Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2023.30.07.044

Molecular analysis of Biofilm genes in Micrococcus luteus Isolated from Pleural Fluid Infections Patients in Al-Najaf Province, Iraq

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Submitted: 20 February 2023; Accepted: 17 March 2023; Published: 10 April 2023

ABSTRACT

Micrococcus luteus is a low-virulence opportunistic pathogen that has been identified as an emerging hospital pathogen. Infections caused by M. luteus have increased over the years due to increased high-risk patients e.g. patients with immune suppression, hematological malignancies, ICU admission. Therefore this study conducted to characterization and investigation of some virulence factor particularly detection of biofilm formation in order to determinants the role of this bacteria in causing infection in Al-Najaf province. Therefore 250 clinical specimens were collected from plural fluid infection 140 (64.9%) of specimens were collected from male and 110 (35.1%) from female, the specimens were cultured on suitable media and cultivated at 37°C. The results showed that 209 (76.9%) gave bacterial growth while 41 (23.1%) appeared no growth. After identification of bacterial isolate, 25 of isolates were identified M. luteus recovered from the pleural fluid infections. TCP method were used to determinant the predominant of the level of biofilm among M. luteus isolates, the results of TCP revealed that all isolate 25 (100%) were biofilm formation. The results of genetic analysis of pml gene revealed that 7 (28%) of M. luteus isolates have this gene.

Keywords: *Pleural fluid, Biofilm, pml, Micrococcus luteus, CRA*

INTRODUCTION

In the pleural cavity of normal human being, there is a small amount of fluid known as a pleural fluid which lubricates the lining of the cavity. Pleural effusion is always abnormal and indicates the presence of an underlying disease (Saladin and Kenneth, 2011). Pleural fluid accumulates when pleural fluid formation exceeds pleural fluid absorption. Normally, fluid enters the pleural space from the capillaries in the parietal pleura and is removed via the lymphatics situated in the parietal pleura. Fluid can also enter the pleural space from the interstitial spaces of the lung via the visceral pleura or from the peritoneal cavity via small holes in the diaphragm. The lymphatics have the capacity to absorb twenty times more fluid than is normally formed (Saladin and Kenneth, 2011). Pleural effusion is defined as an abnormal, excessive collection of fluid in the Pleural space. Two types of effusions can develop, transudative and exudative. Various kinds of pleural effusion, depending on the nature of the fluid and what caused its entry into the pleural space, are hydrothorax (serous fluid), hemothorax (blood), chylothorax (chyle) or pyothorax (pus). Bacterial infection of the pleura was first described in ancient Greece by Hippocrates (Light et al., 2007). Pleural effusions are produced by a wide variety of causes Infectious processes including bacteria, viruses,

tuberculosis, atypical mycobacterium, fungus, as well as parasites account for a substantial percentage of these effusions. The etiologies of pleural effusions as a whole, and then more specifically the various specific findings of pleural effusions resulting from infectious diseases (Light et al., 2007).

Micrococcus luteus is a Gram-positive to Gram-variable, non motile cocci tetra-arranging, pigmented, saprotrophic coccus bacterium in the family Micrococcaceae.(Madigan and Martinko et al., 2005). It is urease and catalase positive. An obligate aerobe, M. luteus is found in soil, dust, water and air, and as part of the normal microbiota of the mammalian skin. The bacterium also colonizes the human mouth, mucosae, oropharynx and upper respiratory tract M. luteus is generally harmless but can become an opportunistic pathogen in immunocompromised people or those with indwelling catheters (Canada, et al., 2011). It resists antibiotic treatment by slowing of major metabolic processes and induction of unique genes. M. luteus is coagulase negative, bacitracin susceptible, and forms bright yellow colonies on nutrient agar M. luteus has been shown to survive in oligotrophic environments for extended periods of time. It has survived for at least 34,000 to 170,000 years, as assessed by 16S rRNA analysis, and possibly much longer (Greenblatt et al., 2004). Its genome was sequenced in 2010 and is one of the smallest genomes of free-living Actinomycetota sequenced to date, comprising a single circular chromosome of 2,501,097 bp (Young et al., 2010). Although generally a harmless saprophyte, M. luteus can act as an opportunistic pathogen. It has been associated with a variety of illnesses including meningitis, septic arthritis, endocarditis, chronic cutaneous infections in HIV positive patients, and catheter infections (Fosse et al.,1985). The main purpose of this study was the detection of antibiotic resistance and virulence factors of M. luteus isolated from pleural fluid patients.

