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Detection of Tetracycline Resistant Gen (*tet K, tet M*) in Some Coagulase Negative Staphylococci Isolated From Different Clinical Sources In Erbil City

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ABSTRACT

Thirty two isolates of coagulase negative staphylococci was isolated and identified from clinical sources ; including eight isolates (12.5%) of each of *Staphylococcus hominis* and *Staphylococcus haemolyticus* from (64) patients urine which suffering from urinary tract infection and eight isolates (16%) of each of *Staphylococcus epidermidi*s and *Staphylococcus auricularis* from (50) ear patients which suffering from otitis in Rizgary teaching hospital in Erbil city were isolated depending on Vitek 2 compact system . The susceptibility tests by using disc diffusion agar method were determined for all isolated coagulase negative Staphylococci to 10 different antibiotics . From obtained results it was observed that all isolates were 100% sensitive to ceftriaxone and novobiocin, while higher resistance was obtained to tetracycline with percentage 50% and they showed differnt resistant to other remaining antibiotics. On the other hand, all isolated *Staphylococcus hominis*, *Staphylococcus haemolyticus* , *Staphylococcus epidermidi*s and *Staphylococcus auricularis* isolates were tested for tetracycline resistant genes *tetK* and *tetM* by using PCR and the results showed that 75% of *Staphylococcus hominis*, *Staphylococcus haemolyticus* and *Staphylococcus epidermidi*s were carried *tetM* gene with product size about 158 bp while 50% of *Staphylococcus auricularis* was carried the above mentioned gen . Also it was found that 50% of *Staphylococcus hominis* and *Staphylococcus haemolyticus* isolates in this study were harboring *tetK* gene with product size 360 bp and no *tetK* gene was observed in *Staphylococcus epidermidis* and *Staphylococcus auricularis* . Also the antibacterial effect of two local species of menthes against resistant isolated bacteria by using the Minimum Inhibitory Concentration (MIC) method were studied . The results showed that the MIC for aqueous extract for both plants *Mentha longifolia* and *Mentha piperita* were 1600µg/ml and it were 1400µg/ml for alcoholic extract of both plants against most Staphylococcus speciese except *Staphylococcus epidermidis* which the MIC was 1200 µg/ml for aqueous and 1000µg/ml for alcoholic extract of *Mentha piperita*, while it was 1200µg/ml for both the aqueous and alcoholic extracts for *Mentha longifolia*..

Introduction

Staphylococci species have become one of the most common causes of nosocomial infections. Staphylococci are members of the family staphylococcaceae which are separated into two large groups on the basis of ability to produce the extracellular enzyme coagulase (1). Among the coagulase negative Staphylococci (CoNS); are *S. hominis*, *S. haemolyticus*, *S. epidermidis* and *S. auricularis.* However, *S. hominis* does not usually cause human disease while it was recognized as a potentially opportunistic and nosocomial pathogen, occasionally cause infection in patients with weak immune systems and it cause some potentially life-threatening infections, such as infective endocarditis (2). While, *S. epidermidis* is a microbiota of the skin, respiratory and gastrointestinal tracts and is principal cause of infection, chiefly in hospitalized patients with indwelling foreign bodies and in immunocompromised patients (3). Also it caused some cases of bacteremia, surgical wound infections (4), conjunctivitis also osteomyelitis, wound infection, otitis media, endophthalmitis, UTI, was reported (5). Although, antibiotic resistance of *S. epidermidis* become an important problem in the recent years due to the spreading of multi-drug resistant and methicillin resistant *S. epidermidis* in the clinical specimens. On the other hand, *S. haemolyticus* is an emerging cause of nosocomial infections, in particular affecting very preterm infants and immunosuppressed patients. Indeed, *S. haemolyticus* is the second most frequently isolated species from human blood cultures, after *S. epidermidis*. However *S. haemolyticus* is often resistant to commonly used antimicrobial agents and was ranked as the most antibiotic-resistant CoNS species (6). In a study it was recognized as the cause of severe infections including meningitis, skin and skin structure infections, prosthetic joint infections, bacteraemia and even endocarditis (7). On the other hand, the *S. auricularis* is isolated almost exclusively from the external auditory meatus. It being commensal bacteria, CoNS was long thought to be non-pathogenic and was regarded as contaminants when found in specimens of human origin (8).

