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Research Article – Metagenomics

Antimicrobial activity evaluation of frankincense oil in domestic water samples by 16s rRNA gene

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Abstract

The domestic water system favors the growth and reproduction of bacterial species. The removal of such contaminant by the application of chemical disinfectants like chlorine may pose health risk. Therefore, the natural products have more authenticity and to be investigated for their antimicrobial activity. The current study investigates the efficiency of frankincense oil as an antimicrobial agent on bacterial species isolated from the domestic water system. In the previous study, 15 bacterial species were isolated based on the colony morphological characteristics and prepared for identification. 16S rRNA gene was amplified using PCR for all bacterial isolates followed by sequencing and identification using BLAST. Thirteen bacterial isolates were identified and represented as *Staphylococcus saprophhyticus, Bacillus pumilus*, *Bacillus safensis and Pseudomonas* sp. and the last one showed more resistance to frankincense oil as the numbers increased with the treatment by 26% and 35%. Other species were either completely or partially eliminated. The results confirmed that the antimicrobial activity of frankincense oil against some bacterial species, mainly exist in storage tank. *Pseudomonas* sp. showed resistance to frankincense oil (10%) used in this study. In addition, *Staphylococcus saprophhyticus* is of a concern to human health; however, it showed sensitivity to frankincense oil and also, its presence in the water tank indicates hygiene issues to the household. The frankincense oil found to be a promising disinfectant, thus further analysis needed to specify the minimum effective concentration to be applied, also to find out whether it is cost effective to be used as a disinfectant.

Key words: Antimicrobial agent; frankincense oil; 16S rRNA gene

Introduction

Bacteriological water analysis is a technique of analyzing water to evaluate the amount of bacterial existence and class of bacteria present in a water body. Bacteriological water analysis normally depends on a very small sample taken from large volume of water and to perform conventional cultural techniques [1]. Water storage tanks may receive microorganisms; few of them might play a role of pathogens. The hygienic precautions are shortly considered in the household areas that continually poses a potential health risk. Use of disinfectants can minimize the pathogens potential however, disinfectants like chlorine has tendency to react with naturally-occurring materials in the water to form by products which may cause major health risks.

Essential oils of frankincense *(Boswellia* sp.) was examined for its antimicrobial activity on number of microbial species including *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Brucella* sp*, Klebsiella pneumoniae,* and *Proteus* sp [2]. The antimicrobial activity of the frankincense oil on bacterial species showed moderate response *against Proteus sp., Staphylococcus aureus* and *Escherichia coli*. Essential oils are odorous compounds that are mainly stored in special plant cells such as glands, glandular hairs, oil and resin ducts. They may occur in flowers, fruits, leaves, roots, wood, stems saps and bark. These oils are responsible for the distinctive aromas associated

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with individual plant species [3]. Essential oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties [4, 5]. Some essential oils have been successfully demonstrated in cancer treatment [6], as a food preservatives, aromatherapy and fragrance industries [7].

This study is a continuation of our previous research work, which investigated the effect of frankincense oil on the growth of heterotrophic bacteria found in diverse water storage tanks located in districts of Fujairah.

The identification and detection of bacteria is required in many different fields of anthropogenic activities. The quick and accurate pathogen identification has crucial role in the fields of medicine, veterinary sciences, ecology agriculture, and food analysis. It is generally assumed that identification of bacteria by using genotypic methods has more accuracy than phenotypic techniques. There are different techniques used for the identification of microbes, such as biochemical testing, microscopic observation and molecular biological techniques. The later one have been developed primarily based on genetic material, DNA profiling that are unique to each source, hence these DNA patterns are then compared to the library of patterns from specific sources to identify isolated bacteria. There are a large number of different molecular methods applied with great accuracy and one of the most frequently used is 16S rRNA sequencing. Identification of bacteria by 16S rRNA gene in recent years featured as an attractive alternative or complement to traditional methods, which are often more time-consuming.