METHODOLOGY

Specimens collection

The study was conducted at the Bacteriology laboratory in Biology Department, Sciences Faculty, Kufa University, Iraq. The specimens 250 plural fluid were obtained from patients who attending to Al-Sadder Medical City hospitals in Al- Najaf suffering from lower respiratory tract infections during the period from January 2021 to December 2022).

Bacterial isolation and identification

M. luteus was isolated and identified according to traditional biochemical diagnostic, by using the routine methods e.g. according to Macfaddin et al.,(2000) ; Collee et al., (1996). These isolates were cultured on brain heart infusion broth at 37°C and on blood agar incubated at 37°C for 24 hours. Inoculate from this broth were streaked onto tryptone soya agar (TSA) (Oxoid, England) and incubated at 37°C for 48 hours (Akayli et al., 2020). The suspected isolates were identified through their morphological characteristics, Gram-staining, motility test, oxidase test and catalase test (Holt et al., 1994) and through Vitek-2 compact system (bioMerieux, France) according to the manufacturer instructions, Vitek-2 was employed for the confirmation of identification. Bacterial strains were maintained on deep Nutrient agar slant (Himedia) for 8-10 weeks with periodic subculture and nutrient broth (Himedia) with 20% glycerol at -20 ◦C. (Funke and Funke-Kissling et al., 2005, Pękalaa et al., 2018).

Antibiotic Resistance in Micrococcus luteus

In this study used 15 types of commonly used antibiotics include: penicillin 10 mg, Ampicillin10, 30mg, Ciprofloxaci µg, Piperacillin 100 µg, Azithromycin 15 µg, Doxycycline 10 µg Canamicin 10 µg, Levofloxacin 5 µg, Cefixime 10µg, Imipenem 10 µg, Amikacin 10µg, Nitrofurantoin 300 µg and Tetracycline 30µg, Rifimpem 5, Doxycycline 10. The antibiotic sensitivity report was performed according to Kirby-Bauer disc diffusion fashion on Mueller-Hinton agar (Morello et al., 2006). It Briefly, the investigated isolates allowed to multiplication for overnight at 37ºC in BHI broth referred to 0.5 McFarland turbidity standard equal to 1.5X108 CFU/ml (Mcfarland et al., 1997), the MH agar plates were fully spreading

with 0.1 ml of growth suspension and then fixed antibiotics disks on the surface. The applied plats left for 10-15 minutes and then incubated for 24 h at 37ºC as a standard cultural condition. The fixed antibiotics were classified as sensitive (S), Intermediate (I), or resistant (R) according to diameters of halo zone in millimeters (mm) around the individual disk, the results were compared with clarifying list of (CLSI et al., 2020).

Biofilm Formation

Tissue Culture Plate Method

We used TCPM as the gold standard test for detection of biofilm formation A loopful of freshly cultured isolates was inoculated in 10 ml of trypticase soy broth with 1% glucose. The inoculated broth was then kept in the incubator at 37°C for 24 hours. Bacterial suspensions were further diluted 1:100 with fresh medium Separate wells of a sterile polystyrene tissue culture plate, composed of 96 flat bottom wells were filled by 200 µl of the prepared bacterial suspension. Similarly, control organisms were put in the tissue culture plate. In addition, only sterile broth was used to ensure sterility and to identify non-specific binding. After incubation at37°C for 24 hours, the plate was gently tapped to remove the content of the wells followed by washing with 200 µl of phosphate buffer saline. The washing step was repeated four times to remove any free bacteria present in the wells. Sodium acetate (2%) were added to the wells and kept for 30 minutes in order to fix the biofilms formed by bacteria attached to the wells. Staining of the fixed biofilms was conducted using crystal violet (0.1%). After 30 minutes, the wells were thoroughly washed by deionized water to remove any extra stain. After drying, a micro-ELISA reader (at 570 nm wave length) was used to measure the optical densities (OD) of stained bacterial biofilms. Test was carried out in triplicate and average of three OD values was taken. Optical densities values indicated bacterial adherence to the wells and biofilm formation. The OD values were calculated and biofilm production was graded into strong, moderate and non/weak as described in previous studies (Panda and Bakir et al., 2016).

