

RPFF, SPGM AND RMLA GENES AND ITS BIOFILM FORMING ABILITY IN *STENOTROPHOMONAS MALTOPHILIA*

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ABSTRACT:

Objectives: Purpose of this study to evaluate antimicrobial profile, association between biofilm formation & biofilm encoding genes among *Stenotrophomonas maltophilia* isolates from various clinical specimens.

Methods: A total of 73 clinical isolates of *Stenotrophomonas maltophilia* were isolated from non-fermentative Gram-negative bacilli from various clinical samples & were identified by VITEK-2 Compact System, Antimicrobial susceptibility testing was done by VITEK-2 compact system & by Kirby Bauer disc diffusion method/ E-test as per CLSI guidelines. Detection of biofilm formation was done by Modified Hucker crystal violet staining method and biofilm encoding genes were detected by PCR.

Result: Antimicrobial susceptibility testing demonstrated that minocycline exhibited the highest activity (90.4% susceptibility), followed by levofloxacin (76.7%) & ticarcillin-clavulanic acid (75.3%). However, high resistance rates were observed against imipenem & meropenem (100% each), chloramphenicol (80.8%), ceftazidime (72.6%), tetracycline (71.2%), and ceftazidime-avibactam (63.1%). Biofilm formation was detected in all isolates (100%), indicating that biofilm production is a common virulence trait among clinical *S. maltophilia* strains. Among the isolates, 35.6% were weak biofilm producers, 47.9% were moderate producers, and 16.4% were strong biofilm producers. Among the 73 *S. maltophilia* isolates, the *rpjF* gene was detected in 48 (65.7%) isolates, *spgM* in 44 (60.3%) isolates, and *rmlA* in 45 (61.6%) isolates.

Conclusion: Antimicrobial resistance and biofilm formation may work together to enhance the persistence and treatment challenges of *S. maltophilia*. Early identification, appropriate antimicrobial susceptibility testing, and better understanding of biofilm-associated mechanisms are important for effective management of infections caused by this emerging pathogen.

INTRODUCTION:

Antimicrobial resistance has become one of the major challenges facing healthcare systems worldwide. The increasing resistance of bacteria to commonly used antibiotics has made the treatment of many infections increasingly difficult. At the same time, only a limited number of new antimicrobial agents are currently being developed, and there are few effective treatment options available for infections caused by multidrug-resistant (MDR) Gram-negative bacteria.[1]

Stenotrophomonas maltophilia is an emerging nosocomial opportunistic pathogen that has received increasing attention in recent years because of its ability to cause difficult-to-treat infections. It is reported particularly among hospitalized and immunocompromised patients, including those with malignancies and cystic fibrosis. The organism is also frequently encountered in clinical laboratories as a non-fermenting Gram-negative bacillus, along with other important pathogens such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. [1–3]

One of the important factors contributing to the persistence and pathogenicity of *S. maltophilia* is its ability to form biofilms. Biofilm formation allows the organism to adhere to both biological surfaces and indwelling medical devices, including urinary catheters and respiratory equipment. Once established, biofilms provide bacteria with increased protection from host immune responses and antimicrobial agents, thereby promoting persistence and chronic or device-associated infections. Biofilm formation is therefore considered an important virulence factor in *S. maltophilia* infections. However, the ability to form biofilms can vary considerably among clinical isolates.[3,4]

Although biofilm formation is well recognized as an important feature of *S. maltophilia*, the molecular mechanisms regulating this process are not yet completely understood. Recent studies have identified several genes that may contribute to biofilm development and regulation, including *rpjF*, *spgM*, and *rmlA*. These genes are involved in different cellular pathways that influence biofilm formation and bacterial persistence.

Considering the increasing clinical importance of *S. maltophilia*, its antimicrobial resistance, and the potential role of biofilm-associated genes in persistence, further investigation is needed. Therefore, the present study aims to evaluate the antimicrobial susceptibility profile of *S. maltophilia* isolated from various clinical specimens and to investigate the association between biofilm formation and the presence of selected biofilm-associated genes, namely *rpjF*, *spgM*, and *rmlA*.

METHODOLOGY

Study Design and Setting

A prospective, cross-sectional study was conducted at MGM Medical College and Hospital, Chhatrapati Sambhajnagar, over a Three-year period from January 2023 to December 2025.

Biosafety Measures

As *Stenotrophomonas maltophilia* is a multidrug-resistant Gram-negative bacillus, appropriate biosafety precautions were followed throughout the study. All procedures involving the organism were carried out in a Class II biosafety cabinet. Appropriate personal protective equipment (PPE) was used during specimen processing, and standard hand-hygiene practices were strictly followed.

