MOLECULAR IDENTIFICATION OF *ROSEOMONAS MUCOSA* AND DETERMINATION THE SOME OF ITS VIRULENCE FACTORS

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ABSTRACT

This study was aimed to characterize *Roseomonas mucosa* isolate using molecular genetics and determination some bacterial virulence factors such as capsular, biofilm formation and antimicrobial susceptibility. By using Polymerase chain reaction (PCR) technique, the molecular characterization of the *R. mucosa* was done by using 16S rRNA gene sequence and phylogenetic analysis. Antimicrobial susceptibility test was carried out by Kirby-Bauer disk diffusion method. Capsular detection was examined using nigrosin pigment. While Congo red agar and microtiter plate using enzyme-linked immunosorbent assay (ELISA) reader methods are used to determined bacterial isolate capability to produce a biofilm. The molecular characterization shows the bacterium that isolated from catheter of a dialysis Iraqi patient is belong to *Roseomonas mucosa*. The isolated *R. mucosa* strain showed resistant to Amoxicillin, methicillin, carbapenemes, bacitracin and Sulfamethoxazole. While it was susceptible to tobramycin, levofloxacin, colisitin sulphate, doxycycline, azithromycin and cephalexin. The results show *R. mucosa* was encapsulated. Furthermore, it has the ability to form weak biofilm. The findings suggest that in spite of this genus seems to have an overall little pathogenic potential for humans, *Roseomonas* sp. in particular, *R. mucosa* may be substantial pathogens in individuals with underlying medical complications.

Keywords: *Roseomonas* sp., antimicrobial susceptibility test, capsular detection and biofilm formation.

التشخيص الجزيئي لبكتيريا ROSEOMONAS MUCOSA وتحديد بعض عوامل الضراوة..

طيبة غزوان البدري مروج عبد الستار العبيدي
مدرس استاذ
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المستخلص

الهدف من هذه الدراسة هو تشخيص بكتيريا ROSEOMONAS MUCOSA العصوية السالبة لصبغة كرام زتحديد بعض عوامل الضراوة البكتيرية مثل تكوين المحفظة وتكوين الاغشية الحيوية وحساسيتها اتجاه بعض المضادات. باستعمال تقنية تفاعل البلمرة المتسلسل (PCR) , تم التوصيف الجزيئي لبكتيريا R.MUCOSA باستخدام تسلسل الجين 16S RRNA وتحليل النشوء والتطور. تم اجراء اختبار الحساسية للمضادات المايكروبية باستخدام طريقة كيربي باور (الأنتشار بالاقراص). تم الكشف عن الكبسولة باستخدام صبغة النيكروسين. بينما تم استخدام اجار الكونغو المحور ولوحة MICRTITER باستخدام قارئ الاليزا لتحديد قدرة البكتيريا المعزولة على انتاج الغضاء الحيوي. يوضح التوصيف الجزيئي ان البكتيريا المعزولة من قسطرة مريض غسيل الكلى العراقي تعود الى ROSEOMONAS MUCOSA. اظهرت سلالة R.MUCOSA المعزولة مقاومة للأموكسسلين والمثيسيلين و الكاربابينيمات والباسيتراسين والسلفاميثوكسازول. بينما كانت حساسة للتوبرمايسين وليفوفلوكساسين وكوليسيتيين سلفات ودوكسايكلين , ازوثرمايسين وسيفاليكسين. وكذلك اظهرت النتائج ان عزلة R.MUCOSA حاوية على غلاف الكبسولة, علاوةً على ذلك, لديها القدرة على تكوين غشاء حيوي ضعيف.

الكلمات المفتاحية: انواع ROSEEOMONAS, فاحص الحساسية الدوائية, الكشف عن الكبسولة وتكوين الغشاء الحيوي.