Congo Red Agar Method

Congo red agar is a specially prepared medium composed of brain heart infusion (BHI) broth (37g/l) supplemented with sucrose (50 g/l), agar No1 (10 g/l) and Congo red (0.8 g/l). We prepared a concentrated aqueous solution of the Congo red stain that was then autoclaved at 121ºC for 15 minutes. Finally it was added to the autoclaved BHI agar with sucrose at 55ºC. Prepared CRA plates were inoculated with the isolated M. luteus and aerobically incubated at 37°C for 24 hours. Appearance of black dry crystalline colonies on the CRA plates indicated biofilm production while the colonies of biofilm non producer remained pink or red colored (Ruchi et al., 2015; Hassan et al., 2011; Freeman et al., 1989).

Molecular Study

Extraction of DNA and PCR Conditions

Total DNA was extracted using the boiling method according to Shah et al., (2017). Thermo cycle PCR was used to use the molecular method to confirm the formation of biofilms once more. This method requires specific primers for the pml gene, which have the sequence F-5' GGATCATCTATAATGAAACTG 3' and R-5' CTGATAATCAACTTGGAAGTT 3', and are 563 bp in size (Abbas et al., 2015). Then, 12 µl of master mix, 5 µl of template DNA, 2 µl of each set of primers, and complete to 25 µl of sterile nuclease-free water were placed in an appropriate PCR tube. The mixture was vortexed well. The PCR designed to amplify pml gene included a primary denaturation step for 2 min at 94°C, 94°C for 40 sec denaturation, 52°C/30 sec for annealing, and followed by extension for 72°C for 60 sec. The reaction mixture was held at 4°C until use while the final extension step took place at 72°C for about 10 minutes (Abbas et al., 2015). Each and every PCR amplification was performed using a Verity Thermal Cycler (Agilent, UK). Then, 1% agarose gel electrophoresis was used to analyze all of the PCR products, and they were all stained with red ethidium bromide dye. Finally, the gel documentation system was used to identify the electrophoresis results.

RESULTS AND DISCUSSION

In this study collected 250 plural fluid specimens from patients attending to Al-Sadder Medical City hospital in Al- Najaf-Iraq, among these 250 specimens 50 specimens not containing on bacteria and 200 specimens gave up a positive culture. A culture analysis based on morphological and biochemical tests revealed a high incidence of Gram positive bacteria included 150 G+ve bacteria and 50 G-ve bacteria showed growth during this time period.

Approximately, 25 of M. luteus were isolated from culturing pleural fluid on blood agar media. The identification of M. luteus was first made by the bacteriological methods including colonial morphology (Figure -1), and biochemical tests. The biochemical tests were included urease positive, catalase positive, non-motile, cocci, tetra arranging, pigmented, saprotrophic coccus. were suspected as M. luteus. M. luteus isolates were confirmed identification using the VITEK-2 compact system.

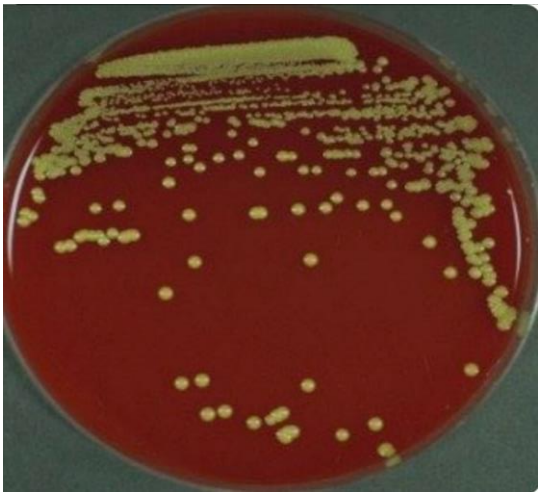

FIGURE 1: Colony of Micrococcus luteus on Blood Agar.

The Antibiotic Susceptibility Test of Micrococcus luteus

The susceptibly of Micrococcus Lutes to common antibiotic that used in treatment bacterial infection were tested using the Kirby-Bauer disk diffusion method according to CLSI et al., (2021) guidelines, which included 11 antibiotic from six antimicrobial categories. Overall, the resistant rate for β-lactams/β-lactamase inhibitor combination antibiotic including Amoxicilin was resistance appeared in 83% of isolates. The resistance bacteria isolates to the third generation of Cefotaxime 100%, resistant to Cefepime was recorded in 83% of isolates, while the resistance to fourth generation Doxycycline were appear in 100% of isolates, the susceptibility results for the tetracycline antibiotics was appear 96%, the evaluated isolates exhibited resistance to SXT 83% of the isolates, and showed resistance to Ciprofloxacin 10%. The resistance of bacterial isolates to Levofloxacin was found 66% of isolates, norfloxacin 83% of isolates, finally the resistance to Penicillin was recorded in 100% of isolates, While resistance to Imipenem represented in 93 %. While resistance to Tobramycin antibiotics was appear 70%. Amakicin 70%, While resistance to Rifampin antibiotics was appear 90%, resistance to Kanamycin was appear 70% (as shown in figure 2).

