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ORIGINAL ARTICLE

Investigation of virulence factors and genes associated with biofilm and protease in *Stenotrophomonas maltophilia* isolates in Bushehr, Iran

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ABSTRACT

Background and Objectives: This study aimed to investigate the virulence factors and genes associated with biofilm and protease in *Stenotrophomonas maltophilia* in Bushehr, Iran.
Materials and Methods: Eighty-seven *S. maltophilia* isolates (67 clinical and 20 environmental isolates) were studied. The isolates were assessed for the production of virulence factors including several enzymes and biofilm. To detect *rmlA, rpfF, spgM, smf-1, StmPr1* 868 bp, *StmPr1* 1621 bp, and *StmPr2* genes, PCR and sequencing were performed.
Results: All isolates (100%) produced DNase, hemolysin, protease, lipase, and hyaluronidase. Seventy-eight (89.7%) isolates were gelatinase producers, and 85 (97.7%) isolates were lecithinase producers. All isolates were biofilm producers: 79 (90.8%) isolates produced strong biofilm, 5 (5.7%) isolates produced moderate biofilm, and 3 (3.5%) isolates produced weak biofilm. The frequency of *smf-1, rmlA, rpfF,* and *spgM* was 93.1%, 86.2%, 26.4%, and 59.8%, respectively. The frequency of protease genes including *StmPr1* 868 bp, *StmPr1* 1621 bp, and *StmPr2* was 12.6%, 41.4%, and 18.4%, respectively.
Conclusion: Our findings revealed a high frequency of isolates that produce DNase, hemolysin, protease, gelatinase, lipase, lecithinase, hyaluronidase, and biofilm. All isolates that harbored *spgM* or *rpfF* or both genes were strong biofilm producers. Notably, the presence of isolates that lacked *spgM* and *rpfF* genes but produced strong biofilm indicates that in addition to these two genes, other genes or factors may play a role in the production of strong biofilm. Based on this research, *S. maltophilia* in our area possesses the capability to produce several factors that could play roles in pathogenicity.

Keywords: *Stenotrophomonas maltophilia*; Biofilm; Virulence; Pathogenicity

INTRODUCTION

Stenotrophomonas maltophilia is a global opportunistic pathogen. This bacterium is classified as a gram-negative obligate aerobe, exhibiting a rod-shaped morphology and motile with a few polar flagella (1) and plays a significant role as an opportunistic pathogen in immunocompromised patients (2). This organism can be isolated from water, soil, sewage, raw milk, frozen fish, and hospital disinfectant solutions (3). *S. maltophilia* is able to attack various systems in the body, including the respiratory system (4, 5), blood (1, 6, 7), skin, soft tissues, and urinary tract (1). The organism is implicated in a variety of

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clinical manifestations such as pneumonia, sepsis, bacteremia, endocarditis, septic arthritis, meningitis, endophthalmitis, and urinary infections (8). *S. maltophilia* infections, particularly in patients who are debilitated or immunocompromised, occur with a significant mortality (8).

S. maltophilia infections are predominantly mediated by medical device or lines connected to patients, for example, endotracheal or tracheostomy tubes and ventilators (2). A notable characteristic of *S. maltophilia* is the ability to establish biofilms on both hospital surfaces and human tissues. Biofilms have been associated with 65% of hospital-acquired infections (8). This microorganism is able to adhere to glass and several types of plastic substances including polyvinyl chloride and Teflon, some of which are used to produce medical equipment. Biofilm increases resistance to host immune defenses and antimicrobial agents (9) and is one of the essential virulence factors of this bacterium (8). *smf-1, rpfF, rmlA*, and *spgM* genes are associated with biofilm formation. The *smf-1* gene encodes fimbriae. *rpfF* gene encodes the enoyl-CoA hydratase. *rmlA* gene encodes the glucose-1-phosphate thymidyltransferase (8) and is necessary for the biosynthesis of the LPS O-antigen. In addition, it has been suggested that the *rmlA* gene is also involved in the biosynthesis of exopolysaccharides in *S. maltophilia* (10). The *spgM* gene, which encodes a bifunctional enzyme with both phosphoglucomutase (PGM) and phosphomannomutase activities, can play a role in the biofilm-forming capability (8, 11) due to the homology with the *algC* gene that is responsible for the synthesis of a PGM associated with LPS and alginate production in *Pseudomonas aeruginosa* (11).