One of the stable parts of the genetic code is a sequencing of the 16S rRNA gene. Prokaryotic ribosomes have small (30S) and a large (50S) subunit. Within the component of the 30S small subunit is 16S ribosomal that is 1500 nucleotides long [9]. The 16S rRNA gene is found in all bacteria and has been used to identify a large number of strains. There are number of reasons to specify the use of 16S rRNA gene sequences to identify the bacterial species. Primary reason, is that 16S rRNA gene sequences exist in almost all bacteria. Secondly, the function of the 16S rRNA gene is permanent. Thirdly 16S rRNA gene sequencing (1,500 bp) is a long enough to perform informatics tests.

One of the major databases in NCBI is a Gene Bank for DNA sequencing. Another important tool developed by NCBI is the Basic Local Alignment Search Tool (BLAST), a program used to search out the sequence similarity. The basic aim of this program is to compare nucleotide or protein sequences to sequence databases by calculating the statistical significance of matches.

The purpose of this study is to identify the bacterial species isolated from natural and treated water samples. A molecular method by using the amplification of 16S rRNA gene sequencing and comparison to a known database of National Center for Biotechnology Information (NCBI) was used.

Since this research is complementary to previous study on the effect of frankincense oil on the growth of heterotrophic microorganisms present in domestic water system. One of the aims of this study was to investigate the effect of frankincense oil on the species diversity of bacteria. Heterotrophic bacteria were cultured on nutrient agar plates followed by enumeration of the colonies that were chosen and isolated based on the phenotypic characteristics of the colony.

Materials and Methods

Total of 15 bacterial isolates were selected for the identification of specific bacterial species from three houses (Table 1). The water samples containing isolates were treated with 10 % (v/v) of frankincense oil.

Culture preparation

Nutrient broth media (Luria Broth, SIGMA) was prepared as per the manufacturer given direction. Using sterile loop, colonies were transferred from the culture plate to the broth media followed by incubation at 37 °C for 24 hours. Glycerol stock was prepared by transferring 50 µL of glycerol solution (SIGMA-ALDRICH) to a microcentrifuge tube and then add 50 µL of culture bacteria. Bacteria glycerol stock tubes were stored in $^{-}$80 °C freeze dried until used. In this study glycerol stock has been made for 15 bacterial isolates only.

Table 1. The number of heterotrophic bacterial count and Total Coliforms species in the control and treated water samples chosen based on the phenotypic characteristics of the colony forming unit (CFU)

House No	House 1		House 2		House 3	
Bacterial group	HBC*	TC*	HBC	TC	HBC	TC
Control sample	Sp1&3&4	Sp1&2	Sp5&6&7&9	Sp8&10	Sp13&14&15	Sp11&12
Treated sample	Sp3&4	Sp2	Sp5 &Sp6	Sp10	Sp13&14	Sp11&12

DNA extraction

Bacterial DNA was extracted and purified using QIAamp DNA Mini Kit, Cat No. /ID: 51304 by Qiagen. The sample (200 µl) was used and 20 µl Proteinase K was added for quality isolation and protein digestion. 200 µl Buffer AL was added followed by rapid vortexing and a subsequent incubation for 10 min at 56°C. 200 µl 96-100% Ethanol was added followed by quick vortex. The mixture was transferred to QIAamp Spin column assembled with 2 ml of the collection tube for DNA purification and centrifuged for 6000 xg for 1 min. The filtrate was discarded and 500 µl Buffer AW1 was added to the column and centrifuged for 6000xg for 1 minute. The filtrate was discarded again and 500 µl of AW2 buffer was added followed by centrifugation at maximum speed (20,000xg) for 3 minutes. The filtrate was discarded and a new collection tube was assembled. 200 µl Buffer AE was added and the sample was incubated at room temperature for 1 minute followed by centrifugation for 6000xg for 1 min.

