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Molecular Analysis of Virulence Genes Lux S, Rsb A and Mrpa in Proteus Mirabilis Isolated from Different Clinical Samples

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Abstract :The research endeavour involved the collection of a total of 100 samples from diverse clinical origins. Specifically, 70 urine samples were obtained from patients, while 20 samples were derived from wounds. Additionally, 10 samples were acquired through ear swabs from patients enrolled in private clinics, Imam Sadiq Hospital, and Al-Hilla Hospitals. The data collection period spanned from November 2023 to December 2024.

Out of the hundred samples that were analyzed, it was observed that a significant majority, specifically eighty samples (80%), exhibited positive bacterial cultures. Conversely, a smaller proportion of the samples, precisely twenty samples (20%), did not exhibit any growth of bacteria. The findings of this study indicate the potential existence of microorganisms, including viruses, fungi, and other anaerobic agents, which could pose challenges in terms of culturing or may be attributed to variations in the sizes and compositions of the samples. Utilizing the Vitek 2 methodology, selective media, and biochemical assays, it was observed that out of the eighty-nine samples analyzed, a subset of twenty samples exhibited positive outcomes, suggesting Proteus mirabilis presence. The detection of luxS, rsmA, and mrpA genes in Proteus mirabilis was accomplished using the polymerase chain reaction (PCR) technique.

Keywords: Proteus mirabilis, lux s, rsm A and mrp A genes detection, Vitek 2 system

1. Introduction :

Rods that are gram-negative and have cilia are characteristic of the genus Proteus and its classification as facultative anaerobes within the Enterobacteriaceae family [Bonnin RA, Girlich D, Jousset AB, Gauthier L et. al. 2020]. Within the human gut microbiota, it has been observed that Proteus species are present in healthy individuals (Li Z, Peng C, Zhang G, Shen Y, Zhang Y et al.,

2022). According to a recent study by Hasan TH, Alasedi KK, and Jaloob AA (2021), Proteus species has been identified as the third most common cause of hospital-acquired infections. According to a recent study conducted by Yuan et al. (2021), it has been observed that the biofilms produced by P. mirabilis can lead to significan

The present study aims to investigate the potential complications that may arise in individuals who undergo long-term bladder catheterization.Opportunistic infections are known to be caused by various factors. One such factor is the formation of biofilms, which play a crucial role in enabling bacterial survival even in unfavourable environments. This phenomenon is closely associated with the persistence of infections, as highlighted in the study conducted by Humphries et al. (2021). Within the realm of Proteus strains, extensive documentation exists regarding biofilm formation by P. mirabilis on catheter material [Shelenkov A, Petrova L, Fomina V, Zamyatin M, et. al. 2021].

Despite extensive research, the precise gene responsible for the development of biofilms remains elusive, it is worth noting that gene products that play a crucial role in biofilm development also have significant implications for pathogenesis. According to a study conducted by Shelenkov et al. in 2020, P. mirabilis is considered to be the predominant pathogen associated with kidney stone-related infections. The emergence of biofilm-producing P. mirabilis strains has been identified as a significant contributor to the rising incidence of catheter-associated urinary tract infections (UTIs) within hospital settings [Mirzaei et al., 2021].

Forming biofilm provides a protective barrier for bacteria, shielding them from the host's immune system and the effects of antibiotics. This phenomenon is particularly relevant in the context of urinary tract infections (UTIs), where the persistence of biofilm-encased bacteria often leads to recurrent infections. The challenge of selecting appropriate antibiotics to effectively treat bacterial infections is further compounded by the fact that many gram-negative bacterial pathogens possess multiple resistance genes. This widespread presence of resistance genes among these pathogens contributes significantly to the global problem of drug resistance (Critchley IA, Cotroneo N, Pucci MJ, Jain A et. al. 2020).

Aim of The Study : Molecular characterization of some virulence genes(lux s ,rsb A ,mrp A) and their role in the pathogenicity

The objectives of study:

1. Isolation and Identification Proteus from different sites of Infection by different methods detection of causative agent by vitek 2 system.

2. Detection of some virulence factors by phenotypic and genotypic levels.

3. Detection of antibiotic susceptibility test.

2. Materials And Methods :A total of one hundred samples were obtained from patients, consisting of 30 males and 70 females, who were admitted to AL-Hilla Teaching Hospital, AL-Immam AL-Sadiq Hospital, and private laboratories. The samples were collected over a period spanning from November 2023 to December 2024.

