing *Pr. mirabilis* was detected by TCP method in rate 70% strains from pus and wound swabs. All the strains under study from catheterized urine samples and wound swab were able to form a biofilm. Similarly, all *Pr. mirabilis* strains from UTIs formed a biofilm better than those isolated from feces as a control group (Elhoshi *et al*.,2023), while in *M. morganii* (62.5%n=5) strong producer,(37.5%n=3) moderate this agreeing with (Zubair and Mohammad,2022). *M. morganii* has a strong ability *t*o produce biofilm in bones as in the case of chronic osteomyelitis (De *et al*.,2016). Biofilm protects bacteria from the host's immune system the effects commonly observed due to the presence of bacteria trapped within biofilms result in a nearly 1000-fold escalation in resistance towards various antimicrobial agents compared to free-floating planktonic bacteria.

Biofilms that develop on surfaces devoid of life are a predominant factor contributing to infections acquired in healthcare settings (Kwiecinska *et al.,* 2014). The likelihood of an increase exists, which may involve the inclusion of other species such as *P. stuartii* and *Klebsiella pneumoniae*, *Morganella morganii, Acinetobacter calcoaceticus, or Enterobacter aerogenes, despite Escherichia coli* commonly being identified as the most prevalent biofilm-forming bacterial species, alongside *Pr. mirabilis, M. morganii, Citrobacter freundii, K. oxytoca, and A. baumann* (Alves *et al*., 2014). Certain strains of *Providencia stuartii* have demonstrated the capacity to develop biofilms on polystyrene microplates, with one strain characterized as highly proficient (Silva *et al.,* 2022). The configuration and composition of biofilms require a wide array of physical, chemical, and genetic constituents. An in-depth analysis of the biofilm of *P.*

Table 5.Number and percentage of biofilm formation by Microtiter Plate Assay

Bacterial strains	Non biofilm formation	Weak	Moderate No.(%)	Strong
Proteus mirabilis	0.0	5(13.5%)	5(13.5%)	27(72.9%)
Morganella morganii		0.0	3(37.5%)	5(62.5%)
Providencia stuartii			1(50%)	1(50%)

Table 6 . Prevalence of biofilm genes in PMP group

Virulance genes / Strains	*mrpA*	Rsm %	Fim A
Proteus mirabilis	100	100	/
Morganella morganii	25	/	/
Providencia stuartii	/	/	100

stuartii revealed that in a planktonic state, this particular species forms aggregates of planktonic cells that precede and coexist with surface-attached biofilms (El-Khatib *et al*., 2018). Another study investigated the versatility of the major porins of this bacterium, namely OmpPst1 and OmpPs2. (Tran *et al.,* 2017).

Molecular detection of biofilm formation

All strains in the present study showed a high prevalence for *mrpA* genes (100% N=10) as in Table 6 and Fig. 5 among *Proteus mirabilis* using specific primers and genomic DNA using Uniplex PCR. These results were similar to those obtained by (ALjeeliz *et al.* (2022), who stated that 100% of *Pr. mirabilis* carry (100%) of *Pr. mirabili*s carrying this gene. Fimbriae are encoded by the *mrp* operon comprising 10 genes situated on the bacterial chromosome (Al-Mayahi, 2017 and Jabber, 2022). It was discovered that all *P. mirabilis* strains tested positive for the mrpA gene. The significance of mrpA genes lies predominantly in their role in adherence, as demonstrated by AL-Dulaimy *et al*. (2023) who reported a 100% positivity rate for mrpA in *Pr. mirabilis*. The MR/P type, a crucial fimbriae type, encodes multiple chromosomal genes present in two copies. The first copy, organized as an operon, encompasses nine genes (mrp A, B, C, D, E, F, G, H, and J) essential for fimbriae synthesis and assembly. The second copy, a single gene known as *mrpI,* regulates operon replication. The initial stage of urolithiasis involves the accumulation of *Pr. mirabilis* in the bladder lumen, leading to increased calcium ion deposition. This process necessitates the presence of urease enzymes and MRP fimbriae. Notably, *mrpA* emerges as a key gene due to its involvement in various virulence factors associated with the configuration and composition of biofilms which require a wide array of physical, chemical, and genetic components. Scrutiny concentrated on the biofilm of *P. stuartii* demonstrated that when existing freely, this particular species establishes clusters of floating cells that come before and exist together with biofilms attached to surfaces (El-Khatib *et al.,* 2018). Another exploration delved into the flexibility of the main porins of this bacterium, specifically *OmpPst1* and *OmpPs2*. The presence of *rsm* genes was notably higher in biofilm producers compared to non-biofilm producers of *Pr. mirabilis*, with the *rsmA* gene being accountable for swarming behavior. An association between *rsmA* and biofilm formation in *P. mirabilis* may exist, supported by our study and the findings of (Sun *et al*.,2020).

