Original Article

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The Frequency of Virulence Factor Production in Clinical Isolates of Stenotrophomonas Maltophilia

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ABSTRACT

BACKGROUND AND OBJECTIVE: Stenotrophomonas maltophilia is known as an emerging bacterium in the world, associated with a wide range of diseases. The aim of this study was to investigate the virulence factors of these bacteria including extracellular enzymes, ability to form biofilm and rpfF gene involved in quorum sensing.

METHODS: This cross-sectional study was conducted using urine samples, blood and sputum samples, swab samples of oxygen manometer system and tap-water of hospitals as well as dental suction. Bacteria identification was done using culture methods and biochemical tests and to confirm the existence of bacteria, presence of 23S rRNA gene was assessed using real-time PCR method. Isolates were studied in terms of gelatinase, hemolysin, hyaluronidase, lecithinase, lipase and protease enzymes using phenotypic method and biofilm formation using microplate method. Moreover, existence of rpfF gene in isolates was investigated using PCR method.

FINDINGS: 100% of isolates contained rpfF gene. Most isolates contained gelatinase (90%), hemolysin (85%), protease (75%), lecithinase (90%), lipase (75%) and hyaluronidase (100%) enzymes. Biofilm formation was not observed in 15% of isolates; 45% of isolates had weak power of biofilm formation; 40% of isolates had moderate power of biofilm formation and none of the isolates had strong power of biofilm formation. Correlation between hemolysin and lipase, hemolysin and lecithinase, and lecithinase and lipase variables was significant.

CONCLUSION: Results of the study demonstrated that bacteria isolates contained various virulence factors including rpfF gene that produce diffusion signal factor which is essential for quorum sensing and were highly capable of producing extracellular enzymes and forming biofilm.

KEY WORDS: *Stenotrophomonas Maltophilia, Rpff Gene, Enzyme, Biofilm.*

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Introduction

Stenotrophomonas belongs to γ-β subclass of proteobacteria (1). It was first introduced under the title of "*Bacterium booker*" in early 1940s (2). In 1981, it was named *Pseudomonas maltophilia*. It was later categorized as *xanthomonas maltophilia* and was finally named *stenotrophomonas maltophilia* (4).

Stenotrophomonas maltophilia is known as a newfound non-fermentative, bacillus-shaped, gram-negative aerobic bacterium in the world. The infection caused by these bacteria is specifically limited to hospitalized patients that suffer from immune disorder. The infection caused by these bacteria in patients with cancer, particularly acute lung cancer, has significantly increased during the last two decades (5-7). The infection caused by these bacteria has been observed in patients with cystic fibrosis, people with burns and people with damaged immune system and susceptible to opportunistic infections (8).

These bacteria along with *Pseudomonas aeruginosa* have been observed in respiratory samples of patients with cystic fibrosis (9). *Stenotrophomonas maltophilia* has been mostly observed in respiratory system infections (10), bacteremia (11), biliary infection (12), infections of bone and transplants, urinary system and soft tissues (13), endophthalmitis (14), eye infections (15), endocarditis (16), blood infection (17) and bacteremia associated with catheters and meningitis (17).

The infection caused by these bacteria may have the risk of death in the cases of malignancy, acute septic shock and organ transplant failure. Factors that influence the virulence of these bacteria include secretion of extracellular enzymes such as proteinase, lecithinase, gelatinase, lipase, hyaluronidase, hemolysin and DNase as well as formation of melanin pigment, bacterial motility and the ability to form biofilm (18).

Enzymes play a key role in the virulence of bacteria, since they can damage host tissue (18). One of the important genes that influence the virulence of bacteria is *rpfF*. This gene is part of a multigenic complex that organizes pathogenic factors and is involved in quorum sensing (19). Considering the often fatal complications of these bacteria for humans, further studies regarding this issue seems necessary. Due to the increased number of cases of infection in recent years, the present study aims to do more investigation regarding these bacteria, assess the pathogenic factors and gain more knowledge about it.

Methods

This cross-sectional study was conducted using 2947 urine samples, 777 blood samples and 100 sputum samples collected from patients admitted to Imam Ali Hospital (Amol), 110 swab samples from oxygen manometer system and 240 samples from tap-water in Hefdah Shahrivar, Imam Ali and Imam Reza Hospitals (Amol) as well as 120 samples from dental suction in dental clinics for six month in 2015.

At first, samples were cultured in Blood Agar culture media (Merck- Germany), Macconkey Agar (Scharlau- Spain) and Eosin Methylene Blue Agar (Merck- Germany) (20) and observing gram-negative bacilli, the bacteria were cultured in Steno medium agar (21). For growing bacteria, biochemical tests including, oxidase, catalase, sugar fermentation in Triple sugar iron agar (Merck- Germany), indole, motility, hydrogen sulfide, MR, VP, lysine decarboxylase, bile esculin (Biolife- Italy), urea, DNase (Himedia- India) were run.

