Investigation of Metallo-Beta-Lactamase Genes (L1 and NDM1) in Isolates of *Stenotrophomonas maltophilia*

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Abstract- *Stenotrophomonas maltophilia* is resistant to a wide range of antibiotics. This study aimed to investigate the beta-lactamase, ESBL, and MBL enzymes and also L1and NDM1 genes in isolates of *Stenotrophomonas maltophilia*. Antibiotic susceptibility test, beta-lactamase, ESBL, and MBL test were done on 23 isolated *Stenotrophomonas maltophilia*. The presence of L1 and NDM1 genes was investigated on isolated MBL bacteria by the PCR method. The results showed the most effective antibiotics were tigecycline and gentamicin (100%), and the highest resistance was observed with aztreonam (56.22%). 39.13% of isolates were ESBL, 82.66% of isolates were beta-lactamase positive, and 60.86% of isolates were Metallo-beta-lactamase positive. 39.13% of MBL-positive isolates were positive for the L1 gene, but the NDM1 gene was not seen. Results of this study showed higher resistance to beta-lactam antibiotics also the presence of the l1 gene. ESBL and MBL producing *S. maltophilia* are frequently resistant to a wide range of antibiotics. Therefore, needs to be done antibiotics sensitivity tests, ESBL, and MBL tests before treatment for this bacterium.

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Introduction

Stenotrophomonas maltophilia is an important gram-negative bacterium in immunocompromised individuals that is resistant to a wide range of antibiotics and has a high intrinsic resistance to antibiotics (1). The molecular mechanisms involved in drug resistance include plasmids, integrons, and transposons (2), the multi-antimicrobial resistance in this bacterium is due to the activity of multidrug efflux pumps (1). *S. maltophilia* can exchange DNA with other bacteria, which is very important in the spread of antibiotic resistance in clinical units. This bacterium receives genes from other bacterial species and can transfer antibiotic resistance to other bacteria. *S. maltophilia* is isolated from the rhizosphere of plants and can be a source of antibiotic resistance. In the rhizosphere of the plant, the horizontal transport of the *S. maltophilia* gene has been observed (1). Beta-lactamases are the main causes of natural resistance to beta-lactams. The resistance is due to two beta-lactams, L1 and L2. L1 is a Metallo-beta-lactamase belonging to Zn^{2+}-B-depended metalloenzymes in class B, which hydrolyzes all classes of β-lactams, except monobactams (3). These two chromosale beta-lactams are produced when cells are placed against beta-lactams. The regulator for the production of both beta-lactamases is identical, but the expression of these two enzymes is different (4). The mechanisms that make the beta-lactamase expression variable are not yet clear, and further research is needed. The two beta-lactamase genes exist on the 200- kb plasmid in *S. maltophilia*, and there is an allelic variation among β-lactamase genes of L1 and L2. In the clinical isolates, it has been shown that with the change in the amino acid units necessary for the binding of β-lactamase L_1 to its substrate, its activity is altered (5). Beta-lactamase activity is not due only to their producing

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genes; and there is probably another mechanism for controlling beta-lactamase gene expression (6). One of the newest Metallo-beta-lactamase factors is the presence of the NDM-1 gene, which has zinc ions in its active site and hydrolyzes all of the antimicrobial compounds of beta-lactam, exception of monobactams. In addition, most bacteria with this gene are resistant to a wide broad of antimicrobial agents and have high resistance mechanisms (for example, aminoglycosides, fluoroquinolones, macrolides, and sulfonamides). The main source of the NDM-1 gene can be the chromosome of bacterial pathogens of plants, including *Pseudomonas aeruginosa* and similar bacteria that are present in the environment. NDM-1 enzymes producing bacteria have been seen in patients with various infections, including respiratory infection, pneumonia, septicemia, wound infections, and related infections with tools and devices used by patients. This enzyme has been detected from bacteria isolated from both types of hospital infections and acquired infections from the community. Among the factors influencing the geographical distribution of the bacteria producing the gene, the increase in travel, including the increase in international travel for medical care and medical treatment, is mentioned (7). The spread of antibiotic resistance genes in *S. maltophilia* requires an antibiotic susceptibility test for patients, and control of clinical isolates may identify the sources of transfer of *S. maltophilia*. Acquiring the gene from bacteria in the environment by *S. maltophilia* increases the importance of controlling the antibiotic resistance of the clinical isolates of *S. maltophilia*. Such controls provide the search for antibiotic resistance genes in environmental resources; provide the spread of these genes in clinical isolates, and it requires programs to reduce antibiotic resistance.

