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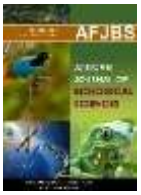

Emergence of *Micrococcus luteus* among hospitalized patients with catheter-associated urinary tract infection

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Abstract

Micrococcus luteus is now increasingly being recognized as an important human pathogen commonly associated with indwelling device. From a total of 24 urine samples collected from patients with urinary catheter, only 3 *M. luteus* were identified by using both VITEK 2 automated system and 16S rRNA. In parallel, the organisms evaluated for their *in-vitro* susceptibility testing and biofilm production. All isolates were found to be susceptible for Chloramphenicol, Ciprofloxacin, Nalidixic acid, Gentamicin, Tetracycline, Novobiocin, Imipenem, Amoxicillin/Clavulanic Acid and Streptomycin antibiotics.

Furthermore, we determined the biofilm production by using Microtiter Plate (96-Well Plate) Assay and surfaces (glass, latex and silicon foley sections). We found that all tested isolates show significant capacity to produce biofilm on different surfaces. To the best of our knowledge, we describe for the first time of *M. luteus* in catheter related urinary tract infection in Iraq.

Introduction

Catheter-associated urinary tract infections (CAUTIs) are the most common nosocomial infection around the world and a leading cause of morbidity. Catheters are one of the most commonly used medical devices in the world and can be characterized as either indwelling (ID) or intermittent catheters (IC) [1][2].

Microbial colonization occurs within five to seven days of catheter placement and is frequently associated with the development of a bacterial biofilm, presumably the source of the CAUTI. A urinary catheter is a long tube that can be made from of different polymers, with silicone being typically used, and latex rubber also common [3][4]. When required, the urinary catheter is inserted into the urethra as far as needed until the urine begins to flow. *Micrococcus luteus* are Gram-positive coccus that is resident on human skin and was initially isolated from the nasal discharge of a patient suffering with acute coryza [5][6]. Rare infectious diseases can develop from *M. luteus* such as prosthetic valve endocarditis and meningitis [7] [8]. Here we identified *M. luteus* among CAUTIs patients [9] [10]. We then tested their susceptibly to antibiotics [11].

Materials and Methods

Study design

Total of 24 patient with indwelling urinary catheters admitted in Sulaymaniyah Surgical Teaching Hospital / Urology and ICU Wards, Kurdistan region-Iraq, during September to October 2019 were included in this study.

Isolation and identification technique

All urine samples were collected from patients who has an indwelling urinary catheter for more than two days. The sample was inoculated onto appropriate culture media, such as blood agar, MacConkey agar and Mannitol Salt Agars and the plates were incubated overnight at 37^{o}C. Any significant colony grown after 24h was isolated and identified according to morphological, cultural and conventional biochemical (catalase, oxidase, and coagulase). All the strains were confirmed to be *M. luteus* via VITEK 2 Compact automated system and 16S rRNA. Improve molecular identification of *M. luteus* by 16S rRNA gene was generated in order to confirm the identification of strains [12][13]. Firstly, the genomic DNA was extracted by standard methods, then we amplified by PCR with universal primers 27F and 1492R [14] [15]. PCRs cycling conditions were conducted in 50 µL reaction mixtures under the following: heating at 94 °C for 2 min, 30 cycles of

95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. PCR product size (about 1500 base pairs). DNA sequencing was performed by the Sanger method using a 3500xl Genetic Analyzer (Applied Biosystems).

Susceptibility testing

Antibiotics susceptibility testing of *M. luteus* was performed using the disk diffusion method as recommended by the Clinical and Laboratory Standards institutes [16]. Antimicrobial disks used were Nitrofurantoin (300 µg), Cefixime (5µg), Metronidazole (50µg), Cephalthin (30mg), Chloramphenicol (30µg), Erythromycin (15µg), Cefixime (10µg), Ampicillin (10µg), Amoxicillin and Clavulanic Acid (20/10µg), Ciprofloxacin (5µg), Azithromycin (15µg), Rifampin (5µg), Ceftriaxone (10µg) Streptomycin (25µg), Vancomycin (10µg), Nalidixic acid (30 µg), Gentamicin (10 µg), Ampicillin (25µg), Tetracycline (30 µg), Tobramycin (10 µg), Cefotaxime (30µg), Novobiocin (30 µg), Imipenem (10 µg) and Amoxicillin (25 µg) .The 0.5 McFarland turbidity was prepared then streaked the swab over the entire surface of the Mueller Hinton agar. The antibiotic discs were applied with sterile forceps onto the surface agar plate then incubated at 37^{o}C for 24 h. The inhibition zone diameter was measured and compared with CLSI guidelines for zone sizes.