Among other virulence factors of *S. maltophilia*, we can mention protease, lipase, lecithinase, gelatinase, DNase, hyaluronidase, and hemolysin, which establish the infection and damage the host tissues (2, 9). Proteinases are essential in the processes of invasiveness, tissue damage, and escaping host immune responses. *StmPr* genes encode proteases. Extracellular lipases help bacteria grow in a carbohydrate-limited environment where lipids serve as the exclusive carbon source. Lecithinase modulates the host immune system. Hyaluronidase facilitates invasion of host tissues (2).

The frequency of virulence factors and their related genes among *S. maltophilia* isolates may vary in different geographical areas. Research on virulence factors and their associated genes and how they are distributed in each geographical region is important to supplement knowledge about the pathogenesis of this organism. Also, this information can be used in future studies to develop new drugs and therapeutic approaches (12). The development of anti-virulence or anti-biofilm agents and anti-virulence therapy are among these new strategies (13). The aim of this study was to investigate the virulence factors and genes associated with biofilm and protease in *S. maltophilia* isolates in the city of Bushehr, southwest of Iran. The genes investigated in this study were *rmlA, rpfF, spgM, smf-1, StmPr1* 868 bp, *StmPr1* 1621 bp, and *StmPr2*.

MATERIALS AND METHODS

***S. maltophilia* isolates.** This study was approved by the Ethical Committee of Bushehr University of Medical Sciences with reference Number IR.BPUMS.REC.1399.031. Sixty-seven *S. maltophilia* clinical isolates recovered from clinical specimens including blood, endotracheal tube (ETT) secretions, urine, and wound were collected from three hospitals in Bushehr, Iran, between January 2015 and December 2018. The clinical isolates were recovered from internal ward, cardiac ward, surgical ward, neonatal ward, gynecology ward, delivery ward, pediatric emergency department, dialysis unit, and intensive care unit (ICU). In addition, 20 *S. maltophilia* environmental isolates were collected from two hospitals and one dental clinic in Bushehr, Iran, between September 2018 and November 2018. The environmental isolates were recovered from various sources including contaminated disinfectant, the external surface of the neonatal incubator in the neonatal intensive care unit (NICU), sinks in various laboratories and wards, toilets, desks of various departments, the floor of the ICU, and the floor of the microbiology laboratory. Identification of the clinical and environmental isolates of *S. maltophilia* was performed by biochemical tests and confirmed by PCR using a primer pair targeting a 278 bp fragment of the 23S rRNA gene (14) (Table 1).

DNase activity. DNase activity was assessed on DNase test agar (Merck, Darmstadt, Germany) supplemented with 0.01% toluidine blue after 72 hours of incubation at 37°C. The activity of DNase was de-

Table 1. Primers used in the present investigation for the identification of *S. maltophilia* and detection of the genes associated with biofilm and protease

Primer	Sequence (5′-3′)	Target gene	Product size (bp)	Reference
Sm-F	GCTGGATTGGTTCTAGGAAAACGC	23S rRNA gene [a]	278	(14)
Sm-R	ACGCAGTCACTCCTTGCG			
rmLA-F	CGGAAAAGCAGAACATCG	*rmlA*	1222	(11)
rmLA-R	GCAACTTGGTTTCAATCACTT			
spgM-F	ATACCGGGGTGCGTTGAC	*spgM*	2750	(11)
spgM-R	CATCTGCATGTGGATCTCGT			
rpfF-F	CACGACAGTACAGGGGACC	*rpfF*	1140	(11)
rpfF-R	GGCAGGAATGCGTTGG			
Fm-F	GGAAGGTATGTCCGAGTCCG	*smf-1*	674	(23)
Fm-R	GCGGGTACGGCTACGATCAGTT			
Pr1a-F	GCCGCAGTGTTGGTTCGATCCA	*StmPr1*	1621	(23)
Pr1a-R	CAGTTCTCGGTGCACGGCTCTT			
Pr1-F	CACGGCGGTCTTGTTGGTCA	*StmPr1*	868	(23)
Pr1-R	CGAGAACGACAACGAGTGCTACA			
Pr2-F	GCCGATTCCGGCATTCACACC	*StmPr2*	1764	(23)
Pr2-R	GGTCAGGCCCGAGAAGGTGCT			

[a] 23S rRNA gene of *S. maltophilia*

tected through the formation of a pink halo around an inoculum spot (2). *Serratia marcescens* (clinical isolate) and *Escherichia coli* (ATCC 25922) were used as positive control and negative control, respectively.

