Bacterial Profile Associated with Pleural Fluid Disease in Al-Najaf Province/Iraq

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Abstract
Stenotrophomonas maltophilia is a low-virulence opportunistic pathogen that has been identified as an emerging hospital pathogen. Infections caused by *S. maltophilia* 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℃. The results showed that 209 (76.9%) gave bacterial growth while 41 (23.1%) appeared no growth. after identification of bacterial isolate, 20 of isolate were identified *S. maltophilia* recovered from the different infections, from those 12 of isolates were recovered from male specimens, while 8 from female. TCP method were used to determinant the predominant of the level of biofilm among *S. maltophilia* isolates, the results of TCP revealed that all isolate 20 (100%) were biofilm formation.
Keywords: Pleural fluid diseases, MDR, *Stenotrofomonus maltophilia*, Biofilm.

1. 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 *et al.*, 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 *et al.*, 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).

S. maltophilia is non fermentative rod Gram negative bacteria and aerobic, they are motile, non-capsulated and non-sporulation grow well on MacConkey agar non-producing pigmented colonies, *S. maltophilia* is catalase-positive, oxidase-negative (which distinguishes it from most other members of the genus) and has a positive reaction for extracellular DNase, it is an uncommon bacterium and human infection is difficult to treat (Neela *et al.*, 2014). Naturally the bacterium *S. maltophilia* is widely distributed in aqueous environments, soil, plants and it has also been used in biotechnology applications (Norton and Dachsetal *et al.*, 2015). *S. maltophilia* can be morbidity and mortality rates among immune compromised patients with multi antibiotics resistant (Chang *et al.*, 2015). The invading pathogen must be able to produce various virulence factors in order to establish infections and this largely depends on environmental conditions and level of micronutrients within the hospital environment (Sritharan *et al.*, 2006).

The newly isolation and identification of *S. maltophilia* and limitation of virulence factors of it, the study aims to determine the biofilm formation among the clinical isolates from pleural fluid infections.

2. Material and Methods

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 hospitals in Al- Najaf (Al-Sadder Medical City) suffering from pleural fluid during the period from January 2021 to December 2022).

Isolation and Identification of *Stenotrofomonus maltophilia*

S. maltophilia was isolated and identified according to traditional biochemical diagnostics, by using routine methods according to Macfaddin (2000); Collee *et al.*, (1996). A Vitek-2 compact system 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 -2℃. The final identification of the

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bacterial isolates has been carried out using Vitek 2 system (Funke and Funke-Kissling *et al.*, 2005).

Antibiotic Susceptibility Test

This study used 15 types of commonly used antibiotics including penicillin 10µg, Ampicillin10µg, 30µg Ciprofloxacin, Piperacillin 100µg, Azithromycin 15µg, Doxycycline 10µg, Canamicin 10µg, Levofloxacin 5µg, Cefixime 10µg, Imipenem 10µg, Amikacin 10µg, Nitrofurantoin 300µg, Tetracycline 30µg and Rifimpem 5µg. The antibiotic sensitivity report was performed according to the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Morello *et al.*, 2006). Briefly, the investigated isolates were allowed to multiplication overnight at 37°C in BHI broth referred to 0.5 McFarland turbidity standard equal to $1.5X10^8$ CFU/ml (Mcfarland, 1907), 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 37°C 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.*, (2021).

3. Biofilm Formation

1- Tissue Culture Plate Method

TCPM was used as the gold standard test for the 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 hrs. 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 filled with 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 at 37°C for 24 hrs, 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%) was added to the wells and kept for 30 minutes to fix the biofilms that bacteria attached to the wells formed. The fixed biofilms were stained using crystal violet (0.1%). After 30 minutes, the wells were thoroughly washed with deionized water to remove any extra stains. After drying, a micro-ELISA reader (at 570 nm wavelength) was used to measure the stained bacterial biofilm's optical densities (OD). The test was carried out in triplicate and an average of three OD values was taken. Optical density 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 *et al.* 2016, Bakir *et al.*, 2016).

2- Congo Red Agar Method

Congo red agar is a specially prepared medium composed of brain heart infusion (BHI) broth (37 g/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 121°C for 15 minutes. Finally, it was added to the autoclaved BHI agar with sucrose at 55°C. Prepared CRA plates were inoculated with the isolated pathogens and aerobically incubated at 37°C for 24 hrs. The appearance of black dry crystalline colonies on the CRA plates indicated biofilm production while the colonies of biofilm nonproducer remained pink or red colored (Ruchi *et al.*, 2015; Hassan *et al.*, 2011).