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INTRODUCTION

The genus *Roseomonas* was established in 1993 by Rihs et al. based on studies of the morphologic features, phenotypes, and genome similarity of 42 strains of pink pigmented, aerobic, and slow-growing gram-negative bacteria (17). This genus includes 6 species: *Roseomonas gilardii* (or genomospecies 1, the type species), *Roseomonas cervicalis* (genomospecies 2), *Roseomonas fauriae* (genomospecies 3), and 3 unnamed *Roseomonas* genomospecies 4, 5, and 6. These organisms have been isolated from the aquatic environment and various clinical Rosemonas spp. can be isolated from some clinical specimens, such as blood, wound, peritoneal dialysis fluid, corneal scrapings, bones, urinary and respiratory specimens, (13). *Roseomonas* usually infects immune compromised patients such as those with leukemia, cancer chemotherapy, malignancy, sepsis, peritonitis, catheter associated infections and pulmonary tuberculosis (9). Some reports describe that the sources of infection are water and soil that infect patients with poor health practices (10). On the other hand, its virulence has been associated to the ''mucoid phenotype'' and it is well known that *Roseomonas* produces a biofilm on foreign materials inside the human body (20). Virulence factors are the molecules produced by pathogenic microorganisms, enhancing their ability to evade their host defenses and cause disease (1). This broad definition comprises secreted products such as toxins, enzymes, exopolysaccharides, as well as cell surface structures such as capsules, biofilms, lipopolysaccharides, glycoprotein and lipoproteins (11). The membrane associated virulence factors aid the bacterium in adhesion and evasion of the host cell. The secretory factors are important components of bacterial armory which help the bacterium wade through the innate and adaptive immune response mounted within the host. In extracellular pathogens, the secretory virulence factors act synergistically to kill the host cells (15).

MATERIAL AND METHODS

Bacterial Culture and molecular characterization : Dependent on Albadri and Alaubydi (21), both standard *R. mucosa* and selected local pink pigment formation *R. mucosa* isolate were propagated using LB agar/ Himedia-India. The extraction kit of DNA was utilized for bacterial DNA extraction (Maxime PCR primex Kit iNtRON. Korea) and the extracted DNA concentration was estimated by nanodrop, UV-spectrophotometer device at two wave length 260/280 nm and then kept at -20°C until used PCR detection. Relies on Hamza *et al.* molecular characterization of the microorganism was made by 16S rRNA gene sequence and phylogenetic analysis (8). 16S designed forward primer (5'-TCCAGAGATGGACTTTCCTAGC-3') an Reverse primer (5'-GGCTACCTTGTTACGACTTCAC-3') were added to 12.5 µl of a PCR master mix,6.5 µl of H_2O and 1.5 µl of boiled bacterial extract and thermo-cycled as follows: 1 cycle of 95°C for 5 min, followed by 30 cycles of 59 °C for 30 sec., and 72°C for 1 min and a final cycle of 72°C for 5 min. The designed primers described above amplify the first 505 bp of the 16S rRNA gene (according to the *Roseomonasmucosa* numbering). Electrophoresis was run for identifying the nucleic acids after DNA extraction in 1% of agarose gel and 1% of TBE (Tris base, borate acid, 0.5 M EDTA solution, 1L ddH2O, pH 8.0). The products were mixed with loading dye buffer (bromophenol blue) in 5:1 ratio and subjected to electrophoresis at 70 volts for 1 h and 45 min., the gel was stained with red safe stain and then photos were taken through UV transillumination 350 nm. Then comparison of the sample 16S rRNA gene sequence with the total nucleotide collection in GenBank using the Basic Local Alignment Search tool (BLAST).

Virulence factors Capsular detection

Capsule staining was used to determine the organism's cellular morphology. While the cells themselves are not stained, their morphology is not distorted in anyway. The nigrosine pigment provides a darkbackgrounds against which are the shapes of unstained cells clearly visible (19).