Biofilm Assay

Microtiter plate assays

Firstly, the Microtiter plate method was used to determine biofilm production. In this method, the *M. luteus* isolates were grown overnight at 37^{o}C in Mueller-Hinton Broth. Then, microtiter plates were inoculated with 100 µl bacterial suspension adjusted to 0.5 McFarland. The inoculated microtiter plate was incubated at 24 hours at 37°C. After incubation, the microtiter plates were washed twice with distilled water and dried. The plates were then stained with 125 µL of 0.1% (w/v) Crystal Violet solution. The crystal violet in the wells was then solubilized using 200µL 30% (v/v) acetic acid and measured for absorbance at 590nm in a BioTek ELx800 microplate reader (Biotek, United States). A high absorbance reading corresponded to a high amount of biofilm and low absorbance readings indicated scarce biofilm formation.

Glass, Silicon Foley Catheter and Latex Foley Catheter

Biofilm on Glass Surface:

Glass kahn tubes were utilized for this procedure:

1ml of **0.5** McFarland bacterial suspensions were incubated for 24 hours at 37°C. After incubation, the glass tubes were washed twice with distilled water and dried. The glass tubes were then stained with 1.25 ml of 0.1% (w/v) Crystal Violet solution. The crystal violet in the test tubes was then solubilized using 2ml 30%

(v/v) acetic acid and measured for absorbance at 590nm by CECIL 7500 [Canada] spectrophotometer. A high absorbance reading corresponded to a high amount of biofilm and low absorbance readings indicated scarce biofilm formation.

Biofilm on Silicon Foley Catheter and Latex Foley Catheter Surfaces:

Sections of urinary catheter were exposed to **0.5** McFarland bacterial suspensions. To maintain consistency identical [225mm^2] surfaces were exposed to bacterial suspensions.

Equation (1) [17]:

$$Tube\ Surface\ Area = 2\pi(R^2 - r^2) + 2\pi h(R + r)$$

Equation (2):

$$h = \frac{Tube\ Surface\ Area - 2\pi(R^2 - r^2)}{2\pi(R + r)}$$

Thus; 10.2mm of 12fr Silicon Foley and 5.3mm of 20fr Latex Foley catheters were cut. Individual catheter sections were submerged in 2ml bacterial suspensions inside separate test tubes. After 24-hour incubation at 37^{o}C the catheter sections were washed twice with distilled water and dried then transferred with care into blank test tubes. The catheter sections were then stained with 2 ml of 0.1% (w/v)

Crystal Violet solution. The crystal violet on the catheter sections was then solubilized using 2ml 30% (v/v) acetic acid and measured for absorbance at 590nm by CECIL 7500 [Canada] spectrophotometer. A high absorbance reading corresponded to a high amount of biofilm and low absorbance readings indicated scarce biofilm formation.

Results

Overall, a total of 24 urine samples collected from patients with urinary catheter only three *M. luteus* (12.5%) isolates were recovered from patients in Sulaymaniyah Surgical Teaching Hospital. The nucleotide sequence was analyzed with BLAST programs, showing the higher degree of homology (100.00%) with *M. luteus* (GenBank/EMBL data library accession number AJ496321.1).

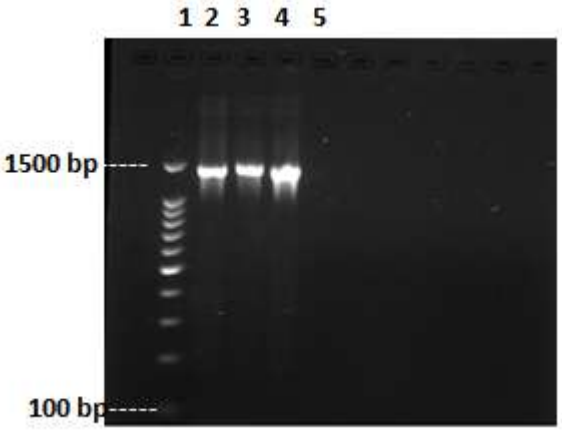

Figure-1: Agarose gel electrophoresis of *M. luteus 16S rRNA* gene amplicons (1500 bp). Lane M: 100bp ladder. Lane 2, 3 and 4: positive *16S rRNA* gene. Lane 5: Negative control

Antibiotics susceptibility

In all the *in-vitro* susceptibility experiments, the positive controls yielded growth of *M. luteus* organism between 24 to 48 hrs. Using the disk diffusion method, we observed that all isolates were found to be susceptible for Chloramphenicol, Ciprofloxacin, Nalidixic acid, Gentamicin, Tetracycline, Novobiocin, Imipenem, Amoxicillin/Clavulanic Acid and Streptomycin antibiotics while they were resistant toward Nitrofurantoin, Penicillin, Cefixime, Metronidazole and Cephalthin.