The samples were obtained from various locations, encompassing 50 urine samples, 20 wound swabs, and 10 ear swabs. The age range of the patients in the study ranged from 17 to 70 years old.

P. mirabilis isolates were identified based on their colony characteristics, such as blood agar and MacConkey agar, observed on media. Additionally, biochemical tests and the vitek 2 system were employed to confirm the identification further.

3. Biofilm Production by the Microtiter Plate Assay: : Furthermore, a volume of 200 µL from a 1:100 dilution of the bacterial culture grown overnight in trypticase soy broth (TSB) and glucose 1% was introduced into the wells of a 96-well flat-bottomed polystyrene plate. After being incubated at 37°C for 24 hours, the cultures were extracted, and the wells were subjected to two washes using 200 µL of phosphate buffer saline (PBS) with a pH value of 7.4. Subsequently, the wells were left to dry at room temperature. The biofilm samples underwent staining using a 0.1% crystal violet solution in water for 15 minutes. Subsequently, each plate was carefully rinsed with distilled water and allowed to air dry at room temperature. An ELISA reader measured the biofilm's optical density (OD) at a 492 nm wavelength. The formation of biofilms has been characterized as negative when the optical densities (ODs) are below 0.120. ODs ranging from 0.120 to 0.240 are considered weakly positive, while ODs exceeding 0.24 are classified as strongly positive (Gupta et al., 2015; Zatout et al., 2022).

Table (1) Analysis of biofilm formation using the TCP technique

Average OD value	Biofilm production
OD≤ODC	No biofilm producer
ODc<OD≤2×Odc	Weak biofilm producer
2×ODc<OD≤4×Odc	Moderate biofilm producer
4×ODc<OD	Strong biofilm producer

4. Molecular study: : The proteus mirabilis lux s, rsm A, and mrpA genes were detected using conventional PCR. The primers utilized for detecting lux s, rsm A, and mrp A genes in the conventional PCR method were specified in Table 2.

Table (2)Primers used in this study.

Gene	Primer Sequence (5' to 3')	Product Size (bp)	Reference
Lux S	F-GTA TGT CTG CAC CTG CGG TA R-TTT GAG TTT GTC TTC TGG TAG TGC	464	Mishu,NJ.,Shamsuzzaman, S. M., Khaleduzzaman, H. M., & Nabonee, M. A. (2022
Mrp A	F-TTC TTA CTG ATA AGA CAT TG R-ATT TCA GGA AAC AAA AGA TG	565	Scavone, P., Iribarnegaray, V., González, M. J., Navarro, N., Caneles-Huerta, N., Jara-Wilde, J., ... & Zunino, P. (2023).
rsmA	F-TAG CGA GTG TTG ACG AGT GG R-AGC GAG GTG AAG AAC GAG AA	562	Mishu,NJ.,Shamsuzzaman, S. M., Khaleduzzaman, H. M., & Nabonee, M. A. (2022

Table (3): A Master Mix Ingredient for PCR Reaction

Components of PCR reaction	Volume
Master mix	10
DNA template	2
Primers forward	1
Primers reverse	1
nuclease-free H2O	5
Total volume	19

Ethical Approval : All participants were furnished with thorough information about the study and its intended goals. Prior to sample collection, verbal consent was obtained from the participants, thereby ensuring that their participation was voluntary. The ethical sanction for the investigation was obtained under reference number BMS/0231/016 from the Committee on Publication Ethics at the College of Medicine, University of Babylon, Iraq.

6. Results and discussion: Among the 100 samples analyzed, 20 showed a positive result for P. mirabilis, as indicated in Table 4. These samples were collected from various sources, including urine, wound, and ear swab specimens.

Table (4): Bacteria distribution according to samples

Clinical Samples	Total Number Of samples	NO.of P.miirabilis isolates	No growth	Male	Female
Urine sample	50(50%)	7 (35%)	3 (15%)	3(15%)	4(20%)
Ear swab	10(10%)	0 (0%)	10(50%)	8	2(10%)
Wound swab	20(20%)	10(50%)	10(50%)	6(30%)	4(20%)
Total	80(80%)	20 (100%)	20(100)	10 (50%)	10(50)

The identification of P. mirabilis was conducted based on its morphological characteristics, biochemical tests, and the Vitek 2 system, as outlined in Table 5.

Table (5) Microscopic and biochemical tests were conducted to identify and characterize p.mirabilis bacteria.