Materials and Methods

Sample collection

A total of 23 *Stenotrophomonas maltophilia* were isolated from clinical and environmental in Amol, Iran was used in this study.

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed according to the Clinical and Laboratory Standard Institute (CLSI) using the Kirby-Bauer disk diffusion method on Mueller Hinton agar (Merck, Germany). Antibiotic disks used were: ciprofloxacin (CIP, 5 μg), gentamicin (GM, 10 μg), ceftazidime (CAZ, 30 μg), aztreonam (ATM, 30 μg), chloramphenicol (C, 30 μg), cotrimoxazole (SXT, 30 μg), minocycline (Min, 30 μg), piperacillin-tazobactam (TZP, 100/10 μg), levofloxacin (LEV, 5μg), cefepime (FEP, 30 μg), tigecycline (TIG, 15 μg).

Identification of beta-lactamase-producing bacteria

For identification of beta-lactamase, producing bacteria was used nitrocefin disk (8).

Identification of ESBL-producing bacteria

The combined disc method consists of comparing the inhibition zone diameter on a disc of a cephalosporin with and without clavulanic acid was used. 10 µg of clavulanic acid are added to the cefotaxime disc (30 µg) and the ceftazidime disc (30 µg). If the strain is an ESBL producer, the inhibition zone for the disc with clavulanic acid increases by≥ 5 mm compared to that of the disc without an inhibitor (5).

Identification of MBL producing bacteria

To identify the MBL, standard microbial suspension, uniform culture was prepared in a Muller Hinton agar medium, then two Imipenem disks 10 µg, spaced 3 cm apart, one of the disks impregnated with 10 µl EDTA 0.5 M and plates were incubated for 18 h at 37° C. Finally, the diameter of the inhibition zone of the bacteria was compared with each other, and the specimens with a diameter of the inhibition zone of the imipenem plus their EDTA were 7≥mm in comparison to the imipenem alone disk, was considered as the metallobetalactamase producer (9).

PCR

DNA was extracted by a High Pure PCR Template Preparation Kit according to the manufacturer's protocol.

The primers related to this gene were designed by AllelID 6 and were synthesized by the Pishgam company.

PCR reaction was done in Thermocycler instruments (TECHNE) with oligonucleotide primers including F: 5'-TCAGGACAAGATGGGCGGTATG-3' and R: 5'-CATTGGCGGCGAAAGTCAGC -3' for *NDM1* gene and L_1 gene F: 5'-ATGCGTTCTACCCTCGCCTTCGCCCTG-3' and R: 5'-AGCGGGCCCCGGCCGTTTCCTTGGCCAG -3', the products size were134 and 245 bp respectively.

Thermal cycler program for NDM_1 gene included

Initial denaturation for 4 minutes under 94º C, 35 times denaturation under 94º C for 30 seconds, Annealing for 1 minute under 55ºC, Extension for 40 seconds under 72º C, and Final Extension for 5 minutes under 72º C.

The thermal cycle for investigation of the L_1 gene included Initial denaturation for 5 minutes under 95º C, 35 times denaturation under 94º C for 30 seconds, Annealing for 30 seconds under 59ºC, Extension for 45 seconds under 72º C, and Final Extension for 5 minutes under 72º C. After the reaction, the production of both genes on Agarose gel 2% became electrophoresis.

