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Biofilm Formation of Stenotrophomonas Maltophilia and the Effect of Antibiotics on Biofilm Formation in vitro

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Abstract: ***Nosocomial infections, which are also commonly referred to as healthcare-related diseases, are a considerable burden on hospital and patient administration around the world. Regular hospital admissions of 10% of the population result in millions of deaths that could have been readily prevented. (CDC, 2020). About 1- 3% of deaths worldwide are caused by nosocomial illnesses (CDC, 2020). The most highly developed nations, such as the USA, have recorded about 2 million nosocomial infection cases, of which 70% were thought to be caused by the medical equipment used during the course of the treatment. The term "biofilm" refers to microbial communities that adhere to surfaces. The aggregation of microorganisms on a surface and their ability to create antibiotic resistance are the principal problems of concern. In this research, biofilm was discovered using the microtitre plate method using 15 isolates of S. maltophilia from different clinical samples. The antimicrobial agents tested against biofilm formation by S.maltophilia included Levofloxacin and Ticarcillin clavulanic acid. MIC is detected by microbroth dilution method. Each drug was tested at one-half, one-fourth, and one-eighth the MIC to study its effect on S. maltophilia biofilm formation. All isolate were found to be biofilm formers by microtitre plate assay. Sub inhibitory concentration of Levofloxacin and Ticarcillin clavulanic acid reduces biofilm production of S. maltophilia***

Keywords: Biofilm formation, Nosocomial infection, *Stenotrophomonas maltophilia*, Minimum inhibitory concentration.

1. Introduction

Stenotrophomonas (Xanthomonas) maltophilia is a gram negative bacillus that is an opportunistic pathogen particularly among hospitalized patients. *S.maltophilia* infections have been associated with high morbidity and mortality in severely immunocompromised and debiliated individuals such as those with malignancies, and implantation of foreign devices (catheters, respiratory therapy equipment etc.) (1-4).

S.maltophilia is a ubiquitous, aerobic, non-fermentative, gram negative bacillus that is closely related to the *Pseudomonas* species.(5) The name signifies “a unit feeding on few substrates,” based on the Greek roots stenos (narrow), trophos (one who feeds), and monas (a unit). Maltophilia means “affinity for malt” based on the Greek roots maltum (malt) and philia (affinity).

S.maltophilia was first isolated in 1943 and, at the time was named *Bacterium bookeri.* It was later classified with *Xanthomonas* and then finally *Stenotrophomonas* in 1993.(4,6,7) *S.maltophilia* is the only species of *Stenotrophomonas* known to infect humans, where closest genetic relatives are plant pathogens.(7,8) It is frequently isolated from soil, water, animals, plant matter, and hospital equipment. *S.maltophilia* has inherent ability to adhere to foreign material and form biofilm, rendering protection from host defenses as well as antimicrobial agents. Factors contributing to this behavior include its positively charged surface and fimbrial adhesions. (7)

S.maltophilia is an obligate aerobe that grows well on commonly used laboratory media, including blood and Mac Conkey agars. Culture from normally sterile body sites is straightforward, and bacteremia and septicemia can be detected using standard blood-culture techniques (9). Selective media can improve culture sensitivity for specimens from non-sterile body sites, such as respiratory secretion from patients with cystic fibrosis (CF) (10). Differentiation of infection from colonization based on clinical criteria may be problematic. It is lactose non fermenting, oxidase negative and catalase positive and can be reliably identified in the laboratory using standard biochemical test. In addition, it is accurately identified by commercially available identification systems such as Vitek compact 2 (11).

S.maltophilia is ubiquitous and can be recovered from almost any clinical site. The most common site for recovery is the respiratory tract, although in most patients these isolate do not appear to be clinically significant. While previous studies of biofilm development and species interaction have focused largely on *Staphylococcus epidermidis, Staphylococcus aureus* and *Pseudomonas aeruginosa* , little is known about *S.maltophilia* (12) .

S. maltophilia is not highly virulent, nevertheless several factors may promote its ability to colonize the respiratory tract and plastic surfaces, such as catheters and endotracheal

tubes. These include a positively charged surface as well as flagella and fimbrial adhesion, the latter have been associated with biofilm formation (14).The outer membrane lipopolysaccharide (LPS) plays a role in colonization and resistance to complement-mediated cell killing, and its lipid A moiety can stimulate peripheral blood monocytes and alveolar macrophages to produce TNFα, which plays a role in the pathogenesis of airway inflammation. *S.maltophilia* produces a number of extracellular enzymes, whose contribution to virulence is currently uncertain (15).