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The antibiotic tetracycline is considering one of the most commonly used therapeutics in human (9). More than 38 tetracycline resistance determinants have been identified in bacterial genera over the last 50 years (10; 11). The two main resistance mechanisms provided by these determinants are efflux pump proteins and ribosomal protection proteins (12). Moreover, the accurate and rapid diagnosis of antibiotic resistance genes in the treatment of staphylococcal infections is extremely important in preventing the spread of staphylococcus infections. PCR-based molecular methods are often preferred for determination of tetracycline resistance genes. On the other hand, the resistance to antimicrobial agents is an increasingly global problem worldwide, especially among nosocomial pathogens and the search for newer and alternative compounds for the treatment of drug resistant infections has led to the increased use of the plant as an alternative for the treatment of infectious disease. The *Mentha piperita* and *Mentha longifolia* (Lamiaceae) species are widely used in conventional medicine, for their antispasmodic, antiseptic and emmenagogue effects (13); moreover, their essential oils are used in chewing gums, alcoholic beverages, cosmetics, perfumes, toothpastes and mouthwashes .Both of them are mainly used as salad, spice and for tea besides mint herbage used for wool dyeing (14 ; 15). Mint oil is widely used for its medicinal properties such as antispasmodic, anti-sickness, anti-helminthic, carminative, stomachic and others (16). This study describes the use of the polymerase chain reaction to detect the *tetK* and *tetM* tetracycline resistance genes in *S. epidermidis*, *S. hominis*, *S. haemolyticus* and S. *auricularis* which isolated from different clinical sources and comparing with phenotypically method by disk diffusion agar. Also

studding the inhibitory effect of the *M. longifolia* and *Mentha piperita* leaves and stems on the growth of isolated bacteria .

Materials and methods

Specimens collection

Between December 2015 and March 2016, One hundred fourteen clinical specimens were collected from Rizgary teaching hospital in Erbil city. Clinical specimens included (64) urine and (50) ear swabs by using sterile containers and swabs respectively. All isolates were identified depending on Vitek 2 compact systems (17).

Antibiotic susceptibility test

Disc diffusion antibacterial susceptibilities of the isolates were tested by the agar disk diffusion method on Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute (18) guidelines. Antibiotic discs (Becton Dickinson, USA) were placed on MuellerHinton agar plates, incubated at 37°C for 24 h, and the diameter of each inhibition zone was measured in millimeters. The following antibiotic discs were used in this study : Ceftriaxone (CRO) 30µg, Ceftazidime (CAZ)10µg, Cefoxitin (FOX) 30µg, Cephalothin (CF) 30µg, Novobiocin (NV) 30µg, Ofloxacin (OF) 30µg, Oxacillin (OX) 10µg, Piperacillin (PRL) 30µg, Teicoplanin (TEC) 30µg and Tetracyclin (TE) 30µg.

Detection of *tet K* and *tet M* genes in isolated bacteria

Extraction of DNA from isolated bacterial cell

The isolation of DNA from bacterial cells was performed by using Presto™ Mini gDNA bacterial kit which included following steps: sample preparation, lysis, DNA binding, washing and elution.

PCR method for *tet K* and *tet M* genes

PCR was used to detect tetracycline resistant genes (*tet K* and *tet M)* in isolated *S. pidermidis*, *S. hominis*, *S. haemolyticus* and S. *auricularis.* The primers were provided by macrogen as shown in table (1). Macrogen synthesized the primer as lyophilized powder, thus the concentration define as pmol. Primers were prepared (Table2) as work stock, then used in PCR reaction volume. The master kit of PCR was contains DNA polymerase, dNTPs, a tracking dye and reaction buffer in a premixed format, freeze-dried into a pellet. Primer (1.3 µl) of each forward and reverse, (2.5µl) of DNA template were added to AccuPower PCR tube then 20µl of distilled water added to AccuPower PCR tubes. After that lyophilized blue pellet dissolved by vortexing. PCR performed for samples, procees in the thermal cycler for 30 cycles as mentioned in table (3). Electrophoresis technique in 2% agarose gel was used to separate DNA molecules according to size as described by (19).

Table (1) : Primers sequences and their product size.