Isolation and Identification of *S. maltophilia*

Non-fermenting Gram-negative bacilli isolated from various clinical specimens, including pus, tracheal aspirates, blood, urine, and other relevant samples, were further processed for identification. *Stenotrophomonas maltophilia* isolates were identified using the VITEK-2 Compact System with the GN ID-341 card. Confirmed isolates were included for further investigation.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility profile of the identified *S. maltophilia* isolates was determined using both automated and conventional methods.

Minimum inhibitory concentration (MIC) results generated by the VITEK-2 Compact System using the NLF AST-406 card were recorded for levofloxacin, minocycline, and co-trimoxazole.

For imipenem (10 µg), meropenem (10 µg), tetracycline (30 µg), chloramphenicol (20 µg), ceftazidime (30 µg), ceftazidime-avibactam, and ticarcillin-clavulanate, antimicrobial susceptibility testing was performed using the Kirby–Bauer disc diffusion method/ E-test. The results were interpreted according to the applicable CLSI guidelines.

Detection of Biofilm Formation

The ability of *S. maltophilia* isolates to form biofilms was assessed using the modified Hucker crystal violet staining method in a 96-well microtiter plate.

An overnight culture of each *S. maltophilia* isolate was prepared and adjusted to the turbidity of a 1.0 McFarland standard. The bacterial suspension was then diluted 1:100 in tryptic soy broth (TSB) supplemented with 1% glucose. Approximately 200 µL of the diluted suspension was transferred into sterile, flat-bottomed 96-well polystyrene microtiter plates.

The plates were incubated at 37°C for 24 hours to allow biofilm formation. Following incubation, the wells were gently washed three times with sterile phosphate-buffered saline (PBS; pH 7.3) to remove non-adherent cells. The adherent biofilms were then fixed at 60°C for 1 hour.

The attached biofilm was stained with 200 µL of modified Hucker crystal violet for 5 minutes at room temperature. The wells were subsequently rinsed with water and allowed to dry. The stained biofilm was then de-stained using 200 µL of 33% glacial acetic acid for 15 minutes.

The optical density (OD) of each well was measured at 492 nm using an ELISA reader. Based on the OD values, the isolates were classified according to their biofilm-forming ability.[3]

Classification of Biofilm Formation

The cut-off optical density (OD_c) was used to categorize the isolates into four groups:

Non-biofilm producer: OD ≤ OD_c (OD ≤ 0.41)

Weak biofilm producer: OD_c < OD ≤ 2 × OD_c (0.41 < OD ≤ 0.82)

Moderate biofilm producer: 2 × OD_c < OD ≤ 4 × OD_c (0.82 < OD ≤ 1.64)

Strong biofilm producer: OD > 4 × OD_c (OD > 1.64)

Detection of Biofilm-Associated Genes

The presence of selected biofilm-associated genes, namely *rpfF*, *spgM*, and *rmlA*, was investigated using the polymerase chain reaction (PCR) technique with specific oligonucleotide primers. The 16S rRNA gene was used as the reference/target for bacterial identification and PCR confirmation.[2]

DNA Extraction

For DNA preparation, the *S. maltophilia* isolates were cultured on Columbia agar supplemented with 5% sheep blood and incubated at 37°C for 24 hours. DNA was then extracted from the resulting bacterial colonies using the High Pure PCR Template Preparation Kit, following the manufacturer's protocol. The concentration and purity of the extracted DNA were assessed using a Nanodrop spectrophotometer.

PCR Amplification

PCR amplification was carried out using an QuantStudio™ 5 Real-Time PCR System in a total reaction volume of 25 µL. Each reaction mixture contained 1 µL of DNA template (approximately 20 ng), 1× PCR buffer, 12.5 µL of 2× Master Mix, 3 mmol/L MgCl₂, 0.4 mmol/L dNTPs, 9.5 µL sterile distilled water, 1 µL of each primer at a concentration of 10 pmol, and 0.08 IU of *Taq* DNA polymerase.

PCR Cycling Conditions

The PCR amplification was performed under the following conditions:

- Initial denaturation: 94°C for 5 minutes
- Denaturation: 94°C for 1 minute
- Annealing: 52–60°C for 60 seconds

- Extension: 72°C for 1 minute
- Final extension: 72°C for 10 minutes

The amplified PCR products were subsequently analyzed to determine the presence or absence of the selected biofilm-associated genes (*rpfF*, *spgM*, and *rmlA*) in the *S. maltophilia* isolates.

