



Detection of Gene Involved in Biofilm Formation in Methicillin Resistance *Staphylococcus aureus* and *Staphylococcus lentus* and Effect of Camphor extract on These Genes

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Abstracts

This research aimed to investigate the antibacterial activity of Camphor extract ,against biofilm formation of clinical isolates *Staphylococcus aureus* and *Staphylococcus lentus* .by detecting the presence and absence of virulence genes that responsible for the biofilm formation after treatment with Camphor extract. thirty isolates were diagnosed initially as Staphylococci then selected five isolates depending on resistance to different type of antibiotics as virulence .After that the isolates test by VITEK-2compact system (ID and AST) to confirm the species of Staphylococci .The diagnostic result showed that three isolates belong to *Staphylococcus aureus* and two to *Staphylococcus lentus*. Investigation of the ability of camphor oil to inhibit the growth of bacteria using diffusion method in Muller Hinton agar ,the results showed the effect of camphor oil to prevent biofilm formation on both bacterial species with concentration (0.1 g/ml). A molecular investigation of susceptibility of Bacteria to biofilm formation was also observe by the presence or absence of virulence gene that responsible of biofilm formation represented by *icaA*, *icaD* gene that responsible for intercellular adhesion by using PCR Technique. And using specialized primer, the results showed that all isolates of *S.aureus* contained both inherited and loss *icaA* in *S.lentus* isolated. This is evidence of the susceptibility of *staphylococcus aureus* for biofilm formation.

Key words : camphor, biofilm ,methicillin ,*Staphylococcus aureus* , *Staphylococcus lentus*.*icaA*,*icaD*.

Introduction

The medicinal plants have been of great interest by researchers in the last few years, despite the development in the manufacture of medicines made of pure chemicals or biological materials such as antibiotics and given the effects of these substances on the public health of the individual, In addition to the increase in the number of micro-organisms resistant mostly for continuous use and without medical consultation (1). The extract of camphor is among the plants belonging to the family Eucalyptus, which has high pharmacological effectiveness because it is rich in essential oils,like cineole With the killer properties of microorganisms (2) In addition to the phenolic compounds that prevent the formation of biofilm impact on the process of Quorum sensing (QS) (3). We can define biofilm by a collection of bacteria attached to living and non-living surfaces, surrounded by substances secreted by bacterial groups, which are extracellular polymers (4),which is used to hide from the host's immune system and resistance to different type of antibiotics (5).*Staphylococcus aureus* and the *Staphylococcus lentus* caused many health problems including skin infections with deep inflammation and inflammation of the bone, chronic osteomyelitis ,endocarditis and other heart diseases that may lead to death (6).

Materials and Methods

Collection and preparation of Camphor Oil extract

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Various forms of camphor were collected from the local markets of Najaf Governorate and the local markets of Baghdad governorate, using hard camphor, which is a crystalline cube from Deer brand/China, The camphor oil was prepared by dissolving 3 g of camphor in 30 ml of sterilized olive oil at 121 ° C and under pressure of 15 lb / kg² for 15 minutes, in a water bath vibrating 95 ° C for 15 minutes until it was confirmed Melting the camphor completely with olive oil then placed in a sterile tube and kept in the refrigerator 4 ° C until it is used and the solution to the storage of pure camphor extract (7).

Collection of specimens

A total 5 specimens which were collected from urinary tract infection, wound, burns and swabs from the indoor environment in hospital of Najaf governorate during a period (from September 2017 to February 2018) , patient's age ranged from (15 years - 35 years). The samples were processed on Blood agar medium ,Nutrient agar and Mannitol salt agar and were incubated at 37°C overnight. The identification of Gram positive bacteria, performed by standard biochemical methods (catalase test, oxidase test, Mannitol Fermentation ,and also diagnosed by using Vitek-2 compact system .

Detection the inhibitory activity of camphor oil towards *staphylococcus aureus* and *staphylococcus lentus*

The well diffusion method was used to study the susceptibility of the camphor oil to the bacterial isolates(8). The isolated bacteria were spread homogenously on the Muller Hinton agar medium and left at room temperature to ensure that the bacterial implants in the center, Then, the center was drilled with a diameter of 8 mm and the camphor oil was added at a concentration of 0.1 g / ml. Excavation of positive control containing olive oil only worked separately to ensure that there was no inhibitory effect of olive oil on developing colonies. Leave the dishes for 10 minutes with room temperature to allow the extract to spread through the middle. It was incubated 37 ° C for 24 hours and the diameters of the drill were measured using a graduated ruler.

DNA template preparation by using boiling method with Tris EDTA-TE buffer :

The total DNA of isolates under study was extracted using the Tris EDTA-TE Buffer method (9) With some modifications made by the researcher to suit the search requirements:, grown Bacterial isolates in BHI Broth at 37 ° C for 24 h.then discarded centrally after placing the tubes in the cannula for 5 minutes at a speed of 5000 rpm. The precipitate dissolved in 1 µl of TE Buffer ,then The tubes were centrifuged centrally at 5000 rpm for 5 minutes, after that re-suspended The percipitate in100 µl of TE Buffer to ensure that all the cells are absorbed, The tubes were placed in a 100 c° water bath for 10 minutes and immediately discarded for 5 minutes at 5000 rpm.,then Collect the supernatant from the tube and keep in freezing at -20 ° C until use.

Primers used for PCR

The genes used in this study are responsible for intercellular adhesion necessary for Biofilm formation of *Staphylococcus aureus* obtained from the Korean Alpha DNA Company as shown in the table1.

Table -1: The primer used in the current study for PCR amplification.