Primers	Primer sequences	Product size
Forward primer *(tetK)*	5'GTA GCG ACA ATA GGT AAT AGT3'	360 bp
Reverse primer *(tetK)*	5'GTA GTG ACA ATA AAC CTC CTA3'	
Forward primer *(tetM)*	5'AGT GGA GCG ATT ACA GAA 3'	158 bp
Reverse primer *(tetM)*	5'CAT ATG TCC TGG CGT GTC TA3'	

Table (2) : *tetK* and *tetM* Primer stock solutions.

Primer name	Volume for 100pmol/µl
Forward primer (*tetK*)	222.0
Reverse primer	248.0

(*tetK*)	
Forward primer (*tetM*)	**226.0**
Reverse primer (*tetM*)	**243.0**

Table (3) : PCR protocol and thermocycling conditions .

Gene name	Initial denaturation	Cycles	Denaturation	Annealing	Extension	Final extension
tetK	95˚C/3min	30	95˚C/30sec	54˚C/30sec	72˚C/30sec	72˚C/4min then 4˚C→∞
tetM	95˚C/3min	30	95˚C/30sec	54˚C/30sec	72˚C/30sec	72˚C/4min then 4˚C→∞

Medicinal plants

Stems and leaves of *M. pipetia* and *M. longifolia* were collected from gardeners in different places in Erbil city, the plants were classified in the Education Salahaddin University Herbarium (ESUH). Then washed with

tap water and after that dried at 40 C for 24 hours and powdered by using coffee machines .

Preparation of aqueous and alcoholic extraction

In present study, 150 ml of distilled water was added to 12 g of ground dried *M. piperita* and *M. longifolia* for each plant separately and each plant was heated below the boiling point and stirred for 1-1/2-3hr, then the mixture was concentrated by oven at 40◦C until dried to obtain the crude extract ,the same extraction was carried out but ethanol used as a solvent (20) .

Antibacterial assay of aqueous and alcoholic extracts of the *M. pipertia* and *M. longifolia*

The ability of the *M. piperita* and *M. longifolia* plants as antibacterial agents were studied and the isolated isolates were separately inoculated in nutrient broth tubes, incubated in incubator for 24hours at 37ºC , then for each bacteria separately 0.1ml of growth culture (comparing with McFarland tube 0.5) was added to 10ml of sterile nutrient broth containing different concentrations of the *M. piperita* and *M. longifolia* (100, 200, 400, 600, 800, 1000, 1200, 1400, 1600,1800 ,2000) µg/ml for both aqueous and alcoholic extracts separately, in addition ,10ml of sterile nutrient broth for each concentration was used as control samples. Later, the cultures were incubated for 24 hours at 37ºC, the bacterial growth was evaluated on the basis of the turbidity of the suspension and all tubes were read by Spectrophotometer at 600nm.

Results and discussion

Thirty two isolates of staphylococci was isolated from clinical sources; including eight isolates (12.5%) of each of *S. hominis* and *S. haemolyticus* from (64) patients urine which suffering from UTI and eight isolates (16%) of each of *S. epidermidi*s and *S. auricularis* from (50) ear patients which suffering from otitis in Rizgary teaching hospital in Erbil city were isolated depending on Vitek 2 compact system and these results agreed with results of (21) . Identification of isolated Gram positive bacteria was depended on Vitek2 analysis with the Gram positive (GP) identification card which designed for using with this system, the colorimetric GP card contain 43 tests as shown in table (4). It is an automated machine designed to provide rapid and accurate phenotypic identification for most clinical microorganism. The percentages of isolation

may be different and may be related to the time of collecting specimens, the number of specimens, the differences in the sources of isolates, hospitals included in each study and the differences in the identification methods. CONS were among the most frequently isolated bacterial species in clinical bacteriology, and most CONS related infections were hospital acquired. Although CONS have long been regarded as nonpathogenic, they had now been recognized as relevant opportunistic pathogens (22).

