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Characterization of Mannitol Fermenter and Salt Tolerant Staphylococci from Breast Tumor Biopsies of Iraqi Women

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Abstract:

The emergence of staphylococci, either coagulase negative (CNS) or coagulase positive (CPS), as important human pathogens has implied that reliable methods for their identification are of large significance in understanding the diseases caused by them. The identification and characterization of staphylococci from biopsies taken from human breast tumors is reported here. Out of 32 tissue biopsies, a total of 12 suspected staphylococci grew on mannitol salt agar (MSA) medium, including 7 fermenters and 5 non-fermenter staphylococci based on traditional laboratory methods. Polymerase chain reaction (PCR) successfully identified seven isolates at the genus level as methicillin resistant *Staphylococcus* spp. by targeting a common region of the *mecA* gene. Only two of the seven bacteria were *S. aureus* based on the three-specific primers designed to amplify the housekeeping gene *recN*, and two of the virulence genes *icaD* and *pvl*. Diagnosing the isolates using the Vitek system revealed different findings. Although 6 of 7 isolates belonged to the *Staphylococcus* genus, including: *S. cohnii* subsp. *cohnii*, 2 isolates; *S. lentus*, 2 isolates; and one isolate for each *S. auricularis* and *S. xylosus*, the last bacterium was completely different (*Aerococcus viridans*). Concerning the two bacteria characterised as *S. aureus* by PCR, they were identified as *S. lentus* by Vitek with comparatively low detection probabilities of 93% and 88%. The data of this study indicate that undoubtedly PCR is a reliable and accurate test for identification of mannitol fermenter and salt tolerant bacteria in comparison with other tests that depend mainly on biochemical characteristics.

Key words: Breast tumor, Mannitol fermenter, PCR, Salt tolerant, *Staphylococcus* spp, Vitek2.

Introduction:

Cancer formation induced by bacteria has been suggested as a consequence of bacterial infection (1, 2), which constitutes one step among multistep process needed for real cell transformation and cancer development. Numerous bacterial mechanisms can contribute to cell transformation. Importantly, chronic bacterial infections increase the possibility to initiate tumorigenesis because of the increasing chance to encountering a pre-transformed cell (3). In this context, *S. aureus* has been suggested to contribute to tumor formation by producing chronic inflammation, which causes production of various cytokines, including tumor necrosis factor that has been associated with all steps of tumor development. Moreover, *S. aureus* has been shown to cause dangerous medical problems in humans by

compromising their genomic integrity through causing DNA double strand breaks (DSBs) (4).

These genetic changes and instabilities might not be the cause, but the result of cancer cell formation. The role played by carcinogens, infectious agents and correlating factors is the induction of cellular senescence instead of mutagenic (5).

Whether microorganisms, including bacteria, are cause or result of carcinogenesis is still under debate. Nevertheless, various Gram-positive bacteria have been found to cause life-threatening infections in cancer patients, especially infections of staphylococci among others (6, 7). Staphylococci belong to a versatile genus of bacteria that can cause acute and chronic infections in a wide range of hosts. Coagulase-negative staphylococci (CNS) are now common opportunistic pathogens in intensive care units and oncology wards (8). The success of these organisms as pathogens is partly due to their ability to alleviate exogenous and endogenous oxidative and nitrosative stress (9). Besides, CNS are associated with numerous

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mechanisms of antibacterial resistance, including methicillin resistance (10).

A reasonable tool for the identification of *S. aureus* is the growth and production of yellow colonies on mannitol salt agar (MSA) due to its ability to withstand high salt content of the medium and to ferment mannitol. These characteristics have long been used to distinguish between CNS and coagulase-positive staphylococci (CPS). However, CNS have also been observed to produce yellow colonies on MSA (11). Thus, the complete and precise identification of *Staphylococcus* spp. is still difficult but has a diagnostic value (12). In this regard, the organism responsible for the infection can be misidentified if only screening tests were employed in clinical laboratories, adding to the other drawbacks of these methods being laborious and time-consuming (13). It has been confirmed that additional tests are needed to differentiate between *S. aureus* and CNS mannitol-fermenting (yellow) colonies on MSA (14). Therefore, many automated commercial systems have been developed and used for routine laboratory diagnosis (15, 16) due to the ability of these new methods to identify microbes at the species-level in a shorter time. One of these systems is the fluorescence-based technology of the VITEK 2 system. This system was used successfully by Ligozzi *et al.*, (17) to identify a large series of Gram-positive cocci, including 100 isolates of CNS. This diagnostic tool has been proved to be able to identify organisms at the species-level faster than traditional methods, however, false-negative reactions have been

reported owing to the short incubation times used by the Vitek system. In comparison, molecular methods, such as polymerase chain reaction (PCR), have been shown to be more reliable for identification of CNS than other commercial systems, such as the API STAPH system and Vitek as well (18).