To extract DNA, boiling method was used. 24-hours bacterial culture was used in LB Broth (Scharlau- Spain). 0.0015 mL of bacterial suspension was centrifuged at 7000 rpm for 1 min. The supernatant was throwed out and adding 100 µL deionized water to the sediment, It heated at 90°C for 30 min and 20 µL Tris HCl 1mol (pH=7.5) was added. It was centrifuged at 10000 rpm for 1 min and finally the supernatant was moved to a sterile microtube. After extracting DNA, *23S rRNA* was examined using real-time PCR. In the final 20 µl volume, the reaction included 5 µl template DNA, 2 µl (10x) buffer, 0.6 µl (50 mM) $MgCl_2$, 0.4 µL (10 mM) dNTP, 0.4 µl F primer and 0.4 µl R primer (22), 0.4 µl ROX dye and 0.9 µl SYBR Green dye, 0.2 µl Taq DNA Polymerase and 9.7 µl deionized water.

Real-time PCR (ABI) was used for the reaction PCR. The temperature program included:

5 min at 95°C in the first stage; 30 seconds at 94°C and 1 min at 59°C in the second stage, which was repeated 45 times. Identification of *rpfF* was done using PCR method.

The final volume was considered 25 µl that consisted of 5 µl template DNA, 0.2 µl Taq DNA Polymerase, 1 µl F primer and 1 µl R primer of *rpfF* gene, 0.5 µl (10 mM) dNTP, 2.5 µl (10x) buffer, 0.75 µl (50 mM) MgCl2 and 14.05 µl deionized water. Thermocycler (TECHNE- England) was used for the reaction. The thermal cycle included the initial denaturation phase at 94°C for 4 min, for 35 times:

denaturation at 94°C for 35 seconds, annealing at 59°C for 1 min, extension at 72°C for 45 seconds and final extension at 72°C for 5 min and the PCR product was electrophoresed. Table 1 shows the specifications of primers and the expected length of fragments in PCR and real-time PCR techniques.

Table 1. Specifications of primers used in this research

bp Size	The gene sequence	Gene Name	Source
278	F: GCTGGATTGGTTCTAGGAAAACGC R: ACGCAGTCACTCCTTGCG	*23S rRNA*	22
145	F: CTGGCTGGCGGTGTAGAGG R:CGAGGAAGGCGTGTTGATGG	*rpfF*	In this study

Enzymatic tests of gelatinase, hemolysis, lecithinase, hyaluronidase, lipases and proteinase were run using bacterial isolates of *stenotrophomonas maltophilia* (18). The bacteria were cultured in LB broth medium (Scharlau- Spain) for 18-24 hours to for investigating the biofilm and after this period, a bacteria suspension with opacity equivalent to a tube of 0.5 Mcfarland standard was prepared.

180 µl of LB broth medium (Scharlau- Spain) was placed in 96-well microplates and then, 20 µl of bacteria suspension was added to the wells and was kept at 37°C for 24 hours. After this period, inoculations were removed and washed with distilled water for 3 times. Then, 200 µl crystal violet was added for 5 min. it was washed with distilled water for 3 times and 200 µl glacial acetic acid was added to each well and light absorption at 492 nm was read using Elisa Reader (Biotek- USA). 3 replicates were considered for each sample and they were categorized as follows:

ODC(Optical Density Cut Off)=(SD×3)+control
OD≤ODC=lack of biofilm formation potential
ODC<OD≤(2×ODC)=Weak biofilm formation potential
(2×ODC)<OD≤(4×ODC)=Moderate biofilm formation potential
(4×ODC)<OD=Strong biofilm formation potential

For statistical analysis, descriptive statistics (frequency, percentage and mean), one-way analysis of variance (ANOVA) and comparison of means using Duncan's new multiple range test were used. To investigate the relationship between studied variables, Pearson and Spearman coefficient tests were used and $p<0.05$ was considered significant.

Results

Overall, 20 bacterial isolates of *stenotrophomonas maltophilia* were isolated; 1 isolate was obtained from tap-water of hospitals (0.05%), 1 from urine samples (0.05%), 1 from blood samples (0.05%), 4 from sputum samples (0.2%), 7 from swab samples of oxygen manometer system (0.35%) and 6 isolates were obtained from dental suctions (0.3%). Real-time PCR results for *23S rRNA* are presented in figure 1.

