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Bactericidal activity of pigments isolated from Fars province (Iran) environmental bacteria on MDR clinical isolates of *Acinetobacter*

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ABSTRACT

Antimicrobial agents are important compounds in reducing infections, but the spread of resistant pathogens diminished the effectiveness of these compounds. This study aimed to isolate the local bacterial pigments with specific antibacterial activity against clinical *Acinetobacter baumannii* isolates in burn centers. For this, pigmented bacteria isolated from environments in Fars province (Iran) and adjoining areas during Jun-July of 2017. Colonies with various pigment colors were isolated in pure cultures on nutrient agar. After identification of the pigmented bacteria, the extracted pigments were evaluated for antibacterial activity against previous confirmed clinical isolates of *Acinetobacter baumannii* with multi-drug resistant specification. Red pigment extract from *Serratia marcescens* (Prodigiosin) has the most proper inhibitory effect on the studied *Acinetobacter baumannii* isolates. The related bacterium was isolated from a salty region entitled Maharlo Lake. The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) value of the pigment extracted from selected bacterium ranged from 2500-5000 µg/ml and 1250-2500µg/ml, respectively. The extracted pigments had a different antimicrobial activity against the studied clinical isolates, but on the basis of MBC/MIC results, the extracted prodigiosin pigment was the most effective anti-Acinetobacter agent. However, further studies are needed to use this pigment as a new antimicrobial agent or disinfectant on burn wounds.

1. Introduction

Various types of diseases are serious threats for human beings; thus, scholars have long been seeking for natural compounds from plants, animals, and other resources for their treatment. Statistically, at least 50 percent of medicines used for treating human diseases are made of natural products which are mostly produced from soil organisms (Joshi & Attri, 2006).

Nowadays, the prevalence of resistant nosocomial infections is going to be a great problem in the health systems in the world. According to the last report of the World Health Organization in 2017, it has been mentioned that many groups of bacteria, especially those which are the most important in hospital-acquired infections, are not controlled by routine anti-microbial agents and they pose the greatest

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threat for the human community (WHO, 2017). According to the importance of infection control in the health systems, it is about more than a century that scientists and clinicians are working on different and new compounds which can inhibit the growth of infectious agents with the minimum side effects on the host. One of these natural compounds is obtained from microorganisms pigments, which have attracted considerable attention today. Overall, it can be stated that pigments produced by microorganisms have medical properties including anti-oxidant, cytotoxic, anti-leishmanial, anti-ulcer, anti-viral, anti-tumor and anti-bacterial effects (Gupta et al., 2011; Joshi & Attri, 2006). For example, Violacein pigment produced by *Chromobacterium violaceum* has medical functions such as antimicrobial activity against microorganisms resistant to the routine drugs (Lopes et al., 2009). The development of anti-bacterial resistance by a bacterial strain is normally due to lack of correct use of these compounds which will subsequently lead to ineffective treatment. Also, it may occur in countries that are economically troubled due to improper access to medicines. The most serious gram-negative infections are those that are associated with the health care system and known as hospital-acquired infections. The most common pathogens in this filed are *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter* ((CDC), 2016). *Acinetobacter* is a non-fermenting gram-negative coccobacilli, and one of the most common causes of nosocomial infections, especially in patients admitted to the ICU and patients with impaired immune systems (Kabbaj et al., 2013). *Acinetobacter baumannii* is the most important clinical species of *Acinetobacter* (Endo et al., 2012). In the past two decades, due to remarkable ability of this bacterium to overcome the most routine and used anti-microbial agents in treatment of hospitalized patients, it has been become a significant clinical problem in hospitals (Boulanger et al., 2012; Hashemizadeh et al., 2010; Pirbonyeh et al., 2016). This bacterium is raised as the second leading cause of nosocomial infections among gram-negative bacteria after *Pseudomonas aeruginosa*, especially in burn centers (Fazeli et al., 2012; Pirbonyeh et al., 2016). At the first detection of *Acinetobacter baumannii* in hospitals, the choice of drug for treatment was carbapenems, while this bacterium is extensively resistant to this group of anti-microbial compound (Higgins et al., 2010a). Due to the occurrence of multi-drug resistance in this microorganism, serious challenges have developed for the treatment of the resulting infections, so the purpose of this study was to evaluate the antibacterial effect of bacterial pigments on *Acinetobacter* sp. isolates from burn wound infections. The group of patients was selected from burn patients hospitalized in Amir-Al-Momenin burn hospital affiliated with Shiraz University of Medical Sciences (SUMS).