Another method was also used to test DNase activity. The isolates were spot inoculated on DNase test agar plates and incubated at 37°C for 72 hours. The emergence of a clear zone surrounding the inoculum spot after adding 1 N HCl indicated DNase activity. *Staphylococcus aureus* (ATCC 25923) and *E. coli* (ATCC 25922) were utilized as positive and negative controls, respectively.

Hemolysin production. *S. maltophilia* isolates were inoculated on tryptic soy agar (Merck, Darmstadt, Germany) containing 5% sheep blood and incubated at 37ºC for 24-96 hours. Hemolysis around the inoculum spot was observed (2, 3). *S. aureus* (ATCC 25923) was used as the positive control, and *Staphylococcus saprophyticus* as the negative control.

Protease activity. Protease activity of the *S. maltophilia* isolates was tested by spot inoculation on Mueller Hinton agar (Merck, Darmstadt, Germany) containing 3% (w/v) skim milk. After 48 hours of incubation at 37ºC, the presence of a clear zone around the inoculum spot indicated a positive test (2). *P. aeruginosa* and *E. coli* were used as positive and negative controls, respectively.

Gelatinase activity. For the detection of gelatinase that can degrade gelatin, a single colony of each test isolate was spot inoculated on nutrient agar (Merck, Darmstadt, Germany) plates containing 3% gelatin (Merck, Darmstadt, Germany). After inoculation, the plates were incubated at 37°C for 20 hours and then cooled for 5 hours at 4°C. The observation of a turbid halo surrounding the inoculum spot was interpreted as positive for gelatinase production (15, 16). *Bacillus cereus* (ATCC 11778) was utilized as the positive control, and *E. coli* (ATCC 25922) as the negative control.

Lipase activity. The isolates were spot inoculated on tryptic soy agar plates supplemented with 1% Tween 80 (2) and incubated at 37°C for up to 7 days (17). The appearance of an opaque zone around the inoculum spot indicated a positive test (17). *Aeromonas hydrophila* and *E. coli* (ATCC 25922) were used as positive and negative controls, respectively.

Lecithinase activity. For the preparation of egg yolk agar, 10 ml of the 50% egg yolk emulsion (Merck, Darmstadt, Germany) was mixed with 90 ml of sterilized tryptic soy agar (2). *S. maltophilia* isolates

were spot inoculated on egg yolk agar and incubated at 35ºC for up to 7 days (17). A white precipitate surrounding or beneath the inoculum spot was indicative of lecithinase production (2). *B. cereus* (18) was used as positive control and *E. coli* (ATCC 25922) as negative control.

Hyaluronidase activity. Hyaluronidase activity was determined by cross-streaking the *S. maltophilia* test isolates with a hyaluronic acid-producing *Streptococcus equi* strain. *Streptococcus equi* subsp. *equi* (ATCC 9528) (prepared by Razi Vaccine and Serum Research Institute, Hesarak, Karaj, Iran) was streaked across the center of the tryptic soy agar plates containing 5% sheep blood. *S. maltophilia* isolates were then streaked across the *S. equi* streak at right angles. The inoculated plates were incubated at 37°C for 24 to 48 hours in a humidified incubator. Hyaluronidase activity was indicated by destruction of mucoid streptococcal growth adjacent to the *S. maltophilia* streak due to degradation of hyaluronic acid (19, 20). *S. aureus* (ATCC 25923), *Streptococcus agalactiae*, and *Streptococcus pyogenes* were used as positive controls and *Staphylococcus epidermidis* was used as negative control.

Biofilm formation assay. The microtiter plate method was used for the quantitative assessment of biofilm. For each isolate, three wells of a sterile 96-well flat-bottomed polystyrene tissue culture plate with a lid (Wuxi Nest Biotechnology Co., Ltd., Wuxi, Jiangsu, China) were filled with 180 µl of tryptic soy broth (TSB) and 20 µl of overnight bacterial culture adjusted to 0.5 McFarland. The negative control wells contained 200 µl of TSB only. The plate was incubated for 18 h at 37°C. After removing the content of the wells, each well was washed three times with 250 µl sterile phosphate-buffered saline (PBS) to eliminate non-adherent bacteria. The plate was dried at 60°C for 1 hour to fix the remaining attached bacteria. The biofilms were then stained with 180 µl of 0.1% crystal violet for 15 min at room temperature, washed with running water, and dried. In the next step, 180 µl of 33% (v/v) glacial acetic acid was added per well to solubilize the dye bound to the biofilms. Each well's optical density (OD) was measured at 570 nm by the use of a microtiter plate reader. The OD cut-off (ODc) was defined as three standard deviations above the mean OD of the negative control. The isolates can be categorized based on adherence capability into the following groups: non-biofilm producers ($OD \leq ODc$), weak biofilm producers ($ODc < OD \leq 2\times ODc$), moderate biofilm producers ($2\times ODc < OD \leq 4\times ODc$), and strong biofilm producers ($4\times ODc < OD$) (21, 22).