Due to the reasons mentioned above, this study was designed and aimed to isolate and characterize mannitol fermenter and salt tolerant bacteria misidentified as *S. aureus* from breast tumor biopsies of Iraqi women using conventional tests, PCR and Vitek2 system.

Materials and methods:

Biopsies

All specimens were handled anonymously. Thirty-two tissue biopsies were obtained aseptically from breast tumors (benign and malignant) of women referred to Baghdad Teaching Hospital from December 2018 to March 2019. In addition, 10 swabs were obtained from different areas and equipment in the surgical hall of the same hospital to evaluate the sterility procedures followed in this room, in particular. These samples were placed into a transport medium (Brain Heart Infusion Broth) and transported within two hours inside a cool box to the laboratory of Zoonotic Diseases Unit, College of Veterinary Medicine, University of Baghdad. Patients' information as stated by specialist physicians are mentioned in Table 1 below.

Table 1. Information of patients from whom the biopsies containing staphylococci were collected.

Patient No.	Age	Type of tumor	Clinical observation	Bacterial growth Characteristics
1	20	Benign	Small mass on the breast	Non-fermenter
2	48	Benign	/	Fermenter
3	65	Malignant, mastectomy (complete removal) of the right breast	Abnormal growth of the breast tissue with abnormal size	Fermenter
4	53	Malignant, mastectomy of the right breast	Abnormal growth and large size of the breast	Fermenter
5	20	Benign (Fibroadenoma)	Small mass at the left breast	Fermenter
6	25	Suspected malignant	Yellow pigmentation, inflammatory cells seen in the left breast (chronic mastitis)	Fermenter
7	52	Malignant (Bilateral)	8 th surgery for the same reason (removal of breast mass) with pus formation in both sides	Non-fermenter L Fermenter R
8	31	Malignant (Bilateral)	Mass at the left breast with pus, and bigger than the right	Fermenter
9	22	Benign (Lipoma)	Small mass at the left breast	Non-fermenter
10	55	Benign (Lipoma)	Mass at the right breast	Non-fermenter
11	38	Probably benign	Mass at the right breast	Non-fermenter

L and R refer to the left or right side of the breast, respectively.

Routine diagnosis

For bacterial isolation, the tissue biopsies were minced with a sterile scalpel into smaller pieces, which were then grinded further with a small amount of normal saline inside a clean and sterile mortar to make a homogenized mixture. Later, a loopful of the homogenate was streaked onto blood agar (to check for the hemolysis ability of the isolates) and mannitol salt agar (MSA) plates, which were incubated aerobically at 36°C for 24 or 48 hr. Distinct well-separated yellow colonies on MSA were chosen for this study, and further characterized. Staphylococci were also identified based on Gram's reaction, catalase, oxidase, coagulase (slide and tube method using rabbit plasma) and DNase tests. In short, DNase test was carried out by inoculating the suspected bacteria on DNase agar plate, the next day a droplet of 1 N HCl was added to the culture, and the positive reaction represented by the formation of wide pale area on the agar. Concerning slide coagulase test, clumping factor was determined, in which loopful of bacterial colonies was homogenized with 50 µl physiological saline, and then mixed with 50 µl of rabbit plasma. The developing coagulation was

determined as positive reaction. Tube coagulase was also performed by adding 0.1 ml of bacterial culture to 0.5 ml rabbit plasma, and appearance of coagulation was regarded as positive reaction (19).

Molecular identification

Primers design

Molecular identification was performed using the primers designed in this study, and manufactured by Macrogen, Korea. PCR assay was conducted to confirm that the suspected isolates belonged to *Staphylococcus* spp. and are methicillin resistant at the same time by designing primers amplify a partial region (280 bp) of the *mecA* gene common to all *Staphylococcus* spp. As we were interested mainly in *S. aureus*, 3 pairs of primers were designed to amplify specifically 3 genes of this bacterium, including the housekeeping gene, *recN*, and other two virulence genes: *pvl* (Panton-Valentine leucocidin) and *icaD* (intercellular adhesion protein), which is responsible for biofilm formation. Accession numbers of genes used to design the primers as well as the amplicon sizes are mentioned in Table 2 below.

Table 2. The genes and their accession numbers used to design the primers.