Most human isolates represent colonization rather than infection, it is however an opportunistic pathogen in highly debilitated patients. (13). The major clinical syndromes are pneumonia and bacteremia. In bacteremia the portal of entry is typically a vascular catheter or is unknown. Other reported clinical syndromes include urinary tract infection, soft tissue infection, ocular infection, endocarditis, and meningitis (16).

A bacterial biofilm refers to a group of bacterial cells that adhere to one another on a surface. These adherent cells are often embedded within a self-produced matrix of an extracellular polymeric substance. Many infectious bacteria, such as *S. maltophilia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, are capable of forming biofilms. Biofilms exhibit greater resistance to antimicrobial drugs than non-biofilm forming bacteria, and are therefore more difficult to treat clinically(17,18) Antibiotics with invitro activity against *S.maltophilia* include trimethoprim-sulfamethoxazole (SXT), fluroquinolones (FQs), tetracyclines, ticarcillin-clavulanic acid, and ceftazidime. Trimethoprim- sulfamethoxazole continues to be primary the choice for the treatment of *S.matophilia*, but fluoroquinolones are an attractive option due to in vitro activity.

In vitro susceptibility testing of *S. maltophilia* poses numerous technical problems, however, the Clinical Laboratory Standards Institute (CLSI) and the British Society for Antimicrobial Chemotherapy (BSAC) have each published standard methods for the susceptibility testing of *S. maltophilia* .(4) The methods cover a limited range of antibiotics and are not interchangeable as they vary in numerous details. *In-vitro* synergy testing, sometimes used for particularly problematic cases, has indicated synergy between certain antibiotic combinations when tested against *S. maltophilia*. The different kinds of synergy tests may either give the same or differing results when testing the same strain-antibiotic combinations and this should be taken into consideration when interpreting the results. (19)

The majority of clinical isolates of *S.maltophilia* infections are resistant to multiple agents used to treat gram-negative infections. Resistance to beta-lactam antibiotics is mediated by two unique, inducible beta-lactamases, a zinc-containing penicillinase and a cephalosporinase. Some isolates appear to produce additional beta-lactamases as well. A TEM-2 beta lactamase has been identified within a transposon in the genome of a clinical isolate of *S. maltophilia* .(5) As a consequence, many strains are resistant to extended spectrum penicillins and third generation cephalosporins, early all strains are resistant to imipenem and meropenem. The majority of strains are susceptible to the combination of ticarcillin and the beta-lactamase inhibitor clavulanic acid *in vitro*; however, the degree of growth inhibition is dependent on testing conditions. (22, 23) In a murine model of *S. maltophilia* pneumonia, the efficacy of ticarcillin/clavulanate acid was similar to that of trimethoprim-sulfamethoxazole (24). Piperacillin/tazobactam is less active in vitro; the majority of strains are resistant. Ampicillin/ sulbactam is generally inactive. Most strains are resistant to aztreonam (7).

The use of two or three antimicrobials to treat *S. maltophilia* infection has become established practice, although there are no clinical trials to support this approach. This strategy is, however, supported by *in vitro* synergy testing . Given the lack of clinical trials, caution must be exercised when attempting to extrapolate *in vitro* synergy testing into clinical practice. Synergistic bacterial killing occurs *in vitro* with ticarcillin/clavulanate plus trimethoprim/ sulfamethoxazole whether or not the isolates are susceptible to either of the two combination agents (25). *In vitro* synergy has also been reported for trimethoprim/sulfamethoxazole combined with ceftazidime, ciprofloxacin, gentamicin, and tobramycin when the strain tested was susceptible to trimethoprim/sulfamethoxazole and susceptible or intermediately susceptible to the second agent. Synergy may also occur with either ticarcillin/clavulanate or ceftazidime plus ciprofloxacin for strains with a ciprofloxacin MIC < 32 ug/ml (26). Although most strains are resistant to aztreonam, the combination of aztreonam and clavulanate in a fixed 2:1 ratio shows synergistic activity (27). The addition of aztreonam to ticarcillin/clavulanate reportedly increases the activity of the latter combination up to 128-fold, with synergy demonstrated by time-kill curves for the majority of isolates tested .(28) These reports of synergy are based on a limited number of strains.