Detection of genes associated with biofilm and protease by PCR and sequencing. The total DNA from *S. maltophilia* isolates was extracted by the use of an extraction kit (GeneAll Biotechnology Co., Ltd, Seoul, South Korea). Specific primers for detecting genes associated with biofilm and protease were utilized (Table 1). Amplification of targeted DNA was performed in 25 µl reaction volumes, each containing 12.5 µl of Taq DNA polymerase 2X Master Mix with 1.5 mM $MgCl_2$ (Ampliqon, Odense, Denmark), 1 µl of each primer, 9.5 µl of nuclease-free water, and 1 µl of DNA template. The PCR products were subsequently analyzed via electrophoresis on 1% agarose gel and visualized using a gel documentation system (UVP, BioDoc-It Imaging System, USA). The PCR products were purified and sequenced by Bioneer Company (Seoul, Korea). Sequence alignment of the nucleotides and deduced protein was conducted online using the Basic Local Alignment Search Tool (BLAST) program provided by the National Center for Biotechnology Information.

The sequences of the genes were submitted to the GenBank database under the following accession numbers: OP555977, OP585155, OP585156, OP651898, OP672368, OQ513990, and OQ513991.

RESULTS

A total of 87 isolates of *S. maltophilia* (67 clinical isolates and 20 environmental isolates) were studied in this project. The clinical isolates were recovered from various clinical specimens mentioned in the Materials and Methods section. Thirty-six (53.7%) clinical isolates were from male and 31 (46.3%) from female patients. The environmental isolates were recovered from several sources in the hospital environment of two hospitals as well as from one dental clinic (see Materials and Methods).

The isolates were phenotypically examined for the production of virulence factors including DNase, hemolysin, protease, gelatinase, lipase, lecithinase, hyaluronidase, and biofilm (Fig. 1). All 87 isolates (100%) produced DNase, hemolysin, protease, lipase, and hyaluronidase. Also, 78 (89.7%) isolates were gelatinase

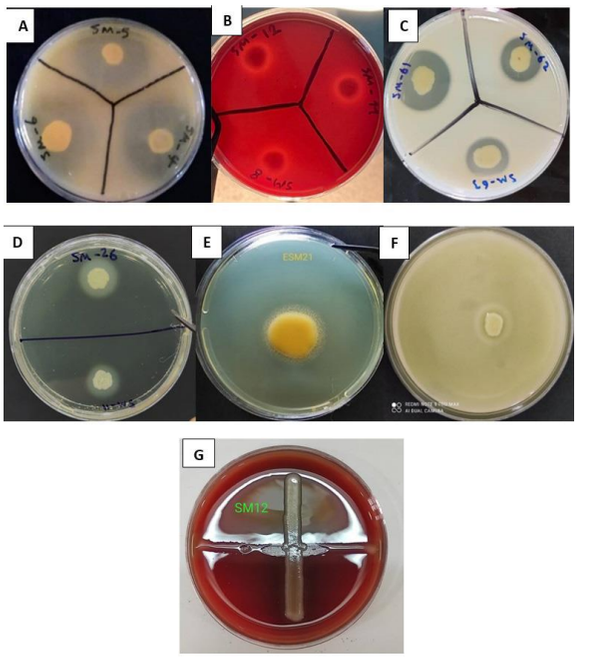

Fig. 1. Phenotypic assessment of virulence factors in *S. maltophilia* isolates. A: The formation of clear zone around the inoculum spot following the addition of 1 N HCl indicates DNase activity, B: The presence of hemolysis surrounding the inoculum spot as a result of hemolysin production, C: The presence of clear zone around the inoculum spot due to protease activity, D: The observation of turbid halo around the inoculum spot as a consequence of gelatinase activity, E: Opaque zone surrounding the inoculum spot by lipase activity, F: A white precipitate around an inoculum spot due to lecithinase production, and G: Diminution in size of the growth of *S. equi* adjacent to the *S. maltophilia* streak due to hyaluronidase activity.