On the other hand, susceptibility tests were determined for all isolated CONS to 10 different antibiotics by disc diffusion method. From obtained results observed various susceptibilities to different antibiotics among isolates and all isolates were 100% sensitive to ceftriaxone and novobiocin, while higher resistance was obtained to tetracycline with percentage 50%, whereas they differ in their resistant to other remaining antibiotics which were oxacillin, teicoplanin, piperacillin, cefoxitin, cephalothin, ofloxacin and ceftazidime with percentage 31.25%, 25%, 21.375%, 18.75%, 18.75%, 9.37% and 3.125% respectively (Table 5), and these results were similar to results of (23). Moreover *S. epidermidi*s was the most resistant bacteria to all studied antibiotics under the study with percentage 23.75%. The resistance to tetracycline occurs by three mechanisms efflux, ribosomal protection, and chemical modification (1). Indeed the antibiotic resistance can be divided in to: Intrinsic resistance in which bacteria may be inherently resistant to an antimicrobial. Acquired resistance this type of resistance results from changes in the bacterial genome. Resistance in bacteria may be acquired by a mutation and passed vertically by selection to daughter cells. More commonly, resistance is acquired by horizontal transfer of resistance genes between strains and species (24). The resistance to antibiotics done through different mechanisms such as inactivation of the drug e.g. production of β- lactamase by staphylococci, the enzyme which is plasmid coded, destroy the β-lactam ring responsible for the antibacterial activity of pencillins. Altered uptake in this mechanism the amount of drug that reaches the target is either reduced or completely inhibited e.g. tetracycline resistance can be either due to altered permeability of the cell wall or to pumping of the drug out of the cell (25).

On the other hand, all isolated *S. hominis*, *S. haemolyticus* , *S. epidermidi*s and *S. auricularis* isolates were tested for tetracycline resistant genes *tetK* and *tetM* by using the primer and PCR conditions as described previously and the amplicons were sized by electrophoresis in 2% agarose gel. PCR method indicated that 75% of *S. hominis S. haemolyticus* and *S. epidermidi*s were carried *tetM* gene with product size about 158 bp while 50% of *S. auricularis* was carried the above mentioned gen (Figure 1). It means that the *tetM* was found also in some sensitive isolates to tetracycline phenotypically. On the other hand, it was found that 50% of *S. hominis* and *S. haemolyticus* isolates in this study were harboring *tetK* gene with product size 360 bp as showen in figure (2), and no *tetK* gene was observed in *S. epidermidi*s and *S. auricularis* . These results were similar to results of (19). In the present study, the results of antibiotic susceptibility by disk diffusion method were compared with gene analysis results in staphylococcal isolates and it was showed that a total of 32 isolates of staphylococci; 20 isolates were found resistant to tetracycline phenotypically, while 22 and 8 isolates were carried *tetM* and *tetK* respectively (Table 6) and comparison of

conventional method and PCR assay did not showed a good agreement and it was found that some isolates were carried either *tetK* or *tetM* or both resistance genes. The antibiotic resistance genes by PCR assay can be done within a few hours, it is rapid and reliable method for antibiotic susceptibility are important to institute appropriate therapy.

On the other hand, the results showed that the MIC for aqueous extract for both plants were 1600µg/ml and it were 1400µg/ml for alcoholic extract of both plants against most isolated staphylococcus (Figure 3) except for *S. epidermidi*s which the MIC for *M. piperita* was 1200 µg/ml for aqueous extract and 1000 µg/ml for alcoholic extract , while the MIC for *M. longifolia* was 1200µg/ml for both the aqueous and alcoholic extracts as it showed in figure (4) .It means that similar results were obtained in present study for both plants and both extractions and these results were similar to that found by (26) when he studied the antimicrobial activity of the plant against seven selected pathogenic and non-pathogenic microorganisms: *Staphylococcus aureus, Streptococcus mutans, Streptococcus faecalis, Streptococcus pyogenis, Lactobacillus acidophilus, Pseudomonas aeruginosa* and the yeast *Candida albicans* and these might be due to that the both plant belong to same family Lamiaceae , which might be have similar chemical composition such as sabinene, linalool, terpinene, eugenol and thymol, menthol, and pulegone. Moreover, thymol previously known for its antimicrobial activity attributed the thymol antimicrobial action to its phenolic character, which can cause membrane-disturbing activities (27; 28). It stimulates bile flow, reduces the tone in the esophageal sphincter, facilitates belching, and has antibacterial properties. Moreover, its used as a local anesthetic agent in cold and cough preparations and in liniments for insect bites, eczema, poison ivy, hemorrhoids, toothaches, and musculoskeletal pain (29).