2. Materials and Methods

2.1. Sampling for pigmented bacteria

In this experimental study, seventy environmental samples were collected from the soil and air in different regions of Fars province, (south of Iran). This region was selected because of its geographical and climatic variation which causes varieties of niches and wildlife of different micro-organisms with different specifications.

Soil samples were collected from 10 cm depth from the surface and for air sampling; active sampling was performed with an Air system sampler (SAS, International PBI, Milan, Italy). Air samples were taken at a flow rate of 180L/min in the middle of the day. All of the samples were cultured on microbiological agar plates for isolating the pigment-producing bacteria. Preparation and cultivation of the samples were performed by the method mentioned before (Caroline et al., 2013; Napoli et al., 2012).

In brief, for soil assay, 1gr of each sample was suspended in 9ml normal saline and 100 microliter of one to one hundred dilutions were inoculated on nutrient agar plates with spread plate method. For Air sampling, active monitoring was used and the air of the selected points was forced on the collection medium (nutrient agar) over a specified time and volume which is mentioned before. Inoculated plates were then incubated for 48 to 72 hours in 37 centigrade degrees. After the incubation time, the colonies with most intense color were selected and a pure new culture was prepared from each one for the identification and pigment extraction tests.

2.2. Identification of Pigmented Bacteria

According to the aim of the study in involving known pigmented bacteria, routine bacteriological procedures were used. In order to identify the selected pigmented bacteria, pure cultures were examined by the method mentioned before (Ahmad et al., 2012). In brief, each culture was tested for color, clonal morphology, Gram staining, and standard microbiological biochemical tests, including Catalase, Oxidase, Phenylalanine deaminase, Triple Sugar Iron Agar pattern, Citrate, SIM, Urease, MR-VP, Ornithine decarboxylase, Oxidative-Fermentative pattern, DNase (Versalovic et al., 2011). To decrease the confirmation steps of the isolates identification, we first tested the determined isolates for anti-microbial activity and selected the isolates with high or proper activity followed by molecular and sequencing base confirmation tests.

2.3. Acinetobacter isolation from burn wounds

According to the importance of *Acinetobacter baumannii* in health settings especially in burn this bacterium was selected for this antibacterial study. Either based on the recent WHO reports this bacterium is almost resistant to all routine antibiotics and is one of the serious threats among nosocomial infections. For this, four multi-drug resistant (MDR) *Acinetobacter baumannii* were nominated for evaluating the antibacterial effect of purified pigments.

Selected bacteria were isolated before from hospitalized burn patients in Amir-Al-Momenin burn hospital. This hospital is the main burn center of the southwest of Iran and is affiliated with Shiraz University of Medical Sciences (SUMS). These isolates were collected from wound samples prepared from routine sampling of hospitalized patients as a part of patient's treatment procedure. Positive growth cultures were tested by routine microbiological tests and primary detected *Acinetobacter* species were confirmed with the molecular method as described before in a study performed in burn and wound healing research center (Dasmeh et al., 2015). Then, the confirmed isolates were checked for Anti-microbial resistant pattern (ARP) for the recommended drugs by clinical laboratory standard Institute (CLSI) (CLSI, 2016a). The ARP test was determined by the Disk diffusion method (Kirby-Bauer) on Mueller-Hinton agar and discs were obtained from MAST (Bootle, UK).

2.4. Pigment Extraction:

For this purpose, the identified pigmented bacteria were separately cultured purely in Mueller-Hinton broth for 48 hours. Then, both the supernatant and bacterial cell pellets were extracted for pigment according to the procedure introduced before (Ahmad et al., 2012). In brief, the supernatant and pellets were extracted separately by using 99.5% acetone. For the supernatant, the extraction was achieved in the ratio of 1:5 (supernatant) and for the bacterial cell pellets, the suspensions were shaken until the pellets were seemed colorless. The extracted pellets were discarded and the supernatant was concentrated by using rotary evaporator (EYELA-SB1200, Japan) at 50°C with chiller temperature set at below 10°C. Concentrated pigments were dried onto glass Petri dishes for three days at 60°C. The purity of the extracted pigments was checked by Thin Layer Chromatography (TLC) with Silica gel aluminum sheets (Merck, $60F_{254}$). The developing process was performed in a mixture of benzene: acetone with the ratio of 2:1. The following procedure was performed by a technician with a protocol recommended in the previous studies (Ahmad et al., 2012; Min-jung et al., 2006).