producers. Out of the 9 gelatinase negative isolates, 8 were clinical (7 isolates were recovered from blood and 1 isolate from ETT secretions) and 1 was environmental (recovered from contaminated disinfectant). Therefore, 59 (88.1%) of the 67 clinical isolates and 19 (95%) of the 20 environmental isolates were gelatinase producers. Moreover, 85 (97.7%) isolates were lecithinase producers. Both lecithinase negative isolates were clinical (recovered from blood). Thus, of the 67 clinical isolates, 65 (97%) were lecithinase producers, but all 20 environmental isolates (100%) were lecithinase producers.

All isolates (100%) were confirmed as biofilm producers: 79 (90.8%) of the isolates produced strong biofilm, 5 (5.7%) exhibited moderate biofilm production, and 3 (3.5%) showed weak biofilm production. The 5 isolates that produced moderate biofilm were clinical, while the 3 isolates that produced weak biofilm were environmental. In other words, none of the clinical isolates produced weak biofilms, and none of the environmental isolates produced moderate biofilms. Of the 67 clinical isolates, 62 (92.5%) were strong biofilm producers, and of the 20 environmental isolates, 17 (85%) were strong biofilm producers (Table 2). The frequency of biofilm production among *S. maltophilia* isolates in relation to the isolation source is presented in Table 3.

Table 2. Biofilm formation ability in the clinical and environmental isolates of *S. maltophilia*

Biofilm	No. (%) of clinical isolates	No. (%) of environmental isolates	Total
Strong	62 (92.5)	17 (85)	79 (90.8)
Moderate	5 (7.5)	0 (0)	5 (5.7)
Weak	0 (0)	3 (15)	3 (3.5)
Total	67 (100)	20 (100)	87 (100)

The presence of biofilm-associated genes including *rmlA, spgM, rpfF,* and *smf-1* was investigated in the 87 isolates. *smf-1, rmlA, spgM,* and *rpfF* genes were detected in 81 (93.1%), 75 (86.2%), 52 (59.8%), and 23 (26.4%) of the 87 isolates, respectively (Table 4). All 8 moderate and weak biofilm-producing isolates were *spgM*-negative and *rpfF*-negative; of these 8 isolates, *rmlA* and *smf-1* genes were detected in 3 and 6 isolates, respectively. The *rmlA, spgM, rpfF,* and *smf-1* genes were not found in 1 moderate biofilm-producing isolate and 1 weak biofilm-producing isolate. Of the 79 strong biofilm-producing isolates, 26 were *spgM*-negative and *rpfF*-negative, of which 1 isolate was negative for the *rmlA, spgM, rpfF,* and *smf-1* genes.

Two allelic variants of the *StmPr1* gene (1621 bp and 868 bp) have been identified in *S. maltophilia.* The presence of *StmPr1* 1621 bp, *StmPr1* 868 bp, and *StmPr2* genes, which encode StmPr1 and StmPr2 proteases was detected in our study (Fig. 2). Among all 87 *S. maltophilia* isolates, 36 (41.4%) carried *StmPr1* 1621 bp, 11 (12.6%) carried *StmPr1* 868 bp, and 16 (18.4%) harbored *StmPr2* gene (Table 4). *StmPr1* 1621 bp variant exhibited a higher frequency among all 87 isolates as well as among clinical isolates compared to *StmPr1* 868 bp and *StmPr2*. Three isolates (2

Table 3. Frequency of biofilm formation among clinical and environmental isolates of *S. maltophilia* in relation to isolation source

Isolation source (no. of isolates)	No. (%) of isolates with biofilm phenotype		
	Strong	Moderate	Weak
Blood (48)	43 (64.1)	5 (7.5)	0 (0)
ETT secretions [a] (14)	14 (20.9)	0 (0)	0 (0)
Urine (3)	3 (4.5)	0 (0)	0 (0)
Wound (2)	2 (3.0)	0 (0)	0 (0)
Total clinical isolates (67)	62 (92.5)	5 (7.5)	0 (0)
Sink (9)	7 (35)	0 (0)	2 (10)
Toilet (3)	3 (15)	0 (0)	0 (0)
Desk (3)	2 (10)	0 (0)	1 (5)
Floor of the ICU [b] (2)	2 (10)	0 (0)	0 (0)
Floor of the microbiology laboratory (1)	1 (5)	0 (0)	0 (0)
Contaminated disinfectant (1)	1 (5)	0 (0)	0 (0)
Neonatal incubator in the NICU [c] (1)	1 (5)	0 (0)	0 (0)
Total environmental isolates (20)	17 (85)	0 (0)	3 (15)