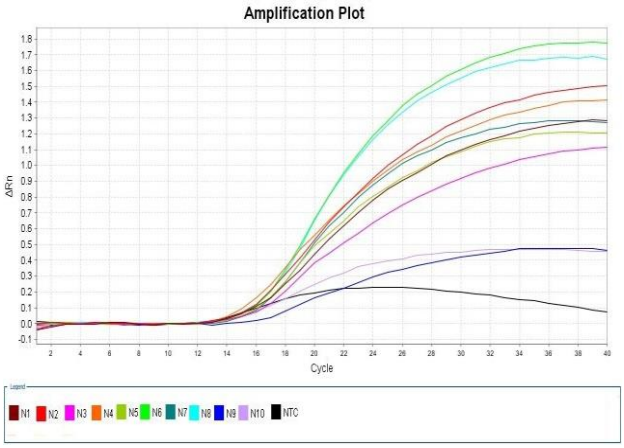

Figure 1. Amplicon plot of positive samples by real-time PCR for *23S rRNA* in *stenotrophomonas maltophilia*

PCR test on *rpfF* gene revealed that all samples, identified as *stenotrophomonas maltophilia* using real-time PCR and culture methods, had *rpfF* gene and formed a band at 145 bp (Fig 2). Most *stenotrophomonas maltophilia* isolates contained gelatinase (90%), hemolysin (85%), protease (75%), lecithinase (90%), lipase (75%) and hyaluronidase (100%). The correlation between variables of hemolysin and lipase ($p=0.000$, $r=0.742$), hemolysin and lecithinase ($p=0.000$, $r=0.781$) and lecithinase and lipase ($p=0.010$, $r=0.574$) was significant.

However, there was no significant correlation between hemolysin and hyaluronidase, hemolysin and gelatinase, hemolysin and protease, lipase and hyaluronidase, lipase and protease, lipase and gelatinase, protease and hyaluronidase, protease and gelatinase, lecithinase and protease, hyaluronidase and gelatinase, hyaluronidase and lecithinase and gelatinase and lecithinase. Biofilm formation was not observed in 15% of isolates, 45% of isolates had weak biofilm formation potential, 40% had moderate biofilm formation potential and none of the samples had strong biofilm formation potential (Fig 3). Similar letters

indicate lack of significant relationship between samples and dissimilar letters indicate significant relationship between samples.

Result of statistical analysis showed that there is no significant relationship between two variables of melanin pigment and biofilm formation (p=0.692, r=-0.094) (data related to pigment formation is not shown). There was a significant relationship between two variables of biofilm formation and swarming motility (p=0.001, r=-0.674). However, there was no significant relationship between biofilm formation and twitching motility (p=0.704, r=-0.091) and swimming motility (p=0.778, r=-0.067) (data related to various motilites is not shown).

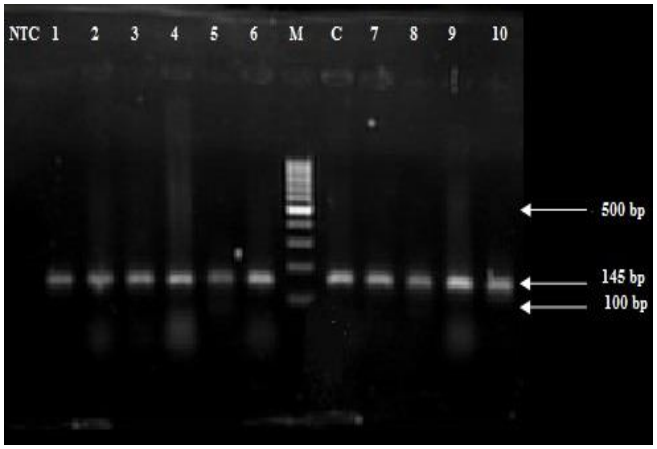

Figure 2. Electrophoresis of PCR product of *rpfF* in *stenotrophomonas maltophilia*. Isolates: 1-10, Negative control: NTC, Positive control: C, 100bp ladder: M

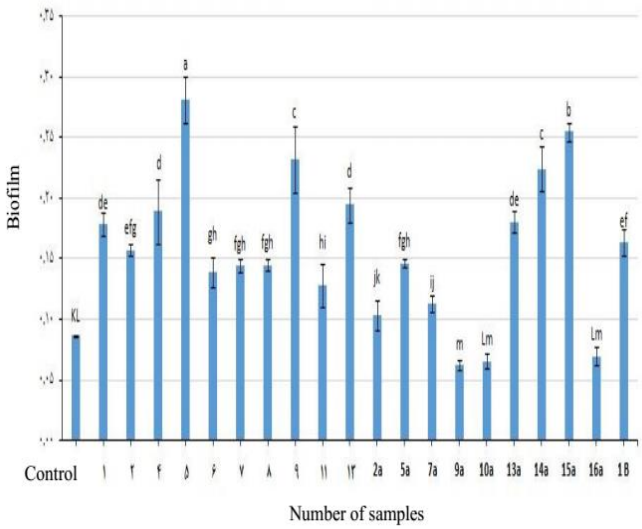

Figure 3. Result of statistical analysis regarding biofilm formation in *stenotrophomonas maltophilia* isolates

