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Methicillin-Resistant *Staphylococcus epidermidis* Isolated from Breast Tumors of Iraqi Patients

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ABSTRACT

Background: *Globally, breast cancer is the second leading cause of death among women in Iraq. Several genetic and environmental factors are associated with the occurrence of the breast cancer.* ***Methods:*** *The aim of this study was to isolate and identify aerobic bacteria from breast tumors. Total 100 samples (50 swabs and 50 biopsies) were collected aseptically from benign and malignant breast tumor specimens of Iraqi patients admitted to Baghdad Medical University Hospital. Bacteria were identified using traditional diagnostic methods, API® Staph as well as VITEK® Card. Furthermore, using species-specific primers, the polymerase chain reaction (PCR) assay followed by electrophoresis verified the most common isolates.* ***Results:*** *Among the isolated bacteria, Staphylococcus epidermidis comprised the highest incidence rate (50.6%) of the 77 culture positive swabs and biopsies. Interestingly, the number of S. epidermidis isolates was 27 out of 50 (54%) in malignant tumors and 12 out of 27 (44.4%) in benign samples. These bacteria were further verified by amplifying recN, which encodes a recombination/repair protein. On disc diffusion antimicrobial susceptibility test, most of the isolated bacteria showed methicillin resistance which was confirmed by amplifying the mecA gene using PCR.* ***Conclusions:*** *These data suggest that S. epidermidis might play an important role in breast carcinogenesis.*

Keywords: *Staphylococcus epidermidis*, Breast tumor, Traditional diagnosis, PCR, recN, mecA genes, Antimicrobial susceptibility test

INTRODUCTION

Microbes inhabiting the human body can influence human health [1]. Many lines of evidence link infections of chronic bacteria to the high frequency of certain types of human cancer [2]. Cancer development and progression at different body regions, such as skin, lung, liver, colon, and stomach has been linked to the microbiome [3]. Roughly, 16% of human cancers all over the world are associated with infectious agents or microbial chronic infections with an increased incidence rate in developing countries (22.9%) compared to the developed ones (7.4%) [4].

Several bacteria either aerobic or anaerobic have been linked or defined as being causative agents of solid tumors [5-8]. The most widely studied bacterium was *Helicobacter pylori*, which has been linked to adenocarcinoma of the distal stomach [9,10]. Another example is *Streptococcus gallolyticus* (formerly known as *Streptococcus bovis*) that is suggested to be associated with an increased risk for colorectal carcinoma [11,12]. Pathogenic *Escherichia coli* is also among many bacterial species that have been linked to increased risk of colon carcinogenesis [13,14]. *Salmonella Typhi* infection has been related to increased risk of gallbladder cancer [15]. Additionally, infections with *Chlamydia pneumonia* were associated with a high risk of lung cancer in certain groups [6,16,17].

The microbial imbalance has been suggested to play a crucial role in the occurrence of multiple diseases including cancer [18]. Microbial alterations that disturb the symbiotic correlation between the microbiota and the host are referred to as dysbiosis [19]. Dysbiosis has been found to impair the control of pathogenic microorganisms and to dysregulate inflammatory or immune response against commensal organisms resulting in severe acute and chronic tissue damage [20]. Dysbiosis or antibiotic treatment has been demonstrated to alter the capability of the microbiome to metabolize estrogen [21]. When dysbiosis takes place, a decrease in the overall number of bacteria and/or the

abundance of specific species, such as *Sphingomonas yanoikuyae*, may cause a reduction in the bacterial-dependent immune cell stimulation, eventually leading to a lenient environment for breast carcinogenesis [1].