[a] endotracheal tube secretions; [b] ICU: intensive care unit; [c] external surface of the neonatal incubator in the neonatal intensive care unit

Table 4. Frequency of biofilm - associated genes and protease genes among 67 clinical and 20 environmental isolates of *S. maltophilia*

Gene	No. (%) of clinical isolates	No. (%) of environmental isolates	Total isolates (%)
rmlA	60 (89.5)	15 (75)	75 (86.2)
spgM	36 (53.7)	16 (80)	52 (59.8)
rpfF	19 (28.3)	4 (20)	23 (26.4)
Smf-1	64 (95.5)	17 (85)	81 (93.1)
StmPr1 1621 bp	33 (49.3)	3 (15)	36 (41.4)
StmPr1 868 bp	9 (13.4)	2 (10)	11 (12.6)
StmPr2	11 (16.4)	5 (25)	16 (18.4)

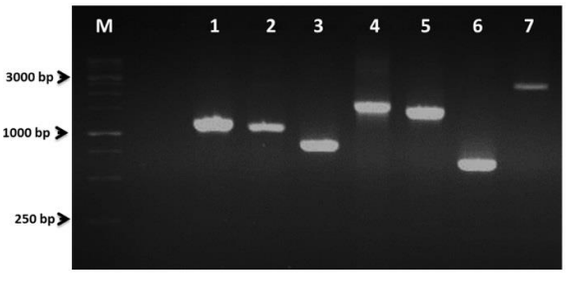

Fig. 2. Agarose gel electrophoresis of PCR products of protease and biofilm-associated genes in *S. maltophilia* isolates. M: 1Kb DNA ladder; lane 1: *rmlA* (1222 bp); lane 2: *rpfF* (1140 bp); lane 3: *StmPr1* (868 bp); lane 4: *StmPr2* (1764 bp); lane 5: *StmPr1* (1621 bp); lane 6: *smf-1* (674 bp), and lane 7: *spgM* (2750 bp).

clinical and 1 environmental) carried both variants *StmPr1* 1621 bp and *StmPr1* 868 bp. The *StmPr1* 1621 bp, *StmPr1* 868 bp, and *StmPr2* genes were not found in 28 isolates (17 clinical and 11 environmental).

DISCUSSION

S. maltophilia is recognized as an opportunistic pathogen inhabiting various places both inside and outside hospitals, usually in moist environments (24). The pathogenesis of *S. maltophilia* is attributed to several virulence factors. This organism possesses the capacity to persist on various surfaces due to its ability to form biofilms (8, 23, 25). We designed this project to study the virulence factors, biofilm-associated genes, and protease genes in isolates of *S. maltophilia* in Bushehr, Iran.

In the present study, all 87 isolates (100%) exhibited production of DNase, hemolysin, protease, lipase, and hyaluronidase. Also, 88.1% of the clinical isolates and 95% of the environmental isolates were gelatinase producers. Moreover, 97% of the clinical isolates and 100% of the environmental isolates were

lecithinase producers. Our data suggest that both clinical and environmental isolates of *S. maltophilia* in Bushehr possess potentially pathogenic enzymes.

In a research conducted by Thomas et al. on 108 *S. maltophilia* clinical isolaes in Malaysia, all isolates showed DNase, gelatinase, hemolysin, lipase, and proteinase activity. Hyaluronidase and lecithinase were produced by 97.2% and 69.4% of the isolates, respectively (2). Like the aforementioned study, in our work DNase, hemolysin, lipase, and protease activity were observed in all the isolates. However, in contrast to their study, in our investigation gelatinase was produced by 89.7% of the isolates. The frequency of lecithinase-producing isolates was higher in our study. In Thomas et al.'s study, none of the urine isolates showed lecithinase and hyaluronidase activity; furthermore, in 56.5% of 39 blood isolates lecithinase activity was not found (2). Contrary to Thomas et al.'s study, in the present project, the urine isolates showed lecithinase and hyaluronidase activity, and only 2 isolates (4.2%) out of 48 blood isolates were found to be negative for lecithinase production.