Phenotypic characterization of biofilm production

The ability of the selected characterized *Roeomonas sp.* isolate to form biofilm was detected via two methods:

Congo red agar test

The evaluation of the capacity of selected

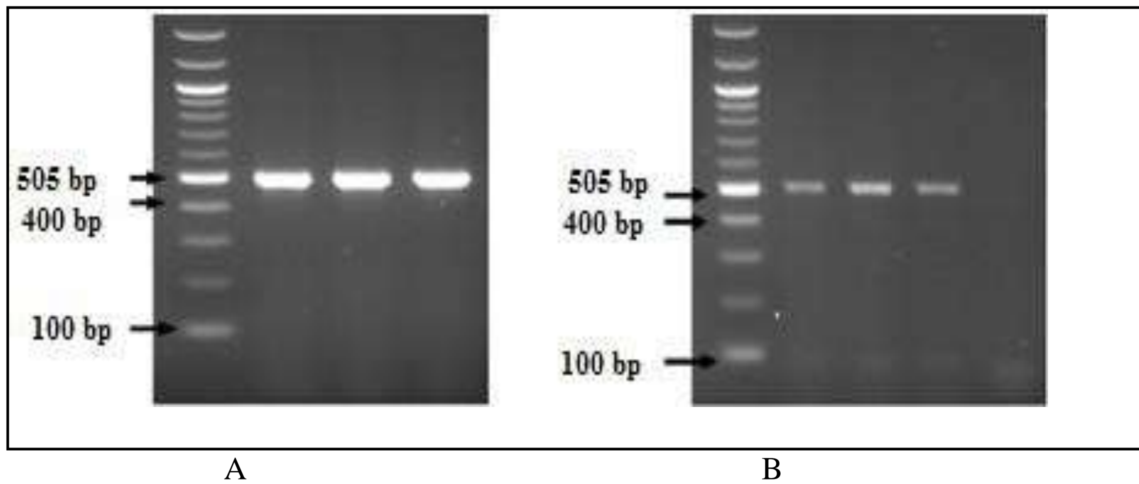
characterized *Roeomonas sp.* isolate to produce a capsule as a presumptive test for biofilm formation was performed using the Congo red agar method following the protocol described in 1989(7). In this test, Congo red dye was used as a pH indicator, showing black coloration at pH ranges between 3.0 and 5.2. Plates with the Congo red agar medium were seeded and incubated in an aerobic environment for 24 to 48 hours at 37℃. After this period, colonies that were dark red or blackish in color, with dry or crystalline consistency, were considered biofilm producers; red colonies with a smooth and darkened appearance in the center were considered biofilm non-producers.

Microtiter plate biofilm production assay. Selected characterized *Roeomonas sp.* isolate was grown in LB broth supplement with 1% glucose for 48hrs at 37°C. A sterile tryptic soya broth (TSB) with 1% sucrose was prepared. Amounts of 180 µl of TSB was added to each well in a microtiter plate, and then 20µl of 48hrs grown selected characterized *Roeomonas sp.* was added. The broth in the well was mixed 10 times by pipette and then incubated for 24 hrs at 37°C without shaking. After incubation, the supernatant was removed and each well was washed 3 times by phosphate buffer. Aliquots of 200µl crystal violet (1%) were added for 15 minutes. The wells were washed 3 times with phosphate buffer and then dried by air for 30 minutes. The supernatant was removed and 200µl of 96% ethanol was added for 15 minutes. Sterile medium was use as a negative control. The result was read using ELISA reader on 630nm (16).

Antimicrobial susceptibility test
Susceptibility to antimicrobials was carried out according to the Kirby-Bauer disk diffusion method (3),by incubation of selected characterized bacterial isolate together with antimicrobial disks included: Amoxicillin, Azithromycin, Bacitracin, Cephalexin, Chloramphenicol, Colisitinsulphate, Doxycycline, Levofloxacin, Methicillin, Sulfamethoxazole, Tobramycin for 48 hr. on Mueller–Hinton agar and results were interpreted using the breakpoints for zone diameters and the susceptibility is measured relies on the European Committee on Antimicrobial Susceptibility Testing (EUCAST,2020). When no breakpoints were available, criteria for related bacteria have been used (19).

RESULTS AND DISCUSSION

The DNA extraction was done and the extracted DNA for both standard and local isolated *R. mucosa* were estimated and shows the concentration of pure DNA are 5 and 9 µg/ml respectively .The PCR technique analysis based on the 16S rRNA gene and maximum-likelihood indicated that bacterial isolate was closely related to standard *Roseomonas mucosa*, Comparison of the sample 16S rRNA gene sequence with the total nucleotide collection in GenBank using the Basic Local Alignment Search tool (BLAST) algorithm was used to assign the bacterial name with 99.99% similarity to other sequences. Based on these results, the isolated bacterium is belongs to *Roseomonas mucosa* and these results confirmed biochemical test done previously in (21).