Gene name	Accession no.	Primer sequence 5'→3'	Product size
<i>mecA</i>	MH188482.1	F: CCACCCTCAAACAGGTGAAT R: AACGTTGTAACCAACCCCAAG	280 bp
<i>recN</i>	CP022894.1	F: GCAACTTGTGGCAAGTGAGA R: GTCCAAATAAAGGGGAACCA	238 bp
<i>Pvl</i>	NC_007795.1	F: CGGCGATGAATTATTGTGTG R: ATGTTTTTGAGGCGTTCAT	214 bp
<i>icaD</i>	CP029474.1	F: ACCCAACGCTAAAATCATCG R: TTCCTCTGCCATTTTTGAA	270 bp

F: Forward, R: Reverse primer, bp: base pair.

DNA extraction

Genomic DNA was extracted from the bacterial isolates according to the protocol of ABIOPure Extraction kit (ABIOPure, USA). Firstly, 7 of the mannitol fermenter isolates with 1 non-fermenter, suspected to be *Staphylococcus* spp., were grown overnight on blood agar plates. Then, three to five colonies were picked up, suspended in 1 ml of phosphate buffered saline (PBS), and spun at 13000 rpm using centrifuge (Fisher Scientific, USA) for 2 min. The supernatant was discarded, and the pellet mixed with 100 µl of lysozyme solution and the same volume of nuclease-free water, then vortexed and incubated in water bath at 37°C for 30 min. Subsequently, the samples were centrifuged for 2 min at 13000 rpm, and the supernatant was discarded. Proteinase K solution (20 µl; 20 mg/ml) and buffer BL (200 µl) were added to each sample for cell lysis and protein

digestion. Later, the tubes were mixed vigorously, incubated at 56°C for 30 min, and then incubated for another 30 min at 70°C for further lysis. Absolute ethanol (200 µl) was added to the samples and vortexed to mix the contents thoroughly. Afterwards, the contents of each tube were transferred carefully to the mini-column, centrifuged at 6,000 xg (>8,000 rpm) for 1 min, and the collection tube was replaced with a new one. Next, 600 µl of buffer BW were added to the mini-column, centrifuged at 8,000 rpm for 1 min, and the collection tube was replaced with a new one. Then, 700 µl from buffer TW were added and spun for 1 min at 8,000 rpm. The flow through was discarded, and the mini-column was introduced back into the collection tube, which was spun at full speed for 1 min to remove residual wash buffer, then the mini-column was placed into a fresh 1.5 ml tube. Finally, buffer AE (100 µl) was added and incubated for 1

min at room temperature, and then centrifuged at 5,000 rpm for 5 min. The concentration of the extracted DNA in each sample was quantified using Quantus Fluorometer (Promega, USA). Briefly, 1 μ l of DNA was mixed with 199 μ l of diluted QuantiFlour dye (Promega, USA), and following 5 min incubation at room temperature, DNA concentration values were determined.

PCR reaction and cycling conditions

The PCR reaction in a total volume of 20 μ l involved the use of 5 μ l of nuclease free water, 1 \times of 10 μ l of GoTag Green Master Mix (Promega, USA) (2 \times stock), 1 μ l of 1 μ M of forward and reverse primers manufactured by Macrogen, Korea (10 μ M stock), and 3 μ l of the DNA sample (10 ng/ μ l). The PCR cycling conditions included: initial denaturation at 95°C for 5 min and 1 cycle. Followed by 30 cycles of each of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension for 30 sec at 72°C. Then, final extension was setup at 72°C for 1 cycle of 7 min.

Agarose gel electrophoresis

Following PCR, agarose gel electrophoresis was performed to confirm the occurrence of DNA amplification. Agarose 1% was prepared by mixing 1 gm of agarose powder with 100 ml of 1 \times TAE buffer in a Duran bottle, which was boiled using Microwave until it was completely dissolved. After that, appropriate volume of Ethidium Bromide (10 mg/ml) was added to the melted gel. The gel was allowed to cool down to 50-60°C before being poured into the gel tray whose sides were sealed with cellophane tapes. After putting the comb into its place, the gel was left to solidify at room temperature for approximately 1 hr. Then, the comb and tapes were carefully removed, and the gel tray was placed in an electrophoresis tank filled with 1 \times TAE buffer that covered the gel. Afterwards, 5 μ l of each PCR product was loaded directly to each well of the gel. Electrical power was turned on at 100 v/mAmp for 75 min. The Ethidium Bromide stained DNA bands in the gel were visualized using gel imaging system (Major Science, Taiwan).