Target	Nucleotide sequences and direction(5'-----3')	Product
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Gene		size (bp)	Reference	
<i>Ica A</i>	F	5 [\] GAG GTA AAG CCA ACG CAC TC 3 [\]	151	(10)
<i>Ica A</i>	R	5 [\] CCT GTA ACC GCA CCA AGT TT 3 [\]		
<i>Ica D</i>	F	5 [\] GAA CCG CTT GCC ATG TGT TG 3 [\]	483	(11)
<i>Ica D</i>	R	5 [\] GCT TGA CCA TGT TGC GTA ACC 3 [\]		

PCR amplification procedure

Five selected isolates were submitted to genotypic study using PCR. The oligonucleotide primers for the *icaA* and *icaD* genes (table 1) were diluted using TE Buffer according to the manufacture company information (Alpha DNA) to get primary concentration equal to 100 µl. The amplification was performed in PCR thermocycler and the reaction mixture was prepared according to the procedure that suggested by the manufacture company (Maxime PCR PreMix kit (i-Taq)). The components of the polymerase chain reaction are shown in the following table 2

Table 2: component and volumes of PCR mixture

PCR Master Mix	Volume
DNA Template	10 µl
Forward primer	4 µl
Reverse primer	4 µl
Nuclease free water	2 µl
Total	20 µl

Conditions for PCR amplification which were set according to this study for the two primers was shown in table 3

Table 3 : Program of thermal cycles to amplify DNA

Gene	Temprature (C) /Time					Cycle No.
	Initial denaturation	Cycling Condition			Final Extention	
		denaturation	Annealing	Extention		
<i>Ica A</i>	94/2 min	94 / 1 min	55/1 min	72/2 min	72/7 min	30
<i>Ica A</i>	94/2 min	94/1 min	60/1 min	72/2 min	72/7 min	30
<i>Ica D</i>	94/2 min	94/1 min	57.5/1 min	72/2 min	72/7 min	30
<i>Ica D</i>	94/2 min	94/1 min	60/1 min	72/2 min	72/7 min	30

The amplified PCR product were analyzed by agarose gel electrophoresis by using 1.5 % agarose supplied with 0.5 µg/ml ethidium bromide for 1 hour(12). DNA ladder (100-1500 bp) were used to assess PCR products were visualized by UV light at 336 nm, and photographs were taken using digital camera.

Results and Discussion

Spread of methicillin –resistance *Staphylococcus aureus* and *Staphylococcus lentus* is an important concern in hospitals and other health care. a total of 5 specimens showed that 3

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isolates belong to *Staphylococcus aureus* and 2 to *Staphylococcus lentus* according to different biochemical test including culturing on Mannitol salt agar and here *S.aureus* isolates able to converting this media color from red to yellow due to Mannitol fermentation, Coagulase test also used as remarkable diagnostic test for *S.aureus* that has the ability to give positive result (13), Another important diagnostic test was performed using Vitek-2 compact system To confirm the diagnosis (14) this two species differ according to source of the isolates ,nature , the Age of the patient ,and geographic location that samples were taken from .By using diffusion method to detect the antimicrobial activity of Camphor oil (0.1 g/ml) ,it was 40mm in diameter for *S. aureus* isolates and 44mm in diameter for *S.lentus* isolates as shown in the following figure1

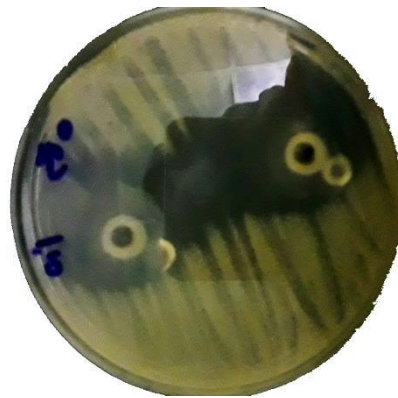


Figure1: shows the anti-microbial effect of camphor oil towards the isolation of *S.aureus* on Muller Hinton agar at concentrated(0.1 g/ml)

The inhibitory efficacy of oil is likely to be due to its active ingredients, notably phenols In addition to volatile oils, especially Cineole oil, which constitutes about 80-90% one of the total active substances of the plant ,Which works to destroy the cell membrane and destroy the bacterial cells (15).then this five isolates were selected for the genotypic study by PCR technique using two different gene sequence Which are important in the process of biofilms formation and it is *ica* gene (inter cellular adhesion) which responsible for adhesion between cell during biofilm formation and it's the first step in this process ,the first one is *icaA* with amplified size equal to 151 bp(10) and the second is *icaD* with amplified size equal to 483 bp(11).The result of PCR experiment revealed that the possession of isolates (*S1 aureus*,*S3aureus*) *icaA* gene with annealing temperature 55 c° ,while the isolates (*S1aureus*,*S2aureus* ,*S3aureus*) possessing *icaA* gene with annealing temperature 60 c° ,but did not show the isolates (*S4 lentus*,*S5 lentus*) the gene in the same temperature as in the figure 2

60 c°

50 c°



Figure 2: Agarose gel electrophoresis (1.5% agarose) of *icaA* gene (S1,S2,S3)is *S.aureus* isolates (,S4,S5) is *S.lentus* isolates ,S6 is control

While the result of *icaD* gene showed that all isolates possessing the gene with two annealing temperature (57.5-60)c° as in figure 3

60 c°

57.5c°



Figure3: Agarose gel electrophoresis (1.5% agarose) for *icaD* gene (S1,S2,S3)is *S.aureus* isolates (,S4,S5) is *S.lentus* isolates ,S6 is control

The presence an absence of virulence genes in the isolates may be due to the sources of collection of samples ,as the use of detergents and disinfectants in hospitals can lead to a change in the nature of genetic materials constituent of the cells and the disappearance of some these genes (17).

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