2. Materials and method

2.1 Selection of isolates

The study was done at tertiary care centre using 15 consecutive isolates of *Stenotrophomonas maltophilia* isolated from various samples such as urine, blood, sputum, pus, ear swabs, BAL, sterile body fluids etc. The all clinical isolates should be gram negative bacilli occurred singly or in pairs, catalase positive, oxidase negative. Other biochemical reactions are in the given table. All isolates were identified with Vitek compact 2 .Only one isolate per patient was collected. (1)

Table 1: Biochemical Reactions of *Stenotrophomonas maltophilia*

Biochemical Reaction	Interpretation
Indole test	Negative
Methyl red test	Negative
Voges Proskauer test	Negative
Citrate utilization test	Variable
Nitrate reduction test	Variable
Mannitol motility	Not fermented , motile
OF Glucose test	Oxidative
Lysine decarboxylase	Positive

Ornithine decarboxylase	Negative
DNAse test	Positive
Esculin hydrolysis	Positive
Gelatine hydrolysis	Positive

VITEK 2 Identification card, ID GN intended for use with Vitek 2 Compact systems for the automated identification of most significant Gram positive organisms and fermenting and non-fermenting Gram negative bacilli.

2.2 Biofilm detection methods

Microtitre plate method

Interpretation

Table 2: Interpretation

Average Optical density	Biofilm formation
≤ OD of cut off value (ODc)	Non
≤ ODc - ≤ 2 X ODc	Weak
≤ 2X ODc - ≤ 4X ODc	Moderate
≥ 4X ODc	Strong

Optical density (OD) of cut off value = Average OD of Negative control + 3X standard deviation of negative control

2.3 Antimicrobial agents and determination of MIC.

The antimicrobial agents tested against biofilm formation by *S.maltophilia* included Levofloxacin and Ticarcillin clavulanic acid. MIC,is detected by microbroth dilution method.

2.4 Effect of MICs and sub-MICs of antimicrobial agents on *S. maltophilia* biofilm formation

Each drug was tested at one-half, one-fourth, and one-eighth the MIC to study its effect on *S. maltophilia* biofilm formation. Various concentrations of antimicrobial agents (Levofloxacin and Ticarcillin-clavulanic acid) prepared in 100 µl of TSB were added to microtiter wells containing 200 µl of the inoculum standardized as described in biofilm formation assay. After 24 hr of incubation, quantitation of biofilms was performed as described for the biofilm formation assay. Drug-free medium was used in control wells.

2.5 Effect of combined MICs and sub-MIC of antimicrobial agents on S.maltophilia biofilm formation.

MICs and sub MICs (½,¼, and ⅛) concentration of both the drugs were prepared and 100 µl of each of these was added to the microtitre plate containing 100 µl of the standardized inoculum. After 24 h of incubation, quantitation of biofilms was performed as described for the biofilm formation assay.

3. Result

3.1 Biofilm formation of *Stenotrophomonas maltophilia*

The results of the biofilm production assay were determined by the spectrophotometric method. All isolate were found to be biofilm formers by microtitre plate assay. Out of two staining methods; 0.1% crystal violet had detected 14 (93.3%) moderate and 1 (6.6%) strong biofilm producers while 0.1% safranine had detected 11 (73.3%) moderate and 4 (26.6%) strong biofilm producers as seen in Table 3 and Figure 1.

Table 3: Comparison of Biofilm detection by two Staining methods

0.1% Crystal violet		0.1% Safranine	
Moderate	Strong	Moderate	Strong
14 (93.3%)	1 (6.6%)	11 (73.3%)	4 (26.6%)