Vitek2 diagnostic system

Vitek2 DensiCHEK standard, Vitek2 cassette and Vitek2 BCL card were used for bacterial identification. The VITEK 2 system (bio-Merieux SA, France) was used according to the manufacturer's instructions. The card consisted of 64 plastic wells, of which 18 empty wells and 46 wells for fluorescent and inhibitory tests that included pH change tests and derivatives to detect aminopeptidases and -osidases. Substrates used for

detection of -osidases were coupled with 4-methylumbelliferone (4MU). While substrates used to detect aminopeptidases coupled with 7-amino-methylcoumarin (7AMC). Substrates used with the 21 tests involved: 4MU- α -D-glucoside, 4MU- α -D-galactoside, 4MU- α -L-arabinofuranoside, 4MU- α -D-N-acetylneuraminic acid, 4MU- β -D-glucoside, 4MU- β -D-galactoside, 4MU- β -D-glucuronide, 4MU-n-acetyl- β -D-glucosaminide, 4MU- β -D-mannoside, 4MU-phosphate, arginine-7AMC, alanine-7AMC, α -glutamic acid-7AMC, histidine-7AMC, urease (butiloxycarbonyl-Val-Pro-Arg-AMC), phenylalanine-7AMC, threonine-7AMC, proline-7AMC, lysine-7AMC, tyrosine-7AMC, and pyroglutamic acid-7AMC. Furthermore, the card contained 16 fermentation tests for the following: D-glucose, D-galactose, D-xylose, lactose, D-mannitol, D-maltose, D-melibiose, D-sorbitol, D-raffinose, D-trehalose, amygdaline, arbutin, salicin, glycerol, N-acetyl-glucosamine, and L-arabinose. In addition, the card included two decarboxylase tests for ornithine and arginine, and six other tests for pyruvate, urease, novobiocin, optochin, 6.5% NaCl as well as polymyxin B sulfate (18).

In this system, the phenotypic identification of bacterial species based on the findings of 43 colorimetric substrates. Before diagnosis, the bacterial isolates were plated onto appropriate media and incubated overnight at 37°C. Then, bacterial suspension was made in saline containing 0.45%-0.50% NaCl inside a polystyrene tube. The suspension's density was adjusted to be equivalent to number 0.5 McFarland standard (1 \times 10⁸ CFU/ml) by using a Densicheck (bioMérieux). Afterwards, the tube and card were inserted into the Vitek2 cassette, and the card was auto-inoculated within the Vitek2 instrument via a vacuum-release method. Wells in the card were optically scanned and read each 15 min, with a total incubation time of approximately 8 hours.

Sequencing

In order to confirm the PCR findings, the amplicons (roughly 270 bp and 400 bp) of the two *S. aureus* isolates were sent to the Macrogen Corporation (Korea) to perform nucleotide sequencing. Sanger sequencing method was used by automated DNA sequencer (ABI3730XL). The sequencing results were analyzed manually and aligned with reference genes sequences deposited on the National Center for Biotechnology Information (NCBI). The DNA sequences obtained from this study were submitted to GenBank of the NCBI to be deposited online under specific accession numbers.

Results:

Routine diagnosis

The 10 swabs obtained from different areas and equipment in the surgical hall of the same hospital from which tumor biopsies were collected showed negative growth on MSA indicating the sterility procedures followed by the hospital.

Concerning the tumor samples, all the suspected fermenter staphylococci isolates shared the Gram's reaction color and arrangement, the growth on MSA (salt resistant), mannitol fermentation (either strong or weak), catalase positive result, oxidase negative, DNase production as well as slide and tube coagulase positive (Fig. 1).

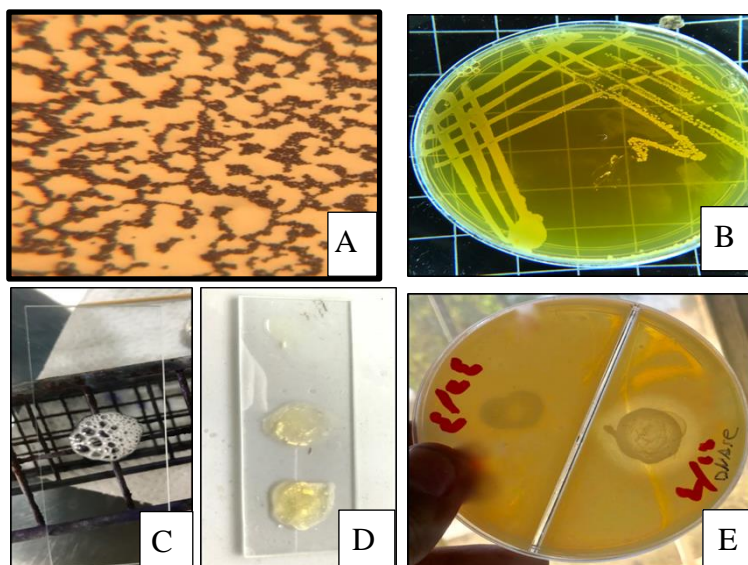


Figure 1. Characteristics of fermenter staphylococci isolates. A- Gram positive grape like cocci. B- Growth on MSA. C- Catalase positive. D- Slide coagulase (upper: negative, middle: weak positive, lower: strong positive). E- DNase positive (right) compared to DNase negative (left).