The capacity of *Pr. mirabilis* to develop biofilms demonstrates a notable association with the expression of *zapA, ureC, rsmA, hmpA, mrpA, atfA, and pmfA*, as indicated. Biofilm formation is linked with the adhesion and aggregation processes of bacteria. A concept highlighted by (Shi -(2016). Although biofilm producers exhibit higher pathogenicity in comparison to non-producers, the heightened pathogenicity seen in producers of biofilms potentially has associations with the

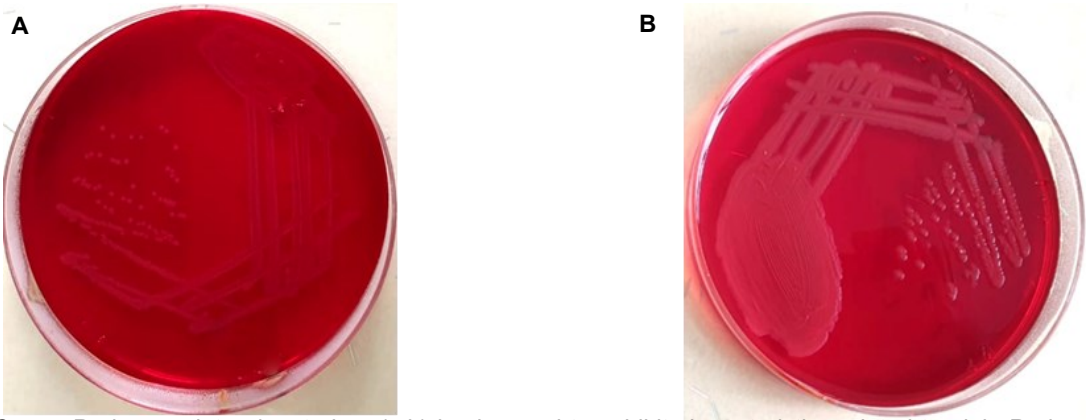

Fig .3. *Congo Red agar plate, denoted as (a,b) is observed to exhibit characteristics related to pink, Red moist, non-biofilm producing colonies of Proteus mirabilis and Morganella morganii*