The use of broad-spectrum antibiotics and an increase in the number of invasive procedures and immunosuppressed patients has caused this intrinsically multidrug-resistant microorganism to emerge as an infectious agent in hospitals, especially in intensive care units (ICUs). Its resistance to many antimicrobial agents, including β-lactams and aminoglycosides, allows patient colonization even when antimicrobial agents are being used (Villarino et al.,1992).

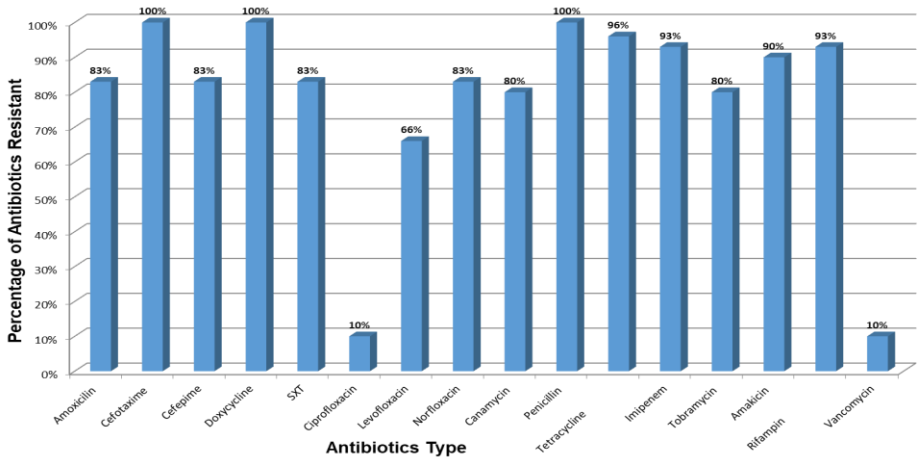

FIGURE 2: The Antibiotic Susceptibility Rates Among Micrococcus luteus.

Biofilm Formation

The result of this study showed the ability of mast M. leteus isolates on biofilm formation through production of crystalline black color on Congo red Agar method (figure 3).

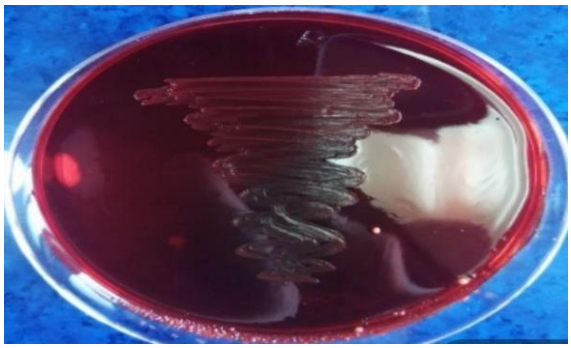

FIGURE 3: Colony of Micrococcus luteus on Congo Red Agar (black colonies of biofilm producer, red colonies of non-biofilm producer).

The results showed that CRA assay is a good method for detection of the ability of slime and biofilm production and agreed with researcher Arciola et al., (2006) who recommend that CRA experiment are a dependable method to determine biofilm production (Cabrera-Contreras et al., 2013). The slime layer works on the bacterial cell packaging, forming thin, living membranes known as biofilm its act as a buffer that inhibits the antibiotic influence within the bacteria cell and thus confers resistance (Al-Khafaji, 2018).

Also, showed the ability of all M. luteus isolation on biofilm formation by Tissue culture plate method (table 1).

TABLE 1: The ability of Micrococcus luteus on biofilm formation by Congo red Agar method and tissue culture plate method

Biofilm formation	CRA No(%)	TCP No (%)
Non	1 (4)	0(0)
Moderate	4 (16)	3 (12)
High	20 (80)	22 (88)

J Popul Ther Clin Pharmacol Vol 30(7):e400–e407; 10 April 2023.