In the end, it closed the tanks and Connected to an electrical power supply. It switched on the power supply and set it to a voltage of 85 mv for 45 minutes. After the end of the electrophoresis, the gel was examined from inside the tank, outside, and with the device (Gel doc). Presence of target bands**.**

For Sequencing was used to confirm strains containing the above genes. Samples were sent to the Macro Corporation in South Korea for sequencing, and the results were analyzed using the NCBI and blasting site, also compared to the Main Workbench5 CLC.

Results

Antibiotic susceptibility results in the disk diffusion method

Antibiotic susceptibility results in the disk diffusion method shown in Table 1. The most effective antibiotics were tigecycline and gentamicin (100%).

Table 1. Antibiotic susceptibility of *Stenotrophomonas maltophilia* isolates by disc diffusion method

Antibiotic	Resistance (R)	Intermediate (I)	Sensitive (S)
Gentamicin	4.34%	-	95.65%
Cefepime	-	-	100%
Cotrimoxazole	30.43%	30.43%	39.13%
Chloramphenicol	4.34%	8.69%	86.95%
Piperacilin-Tazobactam	8.69%	-	91.30%
Minocycline	8.69%	4.34%	86.95%
Aztreonam	-	4.34%	95.65%
Levofloxacin	56.52%	3.04%	30.43%
Ceftazidime	4.34%)	-	95.65%
Tigecycline	26.08%	17.39%	56.52%
Ciprofloxacin	-	-	100%

The following antibiotics included ciprofloxacin (95.65%), minocycline (95.65%), levofloxacin (95.65%), chloramphenicol (91.30%), and piperacillin-tazobactam (86.95%), respectively were.

The highest antibiotic resistance was observed with aztreonam (56.22%), followed by cefepime (30.43%) and ceftazidime (26.08%), respectively. The highest half-sensitivity results were seen with Cephem antibiotics (30.43%), ceftazidime (17.39%), and aztreonam (13.04%), respectively.

After detection of the beta-lactamase enzyme, 19 isolates (82.66%) were positive beta-lactamases, and 4 isolates (17.39%) were negative beta-lactamases. After examining ESBLs, 9 isolates (39.13%) were positive, and after detection of Metallo-β-lactamase enzymes, 14 isolates (60.86%) were positive.

The isolates tested by the phenotypic test for Metallo-β-lactamase were tested for the presence of a new gene for resistance to NDM1. In none of the isolates, the primers designed in these studies, which were shown to be Best (Best) by AllelID 6.0 software, NDM1was not observed. Of the 14 isolates that were positive for the Metallo-β-lactamase test, 9 isolates (39.13%) were negative for this gene, and 14 (60.86%) isolates were positive. The results of the PCR test on the L1 gene formed in the 254 bp region.

The sequencing results confirmed the bacterial gene after blasting the NCBI site. The sequence similarity of the samples was compared with CLC Main Workbench 5 software (Figure 1).

Thirteen isolation of the L1 gene of S. maltophilia were recorded in NCBI site with accession number: KX359183-KX359195 under S. maltophilia Metallo-beta-lactamase L1 gene partial cds.