Table (4) : Identification of some isolated Staphylococci species by Vitek2 system.

Biochemical tests	*S.auricularis*	*S. hominis*	*S.epidermidis*	*S. haemolyticus*
AMY	-	-	-	-
APPA	-	-	-	-
LeuA	-	-	-	-
AlaA	-	-	-	-
dRIB	-	-	-	-
NOVO	-	-	-	-
dRAF	-	-	-	-
OPTO	+	+	+	+
PIPLC	-	-	-	-
CDEX	-	-	-	-
ProA	-	-	-	-
TyrA	-	-	-	-
ILATk	+	-	+	-
NC6.5	+	+	+	+
O129R	+	+	+	-
dXYL	-	-	-	-
AspA	-	-	-	-
BGURr	-	-	-	-
dSOR	-	-	-	-
LAC	+	-	+	+
dMAN	+	-	-	-
SAL	-	-	-	-
ADH1	+	+	+	+
BGAR	-	-	-	-
AGAL	-	-	-	-
URE	+	-	+	-
NAG	-	-	-	-
dMNE	-	-	-	-
SAC	+	-	+	+
BGAL	-	-	-	-
AMAN	-	-	-	-
PyrA	-	-	-	+
POLYB	-	-	-	-
dMAL	+	-	+	+
MBdG	-	-	-	-
dtRE	+	+	-	+
AGLU	-	-	-	-
PHOS	-	-	-	-
BGUR	-	-	-	+

dGAL	+	-	+	+
BACI	-	-	+	-
PUL	-	-	-	-
ADH2s	-	-	-	+

Table (5) : Antibiotics resistant percentage in some Staphylococci species under the study.

Antibiotics	Symbol	Concentration µg	*S. hominis*	*S. epidermidis*	*S. auricularis*	*S. haemolyticus*	Total
Ceftriaxone	CRO	30	0%	0%	0%	0%	0%
Ceftazidime	CAZ	10	0%	12.5%	0%	0%	3.125%
Ofloxacin	OF	30	0%	25%	0%	12.5%	9.375%
Cefoxitin	FOX	30	12.5%	37.5%	12.5%	12.5%	18.75%
Cephalothin	CF	30	25%	12.5%	12.5%	25%	18.75%
Novobiocin	NV	30	0%	0%	0%	0%	0%
Oxacillin	OX	10	37.5%	25%	25%	37.5%	31.25%
Piperacillin	PRL	30	12.5%	37.5%	12.5%	37.5%	25%
Teicoplanin	TEC	30	25%	37.5%	25%	25%	28.125%
Tetracyclin	TE	30	62.5%	50 %	37.5%	50%	50%
Total			17.5%	23.75%	12.5%	20%	

Table (6) : Relationship between tetracycline resistance by phenotypic method and the presence of the tetK and tetM genes by PCR in some Staphylococci species.

Isolated staphylococci	Tetracycline resistance by phenotypic method	The distribution of tetracycline Gene by PCR	
		tetM	*tetK*
S. hominis	**5 (62.5%)**	**6 (75%)**	**4 (50%)**
S. epidermidis	**4 (50%)**	**6 (75%)**	**0(0%)**
S. auricularis	**3 (37.5%)**	**4 (50%)**	**0(0%)**
S. haemolyticus	**4 (50%)**	**6 (75%)**	**4(50%)**
Total	**16**	**22**	**8**

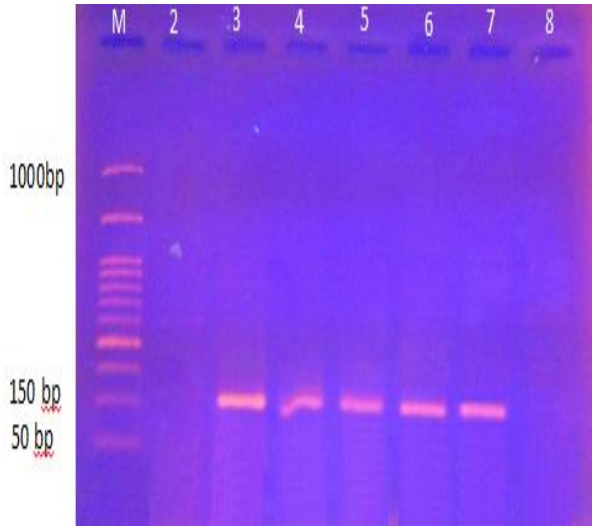

Figure (1) :Polymerase chain reaction products on gel electrophoresis (2%) for *tetM* gene. M: DNA ladder (50 bp). Lanes 2, 8 negative isolates for gene. Lanes 3,4,5,6 7 amplified PCR product of *tetM* gene (158 bp) for positive isolates of Staphylococci species.