RESULTS:

Table-1: Antimicrobial Susceptibility Pattern of *Stenotrophomonas maltophilia* (n=73)

ANTIBIOTICS	Susceptible	Resistant	Intermediate
Minocycline	66 (90.4%)	5 (6.8%)	2 (2.7%)
Levofloxacin	56 (76.7%)	13 (17.8%)	4 (5.5%)
Ticarcillin-clavulanate	55 (75.3%)	18 (24.7%)	00
Co-Trimoxazole	40 (54.8%)	33 (45.2%)	00
Ceftazidime-avibactam	27 (36.9%)	46 (63.1%)	00
Tetracycline	20 (27.4%)	52 (71.2%)	01 (1.4%)
Ceftazidime	20 (27.4%)	53 (72.6%)	00
Chloramphenicol	14 (19.2%)	59 (80.8%)	00
Meropenem	00	73 (100%)	00
Imipenem	00	73 (100%)	00

Table-1 shows the antimicrobial susceptibility pattern of 73 *Stenotrophomonas maltophilia* isolates tested against different antibiotics. Minocycline showed the highest susceptibility, with 66 (90.4%) isolates susceptible, while 5 (6.8%) were resistant and 2 (2.7%) showed intermediate susceptibility.

Levofloxacin showed susceptibility in 56 (76.7%) isolates, with 13 (17.8%) resistant and 4 (5.5%) showing intermediate susceptibility. Similarly, ticarcillin-clavulanate showed susceptibility in 55 (75.3%) isolates, while 18 (24.7%) were resistant and none showed intermediate susceptibility.

For co-trimoxazole, 40 (54.8%) isolates were susceptible and 33 (45.2%) were resistant. Ceftazidime-avibactam showed susceptibility in 27 (36.9%) isolates, whereas 46 (63.1%) were resistant.

Lower susceptibility was observed with tetracycline, where 20 (27.4%) isolates were susceptible, 52 (71.2%) were resistant, and 1 (1.4%) showed intermediate susceptibility. Ceftazidime also showed susceptibility in only 20 (27.4%) isolates, while 53 (72.6%) were resistant. Chloramphenicol showed the lowest susceptibility among the tested antibiotics apart from the carbapenems, with 14 (19.2%) isolates susceptible and 59 (80.8%) resistant.

Notably, all 73 (100%) isolates were resistant to meropenem and imipenem, with no susceptible or intermediate isolates observed for either antibiotic.

Table-2: Distribution of isolates according to Biofilm Phenotype

Biofilm Phenotype	Number of isolates	Percentage
Weak	26	35.6
Moderate	35	47.9
Strong	12	16.4
Total	73	100.0

Table-2 shows the distribution of 73 isolates according to the biofilm phenotype of *Stenotrophomonas maltophilia* isolates.

Among the study isolates, 35 (47.9%) showed a moderate biofilm phenotype, making it the most commonly observed category. Weak biofilm formation was observed in 26 (35.6%) isolates, while 12 (16.4%) isolates showed strong biofilm formation.

Association between biofilm formation and biofilm encoding genes:

Table-3: Distribution of Individual and Combined Biofilm-Encoding Genes According to Biofilm Phenotype among *Stenotrophomonas maltophilia* Isolates (n=73)

Biofilm Encoding Genes	Biofilm formation (n=73)			Total	Chi-square value	P-value
	Strong (n=12)	Moderate (n=35)	Weak (n=26)			
<i>rpjF</i>	0	0	11 (42.3%)	11	1117.3	P<0.0001 S
<i>spgM</i>	0	0	8 (30.8%)	08		
<i>rmlA</i>	0	0	7 (26.9%)	07		
<i>rpjF</i> & <i>rmlA</i>	0	11 (31.4%)	0	11		
<i>rpjF</i> & <i>spgM</i>	0	9 (25.7%)	0	9		
<i>spgM</i> & <i>rmlA</i>	0	10 (28.6%)	0	10		
<i>rpjF</i> , <i>spgM</i> & <i>rmlA</i>	12 (100.0%)	5 (14.3%)	0	17		
Total	12 (100%)	35 (100%)	26 (100%)	73		

S: Significant

Table-3 presents the distribution of *Stenotrophomonas maltophilia* isolates according to their biofilm phenotype and the presence of individual or combined biofilm-encoding genes. Among the 12 isolates with strong biofilm formation, all 12 (100.0%) carried the combination of *rpjF*, *spgM*, and *rmlA*.