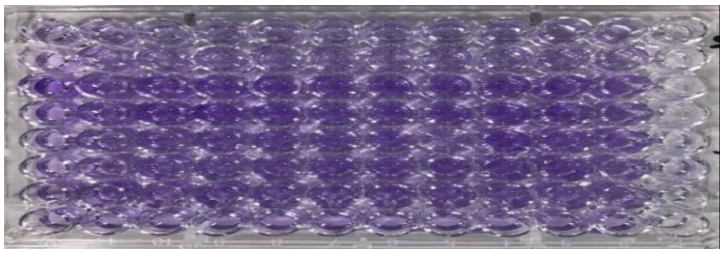

FIGURE 4: Phenotypic Detection of Biofilm Formation of Micrococcus luteus TCP Method.

Biofilms, sessile structured bacterial communities exhibiting recalcitrance to antimicrobial compounds and persistence despite sustained host defenses, are increasingly recognized as a contributing factor to disease pathogenesis in CF and other respiratory tract diseases associated with chronic bacterial infections (Pompilio et al., 2014). Biofilm formation is a complex and multifactorial event. It depends on the surface characteristics, strain motility, genetic factors, and other factors. Biofilm formation correlates with high resistance to antibiotics (Kim et al., 2019).

In recent years, the increase in incidence of disease caused by biofilm-associated organisms has been noted globally. Biofilms pose a serious problem for public health, because biofilm-producing microorganisms exhibit dramatically increased resistance to both antimicrobial agents and host immune response. Of note, the increase in the incidence of MDR bacterial and fungal strains makes many public crises (Ghaly et al., 2020).

Biofilm development and medication resistance make bacterial eradication more challenging in clinical settings. Therefore, The relationship between biofilm formation and drug resistance should be investigated. As previously said (Algburi et al., 2018), our findings revealed that biofilms play a significant role in building antibiotic resistance and that there is a link between biofilm production and antimicrobial resistance.

Molecular Method

Primers for pm1 gene were used for detecting the presence of pm1 gene in M. luteus isolates. It has been found that 7(28%) of these isolates contain the genes with the length of 563 base pairs. The amplicon was detected in gel electrophoresis and compared with allelic ladder. This result was shown in figure (5).

FIGURE 5: Illustrations gel electrophoresis of pm1 gene that the positive result represents 1, 4, 5, 13, 14, 15, 16 isolates from left to right. L: Ladder with 10000bp.

According to Fusco et al., (2017), the PM1 isolate has a high capacity for adhesion in epithelial cells and is distinguished by the homogeneity of its colonies on solid media and the absence of clumping growth in liquid culture media (Brooks et al., 2007). And the development of the biofilm

on biological surfaces after two hours of incubation, up to the full phenotypic characteristics of the biofilm after six hours of incubation due to its high ability to swarm, and using this strain to develop medications aimed at particular virulence factor pathways.

CONCLUSIONS

The outcomes of this study, revealed that a great spread of Micrococcus luteus isolates in Al-Najaf hospitals that produce different virulence factors and resistant to many antibiotics.