2.5. Antimicrobial Activity of the Extracted Pigments

In this step, 5mg of the powdered form of crude extracts was dissolved in 1ml of DMSO and used as the main and strongest solution for each pigment. The measurement of the anti-microbial activity of the extracted pigments, agar disk diffusion method was used. For this, 0.1 ml of above solutions were inoculated to standard blank disks (Padtan Teb Co., Iran) with 6.4 mm sizes separately and dried under sterile conditions. Tested *Acinetobacter* isolates were cultured in Tryptic Soy Broth (TSB) and incubated at 36°C for about 3 hours for a turbidity equivalent to the 0.5 McFarland standards. From these cultures, a spread plate was prepared on Mueller-Hinton Agar with a

sterile swab. The prepared disks were placed on the inoculated plates with 30mm distance from each other and the edge of the plates by using aseptic precautions. According to the recommendation for *Acinetobacter* spp. growth condition, the examined plates were incubated at 36˚C for 20 hours. After the incubation time, the complete inhibition zones around the disks were measured and recorded in diameters. A disk impregnated with methanol was used as negative control for each test isolate.

2.6. Minimum Inhibitory/Bactericidal Concentration (MIC/MBC) of Pigments

The pigments with an appropriate inhibition zone were evaluated either for Minimum Inhibitory Concentration on test *Acinetobacter* isolates using micro-dilution method recommended by CLSI (CLSI, 2016b). The prepared solution in previous step (5mg crude in 1ml DMSO) was used as strength solution and five dilutions of one to two were prepared in the following row wells. Inoculated wells were adjusted to approximately 5×10^5 CFU/ml in a 100µl final volume, and microtiter plates were visually scored after incubation time (36˚C for 20 hours). The results of the MIC were interpreted according to the CLSI breakpoint criteria. To control contamination, two wells were filled in each row with broth medium alone and broth medium with extract, respectively. Either for growth control of the test bacteria (*Acinetobacter*) one well was alone specified to the bacteria and culture medium.

For determining the minimum bactericidal concentration (MBC) of the test compounds, 0.1ml of the culture medium from each well was determined as MIC of each extract. The compound was sub-cultured on fresh Muller-Hinton Agar plates with no inhibitors. Then, the two previous wells with higher concentrations and one well after with lower concentration were tested for bactericidal activity either. The inoculated plates were incubated for 20 to 24 hours at 36˚C and the growth condition was evaluated. No growth of bacteria on agar plates was recorded as bactericidal concentration, while the minimum was considered the MBC of the considered pigment.

2.7. Molecular Identification of Pigmented Bacteria with proper Antimicrobial Activity:

After determining the best pigments with proper anti-*Acinetobacter* activity, the prepared isolates were sub-cultured and extracted for total DNAs. The extraction was performed by Total DNA extraction kit (Accuprep Genomic DNA kit, Bioneer, Alameda, USA) according to the manufacturer's protocol. The extracted DNAs were used for confirmation of the isolates identification with molecular methods. For this step, PCR-sequencing method was used for amplified 16S rDNA genes. The amplification was performed in conventional PCR standard method with two forward (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse (5'-GGTTACCTTGTTACGACTT-3') primers which were introduced by the department of microbiology, Lab016 instruction (Microbiology, 2014). The amplified fragments with confirmed sizes were sliced from agarose gel and purified by gel extraction kit (Bioneer, Alameda, USA). The purified fragments were sent to the 1st step company (Malaysia) for sequencing. Sequencing was performed by the same primers used in amplification PCR, in two forward and reverse directions.

3. Results

In the phenotypic process of the soil and air samples, totally thirty-six isolates were determined with the power of pigment production. According to the conventional microbiological and biochemical tests, six isolates from different genera were selected for the following tests. According to the phenotypic screening tests, identified genera were known as *Pseudomonas* spp., *Serratia marcescens*, *Enterobacter* spp., *Flavobacterium* spp., *Micrococcus luteus* and *Staphylococcus* spp. The total of six pigments with blue-green, red, orange, yellow colors was extracted from these bacteria, respectively (Fig.1). The results for extraction of pigments by acetone solvent and concentration by evaporation apparatus are shown in Fig.2.