Indeed, bacterial mechanisms used to colonize, cause or treat cancer have not been fully discovered [22]. Two mechanisms have been suggested that link bacteria to cancer, these include induction of chronic inflammation, along with the bacterial production of carcinogenic metabolites, which can cause immune evasion and immune suppression [23,24]. However, recent studies demonstrate more complex interactions to be present between bacteria and host. Firstly, the relative abundance of bacterial species and microbial community composition can contribute to tissue health and disease [25-27]. Secondly, not all bacteria are pathogenic; some have probiotic impacts that enable them to maintain host health [28]. Bacteria have been proposed to play a role in maintaining the health of breast tissue via promoting host inflammatory responses [1]. Innate immune sensors and antimicrobial response effectors were found to be expressed at higher levels in healthy breast tissue relative to tumors [1]. Inflammation has been suggested to be implicated in the stimulation of cancer, partly via the production of nitrogen and reactive oxygen species, which in turn provoke the occurrence of single-strand DNA or double-strand DNA breaks or DNA cross-links that can steer genomic instability and mutations within tumor suppressor genes or oncogenes [29-31].

Few researchers have worked on the microbial cause of cancer. They showed that microbes had an extraordinary pleomorphological propensity that was required for the tumor development. Because infections of bacteria can be treated with antibiotics, so identification of bacterial causes of cancer might have essential implications for prevention of cancer. It might be vital to study the link between tumorigenesis and breast microbiota which can be exploited in developing new strategies for cancer treatment, and due to the influence that the microbes can have on the metabolism of pharmaceutical agents used to cure cancer. Taken together, the present study aimed to isolate and identify the bacteria from benign and malignant breast tumors in Baghdad city, with more attention paid to the most prevalent ones.

MATERIALS AND METHODS

Ethics Statement

Before taking the breast tumor samples from patients, approval was obtained from Baghdad Medical University Hospital, Baghdad, Iraq.

Samples Collection and Processing

Important information from each patient was recorded but is not shown because of ethical reasons. Breast tissue biopsies were collected by the surgeon, throughout the period from October 2017 till February 2018, from 50 women in different ages undergoing breast surgery, lumpectomy or mastectomy, for either benign (n=21) or malignant tumors (n=29), respectively at Baghdad Medical University Hospital in Baghdad, Iraq. Pair of swab and biopsy specimens was collected simultaneously and aseptically from inside the breast tissue, i.e., the center of the specimen, of each patient to avoid contamination as far as possible. After excision, the swab was taken and placed in a commercial swab collection tube (Afco, Jordan), and tissue biopsy was placed in a sterile tube containing tryptone soya broth (Salucea, Netherlands) and transported within 2-3 hours on ice-box to the laboratory. As an environmental control, a test tube filled with phosphate-buffered saline was left opened in the surgical room during the time of taking the samples from the surgical operation. In the laboratory, the samples were either cultivated immediately on bacteriological media mentioned below or kept overnight in 4ºC to be cultured the next day.

Tissue specimens were minced by sterile scalpels and homogenized in a suitable volume of sterile phosphate-buffered saline using sterile mortar and pestle inside a sterilized hood or vortexed depending on the size and texture of the specimen. Fresh homogenate and an environmental control were cultivated as below.

Bacterial Isolation and Identification

Firstly, Gram's stain was performed for all the swab and biopsy specimens, and based on the gram's reaction, the samples were plated on blood agar (Oxoid, USA), tryptone soya broth and agar (Salucea, Netherlands), MacConkey's agar (Salucea, Netherlands) or mannitol salt agar (Salucea, Netherlands) for 24-48 hours in an incubator of 37ºC

to obtain bacterial colonies. Biochemical tests (Oxoid, England) used in this study included: catalase, coagulase, clumping factor, urease, indole, alkaline phosphatase, and DNase. Finally, the bacterium was verified using API® Staph strips (Biomerieux, France) as well as VITEK® Card in some suspected cases.