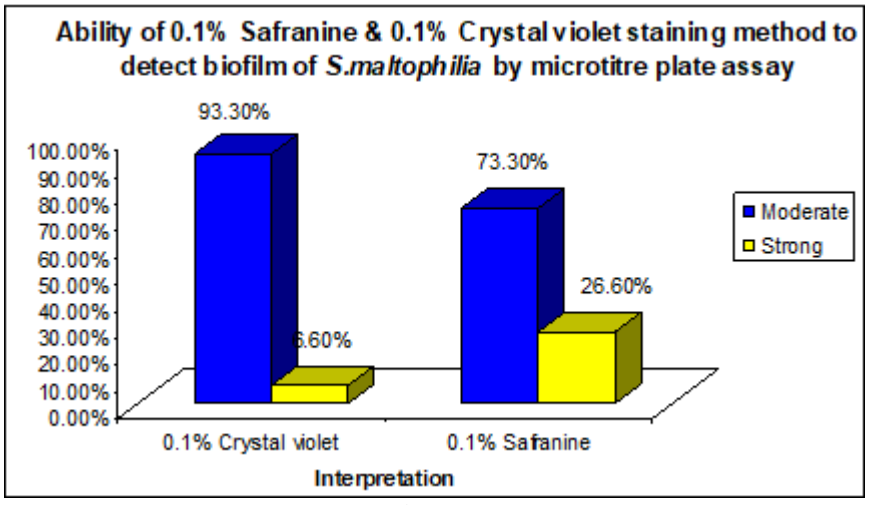

Figure 1

3.2 Detection of minimum inhibitory concentration of Levofloxacin and Ticarcillin- Clavulanic acid against clinical isolate of *S.maltophilia* determined by micro broth dilution method

For planktonic susceptibility studies, MIC of Levofloxacin and Ticarcillin clavulanic acid were determined by broth micro dilution method. Out of 15 isolate 60% were sensitive, 13.3% were resistant, 26.6% were intermediate to Levofloxacin and 66.6% intermediate, 33.3% were resistant to Ticarcillin Clavulanic acid.

3.3 Effect of MIC and sub MICs of Levofloxacin against biofilm formation of *S.maltophilia* by microtitre plate assay

In the presence of ½ MIC of Levofloxacin 46.6% are reduce biofilm formation 53.3% shows no reduction in the biofilm formation. 46.6% are non biofilm producers, 33.3% reduce biofilm formation and 13.3% does not affect the biofilm formation in the presence of ¼ MIC of Levofloxacin. 20% are non biofilm producers and 73% are reduced biofilm formation in the presence of ⅛ MIC of Levofloxacin. No reduction of biofilm was observed in the presence of MIC of Levofloxacin.

3.4. Effect of MIC and sub MICs of Ticarcillin Clavulanic acid against biofilm formation of *S.maltophilia* by microtitre plate assay

The 15 study strains were tested by microtitre plate method for detecting biofilm formation in the presence of Ticarcillin Clavulanic acid . The sub MIC of one fourth of Ticarcillin Clavulanic acid shows 87% completely eradicates biofilm formation and 13% reduce biofilm formation. The presence of one eighth MIC of Ticarcillin clavulanic acid results 100% reduction of biofilm formation. 80% and 73.3% reduction were observed in the presence of one half and MIC of Ticarcillin Clavulanic acid against biofilm formation.

3.5. Effect of combinations of MICs and sub MICs of Levofloxacin and Ticarcillin Clavulanic acid, against biofilm formation of *S.maltophilia* by microtitre plate assay.

To further examine the effect of combination of antibiotics on biofilm formation by *S. maltophilia*, a combination of Levofloxacin and Ticarcillin Clavulanic acid was tested on the 15 clinical isolates of *S.maltophilia* . Combination of one fourth MIC of both Levofloxacin and Ticarcillin Clavulanic acid (100%) was the most active against biofilm formation. Combination of MIC values and one half of the MIC of both antimicrobial agents reduced biofilm formation. The best inhibitory effect was observed when the combination was of one eighth of MIC, compared to other combination of both antimicrobial agents.

4. Conclusion

1) Respiratory tract is the most common site for isolation of *S.maltophilia*
2) *S.maltophilia* is an important causative agent of nosocomial infection
3) *S.maltophilia* causes mono microbial and poly microbial infection
4) Biofilm production was seen in all isolates studied
5) Sub inhibitory concentration of Levofloxacin and Ticarcillin clavulanic acid reduces biofilm production of *S.maltophilia*
6) The concentration of sub-MIC (½ and ¼) of both drugs greatly reduces the biofilm formation of *S.maltophilia*

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