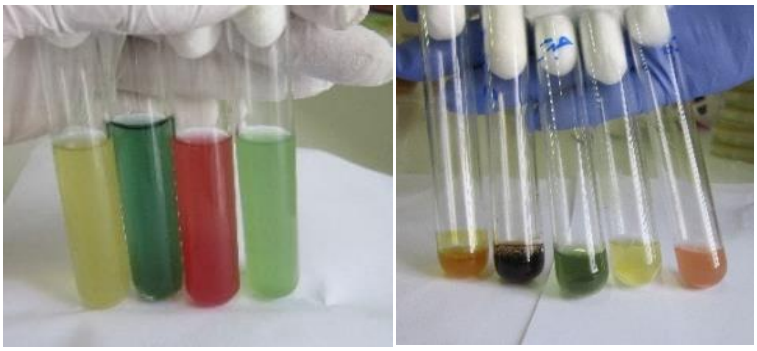
According to the Thin-layer chromatography (TLC) results for purity determination of pigments, as it resulted from one color spot on the TLC sheet in all pigments, it has been proved that all extracts were pure (Fig.3).

Among different pigment extracts, the only red pigment extracted from *Serratia marcescens*

showed a good antibacterial activity against all four tests MDR *Acinetobacter* isolates (Fig.4).

According to the MIC results, it has been shown that the crude pigment extract from *Serratia marcescens* has an inhibitory effect on all four MDR *Acinetobacter* isolates with a range between 2500µ g/ml to 1250µ g/ml, while the MBC activity of the extract was determined in a range between 5000µ g/ml to 2500µ g/ml concentration. In the direct PCR-sequencing step for confirmation of the selected isolate (*Serratia marcescens*) amplified 16S rDNA gene sequence was analyzed using BLAST with nr database of NCBI GenBank. According to these results, it has been confirmed that our selected and primary detected isolate which produced red pigment was *Serratia marcescens* with 100 percent similarity. This bacterium was isolated from a zone around Maharlo Lake. This lake is localized in the southeast of Shiraz (Fars province, Iran), and is rich in potassium and different salts.

Figure 1. Some selected isolates with capability of pigment production in nutrient medium



A **B**

Figure 2. Extraction of pigments by Acetone (A), pigments concentrated by rotary evaporator (B)

Figure 3. Thin layer chromatography results

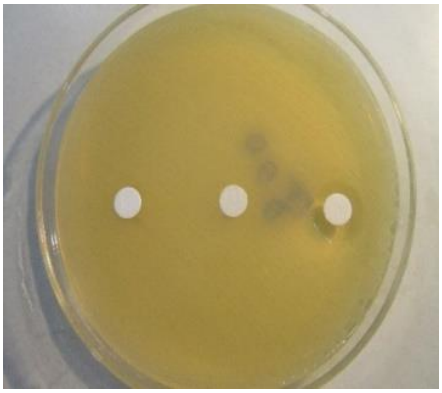


Figure 4. Antimicrobial effect of *Serratia marcescens* pigments on a sample MDR-*Acinetobacter*. First from left side: raw disk, middle disk: Acetone dried disk, and first from right side: impregnated disk with red pigment extract.