Antimicrobial-susceptibility Test

All isolates of *S. epidermidis* were tested for their susceptibility to 11 antimicrobial drugs (Oxoid, England) including: ampicillin 25 µg, gentamicin 10 µg, µg, ciprofloxacin 10 µg, chloramphenicol 10 µg, cefixime 30 µg, ofloxacin 10 µg, methicillin 5 µg, cefotaxime 30 µg, oxacillin 1 µg, penicillin 10 units and amikacin 30 µg. Disc-diffusion method was applied using Mueller-Hinton agar (HIMEDIA, India) according to the method proposed by Wayne in 2017 [32].

Molecular Identification of Isolates

DNA extraction: Five or six colonies of pure isolates already grown on Mannitol salt agar were inoculated into brain heart infusion broth (Oxoid, England) overnight at 37ºC for DNA isolation. Wizard genomic DNA purification kit (Promega, USA) was used to isolate the bacterial genomic DNA from *S. epidermidis* isolates following the instructions of the manufactured company. The concentration and purity of the DNA were estimated by spectrophotometer at 260 nm and 280 nm.

Primer Design

In this study, we used new species-specific primers, designed by us using the Primer3Plus software, to amplify the recN and mecA genes of *S. epidermidis*. Using NCBI-BLAST, those oligonucleotides were checked for their specificity. For both the recN and mecA primer sequences, BLAST showed their presence in *S. epidermidis* strains: FDAARGOS-153, DAR1907, BPH0662, 1457, PM221, 949, SEI, ATCC12228, RP62A, and SR1. The sequences of the recN and mecA genes used to design the oligonucleotide primers were obtained from GenBank sequence databases. Those primers were purchased from Alpha DNA, Canada. Primers designed in this study including recN-Forward (5'-AACCGCGATTCTTTTTGATG-3') and recN-Reverse (5'-GCATTGGATGCCTTGCTTAT-3') were used to amplify a 174 bp recN fragment. In addition, the primers mecA-Forward (5'-GGCGTGGAAGTAACGATTTC-3') and mecA-Reverse (5'-GCGCACGTCTTTGTTGTTTA-3') were used to amplify a 199 bp mecA fragment.

PCR Assay

Amplification of genes fragments was performed on a thermal cycler (BioRad, USA) after optimizing the annealing temperature for the primers. PCR reaction included using 20 µl of PCR mixture containing 6 µl nuclease free water (Promega, USA), 10 µl (2x) of GoTaq® green master mix (a premixed ready-to-use solution containing Taq DNA polymerase, dNTPs, MgCl2 and reaction buffers at optimal concentrations along with blue and yellow dyes for monitoring the progress during electrophoresis) (Promega, USA), 1 µl (10 µM) of recN- or mecA-forward primer, 1 µl (10 µM) of recN- or mecA-Reverse primer and 2 ng/µl of DNA template. Amplification conditions were initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec for denaturation, 58°C for 30 sec for annealing and 72°C for 30 sec for an extension, with a final extension cycle at 72°C for 7 min. Following preparation of 1% agarose gel mixed with 1 µl (10 mg/ml) of ethidium bromide (Promega, USA), PCR amplicons were analyzed by electrophoresis (Thermo, USA), visualized by ethidium bromide staining with UV transillumination and gel images captured using gel imaging system (Major Science, Taiwan). The 100-bp DNA ladder (Promega, USA) was used as a molecular size marker.

RESULTS

Bacterial Isolation and Identification

Regardless of the location of samples within the breast tissue, presence or absence of breast malignancy, age, menopause status, breastfeeding status, parity, and family history (not shown), different bacterial species was detected in breast tissue (not published data). Of 50 biopsies, 45 showed bacterial growth whereas only 32 out of 50 swabs revealed bacterial isolation. Patients who have already taken antibiotics showed negative bacterial isolation (5 malignant and 18 benign samples). The most abundant species was *S. epidermidis* in both benign and malignant tumors and either swab or biopsy. *S. epidermidis* was isolated from 39 out of 77 culture positive samples of benign and malignant breast

tumors, with an incidence rate of 50.6%. They were detected in 27 out of 50 (54%) of malignant samples that showed bacterial growth, and in 12 out of 27 (44.4%) of benign samples (Table 1). API® Staph and VITEK Card verified the findings obtained by traditional diagnostic methods.