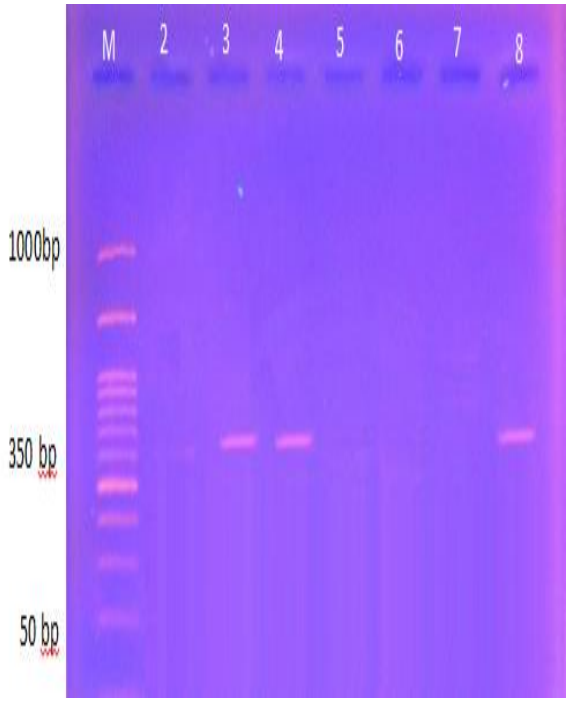

Figure (2): Polymerase chain reaction products on gel electrophoresis (2%) for *tetK* gene. M: DNA ladder (50 bp). Lanes 2, 5,6,7 negative isolates for gene. Lanes 3,4,8 amplified PCR product of *tetK* gene (360 bp) for positive isolates of Staphylococci species.

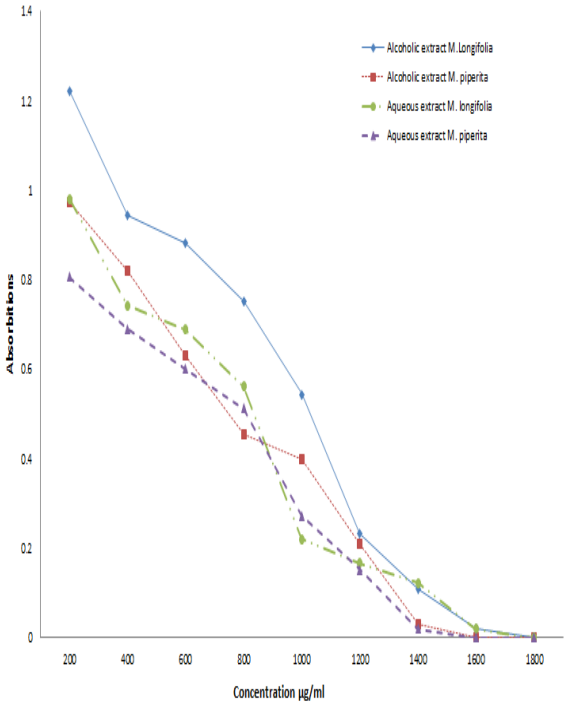

Figure (3): The effect of aqueous and alcoholic extracts of *Mentha piperita* and *Mentha longifolia* on the growth of Staphylococcus species.