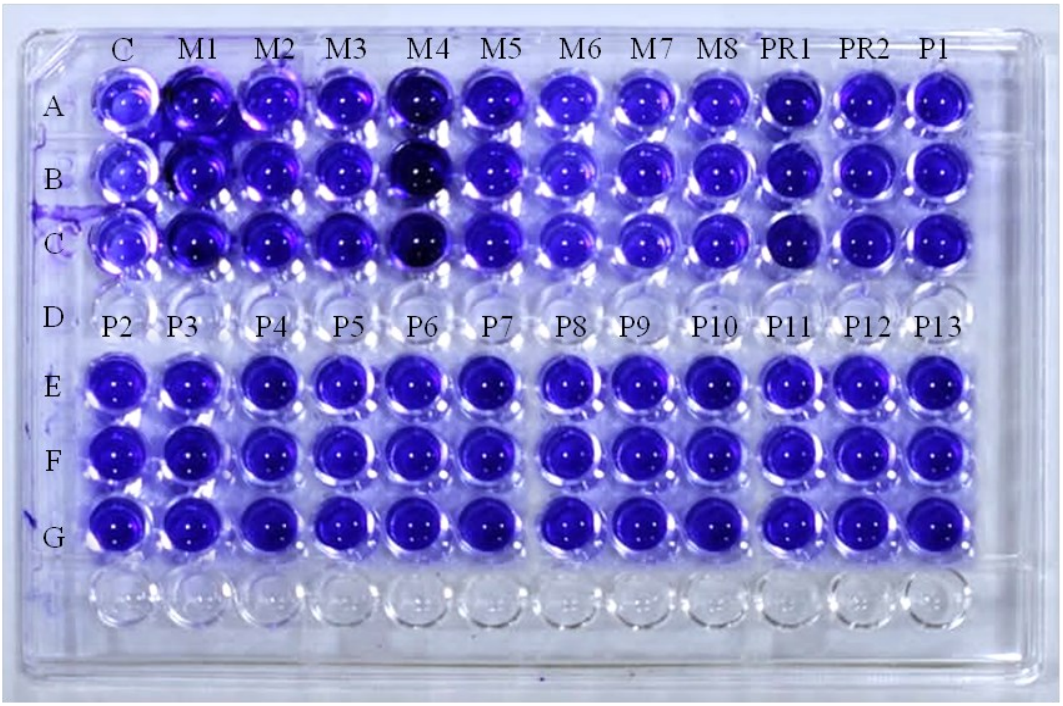

Fig .4. *Microtiter Plate showing biofilmfFormation of PMP Bacterial group*

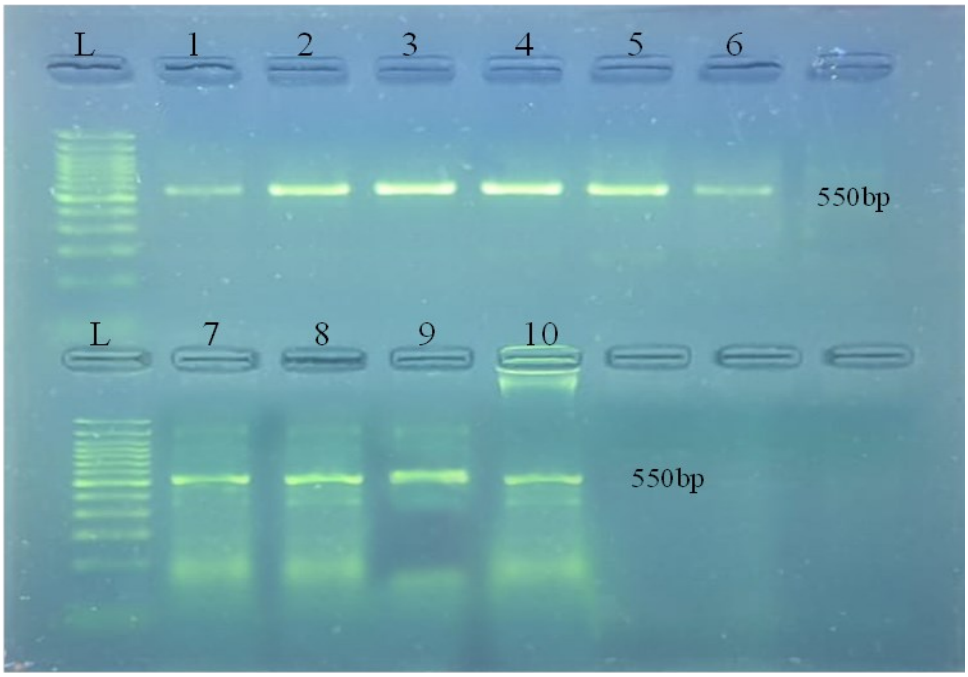

Fig. 5. *Amplification of the rmpA gene in Proteus mirabilis resulting in a product of 550bp. In Lane L, a DNA ladder was observed, while positive results indicated in Lanes 1, 2, 3, 4, 7, 8, and 10.*

capacity of *P. mirabilis* to form biofilms, a phenomenon that is intricately tied to the transcription of *ureC, zapA, rsmA, hmpA* and *mrpA*. In contrast, an investigation into biofilm formation by *M. morganii* indicated that only 25% of isolates tested positive for the *mrpA* gene, as illustrated in Table 6 and Fig 7. This discovery is consistent with Silva *et al.* (2023), who documented that 87% of *M. morganii* strains displayed the mrpA gene, a factor associated with the virulence of *M. morganii* in healthcare and community settings, contributing significantly to biofilm production (Tolulope *et al.,* 2021). The presence of fimbriae genes such as *mrpA* is crucial for initiating infections and facilitating adhesion, a pivotal step in establishing and perpetuating the microorganism within the host environment. Silva *et al*. (2023) emphasized that all *M. morganii* strains capable of adhering to HeLa cells exhibited the *mrpA* gene, regardless of D-mannose utilization. However, the present results