4. 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; *S. maltophilia* isolates were isolated and identified using the VITEK-2 compact system.

The identification of *S. maltophilia* was first made by the bacteriological methods including colonial morphology (Figure 1), and biochemical tests. Approximately, 25 of bacterial isolate were oxidase negative, catalase positive, motile and produce alkaline/alkaline on TSI, as well as non-fermenter lactose were suspected as *S. maltophilia*. Identification of all 25 suspected isolates confirmed by Vitek 2 automated system where it appeared 20 of isolates identified as *S. maltophilia.*

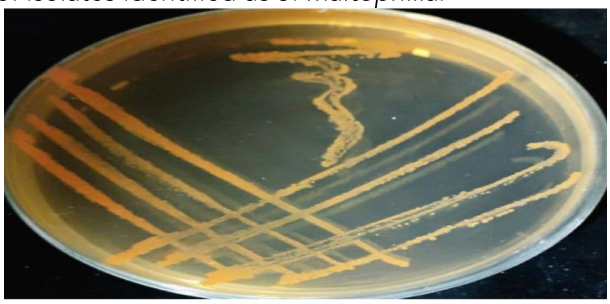

Figure (1): Colony of Stenotrophomonas maltophilia on MacConkey agar.

This result agree with Denton and Kerr *et al.*, (1998), for diagnosis of *S. maltophilia*, it is motile due to polar flagella, and grow well on Alkaline MacConkey agar with lactose non-fermenter. *S. maltophilia* is catalase-positive, oxidase-negative (which distinguishes it from most other members of the genus) in addition to the TSI tests, they were Alkaline/Alkaline.

The Antibiotic Susceptibility Test

The susceptibly of *S. maltophilia* 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 appear in 75% of isolates. The resistance bacteria isolates to the third generation of Cefotaxime 100% represented by Cefepime was recorded in 75% of isolates, while the resistance to fourth generation Doxycycline were appear in 100% of isolates, the susceptibility results for the tetracycline antibiotics was appear 95%, 75% of the evaluated isolates exhibited resistance to SXT, 15% of the isolates showed resistance to Ciprofloxacin. The resistance of bacterial isolates to Levofloxacin was found in 15% while 75% of isolates were resistant to norfloxacin, Penicillin resistant was recorded in 100% of bacterial isolates, While resistance to Imipenem represented in 90% and resistance to Tobramycin antibiotics was appear in 70%. While resistance to Rifampin antibiotics was appear 90%, resistance to Canamycin was appear 70% (Table 1).

Table (1): The Antibiotic Susceptibility Rates Among *S. maltophilia*

Resistant(%)	Sensitive	Intermediate	Resistant	Antibiotic
75%	3	2	15	Amoxicilin
100%	0	0	20	Cefotaxime
75%	4	2	15	Cefepime
100%	0	0	20	Doxycycline
75%	4	2	15	SXT
15%	16	1	3	Ciprofloxacin
55%	6	3	11	Levofloxacin
75%	4	1	15	Norfloxacin
70%	4	2	14	Canamycin
100%	0	0	20	Penicillin
95%	1	0	19	Tetracycline
90%	1	1	18	Imipenem
70%	2	4	14	Tobramycin
25%	1	2	17	Amakicin
90%	2	0	18	Rifampin

R=Resistant , I=Intermediate , S=Sensitive

In previous studies, *S. maltophilia* exhibits resistance to a broad array of antibiotics, including β-lactam antibiotics, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, and polymyxins, the decrease membrane permeability and altered penicillin binding protein (PBP) that contributes to resistance to β-lactams which including cefepime, ticarcillin-clavulanate, ceftazidime, and piperacillin-tazobactam, and the presence of chromosomally encoded multidrug resistance efflux pumps, β-lactamases, and antibiotic-modifying enzymes all contribute to the intrinsic antibiotic resistance of *S. maltophilia* (Brooke *et al.*, 2012). The drug resistance mechanisms are acquired by the horizontal transfer of antibiotic resistance through plasmids, transposons, integrons, integron-like elements and insertion element common region (ISCR) elements (Sanchez *et al.*, 2009).