Discussion

Stenotrophomonas maltophilia isolates have a high level of virulence factors such as extracellular enzymes and biofilm formation. They also contain *rpfF* gene, which plays a significant role in virulence of bacteria. These bacteria are usually identified using selective culture media and biochemical methods. However, using molecular techniques is preferable due to higher accuracy. Both techniques were used in this study. Of 20 samples identified as stenotrophomonas maltophilia using phenotypic methods, 4 samples were negative according to molecular technique.

Therefore, when phenotypic methods have errors, molecular techniques can offer more accurate and reassuring results. In the present study, most *stenotrophomonas maltophilia* isolates contained enzymes of gelatinase (90%), hemolysin (85%), protease (75%), lecithinase (90%), lipase (75%) and hyaluronidase (100%).

In the study of Thomas et al., the activity of gelatinase, hemolysin, lipase and protease was observed in all studied isolates (18). But in the present study, a few isolates lacked this enzyme (gelatinase 10%, hemolysin 15%, protease 25%, and lipase 25%). In the study of Thomas et al., none of the urine isolates contained hyaluronidase and lecithinase enzymes and 56.5% of blood isolates lacked lecithinase enzyme (18). In the present study, urine isolates contained hyaluronidase and lecithinase enzymes, which was different from the study of Thomas.

In addition, according to the present study, blood isolates contained hyaluronidase and lecithinase enzymes, which was not different from the results of this study regarding hyaluronidase enzyme and regarding lecithinase enzyme, some blood samples in the study of Thomas contained this enzyme and some other samples lacked it, which does not show a significant difference.

In the study of Passerini De Rossi et al., of 13 *stenotrophomonas maltophilia* isolates, all isolates had gelatinase enzyme and DNase (23) which was a little different from the present study (90% positive) regarding gelatinase enzyme and the reason for this difference may be related to ecological conditions of bacteria. Biofilm formation is a factor that induces virulence. *Stenotrophomonas maltophilia* can form biofilm on biotic and abiotic surfaces such as glass, plastic and host tissue. Bacterial growth along with biofilm formation is an effective defense mechanism bacteria use to survive competitive environments such

as airways of patients with cystic fibrosis (CF) (24, 25). According to the study of Samantha Flores-Trevin et al. in Mexico, all isolates could form biofilm, 47.9% (57.119) of isolates had weak biofilm formation potential, 38.7% (46.119) of isolates had moderate biofilm formation potential and 13.4% (16.119) of isolates had strong biofilm formation potential (26). In the present study, none of the isolates had strong biofilm formation potential. Moreover, 3 isolates lacked the potential to form biofilm, which is different from the study of Samantha Flores-Trevin. However, in the present study, 45% (9.20) of isolates had weak biofilm formation potential and 40% (8.20) of isolates had moderate biofilm formation potential, which is almost similar to the study of Samantha Flores-Trevin (26). The quorum sensing system in *stenotrophomonas maltophilia* is based on a molecular marker named "diffusible signal factor (DSF)" and formation of DSF is codified by *rpf* gene cluster (19).

In the present study, all isolates contained *rpfF* gene, which was in accord with study of Huedo in 2014 (19). Compared with the biofilm formed by single species of *pseudomonas aeruginosa*, the biofilm formed by mixed species of *pseudomonas aeruginosa* and *stenotrophomonas maltophilia* demonstrated that *stenotrophomonas maltophilia* helps *pseudomonas aeruginosa* to develop a structure with various shapes. These results revealed that DSF is necessary to create a relationship between two bacterial species. *Pseudomonas aeruginosa* and *stenotrophomonas maltophilia* strengthen each other in some environments related to plants and animals (27). Zhuo, et al. demonstrated that prevalence of *rpfF* gene in 24 strains of *stenotrophomonas maltophilia* was 50% and the existence of *rpfF* gene was closely related to biofilm formation, but the effect on biofilm formation was not significant. In the present study, all isolates contained *rpfF* gene, which was different from the study of Zhuo (28). Bacterial isolates contained various virulence factors including *rpfF* gene, which produces sign of essential diffusion for quorum sensing system of bacteria and numerous extracellular enzymes. Most isolates had the potential to form biofilm, which can help them survive and increase their antibiotic resistance. Therefore, antibiotic sensitivity tests before treatment is necessary considering the antibiotic resistance of bacteria.

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