Molecular identification

Thermal cycler successfully amplified a partial region of 280 bp of the *mecA* gene in 7/8 of the tested isolates, in which isolate number 7 was intentionally used as a control that showed methicillin susceptibility on Mueller Hinton agar (data not shown). The *mecA* primers used here had two benefits: identification of *Staphylococcus* spp. and determining the methicillin resistance in the isolates (Fig. 2). Subsequently, the primers specific for *S. aureus* succeeded in its diagnosis through amplification of a specific sequence of the *recN* gene (Fig. 3) along with the virulence gene *icaD* (Fig. 4). However, two bands of approximately 400 bp instead of the expected size of 214 bp were electrophoresed on the agarose gel (Fig. 5).

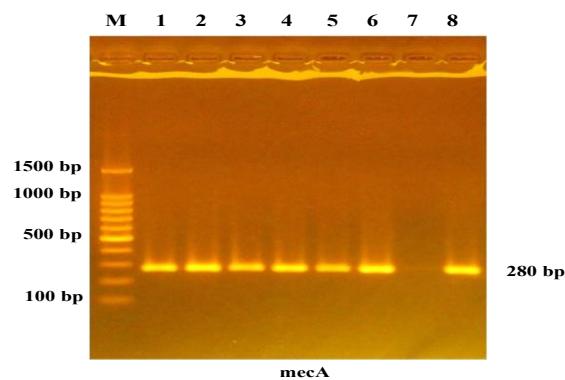


Figure 2. Agarose gel electrophoresis shows bands of the expected size, 280 bp, of the *mecA* gene amplified partially by PCR. Lane M: DNA size marker of 100 bp; lane 1 through 8: PCR products amplified using primers targeting the aforementioned gene and common to many *Staphylococcus* spp.

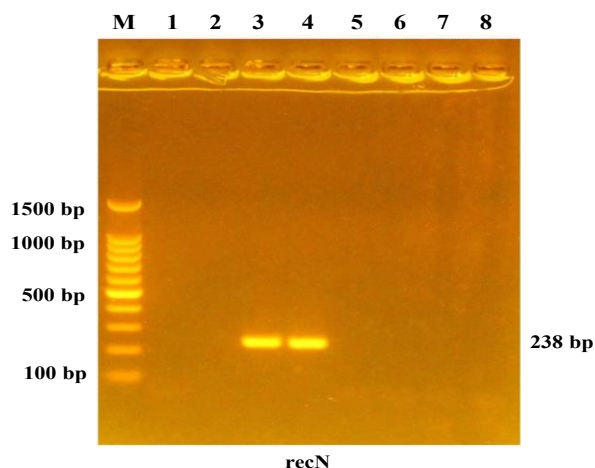


Figure 3. Agarose gel electrophoresis shows bands of the expected size 238 bp of the *recN* gene amplified partially by PCR. Lane M: DNA size marker of 100 bp; lane 1 through 8: PCR amplified products using primers specific for the aforementioned gene in *S. aureus*.

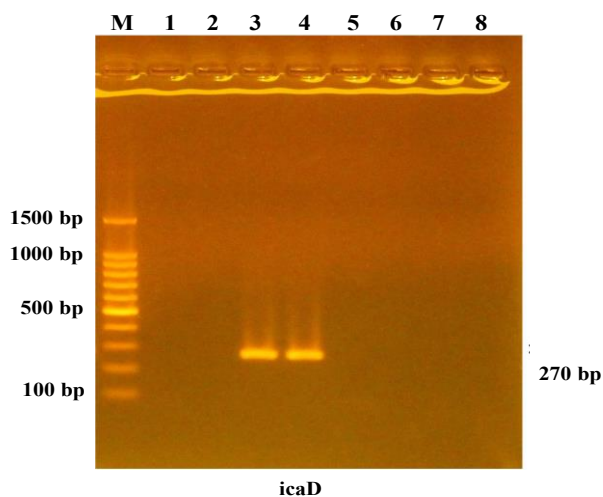


Figure 4. Agarose gel electrophoresis shows bands of the expected size 270 bp of the *icaD* gene amplified partially by PCR. Lane M: DNA size marker of 100 bp; lane 1 through 8: PCR products amplified using primers specific for the aforementioned gene in *S. aureus*.