4. Discussion

According to the increasing prevalence of drug resistance among different bacterial species, especially hospital-acquired infection, nowadays finding new sources for compounds having the ability to inhibit such bacteria and treat resistant nosocomial infections with fewer side effects is a global concern. One of the sources used for finding new anti-microbial agents is the ability of self-bacteria to produce compounds or ingredients which have an inhibitory effect on other genera of micro-organisms. Due to different studies on bacterial mechanisms, it has been proved that the most bacteria have revealed many active mechanisms by which they can impair or kill other microorganisms (Hibbing et al., 2010b). Bacterial pigments are one of these compounds, for which several roles are considered. One of these activities which have been considered recently is the anti-bacterial activity of pigments present by producing bacteria (Kushwaha et al., 2014; Radjasa et al., 2009 ; Venila et al., 2013). According to the natural nature of these compounds and their specific activities, these products may be a good candidate for anti-bacterial agents. According to different studies in our burn center (Amir-Al-Momenin burn hospital, SUMS), *Acinetobacter* sp. is recently increasing according to the resistant properties of this bacterium. Considering the necessity of implementing the new policies for control these kind of infections; this study was designed to find new local compounds against resistant forms of this bacterium. Also in the recent study different local isolates with the property of pigment production was evaluated, but the best isolate which produced a proper pigment with anti-bacterial activity against MDR-*Acinetobacter* isolates was a *Serratia marcescens* isolate from a salty region entitled Maharlo Lake or Daryache-Namak. According to the result of this study, this bacterium has the ability of produce the red pigment (Prodigiosin) with a high purity which is active against the prevalent *Acinetobacter* isolates. In a similar study performed in Iraq, they either reported the antimicrobial activity effect of *Serratia marcescens* red pigment against some Gram-negative and Gram-positive bacteria such as *Klebsiella pneumoniae*, *Proteus mirabilis*, *Escherichia coli Staphylococcus saprophyticus*, and *Streptococcus pyogenes*. In this study, they isolated their study bacterium from a farm soil, while in this study isolated bacterium was perform from a salty soil (Kamble & Hiwarale, 2012). Also, there are several studies performed in relation with bacterial pigments in the world, but they are almost performed on standard pathogenic Gram-positive and Gram negative bacterial species (Rashid et al., 2014), while the remarkable point in our study is evaluation of the effect of the pigments on micro-organisms isolated from clinical samples with Multi-Drug Resistant specifications. In such type of studies we would be able to measure the antibacterial effect of compounds on infections, which are current problem of medical community. In this study like other studies such as those of Goswami, Gallardo, Rashid and others (Gallardo et al., 2014; Goswami & Bhowal, 2014; Rashid

et al., 2014), evaluation of different pigments of different bacteria for antibacterial activity was evaluated. Among blue-green, red and yellow bacterial pigments used in this study, only *Serratia marcescens* red pigment had antibacterial activity against the studied *Acinetobacter* isolates, which is consistent with the findings by Goswami and Gallardo. They showed that red pigment *prodigiosin* had antimicrobial activity against Gram positive and negative bacteria (Gallardo et al., 2014; Goswami & Bhowal, 2014). However in other studies, unlike the current research, antimicrobial effect of other pigments like green-blue pigment of *Pseudomonas aeruginosa* has been reported (Dormanesh et al., 2014). Rashid et al. showed that yellow pigment of *Flavobacterium* has antibacterial effect on gram-positive and negative bacteria (Rashid et al., 2014).

In 2014, Indian scientists, who studied on the red pigment's inhibitory effect on bacteria and fungi, reported inhibitory properties against Klebsiella and Candida. No information on drug resistance in this species has been reported. Also, Lapnda et al. in their study found that red pigment of Serratia has no antimicrobial effect against *Acinetobacter* (Scrascia et al., 2016). This finding is in contrast to those of the present study, where the red pigment inhibited the growth of multidrug resistance in Acinetobacter culture. This shows the high power of the isolated red pigment in this study compared to the work of Lapnda et al. Mekhael and Yousif reported that Serratia marcescens pigment has an antibacterial effect against Klebsiella (Mekhael & Yousif, 2009).

Thus, it can be concluded that antimicrobial effect of different pigments varies due to their different structures. Also, it depends on properties of various microbial specificities with resistance pattern. For example, a pigment may have inhibitory effect on a bacterium in a specific country and it may lack inhibitory effect on the same bacteria in other places. Scientists believe that antimicrobial property of red pigment in Serratia marcescens is due to the ability in passing through the external membrane of microorganisms and their capacity for inhibiting the target enzymes like DNA gyrase and topoisomerase 4, which stop the cell growth (Kushwaha et al., 2014; Mekhael & Yousif, 2009).

Hence, since antimicrobial effect of this compound may be dependent on destruction or inhibition of metabolic reactions in a microorganism, it is important that this pigment is used against the type of microorganism. In this context, there is a need for more studies to obtain acceptable and general conclusion.

Conclusion

Finally, it can be concluded that bacterial pigments can potentially have antimicrobial properties. However, inhibitory capacity of these pigments may considerably vary considering their different types and structures as well as the type of microorganism, so that one pigment from two different structures may have totally different inhibitory effect on the same microorganism. Even one pigment may have different effects, from total inhabitation to lack of growth inhibition, on the same bacterial species, but it may have different drug resistance.

Also, microorganisms isolated from different clinical samples contain various reservoirs of pigments, like microorganisms isolated from environmental and food resources, and various structures of pigment with antimicrobial properties can be searched in them. Hence, further studies are needed for clarification of such variety and microbial effect of pigments, which can provide a new perspective for antimicrobial drugs production in pharmaceutical industry.

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