Table 1 Growth and occurrence of *S. epidermidis* in swabs and biopsies taken from benign and malignant breast tumor

Variables	Bacterial growth	Malignant	Benign
Biopsy (n=50)	45	28	17
S. epidermidis	16	13	3
Swab (n=50)	32	22	10
S. epidermidis	23	14	9
Total Samples	77	50	27
S. epidermidis	39	27	12

Antimicrobial Susceptibility Test of *S. epidermidis*

On disc diffusion antimicrobial susceptibility test, *S. epidermidis* isolates showed multi-drug resistance. Most isolated bacteria from benign and malignant tumors were resistant to Oxacillin, Cefixime, Penicillin and Methicillin. In contrast, most isolates were susceptible to Ofloxacin and Cefotaxime. All isolates were susceptible to Amikacin, Gentamicin, Ampicillin, Chloramphenicol, and Ciprofloxacin.

Molecular Identiication of *S. epidermidis*

Using species-specific primers, the PCR assay verified *S. epidermidis* by amplifying recN, which encodes a recombination/repair protein. Amplicons of 174 bp were observed on agarose gel electrophoresis. Figure 1 reveals that our recN primers are species-specific as there are no bands in lanes 6 and 8 where *S. chromogenes* and *Micrococcus spp*. were loaded.

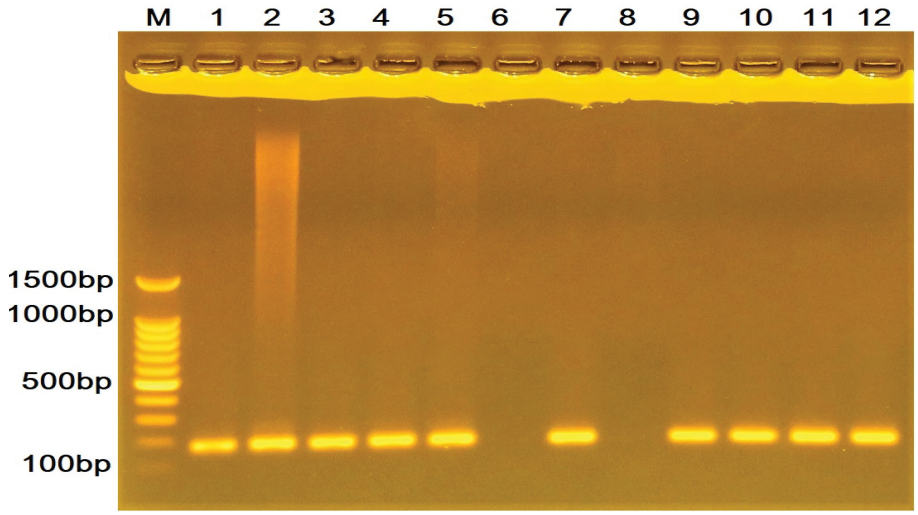

Figure 1 Agarose gel electrophoresis for recN amplicons; agarose gel electrophoresis was performed to verify the PCR amplicons of the recN gene. Bands of the *S. epidermidis* species-specific recN gene fragments (174 bp) are shown. Lane M, molecular size marker (100 bp ladder); all lanes except 6 and 8 (the negative controls) are *S. epidermidis*; Lane 6: *S. chromogenes* and Lane 8: *Micrococcus spp*

Concerning mecA gene, its primers were specific for *S. epidermidis* as shown in Figure 2, where mecA gene fragments were amplified in nine *S. epidermidis* isolates that revealed bands of 199 bp on agarose gel electrophoresis, but no band was observed from one *S. epidermidis* (lane 9), which was phenotypically susceptible to methicillin on disc diffusion antimicrobial susceptibility test. Furthermore, gel electrophoresis showed double non-specific putative bands in lanes 6 and 8, where *S. chromogenes* and *Micrococcus spp*, respectively, were loaded as negative controls (Figure 2).