In the investigation performed by Alcaraz and colleagues on 63 *S. maltophilia* isolates in Argentina, all isolates were lipase and protease producers (26). Also, in another study done by Garcia et al. in São Paulo, Brazil, all 46 studied isolates showed caseinase (protease) activity (3) which is consistent with our finding. But, in contrast to the Garcia et al.'s study, all isolates in the present work showed hemolytic activity.

A notable characteristic of *S. maltophilia* is its capacity to produce biofilm on diverse abiotic surfaces and host tissues (1, 12). This capability confers resistance to various antimicrobial compounds and antiseptics, and protects the microorganism against the host defenses (12). We tested biofilm formation for *S. maltophilia* isolates. All isolates (100%) were biofilm producers. Biofilms were categorized as strong, moderate, and weak. In this study, the intensity of biofilm production (strong plus moderate biofilm producers) was higher in the clinical isolates compared to the environmental isolates, as 100% of the clinical isolates developed strong and moderate biofilms, while 85% of the environmental isolates were able to produce strong biofilms, and it is emphasized that moderate biofilm formation was not found among environmental isolates.

In our investigation, the frequency of biofilm-producing *S. maltophilia* isolates was 100%. This finding was similar to the results of two other investigations in Iran that reported frequencies of 95.73% (8) and 98.7% (27). Also, in the investigations conducted by Flores-Trevino et al. in Mexico (28), Zhuo et al. in China (11), and ElBaradei and Yakout in Alexandria, Egypt (25), all *S. maltophilia* isolates (100%) were biofilm producers. But, contrary to our study, in the research undertaken by Pompilio and colleagues in Italy, 82.5% of *S. maltophilia* strains were capable of forming biofilm (29).

In the present investigation, among all 87 *S. maltophilia* isolates, 84 (96.5%) produced strong (79 isolates; 90.8%) and moderate (5 isolates; 5.7%) biofilms (Table 2). The intensity of biofilm production (strong plus moderate) was higher in our isolates compared to the isolates in the studies performed by Bostanghadiri et al. (strong: 29.87%; moderate: 38.41%) and Azimi et al. (strong: 46.0%; moderate 21.3%) in Iran as well as Flores-Trevino et al. (strong: 13.4%; moderate: 38.7%) in Mexico (8, 27, 28). However, the intensity of biofilm production in our study and the study by ElBaradei and Yakout in Egypt was similar (25).

In this study, the presence of biofilm-associated genes including *smf-1, rmlA, spgM,* and *rpfF* was determined in the isolates. The *smf-1* gene encodes *S. maltophilia* fimbriae 1 (SMF-1 fimbriae), a type-1 fimbriae (23, 30), that is involved both in adherence and initial stages of biofilm development (31, 32). In the present study, the *smf-1* gene was identified in 81 (93.1%) of the total 87 *S. maltophilia* isolates; 64 (95.5%) of the 67 clinical isolates, and 17 (85%) of the 20 environmental isolates were positive for the *smf-1* gene (Table 4).

Azimi et al. studied 150 *S. maltophilia* isolates collected from Tehran and Qazvin, Iran, and reported a frequency of 99.3% for the *smf-1* gene (27). Nicoletti et al. studied 43 clinically isolated strains (41 were from cystic fibrosis (CF) patients and 2 from blood cultures of two non-CF patients) and 7 environmental strains (2 isolated from the hospital environment and 5 reference strains from the LMG collection, Belgium). *smf-1* gene was detected in the 43 clinical isolates and the 2 hospital-isolated environmental strains; however, this gene was not found in the 5 environmental reference strains (23). Contrary to Nicoletti et al.'s study, in our investigation 3 clinical isolates as well as 3 hospital-isolated environmental isolates were *smf-1*-negative. Our findings were similar to the results of the study by ElBaradei and Yakout,

who detected *smf-1* gene in 90% of the isolates (25). In a study conducted by Gallo et al. in Brazil, the *smf-1* gene was identified in 23% and 42% of clinical and hospital environment isolates, respectively, and approximately all (96.8%) of the isolates that carried *smf-1* gene were able to produce biofilms. In their study, most isolates produced weak and moderate biofilms, 48.4% and 45.2%, respectively, while 3.2% produced strong biofilm. None of the *smf-1*-negative isolates were able to produce biofilms (30). As can be seen, the intensity of biofilm production was obviously higher in our study compared to the study by Gallo et al. Moreover, in our work, all 6 *smf-1*-negative isolates produced biofilm (strong: 4 isolates; moderate: 1 isolate; weak: 1 isolate), indicating that *smf-1*-negative isolates may also produce biofilm (even strong biofilm), which could be a warning sign.