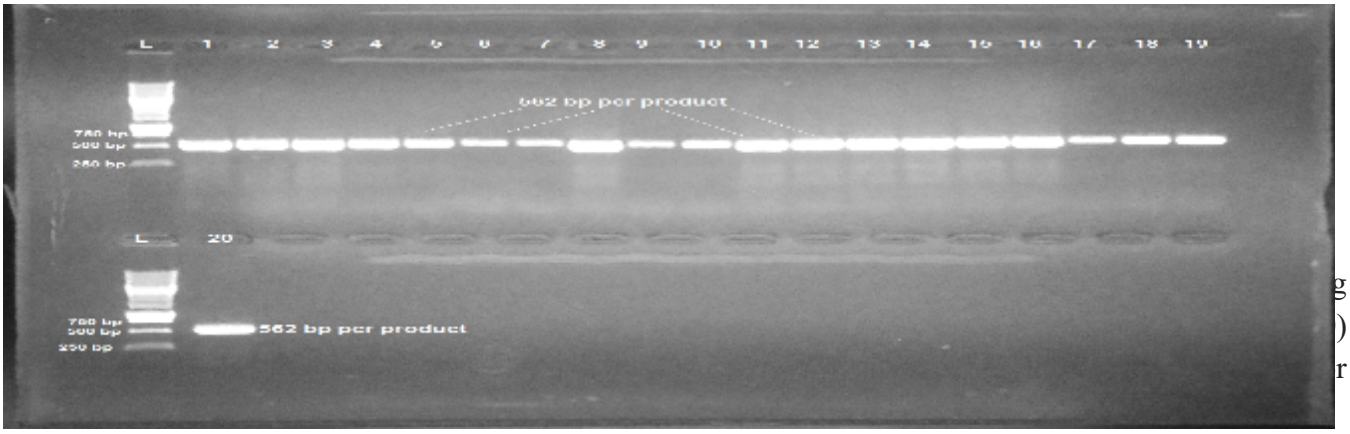
Microscopic and biochemical tests	Tests Response of P.mirabilis
bacteria Gram stain	-
Catalase test	+
Oxidase test	-
Urease test	+
Lactose sugar fermentation	-
Indol test	-

According to Cahan (2023), the pathogenicity of Proteus mirabilis relies on its ability to exhibit various virulence factors, including biofilms, adhesion molecules, urease, proteases, siderophores, and toxins.

Eighty percent of the samples tested in this investigation produced culture-positive findings comparable to those found in DMCH by [Al-Nabhani, N. A. and A. M. J. I. j. o. b. Shami (2023)]. Seventy percent of the samples were culture-positive. P. mirabilis was isolated from wound swabs at a rate of 20%, followed by 10% from urine and blood, comparable to the study conducted by Al-Nabhani, N. A. and A. M. J. I. j. o. b. Shami (2023). The percentage of P. mirabilis isolated from wound samples at DMCH was 10.65%. In contrast, the percentage obtained from wound samples in the study by de Oliveira, Wellington Danilo, et al. 2021 was 13.3%.

Quorum sensing serves as the principal controller of several virulence variables. The regulation of multicellular and coordinated swarming and biofilm formation processes holds great importance in various biological systems. The phenomenon of quorum sensing in P. mirabilis is facilitated by the presence of autoinducer-1, which is regulated by the luxR genes. The process is also influenced by autoinducer-2, which is regulated by the luxS gene [Al-Nabhani, N. A. and A. M. J. I. j. o. b. Shami (2023)]. The research examined the correlation between virulence factors present in clinical samples of P. mirabilis. All isolates exhibited 100% positivity for motility, swarming, and production.

This research demonstrated that the virulence gene rsmA was substantially more abundant in biofilm-producing P. mirabilis compared to non-biofilm producers. Within the scope of this investigation, research revealed that swarming behaviour is caused by the rsmA gene, which was present in 80% of the biofilm producers., in comparison to the rsmA-positive isolates that do not create biofilm. There is a potential correlation between the presence of rsmA and the process of biofilm development in P. mirabilis. The findings align with the data presented by Razzaque (2021), which reported that 80.64% of biofilm-producing P. mirabilis samples tested positive for the rsmA gene. This is illustrated in Figure 1. Additionally, the results indicated that 20 out of 25 samples (80%) were positive, which is in line with the findings of Shelenkov et al. (2020). All the isolates in the present investigation demonstrated a phenotypic trait of swarming, namely the bull's-eye ring. However, it is important to note that although swarming controlled genes play a role in swarming, they are not always essential for the process due to the involvement of several genes and operons [Humphries R, Bobenchik AM, Hindler JA, Schuetz AN(2021)].