A B

Figure 1. The molecular detection of 16S ribosomal RNA gene of Roseomonas isolate (A) show the PCR product with a band (505bp) in 1% agarose gel electrophoresis and 70 volts for 1 h and 45 min., in comparison with standard strain (B).

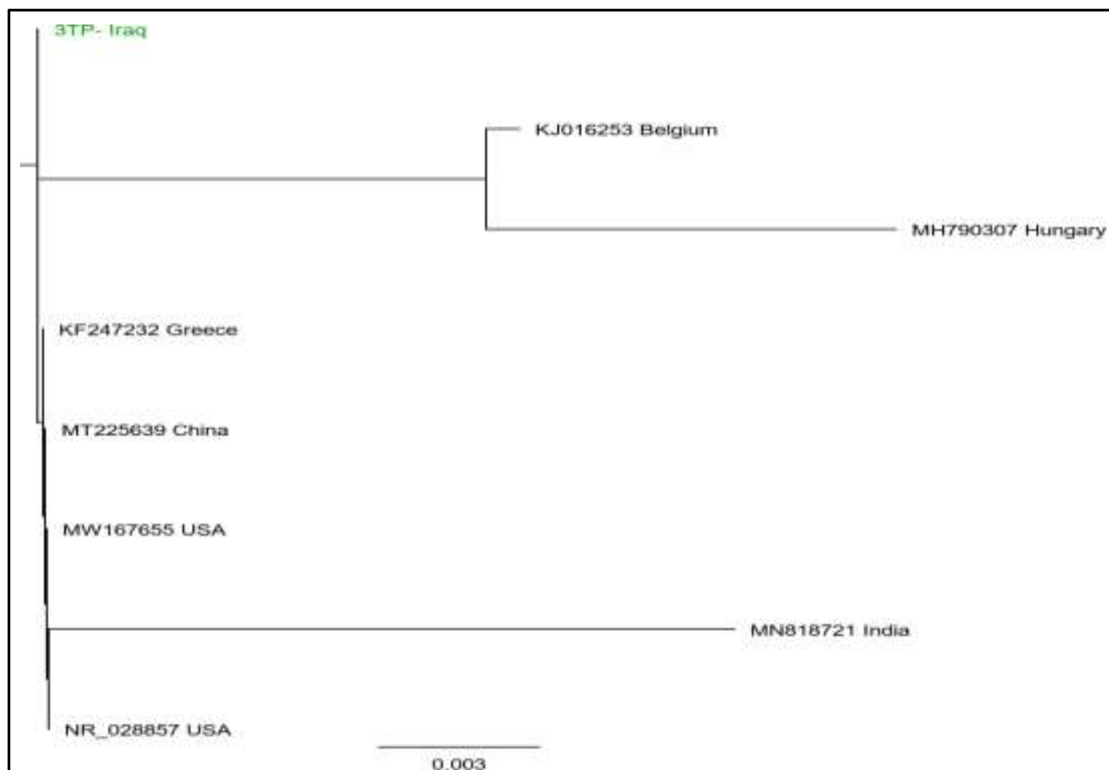


Figure 2. Phylogenetic tree of *Roseomonas mucosa* isolate and related taxa based on maximum-likelihood phylogenetic analysis of 16S rRNA gene sequences available from GenBank (accession numbers in parentheses).

As well as the assigned accession number in GenBank for 16S rRNA sequence of isolate colony was CP044117.1:27, (Figure 1 and 2). Additionally, Phylogenetic analysis revealed the presence of *Roseomonas* genomospecies.A phylogeny is a share identical reading of a family tree. The origin of the tree denotes the inherited lineage, and the tips of the branches signify the progenies of that ancestor. Moving from the origin to the tips, mean moving forward in time. Therefore, it can be suggesting that, the Iraqi *R. mucosa* isolate has an identical root with NR-028857 USA, MW167655 USA, MT225639 China, KF247232 Greece but differ in one nucleotide with each one only.

