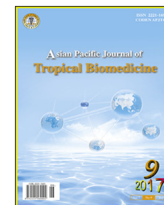
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb



Original article http://dx.doi.org/10.1016/j.apjtb.2017.08.012

Isolation of *Stenotrophomonas maltophilia* from clinical samples: An investigation of patterns motility and production of melanin pigment

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ARTICLE INFO

Article history:
Received 8 Oct 2016
Received in revised form 14 November, 2nd revised form 28 November, 3rd revised form 19 December 2016
Accepted 14 Aug 2017
Available online 18 Aug 2017

Keywords:
Stenotrophomonas maltophilia
23S rRNA
Melanin
Motility

ABSTRACT

Objectives: To investigate possible sources of *Stenotrophomonas maltophilia* (*S. maltophilia*) in the clinical environment.

Methods: Different samples were collected from Amol City of Iran. Steps for the identification of *S. maltophilia* included culturing, biochemical tests, polymerase chain reaction (PCR) of *16S rRNA* gene and *23S rRNA* gene. In addition, production of melanin pigment and patterns of motility of the bacteria, were also investigated.

Results: In our study, 20 *S. maltophilia* strains were isolated from clinical sources, oxygen manometer apparatus of hospitals were 7/110 (6.36%), blood was 1/777 (0.13%), sputum was 4/40 (4%), urine was 1/2 947 (0.03%), tap water was 1/240 (0.42%) and dental suction was 6/120 (5%). The isolated bacteria showed production of melanin pigment with rates of strong, moderate, weak, and lack of pigment. Types of motilities were seen in isolates.

Conclusions: The highest percentage of bacteria is isolated of oxygen manometer system and dental suction, yet has not been reported from oxygen manometer system. These bacteria have also been associated with patients who have respiratory problems, so it is essential for staffs of hospitals to draw attention to this source of bacteria.

1. Introduction

In spite of efforts to survey and treat infectious diseases, opportunistic pathogens still play an important role in disease [1]. *Stenotrophomonas maltophilia* (*S. maltophilia*), a globally emerging non-fermenting gram-negative bacterium, is associated with most human infections [2].

These bacteria are widely found in soil and water, and can cause serious problems in immune compromised patient [2]. Intrinsic and acquired multi-drug resistance have led to many problems in patient treatment and care processes [3]. All in all, multi-drug resistant bacteria are considered as one of the leading factors contributing to patient mortality [2].

This bacterium is regarded with low virulence but can cause serious complications and also has a considerable mortality rate in comparison to other nosocomial infections [4]. *S. maltophilia* is derived from the environment, and isolated from the aquatic environment, the rhizosphere of plant, animal, foods and liquids used in medical care. Infections occur in both children and adults. Transmission to susceptible individuals may occur through direct contact with the source of the bacterium. Hands of health care workers can transmit nosocomial infections from bacteria found in the intensive care unit [5].

Bacteria were isolated from clinical sources such as hospital suction turbine [6], dental suction system [2], dental solid waste [3], hand-washing soap [2], sink drains [1], tap water from the hospital [2], as well as from environmental sources such as tap water [5], river water [2] and plant rhizosphere [2]. The increase in reported cases of infections with this bacterium makes it necessary to study possible sources. Since there is no

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Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

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comprehensive survey on *S. maltophilia* in Iran, identification of *S. maltophilia* and clinical source investigation is an important issue, particularly due to the risk of fatalities.

2. Materials and methods

2.1. Sample collection

Cross-sectional sample collection was performed from January 2015 to June 2015 for 6 months. Samples were collected from urine, blood and sputum of patients who were admitted to Imam Ali hospital in Amol, as well as swabs of oxygen manometer apparatus and tap water in Imam Ali, Imam Reza and 17 Shahrivar hospitals. Also, dental suction samples were taken from the dental offices in Amol City.