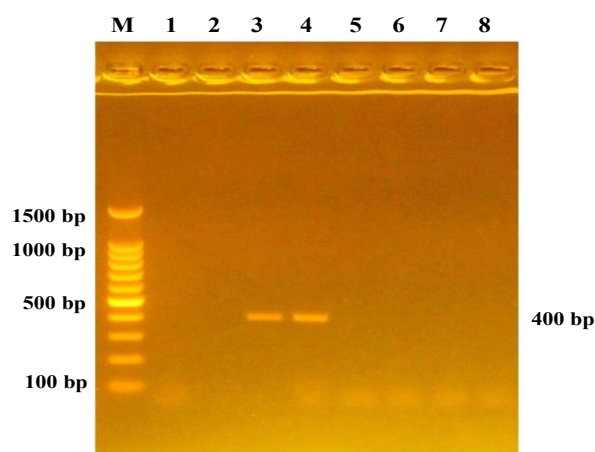


Figure 5. Agarose gel electrophoresis shows bands of approximately 400 bp of unknown gene amplified partially by PCR. Lane M: DNA size marker of 100 bp; lane 1 through 8: PCR amplified genes using primers specific for *S. aureus*.

Vitek 2 diagnosis

Using Vitek2 system, only six samples were diagnosed as staphylococci and one as *Aerococcus*. This system detected the isolates at the species level, with identification probabilities ranged from 88% to 99%. Among the *Staphylococcus* isolates, two belonged to *S. cohnii* subsp. *cohnii* (92% and 99%, respectively), two of *S. lentus* (88% and 93%, respectively), and one of each of *S. auricularis* (92%) and *S. xylosus* (97%). One exception is *Aerococcus viridans* (90%), which was diagnosed as *Staphylococcus* spp. by PCR based on the gene *mecA* specific for staphylococci. Another problem, the *S. aureus* verified by PCR were identified as *S. lentus* with Vitek2 system (Table 3).

Sequencing:

The sequences of the two PCR products sized 270 and 400 bp confirmed the specificity of these primers to *S. aureus*. Upon alignment of sequences obtained from this study with reference genes taken from the NCBI tool using the Basic Local Alignment Search Tool (BLAST), the bands of roughly 270 bp on agarose gel were confirmed to be the *icaD* partial sequence. The sequences of the two *S. aureus* isolates were deposited in the NCBI databases under the GenBank accession numbers MN239185 and MN239186. However, matching of these *icaD* sequences of this study with 10 other highly similar sequences on the BLAST tool showed 99.5% identity between these isolates and the other strains of *S. aureus*.

Whereas the BLAST search for the other bands of approximately 400 bp showed that they were not the *pvl* gene. But, they were found to be 99.5%

identical to the *bglA* gene that encodes for 6-phospho- β -glucosidase of *S. aureus*. The GenBank

accession numbers given to this gene were MN239187 and MN239188.

Table 3. Identification results obtained with the Vitek2 system for mannitol fermenter Staphylococci.

Biochemical details	2 (90%)	3 (88%)	4 (93%)	5 (90%)	6 (92%)	7 (97%)	8 (99%)
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	-	-	-	-	-	-	-

The numbers refer to 2: *S. cohnii* subsp. *cohnii*; 3: *S. lentus*; 4: *S. lentus*; 5: *Aerococcus viridans*; 6: *S. auricularis*; 7: *S. xylosus*; 8: *S. cohnii* subsp. *cohnii*. % refers to the probability of diagnosis, and bold boxes indicate similar results in all the isolates, (-): negative suspected result.

Discussion:

In this study, from 32 biopsies 12 Gram's positive bacterial isolates were isolated and diagnosed by traditional laboratory tests as staphylococci, including 7 were mannitol fermenter and tolerant to the salt content of MSA. Several species of CNS have been reported to ferment mannitol on different media (20). Some of our

isolates were positive for DNase and tube and slide coagulase among other tests. Although other mannitol fermenter staphylococci were coagulase negative, but they were included in the current research. Mostly, mannitol and salt positive CNS are frequently neglected and considered as contaminants, so that they are not identified further by researchers. However, the emergence of CNS as human pathogens and reservoirs of antimicrobial

resistance has made investigators to pay more attention to find a trustworthy method for their identification, which in turn would increase understanding of their potential pathogenicity (21). Interestingly, most isolates of this study showed double zone of hemolysis on blood agar made from human erythrocytes. Production of hemolysin(s) by CPS and CNS has been considered as one of the virulence factors. Moraveji *et al.*, (22) determined hemolytic activities phenotypically in 90% of the total human isolates, and the authors claimed that double hemolysin might indicate that human isolates are highly pathogenic.