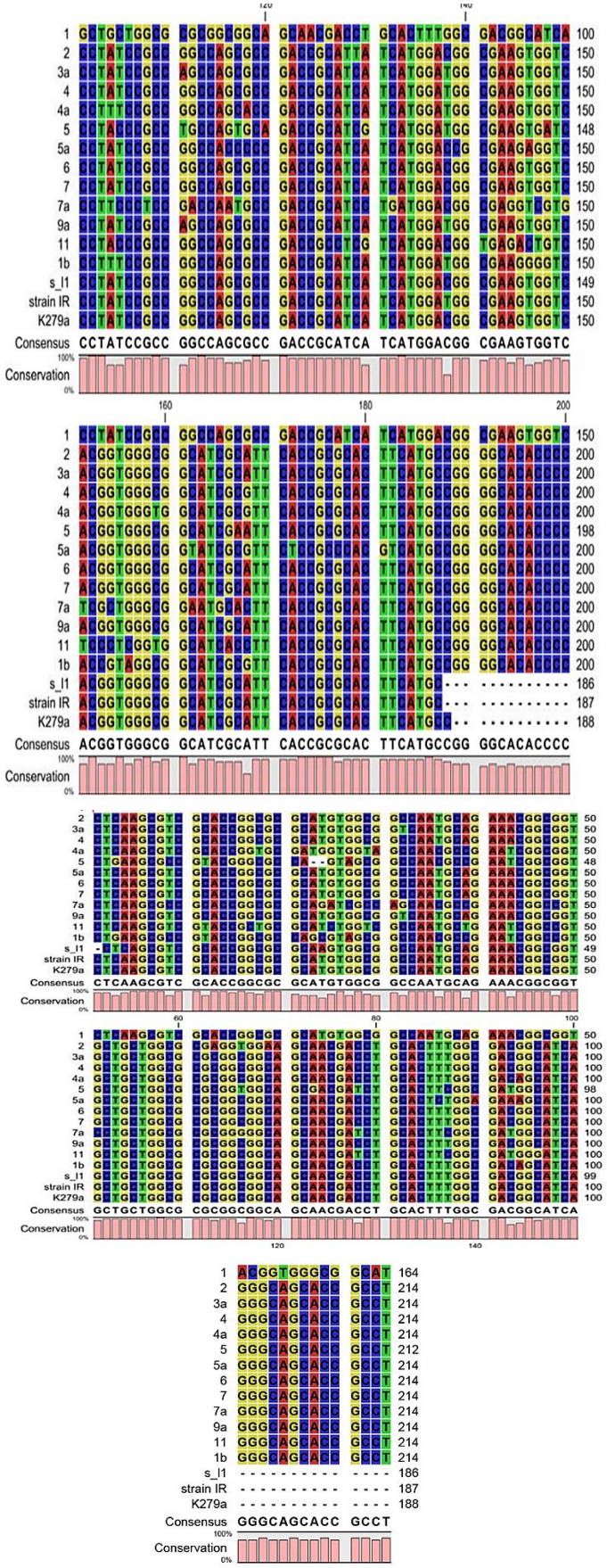

Figure 1. The amount of similarity of the gene nucleotide sequence of Stenotrphomonas maltophilia by CLC Main Workbench 5 software

Discussion

In the present study, the most antibiotics resistant bacteria of *S. maltophilia* by disc diffusion method were aztreonam (56.22%), cefepime (30.43%), and ceftazidime (26.08%). The most effective antibiotics against *S. maltophilia* were tigecycline and gentamicin (100%), and minocycline, levofloxacin, and ciprofloxacin (95.65%).

Also, in this study, 82.76% of the isolates were β-Lactamase positive, 39.13% of isolates were ESBL, 60.86% of the isolates were MBL, and 21/73% of the isolates were both ESBL and MBL.

Increasing and spread of ESBL-producing bacteria causes several problems, such as inappropriate use of antibiotics, inappropriate prescribing of the drug, long-term hospitalization of the patients in the hospital, and finally, the transfer of ESBL-producing genes by transmissive agents such as plasmid and integron in health centers (10).

Beta-lactams, alone or in combination with β-lactam inhibitors, generally exhibit a slight activity against *S. maltophilia* since this organism has a high intrinsic resistance to penicillins, cephalosporins, and carbapenems (11,12).

It seems that intrinsic resistance is obtained from natural environments, and all of them cannot be considered due to the use of antibiotics in clinical and medical centers.

In the environment, due to the use of detergents, herbal toxins, and the use of chemicals in factories, and the entry of these substances into sewages and waters, the bacteria are in contact with these materials and transport the genes of antibiotic resistance. The environmental contamination with antibiotics promotes the development of antibiotic-resistant bacteria and, in addition, provides an opportunity to obtain the drug resistance of other pathogenic bacteria (1).

In the present study, resistance to cotrimoxazole was observed at 34.4%. In a study that obtained results from countries in Europe, Latin America, and North America, the resistance to this antibiotic was reported to be 3.8% (13,14), and Chung *et al*. In 2013 reported resistance rates of 6% (15) that the present study is somewhat similar to the research mentioned.