2.2. Isolation and identification of bacteria

After the initial culture of urine, blood and sputum samples in blood agar, MacConkey agar and EMB agar medium (Merck) [2], the gram negative bacilli was detected and cultured on selective medium agar and steno medium agar [7]. Dental suction swabs and swab samples were transferred to trypticase soy broth at first (Himedia) and after 24 h.

After growth of bacteria, biochemical tests including catalase, oxidase (Patan teb), indole (Merck), motility (Merck), hydrogen sulfide (Merck), methyl red (Merck), voges proskauer (Merck), lysine decarboxylase (Himedia), bile esculine (Biolife), starch, urea, DNase (Himedia) and fermentation of sugars in TSI agar (Merck) was performed.

2.3. DNA extraction

DNA template was extracted by the boiling method. Bacteria were cultured in Luria Bertani broth and incubated at 30 °C for 24 h. The suspension of 1.5 µL was centrifuged at 7 000 rpm for 1 min. The supernatant was discarded and 100 mL deionized water was added to the precipitate, boiled at 95 °C for 30 min. Then 20 µL tris–HCL, 1 moL (pH 7.5) was added and centrifuged at 10 000 rpm for 1 min. The supernatant was transferred to a sterile microtube.

2.4. Materials for PCR reaction

The final reaction volume was 25 µL, including 5 µL of DNA template, 0.2 µL of *Taq* DNA polymerase, 1 µL of each primer forward and reverse [(primers F and R of *16S rRNA* gene) include F: AGTTTGATCCTGGCTC, R: CCTACGTATTACCGCGGC [8] and (primers F and R of *23S rRNA* gene) include F: GCTGGATTGGTTCTAGGAAAACGC R: ACGCAGTCACTCCTTGCG] [9], 0.5 µL of dNTP, 2.5 µL of buffer (10×), 0.75 µL of $MgCl_2$ and 14.5 µL deionized water.

2.5. PCR

Thermo cycler instruments were used. The thermal cycle programme for *23S rRNA* gene included: initial denaturation at 94 °C for 4 min, denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min, extension at 72 °C for 35 s with 35 cycle and final extension at 72 °C for 5 min. For *16S rRNA*: initial denaturation at 94 °C for 4 s, denaturation at 95 °C for 45 s, annealing at 57 °C for 1 min, extension at 72 °C for 1 min with 35 cycle and final extension at 72 °C for 5 min. To evaluate the PCR product, a 2% agarose gel was used with ethidium bromide. For PCR product, the electrophoresis was performed. PCR products of *23S rRNA* gene that were isolated from different sources were sent to the Macrogen Company of Korea for sequencing. The sequences were determined and identified using BLAST, NCBI.

2.6. Melanin production

Bacteria were cultured on LB agar (Scharlau) with 0.2 g L-tyrosine. Results were reported as 0, 1+, 2+ or 3+ in a semi-quantitative way. Samples with greater than 2+ were considered as a high pigment-producing strain [10].

2.7. Motility patterns of *S. maltophilia*

Swimming motility: bacteria were cultured on trypton broth (Biolife) with 0.3% (w/v) agarose (Merck) by a sterile toothpick, as well as swim plates which were inoculated with bacteria from an overnight culture in LB agar plate at 37 °C. Then plates were wrapped with saran wrap to prevent dehydration and incubated at 30 °C for 12–14 h.

For swarming motility, plates were dried at room temperature overnight before being used, and nutrient broth (Himedia) was used that consisted of 0.5% (w/v) agar (Himedia) and 5 g/L Glucose (Merck). After culturing, they were incubated for 24 h at 37 °C.

For twitching motility: bacteria were cultured on LB broth (Scharlau) with 1% (w/v) agar (Himedia) by a sharp toothpick of the plate from on overnight-grown LB agar (Scharlau) and were incubated at 37 °C for 24 h. Results were reported as 0, 1+, 2+, 3+, 4+ as a semi-quantitative procedure and by the basis of measuring the zone of motility in the agar [10].