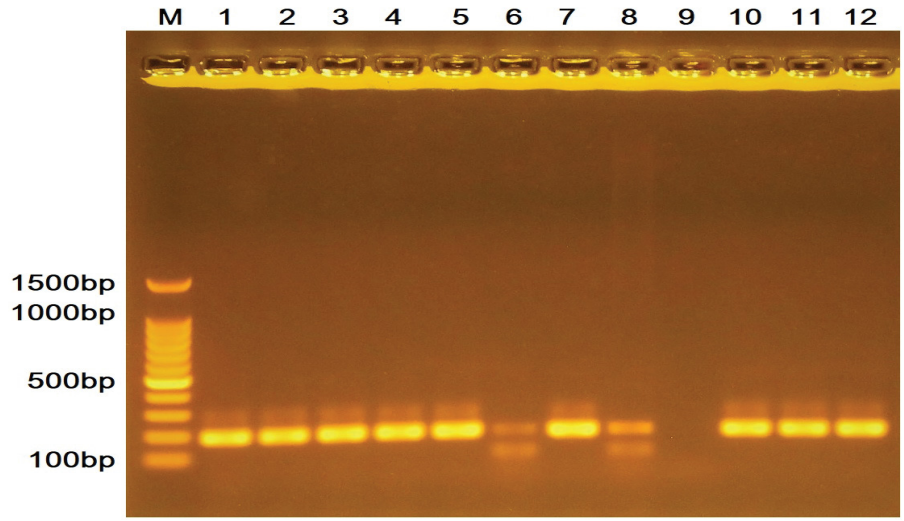

Figure 2 Agarose gel electrophoresis of mecA amplicons; agarose gel electrophoresis was done to confirm the amplification of the species-specific mecA gene of *S. epidermidis* by PCR. Lane M: molecular size marker (100 bp ladder), all lanes (except 6, 8 and 9): *S. epidermidis* specific mecA (199 bp), Lanes 6 and 8: negative control (*S. chromogenes* and *Micrococcus spp*., respectively) and Lane 9: methicillin-susceptible *S. epidermidis* lacking mecA gene.

DISCUSSION

To our knowledge, this is the first study in Iraq where the bacteria were isolated from breast tissue. The latest reports of the Iraqi Ministry of Health refer to the scary increase in the incidence rates of breast cancer with no clear causes, in addition to the limited studies of bacterial isolation from breast tumors worldwide. Several authors who were interested to link carcinogenesis to microbiome worked on DNA extracted from formalin-fixed paraffin-embedded and/or frozen breast tissues, such as in studies of the Irish samples [1,3,33]. Therefore, in our research, we wanted to shed light on microbiome associated with benign and malignant breast tumors. Unfortunately, it was impossible for us to obtain the normal breast tissue for comparison. Clinical data indicate that bacteria may play a protective role against breast tumorigenesis in humans [34]. In the same context, it has been shown that in a mouse model of sporadic breast cancer, treatment of mice with antibiotic resulted in higher risk for tumor development and increased rates of tumor growth [35]. Undoubtedly, specific bacterial species can cause cancer via various and complex mechanisms [36].

In this study, we were able to detect different viable bacteria in 77 samples of malignant and benign breast tissues. Many possible routes have been hypothesized for bacterial access to breast tissue, including bacterial translocation from the gut, a passage from the skin through the nipple-areolar orifices, nipple-oral contact through lactation or sexual contact [37,38]. Many studies support the translocation of beneficial bacteria from the gut to the breast [39-41].