In previous studies in agreement with the current study, the first-line treatment was trimethoprim-sulfamethoxazole, which has been the recommended empiric single agent against *S. maltophilia* infections for many years, it has shown activity against more than 90% of the tested isolates in most studies, though as mentioned above (Church *et al.*, 2013). Due to the existence of a recent study that does not agree with the current study, due to the presence of high resistance trimethoprim/sulfamethoxazole in a combination of environmental and clinical isolates from Mexico (Elufisan *et al.*, 2020). *S. maltophilia* is an opportunistic pathogen of increasing importance. 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). Zhanel *et al.*, (2019) reported that chloramphenicol that has shown promising data against carbapenem-resistant Gram-negative bacteria, including *S. maltophilia*, whereas the current study agree with this reported study, resistance to chloramphenicol represented in (35.7%) of bacterial isolates.

Biofilm formation

This study showed the ability of most bacterial isolation on biofilm formation by Congo red Agar method (figure 2).

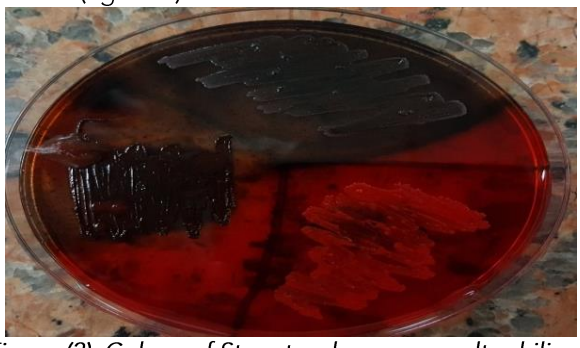

Figure (2): Colony of Stenotrophomonas maltophilia on Congo Red Agar A:black colonies of biofilm producer, B: red colonies of non-biofilm producer.

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 *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 most bacterial isolation on biofilm formation by Tissue culture plate method (table 2).

Table (2): The ability of *Stenotrophomonas maltophilia* on biofilm formation by Congo red Agar method and tissue culture plate method

Biofilm formation	CRA method	TCP method
Non	1 (1)	0%(0)
Moderate	17% (4)	6% (6)
High	66% (15)	14% (14)

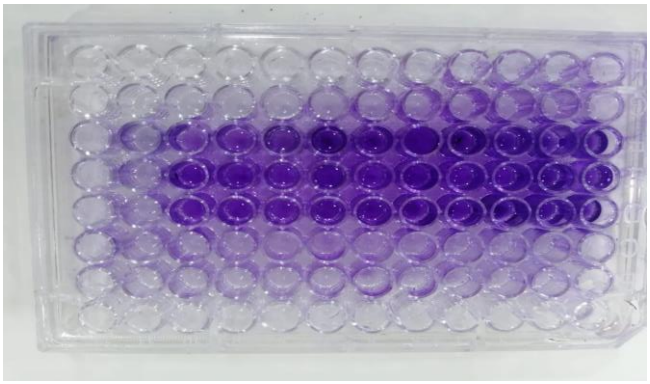

Figure (3): Phenotypic Detection of Biofilm Formation of Stenotrophomonas maltophilia by TCP method.

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).

Relation shape between biofilm forming and antimicrobial resistance

Figure (4) revealed a positive significant high correlation (0.874**) in *S. maltophilia* between the number of antibiotic resistance and the mean of biofilm formation. In this study, the overall biofilm-forming bacterial isolates had higher antimicrobial resistance than that non-biofilm formers. Similar studies carried out in different locations showed that biofilm formers have higher resistance features compared with those of non-biofilm formers (Rabina *et al.*, 2019). Moreover, multidrug resistance was observed among biofilm-forming bacterial isolates than their counterparts to amoxicillin and cephalexin. This higher antimicrobial resistance among biofilm former may emerge from increased properties of the efflux mechanism. In addition, it may be also associated with higher plasmid transfer, modified target genes, and metabolic pathways that allow for resistance to antimicrobials (Netsanet *et al.*, 2019).

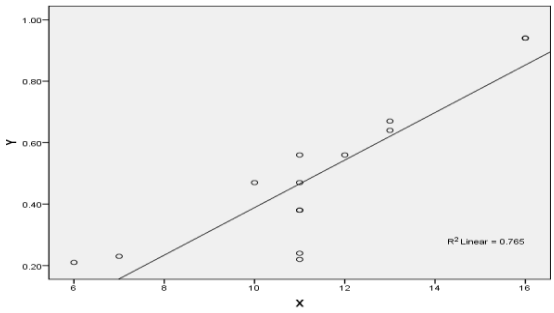

Figure (4): Correlation between number of antibiotic resistance and the mean of biofilm formation in Stenotrophomonas maltophilia isolates (correlation is significant at the 0.01 level). X-Antibiotic resistance number, Y-Mean of biofilm

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.