The results of this study showed that resistance to levofloxacin was 4.34%. In a study by Kanamori *et al.*, in 2015 (16), the resistance to levofloxacin was 6.1%, and similar to the results of Zhang *et al*., 2012 in China (17), the results of the present study are similar to the results of the two studies are mentioned.

The results of this study showed that the isolates were sensitive to antibiotic minocycline (95.62%). In a study by Chang *et al*., (2012) (18), as well as Neela et al. In 2012 (19), the isolates of *S. maltophilia* were sensitive to minocycline, as well as in studies on this bacterium in Taiwan (20), Brazil (14), Spain (21), and the United States (7). The same results were obtained for minocycline sensitivity. All the results are much similar to the results of this study.

Another antibiotic used in this study was tigecycline, the first glycylcycline that has a clinical application (22). In the present study, the sensitivity to this antibiotic was 100%. These results were consistent with the report given by Chang *et al*., in 2012 (18), tigecycline and minocycline and the active agents against *S. maltophilia*, these antibiotics, which are newer and less commonly used, can be considered for treatment.

Few isolates are susceptible to beta-lactam due to the lack of β-lactamase enzymes. In 20% of cases, resistance is due to the creation of mutations that are still not recognizable and lead to high levels of β-lactamase production, and in other cases, there is no reason to produce β-lactamases and, like other bacteria, may be involved diffusion or efflux pump mechanisms (23).

In the present study, all isolates of Metallo-β-lactamase-positive were tested for the L1 gene by PCR method, and 60.86% of the isolates were positive for this gene. In a study by Samanatha *et al*., in 2013, the L1 gene was found to be 71.1% in the isolates of *S. maltophilia* (24).

A similarity was found between the two studies. In a study by Yang *et al*., in 2014, patients (20.95%) were positive for the L1 gene (25). The present study showed a higher level of the L1 gene due to the higher prevalence of this gene in bacteria in this region and its spread through various sources. These bacteria are often found in the water resources of hospitals and can infect hospital devices and patients. Therefore, need for an antimicrobial resistance program for the presence and spread of *S. maltophilia* in the community and health care units.

A study by Berrazeg *et al*., in 2014 (26), summarized the results of various studies from December 2009 to December 2012 in 55 different countries. Of the 950 isolates of *S. maltophilia*, 5 samples (0.5%) had this gene.

Most isolates of the NDM-1 enzyme producer were from India, Pakistan, and China, and from Iran; one case of this gene was reported in Tehran in 2011, which was found in *Klebsiella pneumonia*. The actual amount of the NDM-1-producing bacteria will likely be more than that

reported since most countries have not systematically monitored programs for testing of infections caused by relatively high resistance bacteria, and also, many bacteria have not been tested for NDM-1 enzyme production (26).

Fortunately, in this study, this resistance gene (NDM-1) was not detected by PCR in any of the isolates, which may be because of this tourist less this area. Also, this study was carried out on a small sample and was a cross-sectional study.

Given that *S. maltophilia* appears to be a major pathogenetic agent worldwide must be considered antibiotic resistance control, survival, and development and health care programs in the community. An increase in people with an immunocompromised in the world due to AIDS and chemotherapy is also expected and necessary for the global control of the drug resistance of opportunistic pathogens such as *S. maltophilia* and the identification of the genetic transfer pathway that occurs between different species of bacteria

Also, with ESBL-positive isolates, this issue should be taken into account as it increases antibiotic resistance. Due to antibiotic resistance, is recommended antibiotic susceptibility testing for treatment.

In this study, the best antibiotics were tigecycline and minocycline, and it is better not to use beta-lactam antibiotics. In patients with cystic fibrosis who use more antibiotics, alternative therapies should be considered. Highly osmotic saline solutions and regular treatment with the recombinant DNase enzyme (rhDNase) can be considered for the treatment of this issue should be taken into account as it increases antibiotic resistance. Due to antibiotic resistance, is recommended antibiotic susceptibility testing for treatment in patients with cystic fibrosis.

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