Data was analysed by SPSS software version 16. To analyse the data descriptive statistics (frequency, percentage, mean), analysis of variance (One-Way-ANOVA) and comparison of means using Duncan's multiple ranges ($P < 0.05$) tests was performed. The relationship between the variables was performed through Pearson correlations coefficient test, P values of <0.05 were considered as significant.

3. Results

In this study, 20 *S. maltophilia* strains were isolated from multiple clinical sources, including oxygen manometer apparatus of hospitals: 7/110 (6.36%), blood: 1/777 (0.13%), sputum: 4/40 (4%), urine: 1/2 947 (0.03%) and dental suction: 6/120 (5%). The results were negative for all of the following tests: indole, methyl red, voges proskauer, hydrogen sulfide, gas production of sugar, urea, starch, fermentation of sugars in TSI agar. The results were positive for all tests including catalase, bile esculine, DNase and lysine decarboxylate. Only the oxidase tests were both positive and negative, 4/20 (25%) of samples were oxidase positive and 15/20 (75%) of samples were oxidase negative.

3.1. PCR products

The PCR products from the samples identified by phenotypic methods as *S. maltophilia* were later confirmed with the molecular analysis based on *16S rRNA* gene that formed 569 bp fragments. The results of PCR with targeting *23S rRNA* formed 278 bp fragments.

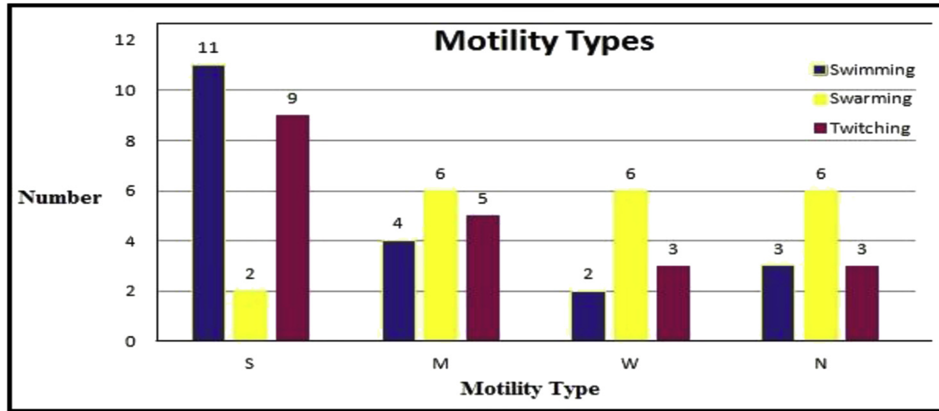

Figure 1. Comparision of motility types of *S. maltophilia* isolates.
S: Strong; M: Moderate; W: Weak; N: Negative.

Four out of twenty-four of samples were identified as *S. maltophilia* by culturing and PCR for *16S rRNA* gene, in next step were not confirmed by the molecular method with targeting *23S rRNA*. After blasting of *23S rRNA* gene sequencing of different samples in NCBI site, the results confirmed the bacteria as *S. maltophilia*.

3.2. Results of melanin production

Three samples had strong melanin production: 2 from oxygen manometer and 1 of sputum.

Eleven samples had moderate melanin production: 5 from dental suction, 1 of blood, 1 of urine, 2 of oxygen manometer and 2 of sputum.

Two samples had weak melanin production: 1 of dental suction and 1 of oxygen manometer.

Four samples had negative melanin production: 2 from oxygen manometers and 2 of sputum.

3.3. Results of patterns motility

The results of patterns motility were shown in Figure 1. The correlation between two types of swarming and twitching motility showed significant difference ($P < 0.05$). While, among the other types of motility there was no significant correlation ($P > 0.05$). The correlation between the two variables, swimming motility and melanin pigment production, showed that variables were independent ($P = 0.848$). The correlation between the two variables, swarming motility and melanin pigment production, showed that variables were independent ($P = 0.092$). The correlation between the two variables, twitching motility and melanin pigment production, showed that variables were independent ($P = 0.163$).