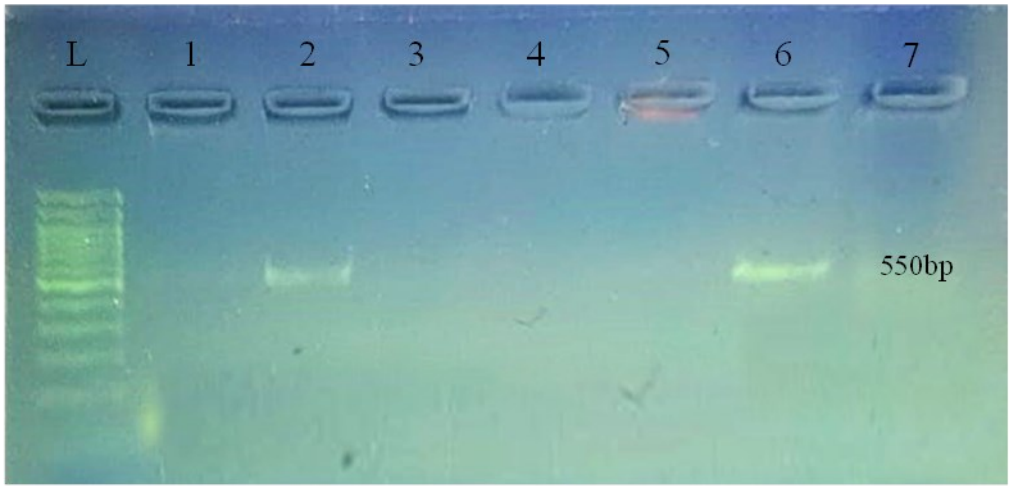

Fig .6. *Amplification of rmp Agene in Morganella morganii with product 550 bp . In Lane L, a DNA ladder, while positive results indicated in Lanes (2,6)*

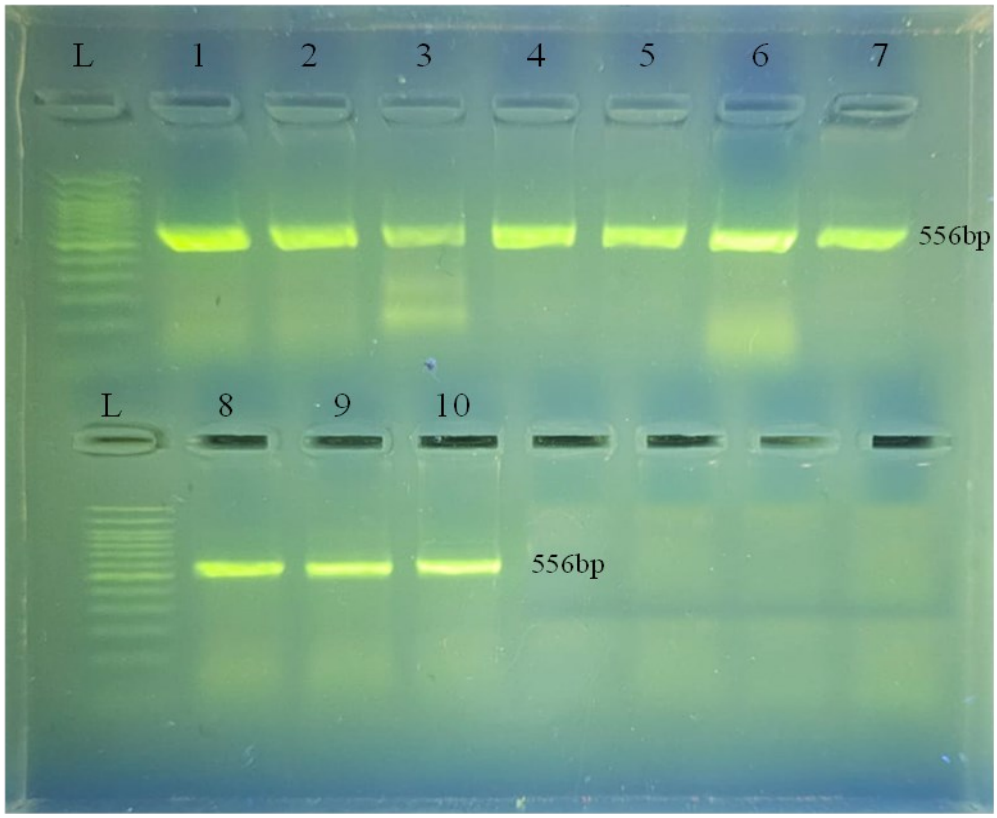

Fig. 7. *Amplification of rsm gene in Proteus mirabilis with product 556 bp . In Lane L, a DNA ladder was observed, while positive results indicated in Lanes (1,2,3,4....10)*

indicated the detection of only type 1 fimbria in the bacterial adhesion assay, utilizing the same primer for the mrpA gene in both *Pr. mirabilis and M. morganii.* The mrp operon, situated immediately adjacent to *mrpJ*, was labeled as *mrp*. This configuration resembles the duplicated operon identified in *Pr. mirabilis*, albeit with unique annotation and gene orientation (Pearson and Sebaihia, 2008). Both *M. morganii KT and Pr. mirabilis* possess duplicated MR/P fimbrial operons at the transcriptional level, albeit differing in the number of genes present (Pearson and Mobley, 2008). In two strains of *Providencia stuarti*, both were positive for the fimA gene at a rate of 100%, as shown in Table 6 and Fig. 8. This finding agrees with Guidone *et al.* (2023) that reported *P. stuarti* strains carry the two fimbriae genes, namely *fimA* and *mrkA*, potentially contribute to the pathogenesis in humans by 93.5%. The *fimA* gene encodes the largest subunit of type 1 fimbriae, which is crucial for host cell adhesion and colonization, highlighted by Promite (Saha,2020).