The LuxS gene was found in 95% of biofilm-producing P. mirabilis isolates, whereas the mrpA gene was present in 25%. Neither the mrpA gene nor the LuxS gene was shown to be significantly associated with biofilm formation. Figures (2 and 3).

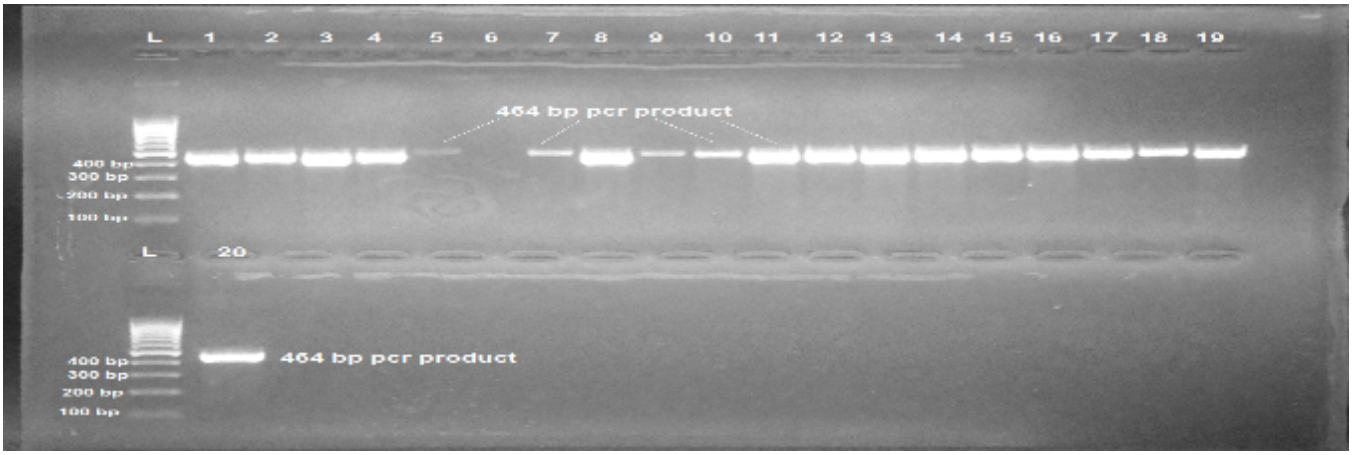


Figure (2): An agarose gel stained with ethidium bromide displays PCR amplification results using 464bp gene primers for extracted DNA of p. mirabilis. The gel includes a ladder (L) and 20 samples (1, 2, 3, … 20) of p. mirabilis positive for the lux s gene.