4. Discussion

S. maltophilia is found in some of the environmental resources such as surface water, waste water, soil and rhizosphere of plant, and is also seen in food, drinking water and contaminated liquids which used for medical care [11]. This bacterium is also found naturally in the air and on humid and dry surfaces, so there is a greater chance of patients' infection in hospitals. The major difficulty with these bacteria is its association with nosocomial infections as opportunistic pathogen [12].

Infection rate is low and it seems dependent on the patient's previous care such as using prosthetic devices [11]. Acquired infections from population are rare but have been observed. Skin is not thought of as a favourable environment for spread of the bacterium, and carrying of bacterium on the hands of hospital staff has not been proven. Although, it is commonly considered hands of hospital staff have no role in bacterium transmission, but *S. maltophilia* may accidentally be on the hands of staff and transferred to patient during care [13].

So for the patients in ICU who commonly are sensitive and at high risk, stopping the spread of the bacterium in the colonization stage is vital. Continuously improving health care measurements can lead to a diminished carrier and finally can reduce spread of bacteria in hospitals [13].

Infection with *S. maltophilia* is more likely with long-term mechanical ventilation, tracheotomy, and central venues, arterial and urinary catheters [14].

Based on these observations and the high levels of hospital infections, it seems a necessity to control the processes which will require a systematic program [13]. The necessity for the identification of pathogenic bacteria like *S. maltophilia* shows the need to boost improvement of health measurement and protection.

S. maltophilia are generally identified by selective media and biochemical methods, but sometimes, these tests are not able to detect these bacteria from other gram negative bacteria, therefore, the application of molecular methods in this field have special value [9].

Conventional PCR is highly sensitive and can detect infections which are caused by gram negative bacteria such as *S. maltophilia* [9], especially when the bacteria are causing infections in cancer patients. In such a circumstance, the correct diagnosis is very useful for controlling disease and reducing mortality.

The application of PCR has a limitation to distinguish *S. maltophilia* with *16S rRNA* gene as target. Due to considerable genetic similarity between *S. maltophilia* and other nonfermentative gram negative bacilli, this method shows low specificity [9], but the use of PCR to identify *23S rRNA* is more efficient. This is because the *23S rRNA* gene shows more variety in this area between *Stenotrophomonas* species. The primers which were used in this study, are not only useful to identify *23S rRNA* gene, but are also useful for accurate diagnosis [9].

Molecular methods can provide a fast identification result, using appropriate antimicrobial therapy, which leads to effective treatment of infections caused by these microorganisms.

In this study, at first, the *16S rRNA* gene was used to identify the bacteria, which confirmed presence and DNA extraction of bacteria. But for decisive and correct diagnose of bacterium, *23S rRNA* gene was studied by conventional PCR.

Bacteria identification steps were performed by culturing, biochemical tests and finally molecular methods. Three out of twenty-three samples that were identified as *S. maltophilia* by culture methods and biochemical test were not confirmed by any molecular method, which highlighted the problems with false positive with the culture method.

In 2013, Wagner Gallo *et al.* [9] completed a survey on *S. maltophilia*, which is a hospital pathogenic bacterium with multi-drug resistance. They used conventional PCR and real-time PCR to detect *23S rRNA* for identification of *S. maltophilia*. And the results showed when bacteria were detected with Vitek system, PCR method showed high efficiency [9].

In our research, 4/20 (20%) of isolates were oxidase positive, akin to results found by the Carmody study and colleagues showed that almost 20% were oxidase positive from 766 isolates of *S. maltophilia*. Some researchers have shown that *S. maltophilia* can be isolated with negative oxidase [15,16].

In a study carried out by Lanotte *et al*. [13], there was a considerable increase of *S. maltophilia* colonization in tracheal tubes. Cross-colonization was also confirmed with molecular methods. With the improvement of health assessments, carriers are isolated, although environmental sources of bacteria have not been known; these measurements could stop the spread of these multi-resistance bacteria among PICU patients in hospital. Frequently reports, once every two weeks, to look for bacteria colonization in tracheal tubes, will determine more pulmonary pathogenic bacteria. And reports should be more frequent in outbreak periods [13].