Among the 35 moderate biofilm-forming isolates, the most frequently observed gene combination was *rpjF* & *rmlA* in 11 (31.4%) isolates, followed by *spgM* & *rmlA* in 10 (28.6%) and *rpjF* & *spgM* in 9 (25.7%) isolates. The combination of all three genes, *rpjF*, *spgM* & *rmlA*, was present in 5 (14.3%) moderate biofilm-forming isolates.

Among the 26 weak biofilm-forming isolates, *rpjF* was the most frequently detected gene, present in 11 (42.3%) isolates, followed by *spgM* in 8 (30.8%) and *rmlA* in 7 (26.9%) isolates.

The findings show that strong biofilm formation was observed exclusively among isolates carrying all three genes, whereas weak biofilm formation was mainly seen in isolates carrying individual biofilm-associated genes. Moderate biofilm formation was more commonly associated with combinations of two genes. This pattern indicates that the presence of multiple biofilm-associated genes may contribute to stronger biofilm formation in *S. maltophilia* isolates.

Table-4: Association between biofilm formation and biofilm encoding genes (n=73)

Biofilm Genes	Encoding	Biofilm formation (n=73)			Chi-square value	P-value
		Strong (n=12)	Moderate (n=35)	Weak (n=26)		
<i>rpjF</i>	Present	12 (100%)	25 (71.4%)	11 (42.3%)	13.09	P=0.0014 S
	Absent	0 (0%)	10 (28.6%)	15 (57.7%)		
<i>spgM</i>	Present	12 (100%)	24 (68.6%)	08 (30.8%)	18.37	P<0.0001 S
	Absent	0 (0%,)	11 (31.4%)	18 (69.2%)		
<i>rmlA</i>	Present	12 (100%)	26 (74.3%)	07 (26.9%)	24.02	P<0.0001 S
	Absent	0 (0%)	9 (25.7%)	19 (73.1%)		

S: Significant

Table-4 presents the association between biofilm formation phenotype and the presence of biofilm-encoding genes among 73 *Stenotrophomonas maltophilia* isolates.

For the *rpjF* gene, the gene was present in 12 (100%) strong, 25 (71.4%) moderate, and 11 (42.3%) weak biofilm-forming isolates. There was statistically significant association between biofilm formation and biofilm encoding *rpjF* gene in *Stenotrophomonas maltophilia* isolates (p=0.0014).

The *spgM* gene was detected in 12 (100%) strong, 24 (68.6%) moderate, and 8 (30.8%) weak biofilm-forming isolates. A statistically significant association was observed between biofilm formation and biofilm encoding *spgM* gene in *Stenotrophomonas maltophilia* isolates (p<0.0001).

Similarly, the *rmlA* gene was present in 12 (100%) strong, 26 (74.3%) moderate, and 7 (26.9%) weak biofilm-forming isolates. The association was statistically significant between biofilm formation and biofilm encoding *rmlA* gene in *Stenotrophomonas maltophilia* isolates ($p < 0.0001$).

All biofilm encoding three genes *rpjF*, *spgM* and *rmlA* were found more frequently among isolates showing strong and moderate biofilm formation compared with weak biofilm formation. The statistically significant *p*-values indicate that the presence of these biofilm-encoding genes was significantly associated with biofilm-forming phenotype. Among the three genes, *rmlA* showed the highest chi-square value, suggesting the strongest association with biofilm phenotype in this study.

DISCUSSION:

Stenotrophomonas maltophilia has become an important opportunistic pathogen in hospital settings because of its intrinsic resistance to several antimicrobial agents and its ability to persist in the environment and form biofilms. The present study was undertaken to evaluate the antimicrobial susceptibility pattern of *S. maltophilia*, determine its biofilm-forming ability, and investigate the relationship between biofilm formation and selected biofilm-associated genes, namely *rpjF*, *spgM*, and *rmlA*. The findings of the present study demonstrate possible relationship between stronger biofilm formation and the presence of these genes.

Antimicrobial susceptibility pattern

In the present study, minocycline showed the highest overall activity against *S. maltophilia*, with 90.4% of isolates being susceptible. Levofloxacin and ticarcillin-clavulanate also showed relatively good activity, with susceptibility rates of 76.7% and 75.3%, respectively. Co-trimoxazole showed a comparatively lower susceptibility rate of 54.8%.

The high activity of minocycline observed in our study is consistent with previous reports. Bostanghadiri et al. reported minocycline susceptibility of 100% among their *S. maltophilia* isolates, while levofloxacin and trimethoprim-sulfamethoxazole also showed high susceptibility rates. Their study similarly demonstrated complete resistance to imipenem and meropenem. These findings support the observation that minocycline may retain useful activity against *S. maltophilia*, although susceptibility patterns can vary according to geographical location, patient population, and local antimicrobial exposure.