REFERENCE

1. Akayli, T., Yardimci, R. E. and ÜRKÜ, Ç. (2020). Diagnosis of Micrococcus luteus infection in cultured sharpsnout sea bream Diplodus puntazzo Cetti, 1777). Journal of Institue of Science and Technology, 36 (2):214-220.
2. Algburi, A;Zehm, S; Netrebov, V; Weeks, R; Zubovskiy, K and Chikindas, M. L. (2018). Benzoyl peroxide inhibits quorum sensing and biofilm formation by Gardnerella vaginalis 14018. Infectious diseases in obstetrics and gynecology.
3. Al-Khafaji, A. N. (2018). Isolation and Identification of Methicillin Resistance Staphylococcus aureus and Detection their Ability to the Production of Virulence Factors. Journal of University of Babylon for Pure and Applied Sciences, 26(8), 100-111.
4. Arciola, C.R.A.; Campoccia, D.; Baldassarri, L.; et al (2006).:" Detection of biofilm formation in Staphylococcus epidermidis from implant infections. Comparison of a PCR-method that recognizes the presence of ica genes with two classic phenotypic methods. J Biomed Mater Res A 76: 425–430.
5. Bakir SH, Ali FA. (2016).Comparison of Different Methods for Detection of Biofilm Production in Multi-Drug Resistance Bacteria Causing Pharyngotonsillitis IJRPB 2016;3:13-22
6. Cabrera-Contreras, R.; Morelos-Ramírez, R.; Galicia-Camacho, A. N. and Meléndez-Herrada, E. (2013). Antibiotic resistance and biofilm production in Staphylococcus epidermidis strains, isolated from a tertiary care hospital in Mexico City. International Scholarly Research Notices.
7. Canada, Public Health Agency of (19 April 2011). "Pathogen Safety Data Sheets: Infectious Substances – Micrococcus spp.canada.ca. Retrieved 5 January 2023.
8. CLSI. (2020). Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. M100- 30th ed CLSI, Wayne, PA, USA, 2020.
9. Fosse T, Toga B, Peloux Y, Granthil C, Bertrando J, Sethian M. Meningitis due to Micrococcus luteus. Infection. (1985);13(6): 280–281..
10. Freeman J, Falkiner FR, Keane CT.(1989) New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol; 42:872-4.17- Greenhalgh T. How to read a paper: papers.
11. Funke, G., & Funke-Kissling, P. (2005). Performance of the new VITEK 2 GP card for identification of medically relevant gram-positive cocci in a routine clinical laboratory. Journal of clinical microbiology, 43(1): 84-88.
12. Ghaly, M. F.; Shaheen, A. A.; Bouhy, A. M. and Bendary, M. M. (2020). Alternative therapy to manage otitis media caused by multidrugresistant fungi. Archives of microbiology, 202(5), 1231-1240.
13. Greenblatt, C.L.; Baum, J.; Klein, B.Y.; Nachshon, S.; Koltunov, V.; Cano, R.J. (2004). "Micrococcus luteus – Survival in Amber48 (1): 120–127.
14. Hassan A, Usman J, Kaleem F, Omair M, Khalid AIqbal M. (2011),Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis;15:305-11.
15. Holt, J. G., Krieg, N. R., Sneath, P. H., Staley,J. T. and Williams, S. T. (1994). Bergey's
16. Kim, E. J., Kim, Y. C., Ahn, J. Y., Jeong, S. J., Ku, N. S., Choi, J. Y., ... & Song, Y. G. (2019). Risk factors for mortality in patients with Stenotrophomonas maltophilia bacteremia and clinical impact of quinolone–resistant strains. BMC infectious diseases, 19(1), 1-8
17. Light RW.(2007). Para pneumonic Effusions and Empyema In: Light RW. (Editor). Pleural Diseases, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.;179-210.
18. MacFaddin, J. F. (2000). Biochemical tests for identification of medical bacteria, williams and wilkins. Philadelphia, PA, 113.
19. Madigan M; Martinko J, eds. (2005). Brock Biology of Microorganisms (11th ed.). Prentice Hall. ISBN 978-0-13-144329-7.
20. McFarland, J. (1907). The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. Journal of the American Medical Association, 49(14): 1176-1178.

21. Morello, R., Bertin, T.K., Chen, Y., Hicks, J., Tonachini, L., Monticone, M., & Lee, B. (2006). CRTAP is required for prolyl 3-hydroxylation and mutations cause recessive osteogenesis imperfecta. Cell, 127(2): 291-304.
22. Panda PS, Chaudhary U, Dube SK.(2016) Comparison of four different methods for detection of biofilm formation by uropathogens. Indian J Pathol Microbiol;59:177-9.
23. Pękalaa, A., Paździora, E., Antychowiczb, J., Bernadc, A., Głowackad, H., Więceka, B.
24. Pompilio, A., Ciavardelli, D., Crocetta, V., Consalvo, A., Zappacosta, R., Di Ilio, C., & Di Bonaventura, G. (2014). Stenotrophomonas maltophilia virulence and specific variations in trace elements during acute lung infection: implications in cystic fibrosis. PloS one, 9(2), e88769.
25. 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;4 840-849.
26. Saladin, Kenneth S. (2011). Human anatomy (3rd ed.). New York: McGraw-Hill. pp. 643–644
27. Villarino, M.E.; Stevens, L.E. and Schable, B.(1992). Risk factors for epidemic Xanthomonas maltophilia infection/colonization in intensive care unit. Infect Cont HospEpidemiol;13:201–206.
28. Walbaum, 1792). Aquaculture, 486:285-0
29. Young M, Artsatbanov V, Beller HR, Chandra G, Chater KF, Dover LG, Goh EB, Kahan T, Kaprelyants AS, Kyrpides N, Lapidus A, Lowry SR, Lykidis A, Mahillon J, Markowitz V, Mavromatis K, Mukamolova GV, Oren A, Rokem JS, Smith MC, Young DI, Greenblatt CL 192 (3): 841–860. (2010).