In the present study, *rmlA*, *spgM*, and *rpfF* genes were detected in 86.2%, 59.8%, and 26.4% of the total 87 isolates, respectively. Various results regarding the frequency of *rmlA*, *spgM*, and *rpfF* genes have been reported in other studies (8, 11, 25, 27, 33). In the study conducted by Bostanghadiri and colleagues, isolates exhibiting the *rpfF+/spgM+/rmlA+* genotype were correlated with production of moderate or strong biofilm (8). However, in our study, out of 5 moderate biofilm-producing isolates, 1 isolate showed *rpfF–/spgM–/rmlA+/smf-1+* genotype, 3 isolates showed *rpfF–/spgM–/rmlA–/smf-1+* genotype, and 1 isolate showed *rpfF–/spgM–/rmlA–/smf-1–* genotype. Therefore, since the *smf-1* gene was present in 4 of these 5 isolates, this gene may have played a role in biofilm production in the 4 isolates mentioned. Azimi et al. reported that the presence of *rpfF* and *smf-1*, but not *spgM*, could be associated with biofilm formation (27). Zhuo et al. reported that strains with the *spgM+/rpfF+/rmlA+* genotype were easier to exhibit a strong or moderate biofilm producer phenotype compared to strains possessing the *spgM–/rpfF–/rmlA+* genotype which was frequently observed in weak biofilm producer phenotype (11). Pompilio et al. found a significant association between the presence of the *spgM* gene and strong biofilm formation (33). In our study, all isolates that harbored *spgM* or *rpfF* or both genes, were strong biofilm producers. Also, it should be emphasized that in our study 26 strong biofilm-producing isolates were *spgM*-negative and *rpfF*-negative, indicating that in addition to these two genes, other genes or factors could contribute to the formation of strong biofilm.

The major extracellular protease of *S. maltophilia* (StmPr1) acts as a virulence factor that is able to degrade connective tissue and several human serum proteins, and also inactivates immune system components. StmPr2 is the minor extracellular protease (12, 23). It has been reported that serine proteases StmPr1 and StmPr2 contribute to the degradation of extracellular matrix proteins and interleukin-8. A third serine protease (StmPr3) has also been identified that contributes to the above described activities. In addition, a fourth serine protease (StmPr4) has been recognized in fewer strains. But, StmPr1 has a dominant role in the proteolytic effects of this bacterium (possibly due to its large amount), followed by StmPr2 and StmPr3 (12).

Protease *StmPr1* is encoded by the *StmPr1* gene. There is some degree of variability in the nucleotide sequences of *StmPr1* genes among different strains, while *StmPr2* gene which encodes protease StmPr2 is highly conserved. Two allelic variants of *StmPr1* gene have been recognized, *StmPr1* 1621 bp and *StmPr1* 868 bp (*S. maltophilia* isolates harbor either the first or the second variant, both of them or none) (12, 23). In the present study, among all *S. maltophilia* isolates, 41.4% carried *StmPr1* 1621 bp, 12.6% carried *StmPr1* 868 bp, and 18.4% harbored *StmPr2* gene. The *StmPr1* 1621 bp, *StmPr1* 868 bp, and *StmPr2* genes were not found in 28 isolates, however, these isolates were protease producers by phenotypic test. These 28 isolates probably harbored *StmPr3* or *StmPr4* gene that have not been investigated in our study. The frequency of protease genes was lower in our study compared to the study done by Nicoletti et al. (23) and Duan et al. (34). Also, the frequency of *StmPr1* 1621 bp and *StmPr2* genes in our work was lower than the frequency reported by ElBaradei and Yakout; however, the frequency of *StmPr1* 868 bp was similar (25).

CONCLUSION

The results of our investigation revealed that the frequency of isolates producing potentially pathogenic enzymes and biofilms is high in Bushehr which could be a warning sign. All isolates possessing the *spgM* or *rpfF* genes, or both, were identified as strong biofilm producers. It is imperative to highlight that the existence of isolates that lacked *spgM* and *rpfF* genes, but produced strong biofilm, shows that

in addition to these two genes, other genes or factors may play a role in the production of strong biofilm. According to the findings of this study, *S. maltophilia* in our region can produce several factors including enzymes and biofilm which could have roles in pathogenicity. Detection of potentially pathogenic *S. maltophilia* isolates plays a major role in preventing their spread as well as the successful treatment of their infections.

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