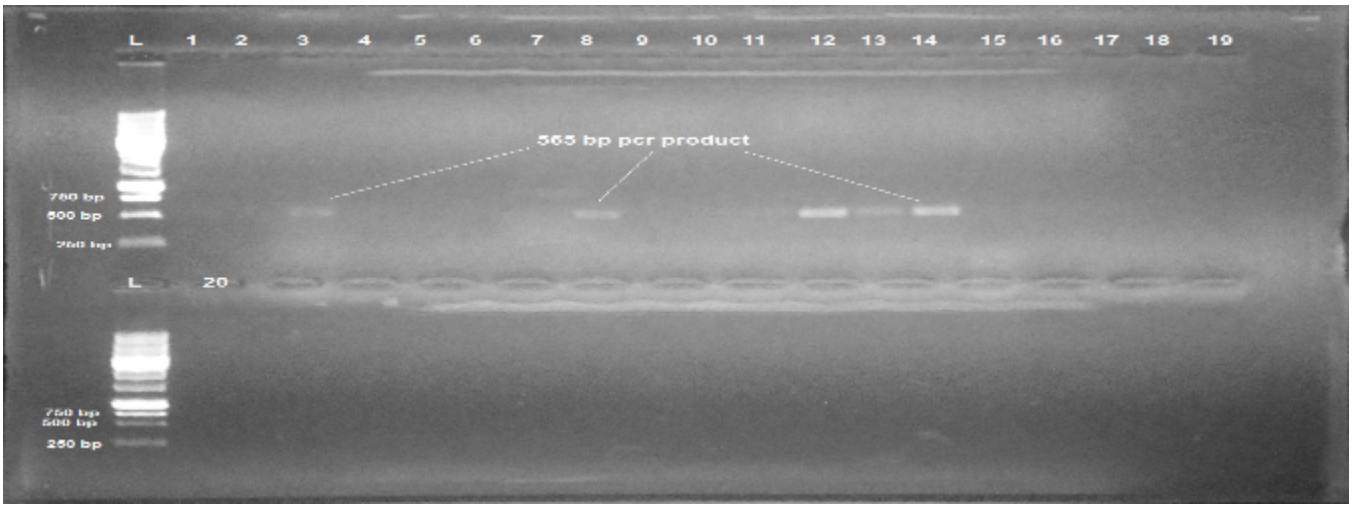


Figure (3): An agarose gel stained with ethidium bromide displays PCR amplification results using 565bp gene primers for extracted DNA of p. mirabilis. The gel includes a ladder (L) and 20 samples (1, 2, 3, … 20) of p. mirabilis positive mrp A gene.

The study's findings indicate that all clinical isolates of P. mirabilis have a robust ability to generate biofilm. The results were consistent with Ali's (2012) findings, which showed that out of 48 P. mirabilis isolates, 45 (93.75%) exhibited robust biofilm development, whereas 3 (6.25%) did not produce biofilms.

Biofilm formation enables bacteria to be protected against a range of stressors, such as immunological reactions and antimicrobial treatments. Bacteria's ability to form biofilms has been linked to heightened antibiotic resistance and the development of chronic recurring infections. Many microorganisms may survive independently or as collective micro-communities within a matrix of extracellular polymeric substances known as biofilm. A crucial pathogenicity determinant is the microorganism's capacity to generate biofilm, which offers a protective habitat for the species to endure and elude antibiotics (Dincer et al., 2020).

Gram-negative bacteria are categorized as biofilms, which protect against environmental stress and bactericides within microbiological classes (Garde et al., 2015). The biofilm contains sessile bacteria, which are in a stationary or latent state of growth and exhibit phenotypes different from planktonic bacteria (Muhammad et al., 2020).

The production of different virulence factors is regulated by quorum sensing systems that rely on N-acyl-l-homoserine lactones (AHLs). Cell-cell communication via quorum sensing is believed to be mainly facilitated by AHLs, which operate as signalling molecules (Scoffone et al., 2019; Venkatramanan et al., 2020).

The study's findings were consistent with the results obtained by Hadjifrangiskou et al., (2012) and Holling et al., (2014), which demonstrated that all Proteus isolates could produce biofilms. The information is shown in Table 6.

Table(6) biofilm producer of p.mirabilis isolates (N20) Protocol and calculation according to:Stepanović, S., Ćirković, I., Ranin, L., & S✓ vabić-Vlahović, M. (2004). Biofilm formation by Salmonella spp. and Listeria monocytogenes on plastic surface. Letters in applied microbiology, 38(5), 428-432

Sa	n2	n3	n1	10	30	24	19	7	2	21	11	12
r1	0.342	0.687	0.405	0.176	0.234	0.183	0.047	0.214	1.157	0.16		
r2	0.229	0.331	0.317	0.193	0.222	0.236	0.06	0.3	0.643	0.145		
r3	0.302	0.265	0.314	0.268	0.2	0.234	0.059	0.311	0.692	0.176		
Mean	0.291	0.427667	0.345333	0.212333	0.218667	0.217667	0.055333	0.275	0.830667	0.160333		
Std	0.05729747	0.227001	0.051695	0.048952	0.017243	0.030039	0.007234	0.053113	0.283673	0.015503		
Sa	20	15	17	18	14	19(2)	16	12	13	11		control
r1	0.305	0.22	0.236	0.15	0.36	0.327	0.241	0.276	0.264	0.451		0.049
r2	0.282	0.216	0.195	0.137	0.288	0.281	0.208	0.278	0.285	0.577		0.05
r3	0.2935	0.218	0.2155	0.1435	0.324	0.304	0.2245	0.277	0.2745	0.514		0.048
Mean	0.2935	0.218	0.2155	0.1435	0.324	0.304	0.2245	0.277	0.2745	0.514		0.049
Std	0.0115	0.002	0.0205	0.0065	0.036	0.023	0.0165	0.001	0.0105	0.063		0.001

Green = weak biofilm producer

Yellow= moderate biofilm producer

Red = robust biofilm produce

Conclusion:

Among the virulence genes examined in this investigation, the prevalence of the rsb and lux s genes was higher in isolates that produced biofilm than in isolates that did not. P. mirabilis strains that produced biofilm exhibited more excellent resistance to all antibiotics tested than non-biofilm producers; however, the observed differences did not reach statistical significance. The presence of these genes enhances the pathogenicity of p. mirabilis.

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