The polymerase chain reaction (PCR) was carried out using PCR master mix received from Qiagen (Taq PCR Master Mix Kit, Cat No. / ID: 201443), which is a premix solution containing Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers. The Bacterial DNA (3µl) along with primer sequence (forward 5'AGAGTTTGATCCTGGCTC3' and reverse 5'GGTTACCTTGTTACGACT3') were used for PCR reaction. The PCR was carried out in a thermal cycler (Biorad T100) using cycling conditions consisting of; initial denaturation at 94 °C for 4 min, followed by annealing at 71°C for 1 hr. and 30 min and extension at 72 °C for 7 min. The PCR was run for a total of 30 cycles and the expected product size was 1439bp. After that, The PCR products were analysed using 1.8 % Agarose gel electrophoresis.

The reaction products were purified using ethanol/EDTA precipitation. After that, 65 µl from 125 Mm EDTA and absolute ethanol mixture were added to the cycle sequencing reaction products and incubated at room temperature for 15 min followed by 20 min spinning at 14000 rpm. The supernatant was slowly aspirated from the pellet and 120 µl of 70% ethanol were added to the tubes, then spin for 5 minutes at 14000 rpm. The remaining solutions were removed and the pellets were dried at 37°C. The pellets were re-suspended in 10 µl of Hi-Di Formamide. The DNA samples were denaturing at 95°C for 2 minutes and immediately placed on the sequencer (Genetic analyzer 3500, Applied Biosystems).

Results and Discussion

Total of 15 isolates were used for this study from that only 13 isolates were shown amplification with the correct product size of 1439 bp (Figure 1). However, 16s rRNA analysis using BLAST resulted 4 group of bacteria such as, marine bacterium, *psuedomaonas* sp, *staphylococcus sp* and *bacillus* sp. found in 15 samples with significant e-value support (Table 2).

As depicted in the table, the isolates indicating Sp1, Sp2, Sp3 and Sp4 were collected from the house 1. From the house 2, other isolates such as Sp5, Sp6, Sp7, Sp8, Sp9 and Sp10 were collected. Similarly Sp11, Sp12, Sp13, Sp14 and Sp 15 were collected from House 3.

Table 2. BLAST result for the 13 bacterial isolates

Sample Number	Species name	Query coverage	Similarity % (Max ID)	E Value	Max score
1	Marine bacterium	96%	99%	0	1402
2	*Pseudomonas* sp.	96%	99%	0	1404
3	Marine bacterium	96%	97%	0	1315
4	*Bacillus stratosphericus*	98%	98%	0	1417
5	*Bacillus pumilus*	95%	99%	0	1428
6	*Bacillus pumilus*	96%	97%	0	1317
7	*Staphylococcus saprophhyticus*	97%	98%	0	1406
8	*Staphylococcus saprophhyticus*	96%	98%	0	1386
9	*Bacillus pumilus*	96%	99%	0	1404
10	*Pseudomonas sp.*	96%	99%	0	1404
11	*Bacillus pumilus*	99%	97%	0	1351
12	*Bacillus pumilus*	96%	99%	0	1402
13	*Bacillus pumilus*	98%	98%	0	1417
14	*Bacillus safensis*	88%	98%	0	1267
15	*Bacillus pumilus*	97%	98%	0	1497