Primers specific for staphylococci were applied in this study to identify this important genus using PCR, in which the *mecA* gene was used for genus identification as well as confirmation of methicillin resistant. The *mecA* gene has been considered as a useful molecular marker for detecting methicillin resistance in *Staphylococcus* spp. in general (23). Here, seven of eight tested isolates were identified as methicillin resistant staphylococci. Methicillin resistance has been considered as a marker of resistance to other beta-lactams and is awarded by various mechanisms. This resistance occurs due to the presence of the *mecA* gene that is located on a staphylococcal chromosomal cassette (SCC). This gene codes for an altered penicillin binding protein (PBP2a) with low affinity for all beta lactams. Other genes regulating the expression of *mecA* are located on SCC*mec* as well. In addition, various other genes such as *aux* and *fem*, in *S. aureus* for example, are able to regulate *mecA* expression. The *blaZ* gene that resides on the transposon Tn552 encodes beta lactamase and could mediate methicillin resistance, and it has regulatory genes resemble those of *mecA* (10).

Because of the importance of *S. aureus* as a serious pathogen, primers specific for the *recN* gene and the virulence factors *icaD* and *pvl* were designed for this bacterium to be used in the PCR assay. The first gene was successfully amplified in this study indicating that this bacterium is *S. aureus*. Similarly, the *icaD* gene was found in two of the isolates, which further confirmed the identity of *S. aureus*. This gene was shown to be present in all biofilm producing strains, indicating the importance of *ica* genes as virulence markers in infections of staphylococci (24). In contrast, the PCR assay failed to amplify the *pvl* gene because of its absence in the isolates of this research. The *pvl* gene codes for Pantone-Valentine leukocidin (PVL), which is a cytotoxin that causes leukocyte destruction and tissue necrosis. It was found that PVL produced by less than 5% of *S. aureus* strains, and was related

with necrotic lesions involving mucosa or skin (25) and associated with recurrent skin and soft tissue infections (26). Nucleotide sequencing verified the PCR product of the partial *icaD* gene, which was deposited in GenBank under the Accession Numbers MN239185 and MN239186. While sequencing of the other gene of approximate size of 400 bp was found to be identical to the *bglA* gene (sequences of this study were deposited under the Accession Numbers MN239187 and MN239188), which encodes for the 6-phospho- β -glucosidase (BglA) from glycoside hydrolase family 1 (GH-1). This gene product catalyzes the hydrolysis of β -1,4-linked cellobiose 6-phosphate to yield glucose and glucose 6-phosphate. Both reaction products are further metabolized by the energy-generating glycolytic pathway (27). In a study, it has been indicated that β -glucoside metabolism by 6-phospho- β -glucosidase might be important for survival and virulence of pneumococci (28).

Significantly, this bacterium was grown from patients number 3 and 4 (Table 1) where malignant breast cancer was confirmed, and the women subjected to mastectomy. This reflects the seriousness of this bacterium. Lodhi and co-workers (29) stated that methicillin resistant *S. aureus* (MRSA) is the most common pathogenic organism in breast abscesses and can lead to worse outcome. The virulence of *S. aureus* is determined by a variety of secreted proteins that are toxic to humans. In one study, one third of the virulence genes were found only in *S. aureus* isolates, which contained approximately 100 of these genes (30). *S. aureus* secretes a number of hemolysins, such as β -toxin and δ -toxin (31). Thus, the presence of *S. aureus* inside tumorous tissue cannot be excluded from being the cause of deteriorating women cases leading to complete excision of the breast, and this organism may be the principal cause of cancer occurrence. Originally, *S. aureus* was considered as stringent extracellular pathogen, but in the last decade it has been classified as facultative intracellular pathogen (32). This bacterium can be ingested by many cell types, within which it persists for varying periods of time (33). Internalization of *S. aureus* in endothelial cells has been suggested to trigger its persistence, and those endothelial cells could establish an intracellular persistence niche responsible for infection deterioration post antibacterial treatment (34, 35).

Using Vitek2 system to identify fermenter bacteria showed consistent results at the genus level, in which six of seven isolates were staphylococci (5 out of 6 were mannitol fermenters), but one was *Aerococcus viridans* (mannitol fermenter). However, the *S. aureus*

isolates confirmed by PCR using three sets of specific primers were misidentified as *S. lentus* by Vitek2 system with somewhat low detection probabilities of 88% and 93%. *Staphylococcus lentus* has been associated with animal infections and has rarely been reported as a human pathogen (36). Those staphylococci have been isolated from chickens, rodents, mammals and in farm water and soil (37). Nevertheless, *S. lentus* is considered as a true pathogen that deserves more attention. In the last years, *S. lentus* has been reported to cause serious human infections, including wound infections, endocarditis, endophthalmitis, urinary tract infections, pelvic inflammatory disease, and peritonitis (38). In addition, *S. lentus* has also been isolated from hospitals, and it showed multiple resistance to antibiotics (39).