The study conducted by Daga *et al*. (2019) revealed a 95.8% prevalence of genes encoding type 1 fimbriae observed in the *E. coli* isolates under analysis. Similarly, Kakian *et al*. (2019) reported a fimA gene frequency of 74% in isolates obtained from hospital inpatients and 76% in those from outpatients. These fimbriae serve a critical function in facilitating attachment to epithelial cells of the host and are notably involved in the formation of biofilms on living surfaces, as discussed by (Ghasemian *et al.*2019). A comprehensive understanding of the potential mechanisms underpinning the formation of bacterial biofilms is paramount for elucidating the structure of the extracellular matrix, various subtypes, and cellular responses. Furthermore, the adhesion mechanisms are vital for the persistence and survival of *P. stuartii* in the initial stage of colonization, intending to potentially prevent or control persistent infections caused by this particular pathogen. The information provided underscores the critical importance of additional research endeavors, considering the distinctive characteristics observed in this species indicating an escalated resistance profile and enhanced survival of this bacterium within the hospital setting. Consequently, this scenario poses significant challenges in mitigating potential infections, as underscored by (Silva *et al*. 2022).

Conclusion

The present study concluded that the PMP bacterial group possesses various virulence characteristics that contribute to their success as pathogens. There exist precise methods for detecting biofilms, such as molecular techniques. The current necessity is for a cost-effective, user-friendly method that requires minimal technical knowledge; the most suitable option is a method that is a specific, reproducible, and reliable method, offering both qualitative and quantitative analysis without subjective errors. Given the convenience, speed, and cost efficiency, a Microtiter plate test and CRA methods could be deemed viable options for detecting biofilms in resource-limited settings.

ACKNOWLEDGEMENTS

Thanks, and appreciation to the University of Mosul and the College of Science for assisting in completing this research.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

1. AL-Dulaimy, I. M., Saleem, A. J. & Al-Taai, H. R. R. (2023). Detection of flaA, fliC, mrpA, and rsbA Gene in *Porteus mirabilis* Multidrug Resistance Isolated from Different Clinical Sources in Baquba City. *The Egyptian Journal of Hospital Medicine*, *90*(2): 2831-283 DOI: 10.21608/EJHM.2023.287337
2. Algammal, A. M., Hashem, H. R., Alfifi, K. J., Hetta, H. F., Sheraba, N. S., Ramadan, H. & El-Tarabili, R. M. (2021). ATP D gene sequencing, multidrug resistance traits, virulence-determinants, and antimicrobial resistance genes of emerging XDR and MDR-*Proteus mirabilis*. *Scientific Reports*, *11*(1):9476. DOI: 10.1038/s41598-021-88861-w
3. ALjeeliz, Z. A. H., Raheema, R. H. & Abood, S. F. (2022). Relationship between antibiotic resistance, biofilm-formation and virulence factors among multidrug-resistant Proteus mirabilis isolated from UTI patients in Wasit province, Iraq. *HIV Nursing*, *22*(2): 2082-2088. https://hivnursing.net/index.php/hiv/article/view/709/614
4. Al-Mayahi, F. (2017). Phenotypic and Molecular detection of Virulence factors in Proteus mirabilis isolated from different clinical sources. *Bas J Vet Res*, *16*(1): 369-88. https://www.researchgate.net/publication/316120589
5. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3): 403-410. DOI: 10.1016/S0022-2836(05)80360-2
6. Alves, M. J., Barreira, J. C., Carballo, I., Trinta, L., Perreira, L., Ferreira, I. C. & Pintado, M. (2014). Propensity for biofilm formation by clinical isolates from urinary tract infections: developing a multifactorial predictive model to improve antibiotherapy. *Journal of Medical Microbiology*, *63*(3): 471-477. https://doi.org/10.1099/jmm.0.071746-0
7. Armbruster, C. E., Mobley, H. L. & Pearson, M. M. (2018). Pathogenesis of infection. *Eco Sal Plus*, *8*(1): 10-1128. doi: 10.1128/ecosalplus.ESP-0009-2017.
8. Al□Gburi, N. M. (2020). Isolation and molecular identification and antimicrobial susceptibility of Providencia spp. from raw Cow's Milk in Baghdad, Iraq. Veterinary Medicine International, 2020(1), 8874747. https://doi.org/10.1155/2020/8874747
9. Behera, D. U., Dixit, S., Gaur, M., Mishra, R., Sahoo, R. K., Sahoo, M. ... & Subudhi, E. (2023). Sequencing and Characterization of M. morganii Strain UM869: A Comprehensive Comparative Genomic Analysis of Virulence, Antibiotic Resistance, and Functional Pathways. *Genes*, *14*(6): 1279. *4*(6), 1279; https://doi.org/10.3390/genes14061279
10. Bujňáková, D., Puvača, N. & Ćirković, I. (2022). Virulence factors and antibiotic resistance of Enterobacterales. *Microorganisms*, *10*(8) :1588 DOI: 10.3390/microorganisms10081588
11. Daga, A. P., Koga, V. L., Soncini, J. G. M., de Matos, C. M., Perugini, M. R. E., Pelisson, M. ... & Vespero, E. C. (2019). *Escherichia coli* bloodstream infections in patients at a university hospital: virulence factors and clinical characteristics. *Frontiers in Cellular and Infection Microbiology*, *9*:191. DOI: 10.3389/fcimb.2019.00191
12. De, A., Raj, H. J. & Maiti, P. K. (2016). Biofilm in osteomyelitis caused by a rare pathogen, Morganella morganii: A case report. *Journal of Clinical and Diagnostic Research: JCDR*, *10*(6):DD06 DOI: 10.7860/JCDR/2016/18666.7990
13. Dhanalakshmi, T. A., Venkatesha, D., Nusrath, A. & Asha-