5. Conclusions

The outcomes of this study revealed a great spread of *S. maltophilia* isolates in Najaf hospitals that produce virulence factor and are resistant to many antibiotics.

References

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.

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.

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.

Bakir SH, Ali FA. Comparison of Different Methods for Detection of Biofilm Production in Multi-Drug Resistance Bacteria Causing Pharyngotonsillitis IJRPB 2016;3:13-22.

Brooke, J. S. (2012). *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. Clinical microbiology reviews, 25(1), 2-41.

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.

Chang CC, Chen TP, Yeh CH.(2015). A simple weighted scoring system to guide surgical decision-making in patients with parapneumonic pleural effusion. J Thorac Dis;8:3168-74.

Chang, Y. T.; Lin, C. Y.; Chen, Y. H. and Hsueh, P. R. (2015). Update on infections caused by *Stenotrophomonas maltophilia* with particular attention to resistance mechanisms and therapeutic options. Frontiers in microbiology, 6, 893.

Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol, 996:1006-22.

Church, D.; Lloyd, T.; Peirano, G.and Pitout, J.(2013). Antimicrobial susceptibility and combination testing of invasive *Stenotrophomonas maltophilia* isolates. Scand J Infect Dis. Apr;45(4):265-70.

CLSI. (2021). Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. M100- 31th ed CLSI, Wayne, PA, USA, 2021.

Elufisan ,T.O.; Luna, I.C.; Oyedara, O.O.; Varela, A.S. ;García, V.B.; Oluyide, B.O.; Treviño ,S.F. ; López, M.A. and Guo, X.(2020). Antimicrobial susceptibility pattern of *Stenotrophomonas* species isolated from Mexico. Afr Health Sci. Mar;20(1):168-181.

Freeman J, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol 1989; 42:872-4.

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.

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.

Hassan A, Usman J, Kaleem F, Omair M, Khalid Alqbal M. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis 2011;15:305-11.

Light RW. Para pneumonic Effusions and Empyema In: Light RW. Pleural Diseases, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA. 2007;179-210.

MacFaddin, J. F. (2000). Biochemical tests for identification of medical bacteria, williams and wilkins. Philadelphia, PA, 113.

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.

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.

Neela, V. K. (2014). "Could clinical *Stenotrophomonas maltophilia* be a potential pathogen in clinical setting?" in 3rd International Conference on Clinical Microbiology and Microbial Genomics (Valencia), 53.

Netsanet A, Tesfaye K, and Lule T.(2019). Magnitude of Biofilm Formation and Antimicrobial Resistance Pattern of Bacteria Isolated from Urinary Catheterized Inpatients of Jimma University Medical Center, Southwest Ethiopia. International Journal of Microbiology.

Norton, J. and Dachs, R. (2015). "*Stenotrophomonas maltophilia*: culprit or colonizer?" in Southern Medical Association (Destin, FL), 30.

Panda PS, Chaudhary U, Dube SK. Comparison of four different methods for detection of biofilm formation by uropathogens. Indian J Pathol Microbiol 2016;59:177-9.

Rabina D., Ratna B. and Lok B. S.(2019). Study of biofilm formation and antibiotic resistance pattern of gram-negative Bacilli among the clinical isolates at BPKIHS, Dharan. BMC Research Notes. 12(38).

Ruchi T, Sujata B, Anuradha D. 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 2015;4: 840-849.

Sánchez, M. B. (2015). Antibiotic resistance in the opportunistic pathogen *Stenotrophomonas maltophilia*. Frontiers in microbiology, 6, 658.

Senol, E. (2004). *Stenotrophomonas maltophilia*: the significance and role as a nosocomial pathogen. J. Hosp. Infect. 57, 1–7.

Sritharan, M. (2006). Iron and bacterial virulence. Indian J. Med. Microbiol. 24, 163–164.

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.

Zhanel, G.G.; Golden, A.R.; Zelenitsky, S. Wiebe, K.; Lawrence, C.K.; Adam, H.J. and arlowsky, J.A.(2019). Cefiderocol: A Siderophore.