Looney and colleagues, in 2009, showed although *S. maltophilia* is not pathogenic, it can be present in epithelial cells of the respiratory tract, on medical equipment surfaces and infected hospital patients [17]. In this study, these bacteria were isolated from 4% sputum samples that compatible with Looney study.

In a study by Adjidé *et al.* [18] on *S. maltophilia* and *Pseudomonas aeruginosa*, these two bacteria were known as the major pathogenic and opportunistic bacteria originating from a water source, which play an important role in nosocomial infection. These two bacteria were seen in 10.7% and 21.3% of swabs from tap water respectively. *P. aeruginosa* was found at 7.4% swab samples and *S. maltophilia* in 2.5% of swab samples [18].

In a recent study, *S. maltophilia* is isolated from 0.42% of swab of tap water, which is lower than the results from the Adjidé study.

In a study performed by Di Bonoventura and colleagues in 2006 in France, all of 40 isolated *S. maltophilia* showed the swimming motility, but the swarming and twitching motility was not seen in all bacteria [19].

In this study, 3/20 (15%) of those isolated were lacking swimming motility, 6/20 (30%) were lacking swarming motility and 3/20 (15%) showed lacking of twitching motility.

Adamek and colleagues in Switzerland reported a significant difference between swimming and twitching motility in 2011 [20]. In this research, a significant difference between swarming and twitching motility was seen ($P < 0.05$), but difference between other types of motility were not significant ($P > 0.05$).

In this study, motility was divided into strong, moderate, weak and negative. The numbers of bacteria were highest for strong swimming motility and were lowest with strong swarming motility. The numbers of samples were highest for bacteria with weak swarming motility and the least number of samples was found for bacteria with weak swimming motility. The numbers of bacteria that have no swarming motility were greatest and bacteria with no swimming and twitching motility were similar.

In a study conducted in 2014 by Thomas and colleagues, all of the 108 bacteria were able to move. According to their results, swimming and swarming motility were usual but, there was not any certain twitching motility [10].

In a study conducted in 2011 by Pompilia *et al*., *S. maltophilia* that were isolated from clinical and non-clinical sources, out of 89 samples, 5 clinical samples and 4 non-clinical samples had no movement. Of course, only swimming and twitching motility were examined [21]. According to the results of different researchers, different motility patterns were observed.

Regarding the melanin pigment, our study showed 4/20 (25%) isolates of *S. maltophilia* had no melanin pigment; 3/20 (15%) had strong melanin pigment production; 11/20 (55%) had moderate melanin pigment and 2/20 (10%) had weak melanin pigment production. The isolates lacking in melanin pigment on blood agar was unlikely to have other strains. No green pigment was found and white, milky, yellow and grey were seen.

In a study conducted in 2014 by Etinosa *et al*., 51 of the 96 samples made strong melanin pigment, and 45 of the 96 samples produced weak melanin pigment, which is in contrast to our study [22].

Our studies showed the *S. maltophilia* were isolated from various sources with differing percentages. The highest percentage of bacteria was isolated from oxygen manometer system and dental suction, yet has not been reported from oxygen manometer system. As these bacteria have also been associated with patients who have respiratory problems, it is essential for staffs of hospitals to draw attention to this source of bacteria. The molecular methods, with *23S rRNA* gene as target, are more specific according to our work. The relation between different patterns of motility and the production proportion of melanin pigment with pathogenesis of bacterial is necessary to be investigated.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was funded by Islamic Azad University Karaj Branch (grant number: 1193). We are grateful to Imam Ali, Imam Reza and the staff from the 17 Shahrivar hospitals, and also the molecular lab staff from the Pasteur Institute of Iran (North research Center) for their sincere cooperation.

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