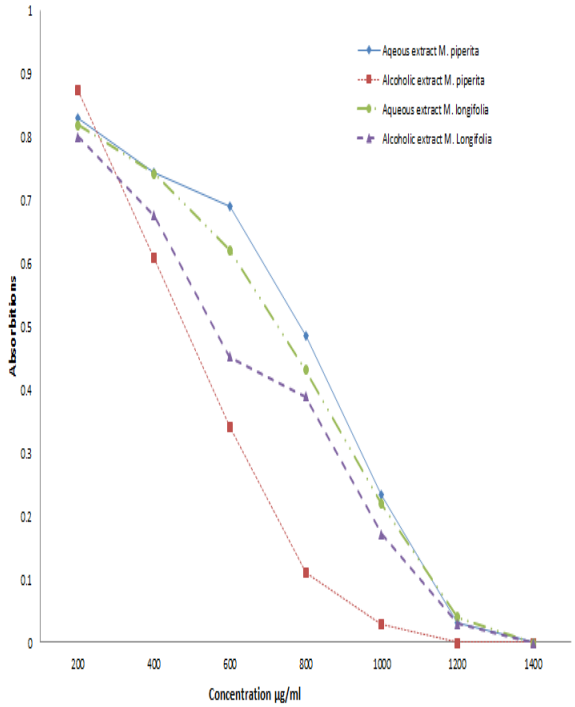

Figure (4): The effect of aqueous and alcoholic extracts of *Mentha piperita* and *Mentha longifolia* on the growth of *Staphylococcus pidermidi*s.

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التحري عن جينات المقاومة للتراسايكلين (tet K ,tet M) في بعض المكورات العنقودية السالبة لانزيم التجلط المعزولة من مصادر سريرية مختلفة في مدينة اربيل .

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الخلاصة:

تم عزل وشخيص اثنين وثلاثون عزلة من المكورات العنقودية السالبة لانزيم المخثر للبلازما من عينات سريرية ؛ والتي تشمل ثمانية عزلات(12.5 %)من كل من *Staphylococcus hominis* و *Staphylococcus haemolyticus* من ادرار (64) مرضى يعانون من التهاب المثانة و ثمانية (16%)عزلات اخرى من *Staphylococcus epidermidis* و *Staphylococcus auricularis* من اذن (50)مرضى يعانون من التهاب الاذن في مستشفى اربيل التعليمي في مدينة أربيل وذلك باستخدام جهاز VITEK 2 Compact System. ثم تم إختبارالحساسية لعشرة مضادات حيوية وباستخدام طريقة أنتشارالاقراص لجميع المكورات العنقودية السالبة لانزيم المخثر للبلازما. وقد اظهرت النتائج بان جميع العزلات كانت 100% حساسة ceftriaxone و novobiocin ، في حين اعلى مقاومة(50%) كانت tetracycline وايضا اظهرت العزلات مقاومة متفاوتة للمضادات المتبقية .ومن جهة اخرى فحصت جميع عزلات *Staphylococcus epidermidis* , *Staphylococcus haemolyticus* ,*Staphylococcus hominis* و *Staphylococcus auricularis* في احتوائها على الجينات المقاومة لـtetracyclin والتي هي *tetK* و *tetM* وباستخدام تقنية PCR وقد اظهرت النتائج بانه 75% من *Staphylococcus hominis* ، *Staphylococcus haemolyticus* و *Staphylococcus epidermidis* تحمل جين *tetM* وكان حجم تضخيم 185 زوج قاعدة ،بينما كانت تحمل *Staphylococcus auricularis* 50% من الجين المذكور. ايضا وجد بانه 50% من عزلات *Staphylococcus hominis* و *Staphylococcus haemolyticus* تحمل جين *tetK* مع حجم تضخيم 360 زوج قاعدة ولم يلاحظ جين *tetK* في كل من *Staphylococcus epidermidis* و*Staphylococcus auricularis* . كما درس ايضا تاثير المضاد البكتيري لنوعين محليين من نبات النعناع ضد العزلات البكتيريه المقاومة وباستخدام طريقة التركيز المثبط الادنى (MIC). وقد اظهرت النتائج بان MIC للمستخلص المائي لكلتا النباتين *Mentha longifolia* و *Mentha piperita* كانت 1600ميكروغرام/مل ،اما للمستخلص الكحولي MIC كانت1400 ميكروغرام/ مل لكلتا النباتين ولمعضم انواع المكورات العنقودية باستثناء *Staphylococcus epidermidis* التي كانت MIC للمستخلص المائي 1200ميكروغرام/ مل و 1000 ميكروغرام/ مل للمستخلص الكحولي لنبات *Mentha piperita* ، بينما كانت 1200ميكروغرام/ مل لكلتا المستخلصين المائي والكحولي لنبات *Mentha longifolia*.