Additionally, we analyzed and compared the M. *luteus* for biofilm formation with others relative biofilm formation bacteria (such as: *Pseudomonas aeruginosa*, *Proteus sp* and *Staphylococcus epidermidis*), all tested M. *luteus* shown strong biofilm formation on methods (Figure 2, 3, 4 & 5).

Microtiter Assay has shown the significant potential of *M.luteus* to produce biofilm as illustrated in figure-1.

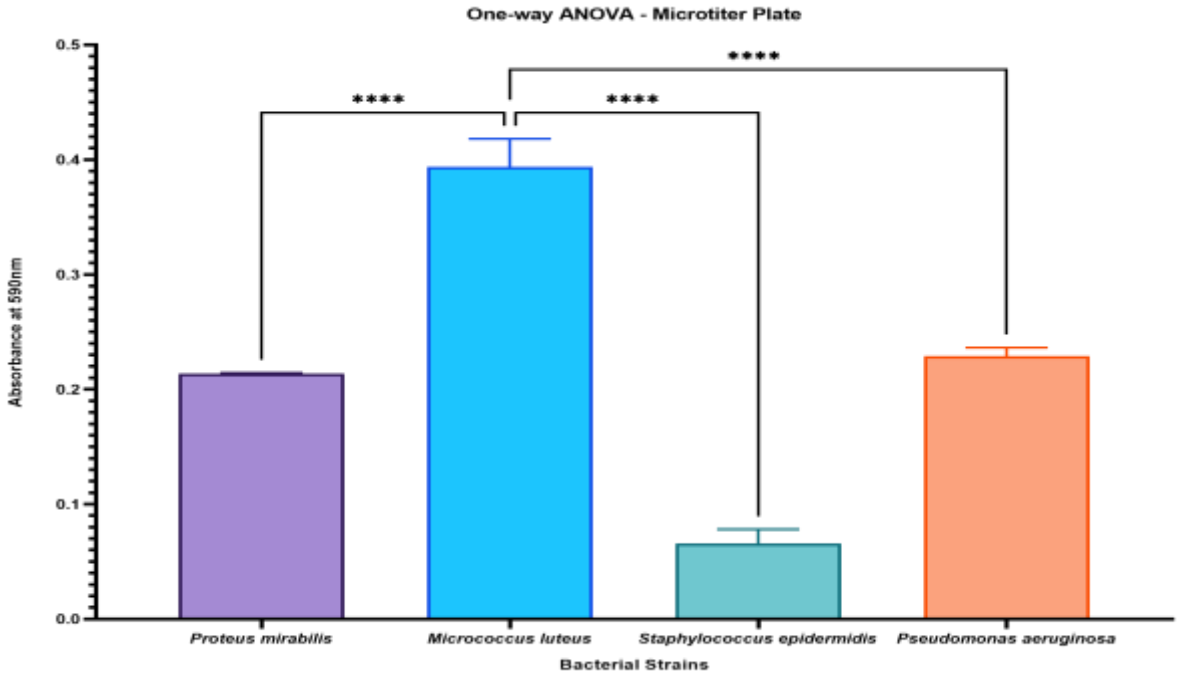

Figure-2 illustrates the biofilm formation on 96-well microtiter assay

Results of biofilm production on glass surfaces reveal similar significance of *M. luteus* capacity to form biofilm, shown in figure-1.