rani, N. (2018). Evaluation of Phenotypic Methods for Detection of Biofilm Formation in Uropathogens. *National Journal of Laboratory Medicine.* 7(4): MO06 DOI:10.7860/NJLM/2018/35952:2321
14. Drzewiecka, D. (2016). Significance and roles of *Proteus spp.* bacteria in natural environments. *Microbial Ecology*, *72*, 741-758 doi: 10.1007/s00248-015-0720-6
15. Elhoshi, M., El-Sherbiny, E., Elsheredy, A. & Aboulela, A. G. (2023). A correlation study between virulence factors and multidrug resistance among clinical isolates of Proteus mirabilis. *Brazilian Journal of Microbiology*, *54*(3): 1387-1397. doi: 10.1007/s42770-023-01080-5
16. El-Khatib, M., Nasrallah, C., Lopes, J., Tran, Q. T., Tetreau, G., Basbous, H. ... & Colletier, J. P. (2018). Porin self-association enables cell-to-cell contact in *Providencia stuartii* floating communities. *Proceedings of the National Academy of Sciences*, *115*(10): E2220-E2228. doi: 10.3904/kjim.2015.30.2.167
17. Ferheen, I., Spurio, R., Mancini, L. & Marcheggiani, S. (2023). Detection of *Morganella morganii* bound to a plastic substrate in surface water. *Journal of Global Antimicrobial Resistance*, *32*: 104-107 DOI:10.1016/j.jgar.2023.01.008
18. Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A. & Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9): 563-575. DOI: 10.1038/nrmicro.2016.94
19. Ghasemian, A., Mobarez, A. M., Peerayeh, S. N. & Abadi, A. B. (2019). The association of surface adhesin genes and the biofilm formation among Klebsiella oxytoca clinical isolates. *New Microbes and New Infections*, *27*: 36-39. doi: 10.1016/j.nmni.2018.07.001
20. Gopinathan, H., Johny, T. K. & Bhat, S. G. (2021) Enhanced CAUTI Risk due to Strong Biofilm Forming MDR Bacteria in Indwelling Urinary Catheters. 6(1): id1030 –1-https://doi.org/10.1371/journal.pone.0023500
21. Gopinathan, H., Johny, T. K. & Bhat, S. G. (2021) Enhanced CAUTI Risk due to Strong Biofilm Forming MDR Bacteria in Indwelling Urinary Catheters. 6(1): 1-8 DOI:10.26420/austinjmicrobiol.2021.1030
22. Guidone, G. H. M., Cardozo, J. G., Silva, L. C., Sanches, M. S., Galhardi, L. C. F., Kobayashi, R. K. T. ... & Rocha, S. P. D. (2023). Epidemiology and characterization of *Providencia stuartii* isolated from hospitalized patients in southern Brazil: a possible emerging pathogen. *Access Microbiology*, *5*(10): 000652-v4. https://doi.org/10.1099/acmi.0.000652.v4
23. Hall□Stoodley, L. & Stoodley, P. (2009). Evolving concepts in biofilm infections. *Cellular Microbiology*, *11*(7), 1034-1043. DOI: 10.1111/j.1462-5822.2009.01323.x
24. Jaber, A. S. (2022). Detection of Virulence Genes of Proteus Mirabilis Isolated from Clinical Samples of some Hospitals in Nasiriyah City. *Pakistan Journal of Medical & Health Sciences*, *16*(10), 433-433. https://doi.org/10.53350/pjmhs221610433
25. Kaiser, T.D., Pereira, E.M., Dos- Santos, K.R., Maciel,E .L., Schuenck, R.P., Nunes, A.P. (2013).Modification of the Congo red agar method to detect biofilm production by Staphylococcus epidermidis. Diagnostic Microbiology and Infectious Disease. 75: 235-239. https://doi.org/10.1016/j.diagmicrobio.2012.11.014