This inconsistent finding of the Vitek2 system might represent another weakness of this system to be added to other reported incorrect identifications. Misidentification of species with decreased probability has been reported by many authors (18, 20) who used Vitek2 system. It was found that of 37 *S. hominis* isolates, seven were erroneously diagnosed as either *S. epidermidis* (43%), *S. saprophyticus/S. epidermidis* (43%), or *S. warneri* (14%). The possibility of false-negative reactions of Vitek2 can be attributed to the short incubation times used by this system (18). Moreover, the poorer performance of this system with CNS has been hypothesized to be related to the relatively decreased metabolism rates of these species. The same problem for the same reason has also been met with the use of the Vitek2 system for identification of some Gram-negative bacteria (40).

Interestingly, this study reports for the first-time isolation of certain bacterial spp. from breast biopsies where they have not been reported before. For instance, *A. viridans*, which recently has been recognized as human pathogens capable of causing variety of infections (41). It has occasionally been encountered as human pathogens leading to endocarditis, meningitis, osteomyelitis, septic arthritis, urinary tract infections, bacteremia, and cutaneous vasculitis (42). These bacteria are easily misidentified as streptococci or staphylococci; therefore, their infections have long been underestimated (43). Although identification of this species based on Vitek method was proved to be error-prone, in which there is a possibility of identifying *A. sanguinicola* as *A. viridans* (44).

Conclusion:

In conclusion, the existence of methicillin resistant staphylococci, including the most pathogenic *S. aureus*, in the tissue of breast cancer

can predict worse prognosis. Consequently, accurate identification of *Staphylococcus* spp. is critical for the correct management of their infections. Although commercial rapid tests are available, yet their results are meaningless if they cannot be considered reliable. Hence, future studies related to staphylococci identification need to be based on the most reliable molecular techniques, including PCR.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

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توصيف المكورات العنقودية المخمرة للمانتول والمقاومة للأملاح المعزولة من خزع اورام الثدي

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الخلاصة:

نشوء جراثيم المكورات العنقودية (Staphylococci) سواء كانت موجبة او سالبة لخميرة التجلط، كمسببات مرضية مهمة للإنسان، يتطلب استخدام طرائق موثوقة لتشخيصها لغرض فهم الامراض التي تسببها. سجلت الدراسة الحالية توصيف وتشخيص المكورات العنقودية في خزع مأخوذة من اورام الثدي في النساء. من مجموع ٣٢ خزعة نسيجية، نمت ١٢ عزلة على وسط املاح المانتول الصلب وكان مشكوكا بها على أنها المكورات العنقودية، ضمنها ٧ مخمرة للمانتول و ٥ عزلات أخرى غير مخمرة اعتمادا على طرائق التشخيص الروتينية في المختبر. نجح تفاعل سلسلة خميرة البلمرة بالتعرف على ٧ عزلات على مستوى الجنس كونها تعود لمجموعة المكورات المقاومة للمثسلين من خلال استخدام بوادئ استهدفت منطقة عامة في جين *mecA*. جرى تشخيص اثنان من المكورات العنقودية الذهبية *S. aureus* من بين العزلات السبعة وذلك باستخدام ٣ ازواج من البوادئ خاصة بالجرثومة (*recN*, *icaD*, *pvl*). أظهر نظام الفايك (Vitek2 system) نتائج مختلفة بالمقارنة مع النتائج أعلاه. مع ذلك، استطاع نظام الفايك التعرف على ٦ عزلات من ٧ كونها تعود الى جنس المكورات العنقودية، ضمنها: عزلتان تعود لكل من النوع *S. cohnii* subsp. *cohnii* و *S. lentus*، وعزلة واحدة لكل من *S. xylosum* و *S. auricularis*، بينما كان تشخيص العزلة الأخيرة مختلفا تماما (*Aerococcus viridans*). فيما يخص جرثومتي المكورات العنقودية الذهبية التي شخصها تفاعل سلسلة خميرة البلمرة، فقد شخصت على انها نوعا اخر (*S. lentus*) بواسطة نظام الفايك وبنسب احتمالية منخفضة نسبيا ٩٣٪ و ٨٨٪. تبين الدراسة انه بلا شك نتائج تفاعل سلسلة خميرة البلمرة موثوقة ويمكن التعويل عليها للتعرف على الجراثيم المخمرة للمانتول والمقاومة للأملاح بالمقارنة مع الاختبارات الأخرى التي تعتمد بصورة رئيسية على الخواص البيوكيميائية للأحياء المجهرية.

الكلمات المفتاحية: اورام الثدي، مخمرات المانتول، تفاعل سلسلة خميرة البلمرة، الصامدة للأملاح، المكورات العنقودية، الفايك.