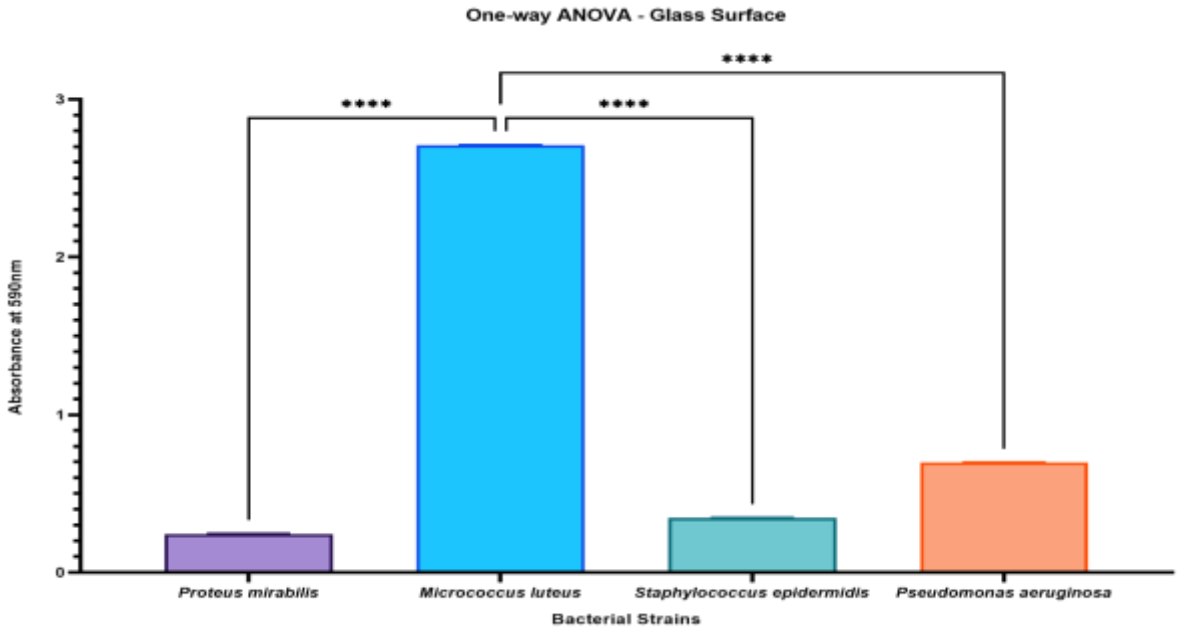

Figure-3 illustrates the biofilm formation on glass surface

Silicon Foley Catheter were providing less favorable surface to allow the production of biofilm by Micrococcus *luteus* when compared to Latex Foley Catheter as shown in figure-3.