26. Kakian, F., Abadi, M. S. S., Gholipour, A., Fadaie, M., Zamanzad, B., Khairi, S. ... & Damavand, M. S. (2019). Evaluating the prevalence of virulence genes of *Escherichia coli* in patients affected by urinary tract infection. *Gene 16*:100433. https://doi.org/10.1016/j.genrep.2019.100433Get rights and content
27. Kwiecinska-Piróg, J., Bogiel, T., Skowron, K., Wieckowska, E. & Gospodarek, E. (2014). Proteus mirabilis biofilm-qualitative and quantitative colorimetric methods-based evaluation. *Brazilian Journal of Microbiology*, *45*: 1423-1431. https://doi.org/10.1590/S1517-83822014000400037
28. Milo, S., Thet, N. T., Liu, D., Nzakizwanayo, J., Jones, B. V., & Jenkins, A. T. A. (2016). An *in-situ* infection detection sensor coating for urinary catheters. *Biosensors and Bioelectronics*, *81*: 166-172. doi: 10.1016/j.bios.2016.02.059
29. Minnullina, L., Pudova, D., Shagimardanova, E., Sharapova, L., Sharipova, M. & Mardanova, A. (2019). Comparative genome analysis of uropathogenic *Morganella morganii* strains. *Frontiers in Cellular and Infection Microbiology*, *9*:167. doi: 10.3389/fcimb.2019.00167
30. Mishu, N. J., Shamsuzzaman, S. M., Khaleduzzaman, H. M. & Nabonee, M. A. (2022). Association between biofilm formation and virulence genes expression and antibiotic resistance pattern in Proteus mirabilis, isolated from patients of Dhaka Medical College Hospital. *Archives of Clinical and Biomedical Research*, *6*(3): 418-434. DOI:10.26502/acbr.50170257
31. Mukhtar, A. A., Abd Alfadil, N. A., Mohamed, M. S., Altayb, H. N., Elzak, S. G. & Hassan, M. S. (2018). Identification of *Proteus mirabilis* on banknotes using 16s rRNA gene in Khartoum State. *Sudan Journal of Medical Sciences*, *13*(3): 175-186. DOI: 10.18502/sjms.v13i3.2955
32. Oliveira, A., Maria de Lourdes, R.S. (2010). Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci. BMC Research Notes, 3: 1-8. doi: 10.1186/1756-0500-3-260
33. Pearson, M. M. & Mobley, H. L. (2008). Repression of motility during fimbrial expression: identification of 14 mrpJ gene paralogues in Proteus mirabilis. *Molecular Microbiology*, *69*(2): 548-558. DOI: 10.1111/j.1365-2958.2008.06307.x
34. Pearson, M. M., Sebaihia, M., Churcher, C., Quail, M. A., Seshasayee, A. S., Luscombe, N. M. ... & Mobley, H. L. (2008). Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and motility. *Journal of Bacteriology*, *190*(11): 4027-4037.DO i: 10.1128/JB.01981-07.
35. Pelling, H., Nzakizwanayo, J., Milo, S., Denham, E. L., MacFarlane, W. M., Bock, L. J... & Jones, B. V. (2019). Bacterial biofilm formation on indwelling urethral catheters. *Letters in Applied Microbiology*, *68*(4): 277-293 DOI: 10.1111/lam.13144
36. Promite, S. & Saha, S. K. (2020). *Escherichia coli* in respiratory tract infections: evaluating antimicrobial resistance and prevalence of fimA, neuC, and iutA virulence genes. *Gene Reports*, *18*: 100576. https://doi.org/10.1016/j.genrep.2019.100576
37. Ruchi, T., Sujata, B. & Anuradha, D. (2015). Comparison of phenotypic methods for the detection of biofilm production in uro-pathogens in a tertiary care hospital in India. *Int*