In this study, resistance to co-trimoxazole was observed in 45.2% of isolates. This is relatively high compared with earlier studies in which co-trimoxazole retained greater activity. Bostanghadiri et al. reported 97.65% susceptibility to trimethoprim-sulfamethoxazole in one study. The comparatively high resistance observed in the present study may reflect differences in antimicrobial use, local resistance mechanisms, or the characteristics of the hospital population from which the isolates were obtained. This finding is clinically important because co-trimoxazole has traditionally been considered one of the major treatment options for *S. maltophilia*.

A high level of resistance was observed to ceftazidime, tetracycline, chloramphenicol, and ceftazidime-avibactam. The most striking finding was the 100% resistance to both imipenem and meropenem. This observation is consistent with the well-recognized intrinsic resistance of *S. maltophilia* to carbapenems. The resistance of *S. maltophilia* to carbapenems is associated with intrinsic mechanisms including β -lactamases and other resistance determinants, making carbapenems unreliable choices for treatment of infections caused by this organism.

Association between biofilm formation and biofilm-associated genes

One of the important findings of the present study was the detection of the three selected biofilm-associated genes. The *rpjF* gene was detected in 48 (65.7%) isolates, *spgM* in 44 (60.3%) isolates, and *rmlA* in 45 (61.6%) isolates.

A clear difference was observed when the distribution of these genes was compared with biofilm-forming ability. All 12 strong biofilm producers carried *rpjF*, *spgM*, and *rmlA*. Among moderate biofilm producers, these genes were detected in 71.4%, 68.6%, and 74.3% of isolates, respectively, whereas their frequencies were considerably lower among weak biofilm producers.

Our findings are broadly consistent with previous studies investigating the molecular basis of biofilm formation in *S. maltophilia*. Zhuo et al. investigated *spgM*, *rpjF*, and *rmlA* and found that these genes were associated with biofilm formation, although the presence of the genes alone did not fully explain the amount of biofilm produced. Their findings suggested that genetic variation and mutations may influence the functional contribution of these genes to biofilm formation.

Similarly, a study by Azimi et al. examined the distribution of *smf-I*, *rmlA*, *spgM*, and *rpjF* in relation to biofilm-forming capacity and reported that the presence of *rpjF* was associated with biofilm formation. The study also reported an association between strong biofilm production and resistance to certain antimicrobial agents.

The findings of Bostanghadiri et al. also support the importance of biofilm-associated genes in clinical *S. maltophilia* isolates. Their study demonstrated a high prevalence of biofilm-associated genes among clinical isolates, together with a high frequency of biofilm production.

In our study, 17 isolates carried all three genes, of which 12 (71%) were strong biofilm producers and 5 (29%) were moderate biofilm producers. None of the weak biofilm producers carried all three genes. This pattern is particularly interesting because it suggests that the combined presence of *rpjF*, *spgM*, and *rmlA* may be associated with stronger biofilm formation. However, the results should not be interpreted as evidence that these

genes alone determine biofilm strength. Biofilm development is a complex, multifactorial process influenced by genetic regulation, environmental conditions, quorum sensing, surface properties, nutrient availability, and other bacterial factors.

Routine identification of *S. maltophilia*, appropriate antimicrobial susceptibility testing, and awareness of its biofilm-forming potential may therefore help in better management of infections caused by this organism. Further molecular studies involving gene expression and functional analysis would be useful to determine whether *rpjF*, *spgM*, and *rmlA* directly influence the quantity and structure of biofilm formation.

CONCLUSION:

The present study highlights *Stenotrophomonas maltophilia* as an important multidrug-resistant pathogen with a strong ability to form biofilms. Among the 73 isolates studied, minocycline showed the highest susceptibility (90%), while 100% resistance was observed to imipenem and meropenem.

Biofilm formation was observed in all isolates, with moderate biofilm producers being the most common. The biofilm-associated genes *rpjF*, *spgM* and *rmlA* were detected more frequently among strong biofilm producers, and all three genes were present in all strong biofilm-producing isolates.

Overall, our findings suggest that antimicrobial resistance and biofilm formation may work together to enhance the persistence and treatment challenges of *S. maltophilia*. Early identification, appropriate antimicrobial susceptibility testing, and better understanding of biofilm-associated mechanisms are important for effective management of infections caused by this emerging pathogen.

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