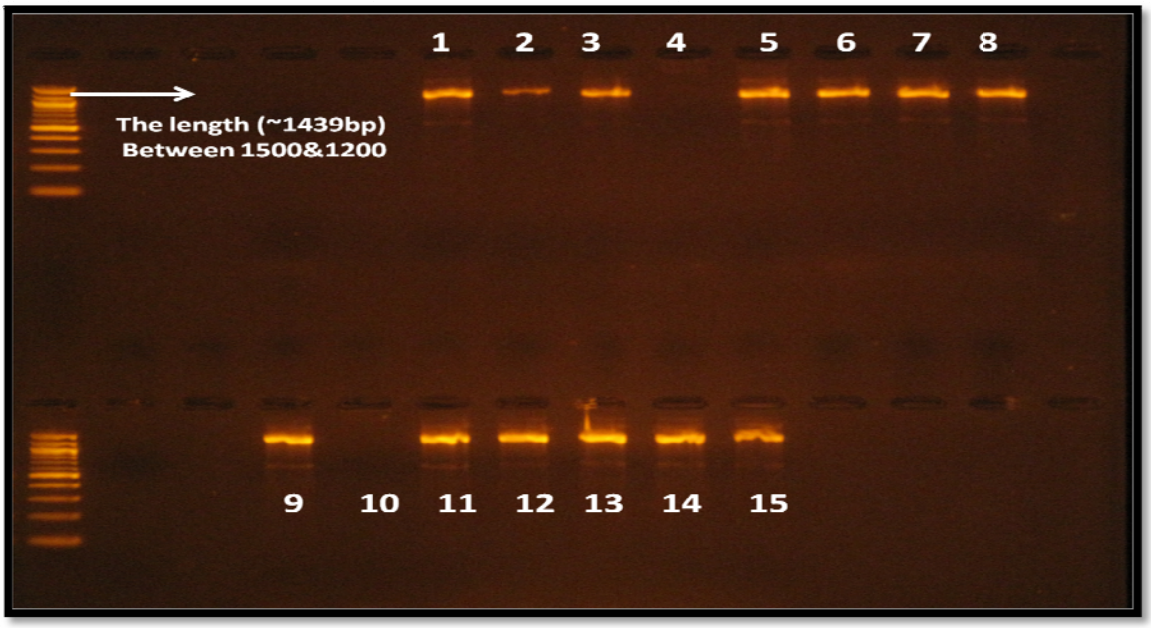

Fig. 1. Gel showing the bands obtained for 13 bacterial isolates using 16S rDNA Primer

Table 3. The effect of Frankincense oil on the growth of 15 bacteria species collected from different water storage tanks at different houses in Al Fujairah

House Nos.	House 1 (CFU/1ml)				House 2 (CFU/1ml)						House 3 (CFU/1ml)				
Species	Sp1	Sp2	Sp3	Sp4	Sp5	Sp6	Sp7	Sp8	Sp9	Sp10	Sp11	Sp12	Sp13	Sp14	Sp15
Control	97.5	270	325	315	200	340	70	70	95	170	70	180	605	287.5	350
Treatment	0	340	65	120	32.2	195	0	0	0	230	45	130	165	40	0
Percentage of removal (%)	100	↑**21***	77	62	84	43	100	100	100	↑**35***	36	28	73	86	100

The effect of frankincense oil on the growth of bacterial isolates were determined by comparing the viability of the isolates, both in control and treated water samples. It was found that marine bacterial isolates (Sp1) were completely removed (100%) or showed no growth in the samples treated with frankincense oil. In previous studies, the effect of frankincense oil was evaluated on *psuedomaonas spp*, *staphylococcus aureus* and demonstrated the highest degree of activity against them (10, 11). However, there was no previous reports of frankincense oil activity against isolates

such as *staphylococcus saprophhyticus, bacillus pumilus, bacillus stratosphericus* and *bacillus safensis.* Moreover, *staphylococcus saprophhyticus* (Sp7 & Sp8*)* and *bacillus pumilus* (Sp15) showed the same response against frankincense oil with 100 % removal or no growth (Figure 2). However, Isolates indicates Sp5, Sp6, Sp11, Sp12 and Sp13 which represents *Bacillus pumilus* showed partial removal of 84%, 43%, 36%, 28% and 73% respectively. Similarly, *Bacillus stratosphericus* (Sp4) was another identified isolate, commonly found in the atmosphere showed 62 % removal with frankincense oil treatment. It should also be noted that the data using *pseudomonas sp* (Sp2 and Sp10) against frankincense oil were found highly resistant. It attributes the concentration might not be sufficient that they are more susceptible to the activity of these particular essential oils.