Virulence factors Capsular detection

After two days' cultured colonies morphology of *R. mucosa* was proposed to denote its prominent mucoid, almost runny colonies. This appearance as a result of a luxuries capsule detection of *Roseomonas mucosa* are

surrounded the bacterial cells (figure 3), whichcan be considered as a first virulence trait for this isolated bacteria because such capsule act as adhesion factor increases the bacterial ability to adhere with different materials whether it is alive such as skin or not alive e.g. different types of catheters (6). In addition, capsule is one such bacterial component, which exhibits many roles that include defense against environmental factors and resistance to innate immune clearance as Venkataramana documented (22).

Figure 3. Capsular detection of *Roseomonas mucosa* isolate.

Phenotypic characterization of biofilmproduction

Congo red agar test

The result of culturing selected *Roseomonas mucosa* isolate on Congo red agar showed that the isolated bacteria has weak capability to produce biofilm on Congo red agar as showed in figure 4. Non-biofilm producer colonies usually remained pink to red on Congo redagar as Freeman et al documented (7).

Figure 4. Non-Biofilm producer R mucosa on congo red agar.

Microtiter plate biofilm production assay. Biofilm quantification analysis was fixated the previous result for isolated *R. mucosa* bacterium, its shows that this bacterium isolate has a weak capability to produce biofilm, indicating that this technique wasmore efficient than Congo red agar for the detection of biofilm production. However, in 2017, Diesendorf1 et al. observation that *R. mucosa* isolated from root canal has the ability to produce spotty localized biofilm on solid glass surface, additionally was able to form biofilm on dentin (5). As well as Katsunori et al. reported that *Roseomonas* sp. that isolated from cooling tower in Tokyo has the ability to produces biofilm (12).

Determination of Antimicrobials susceptibility

Antimicrobial resistance is one of the worldwide problems. Microbial gaining a resistant against any antimicrobial agent is considered an epidemiological marker

changing for this reason; determination of antimicrobials susceptibility is act as one of microbial virulence factor. The results revealed that *R. mucosa* isolates were resistant to some empirical antimicrobial agents such as amoxicillin, methicillin, carbapenemes, bacitracin and Sulfamethoxazole. While sensitive to tobramycin, levofloxacin, colisitin sulphate, doxycycline, azithromycin and cephalexin (figure 5). Some of these results are in agreement with Diesendorf and her coworkers (5) documentation that *R. mucosa* isolated from tooth root canal revealed strong slime layer formation and resistant to most β-lactam antibiotics, whilst this isolate was susceptible to aminoglycosides, polymyxines, sulfonamides, carbapenemes, fluorochinolones and tetracyclines. As well as these results are confirmed with Dien et al. who demonstrated that *R. mucosa* is varies between sensitive to intermediates susceptibilities for most antimicrobial agents including aminoglycoside, most beta lactam, quinolones and Sulfamethoxazole (5). Also Shayuan and his coworkers reported that, *R. mucosa* isolated from patient with systemic lupus erythematosus exhibited large inhibition zone for most of antimicrobials tested (22). On another hand in 2018, Koh *et al.* reported a genus belong to *Roseomonas*. A species of pink-pigmented bacteria has been related with numerous primary of hospital-acquired infections; though, its nosocomial infection had never been reported before and the sources of these isolates were from the different samples collect from the hospital environment (14). The global research in addition to the recent finding may reflect the increasing bacterial resistant against empirical antimicrobial agents. Thus, real treatment of such bacterium particularly in immunocompromised patients by catheter removal may be needed. Antimicrobials alone can fail to eliminate catheter related sepsis regardless of achieving therapeutic levels as Alcala *et al.* mentioned (2).

Figure 5. Disc diffusion method of antimicrobials susceptibility test of *R.mucosa* isolates.

CONCLUSION

From the outcome of the present investigation, it is possible to reveal that;

This study is considered as an improvement that, *R. mucosa* has a virulent factors enables this bacterium to invade vital or solid materials. In spite of this genus seems to have an overall little pathogenic potential for humans, Roseomonas sp. in particular, *R. mucosa* may be substantial pathogens in individuals with underlying medical complications.

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