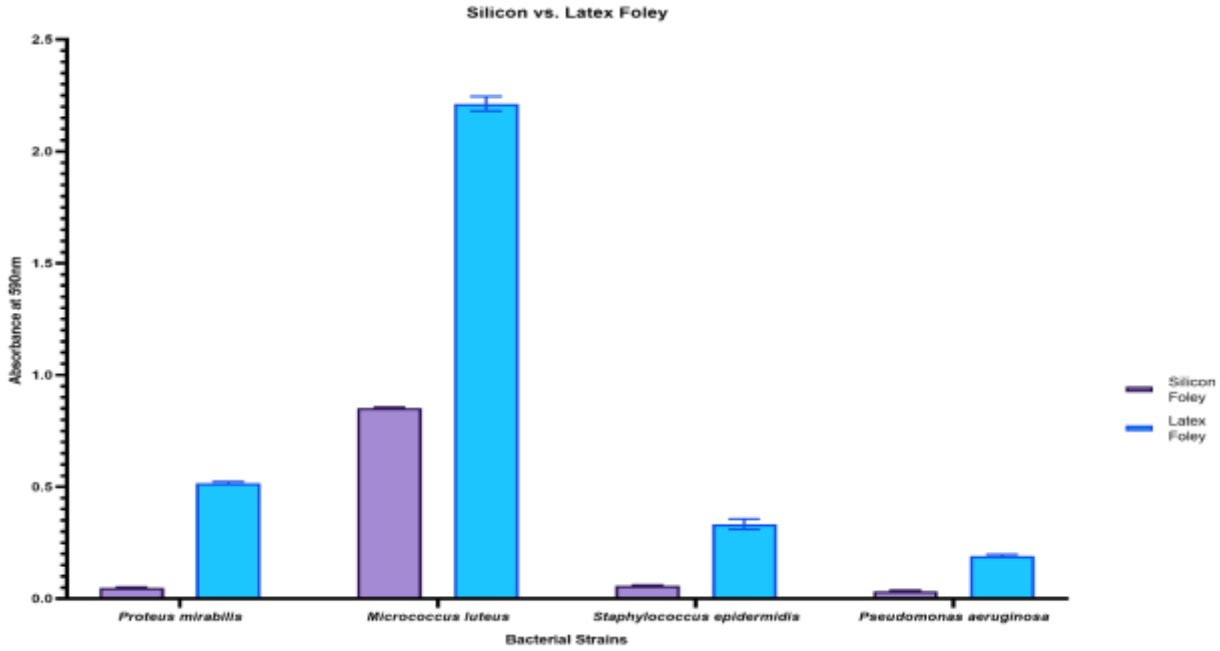

Figure-4 illustrates the biofilm formation on catheter

Discussion

The *in-vitro* susceptibility and sequencing data herein presented were interpreted as authentic, since the negative controls used in both culture-based and PCR-based experiments remained negative. *Micrococcus* sp., members of the family Micrococcaceae, are Gram-positive cocci, catalase-positive, arranged in tetrad

clusters and one of the most frequent causes of many common bacterial infections, including acute urinary infection in young women [18]. Ashwag et al. previously reported in a similar study conducted in Saudi Arabia on 120 urine specimen from young females, they found that one of the top frequently isolated bacteria are *Micrococcus* sp. (16%) [19]. Another study [18] of 55 male students of urinary infection gave *Micrococcus luteus* (40%). Both these studies produced strong evidence which proposed that the micrococcus was more creative of inflammatory exudate than any other urinary bacterial pathogens [20]. Here, we observed that all isolates are sensitive to most of selected antibiotics. This result agrees with the results previously reported in a study conducted in Pakistan, in which they found that *M. luteus* showed sensitive to most of the antibiotics used including, tetracycline, ampicillin, gentamicin, kanamycin, neomycin, baquiloprim sulphadimidine, chloramphenicol and sulphamethoxazole trimethoprim [21]. In spite of infrequent information is available on the pathogenesis of *Micrococcus* sp. and adequate novel studies to specify the pattern of Micrococcal response to antibiotics but in general they rarely produced beta-lactamases [22]. Furthermore, Dürst et al. found that *M.luteus* was susceptible and showed the response to penicillin [23]. Significantly all of these bacteria were capable to form biofilm on different tested surfaces [24][25]. This is consistent with previous findings for the formation of biofilms on medical devices [7][26]. Ianniello et al. report a case of native valve infective endocarditis

due to *M. luteus* and capability to produce biofilms with a prosthetic material [27]. This study is significant to note that patients with urinary catheters are susceptible to life-threatening infections caused by *M. letues* that are normally deemed harmless. Clinicians should be conscious of early detection for treatment of a real infection caused by these bacteria that could be important for this patient population. Additional experimental methods of antibiotic sensitivity tests are yet to be implemented to assess the potential versus feasibility of preferred drugs on biofilms [28]. It is inevitable that higher concentration of antibiotic(s) is needed to eradicate colonized biofilm infections. However, the dosage-related toxicity is another obstacle to overcome [29]. Implementing different drug delivery methods might possibly resolve the toxicity issues [30][31].

Conclusion:

In addition to being one of the leading causes of patient morbidity and mortality, healthcare-associated infection (HAI) is one of the most serious dangers to patients' safety. The most frequent HAI in the world is catheter-associated urinary tract infection (CAUTI). In addition, it is the most neglected infections that could make the post hospitalization experience not so pleasant for the patients. It also increases the re-admissions of the patients with unrelated side-effects. There is a great necessity to evaluate and analyze the types of various CAUTIs to have complete post hospitalization care that could help both the patients and the physicians to

avoid un-necessary re-admissions of the patients. In our present research investigation, we have made an attempt to spread awareness about the potential of Micrococcus luteus to implicate patients' health. In addition, our team emphasized the Micrococcus luteus isolates follow up, treatment and patient prognosis to avoid colonized infections. These Micrococcaceae are reported to be one of the most frequent infections in the urinary tract. Our research findings are in line with the previous research reports that confirm the ability of the formation of biofilms on medical devices, especially catheters. Hence emphasizing the importance of understanding the risks of urinary catheter induced infections which might prove life threatening in the case of the long-term hospitalized patients. Our team recommends the necessity of early detection methods, appropriate choice of antibiotics to control the urinary catheter associated bacteria to reduce the risk of morbidity and mortality among the hospitalized patients. Despite the fact that *M. letues* has significantly shown its potential to form biofilm on different surfaces.

Ethics Statement

Ethical approval was granted from Sulaymaniyah Surgical Teaching Hospital. Informed assent was obtained from each patient with indwelling urinary catheters regarding demographic characteristics.

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