J Curr Microbiol App Sci, *4*(9): 840-49 http:// www.ijcmas.com.

38. Scavone, P., Villar, S., Umpierrez, A. & Zunino, P. (2015). Role of *Proteus mirabilis* MR/P fimbriae and flagella in adhesion, cytotoxicity and genotoxicity induction in T24 and Vero cells. *Pathogens and Disease*, *73*(4): ftv017. https://doi.org/10.1093/femspd/ftv017
39. Schulze-Schweifing, K., Banerjee, A. & Wade, W. G. (2014). Comparison of bacterial culture and 16S rRNA community profiling by clonal analysis and pyrosequencing for the characterization of the dentine caries-associated microbiome. *Frontiers in Cellular and Infection Microbiology*, *4*: 164. | https://doi.org/10.3389/fcimb.2014.00164
40. Shi, X., Lin, Y., Qiu, Y., Li, Y., Jiang, M., Chen, Q. ... & Huang, S. (2016). Comparative screening of digestion tract toxic genes in *Proteus mirabilis*. *PlOS One*, *11*(3): e0151873 https://doi.org/10.1371/journal.pone.0151873
41. Silva, L. C., Sanches, M. S., Guidone, G. H. M., Montini, V. H., de Oliva, B. H. D., do Nascimento, A. B. ... & Rocha, S. P. D. (2023). Clonal relationship, virulence genes, and antimicrobial resistance of *Morganella morganii* isolated from community-acquired infections and hospitalized patients: a neglected opportunistic pathogen. *International Microbiology*, 5:1-12. Doi: 10.1007/s10123-023-00400-x
42. Silva, S. M. D., Ramos, B. A., Sá, R. A. D., Silva, M. V. D., Correia, M. T. & Oliveira, M. B. D. (2022). Investigation of factors related to biofilm formation in *Providencia stuartii*. *Anais da Academia Brasileira de Ciências*, *94*(3): e20210765. • https://doi.org/10.1590/0001-37652022202 10765
43. Sun, Y., Wen, S., Zhao, L., Xia, Q., Pan, Y., Liu, H. ... & Wang, H. (2020). Association among biofilm formation, virulence gene expression, and antibiotic resistance in *Proteus mirabilis* isolates from diarrheic animals in Northeast China. *BMC Veterinary Research*, *16*:1-10. DOI:10.1186/s12917-020-02372-w
44. Tabatabaei, A., Ahmadi, K., Shabestari, A. N., Khosravi, N. & Badamchi, A. (2021). Virulence genes and antimicrobial resistance pattern in *Proteus mirabilis* strains isolated from patients attended with urinary infections to Tertiary Hospitals, in Iran. *African Health Sciences*, *21*(4), 1677-84. doi 10.4314 /ahs .v21i4.22.
45. Temkin, E., Adler, A., Lerner, A., and Carmeli, Y. (2014) Carbapenem-resistant Enterobacteriaceae: biology, epidemiology, and management. *Ann NY Acad Sci* 1323: 22–42. https://doi.org/10.1111/nyas.12537
46. Tolulope, A., Ewaoche, I. S. & Ibemologi, A. (2021). UreC and ZapA virulence genes amplification in clinical specimen of *Proteus mirabilis* in Bayelsa state, Nigeria. *J Microbiol. Exp.*, *9*(1):15-20. DOI:10.15406/jmen.2021.09.00317
47. Tran, Q. T., Maigre, L., D'Agostino, T., Ceccarelli, M., Winterhalter, M., Pagès, J. M., & Davin-Regli, A. (2017). Porin flexibility in *Providencia stuartii*: cell-surface-exposed loops L5 and L7 are markers of Providencia porin OmpPst1. *Research in Microbiology*, *168*(8), 685-699. https://doi.org/10.1016/j.resmic.2017.05.003
48. Wie, S. H. (2015). Clinical significance of *Providencia* bacteremia or bacteriuria. *The Korean Journal of Internal Medicine*, *30*(2): 167. doi: 10.3904/kjim.2015.30.2.167
49. Wu, M. C., Lin, T. L., Hsieh, P. F., Yang, H. C., and Wang, J. T. (2011). Isolation of genes involved in biofilm formation of a *Klebsiella pneumoniae* strain causing pyogenic liver abscess. PlOS One, 6(8): e23500 DOI: 10.1371/journal.pone.0023500
50. Zisman, A. L. (2017). Effectiveness of treatment modalities on kidney stone recurrence. *Clinical Journal of the American Society of Nephrology*, *12*(10): 1699-1708. DOI: 10.2215/CJN.11201016
51. Zubair, M. & Mohammad, I. (2023). Interrelationship of extended spectrum beta-lactamase producers and biofilm formation among the Gram-Negative Bacteria from Tabuk, KSA. *Open Access Macedonian Journal of Medical Sciences*, *11*(A): 15-22. DOI:10.3889/oamjms.2023.11101
52. Zunino, P., Geymonat, L., Allen, A. G., Preston, A., Sosa, V. & Maskell, D. J. (2001). New aspects of the role of MR/P fimbriae in *Proteus mirabilis* urinary tract infection. *FEMS Immunology & Medical Microbiology*, *31*(2):113-120