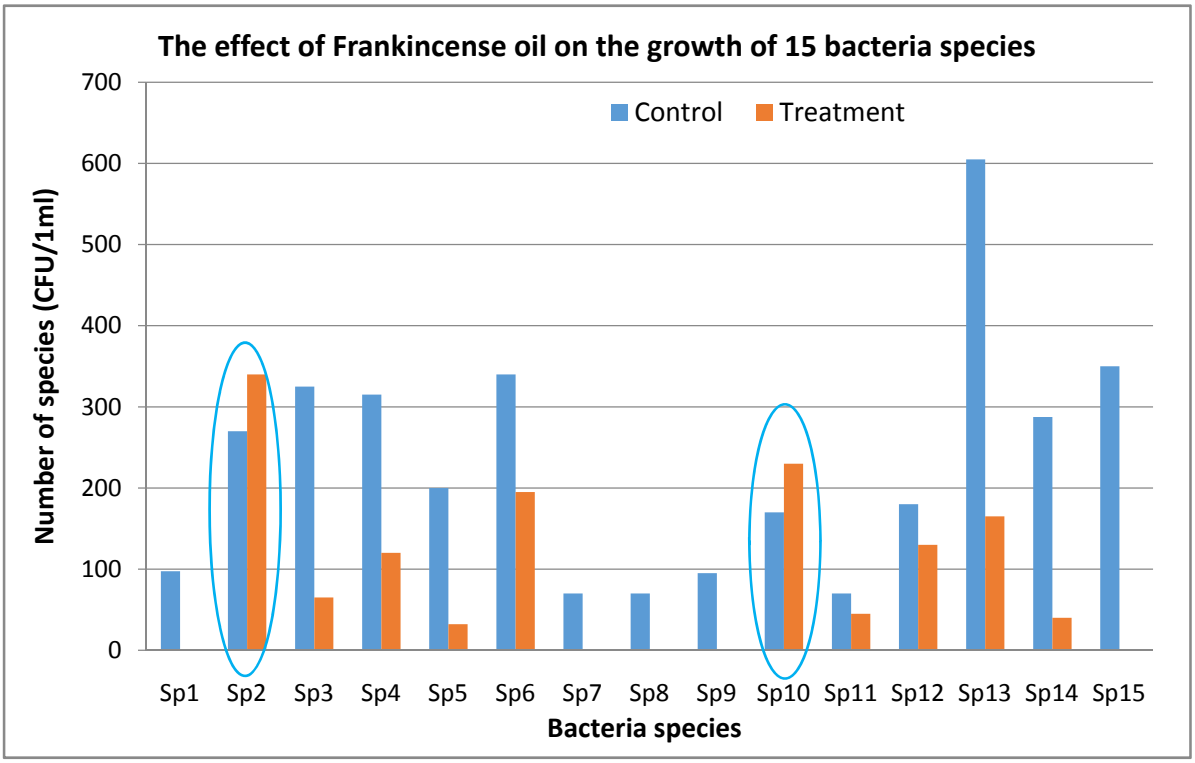

Fig. 2. The effect of Frankincense oil on the growth of 15 bacteria species collected from different water storage tanks at different houses in Al Fujairah.

Conclusion

The current study investigated the effect of frankincense oil on the growth of bacterial species. Three bacterial species were identified including *Bacillus*, *Pseudomonas*, and *Staphylococcus*. The result showed that *Pseudomonas* was highly resistant to the treatment with frankincense oil. On the other hand the 10 % frankincense oil completely or partially eliminated *Bacillus pumilus* and *Staphylococcus saprophyticus*. Furthermore, *Staphylococcus saprophyticus* might be of a concern to human health as it causes urinary tract infection. Finally, frankincense oil affected the abundance and diversity of the bacterial species.

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