Regarding the replication of bacteria within tumors, many mechanisms have been proposed to be involved. One main mechanism is assumed to occur via the hypotoxic nature of solid tumors leading to decreased oxygen levels in comparison with normal tissues, providing an environment suitable for growth of anaerobic and facultative anaerobic bacteria [42]. Another way involves the availability of nutrients such as purines within the necrotic area [43]. Furthermore, other elements are thought to play a role in bacterial replication that is tumor-specific, such as local immune suppression and abnormal neovasculature (formation of new blood vessels) [43]. Nevertheless, these new blood vessels are vastly disorganized with incomplete endothelial linings and blind ends, which can lead to slow blood flow along with leaky blood vessels. These leaky vessels could permit circulating bacteria to enter tumor tissues and become embedded locally [43]. Moreover, different mechanisms are utilized by tumor cells to elude recognition by the immune system thereby providing a refuge for bacteria to avoid immune clearance [44,45].

Our findings refer to the predominant isolation of *S. epidermidis*, the coagulase-negative staphylococci (CNS), from benign and malignant breast tumors. Although the *S. epidermidis* diagnosis is based on time-consuming traditional

biochemical methods, which identify the bacterium at the species level, these tests have low accuracy [46]. Therefore, API® Staph and VITEK were applied to confirm the isolates. In addition, accurate PCR test was carried out here for the identification of the isolates using primers specific for *S. epidermidis*-recN gene, which encodes a repair and recombination protein. This gene could be used to forecast whole-genome relatedness with high accuracy [47].

We found results of other studies are similar to ours, such as that of where bacteria were isolated from nine of ten different malignant tissues, and *S. epidermidis* strains were detected in four of five breast cancers [48]. *S. epidermidis* has been isolated from lung cancer and breast cancer [5,8]. Moreover, in the study conducted by Brook the most frequently isolated aerobic bacteria from various necrotic tumors were *S. aureus*, although *S. epidermidis* was also detected. Interestingly, those papers share our finding in that the isolated bacteria are commonly associated with the flora of the human body.

An important role of certain strains of commensal skin *S. epidermidis* has been discovered through its production of 6-N-hydroxyaminopurine (6-HAP), a molecule that inhibits DNA polymerase activity thereby impairs tumor growth [49,50]. We propose herein a protective role of this bacterium in healthy breast tissue, which needs to be investigated. However, it has been shown that breast tissue is richer than the skin tissue in bacterial species [3]. On the other hand, it has been suggested that colonization with secondary bacteria mainly CNS in the long-lasting inflammatory environment could play a role in tumorigenesis [51]. Establishing a tumor infection has been proposed to occur even with very low figures of viable bacteria [52]. Although an opposite correlation was suggested to exist between bacterial load at the tumor region and severity of breast cancer [1]. Furthermore, cancer-associated microorganisms including *S. epidermidis* were found to be able to produce human chorionic gonadotropins (hCG) like substance, but in varying amounts (due to differences in growth rates of the microorganisms leading to differences in cell numbers) compared to organisms isolated from non-tumorous tissues [48]. In a larger study performed on CNS strains, including 23 from cancer patients, to check the expression of hCG-like material. The expression of this substance was found to be a strain, not a species characteristic and has molecular similarity to the human hormone [53]. Interestingly, ectopic expression of β-hCG in various cancers including breast has been associated with poor prognosis owing to its tumor-promoting function. While targeting β-hCG expressing cancer cells appears to be a good strategy for breast cancer treatment [54]. These results agree with the previous findings of other workers concerning the possibility of the existence of bacteria-tumor relationships.

Recently, *S. epidermidis* has emerged as a pathogen that is resistant to many antibiotics including methicillin [55]. *S. epidermidis* isolated in this study showed resistance to methicillin, oxacillin, and penicillin using disc diffusion method. Although, the detection of methicillin resistance by traditional tests based on phenotypic expression is rather heterogeneous [56]. Consequently, in our study species-specific, the mecA gene was amplified in *S. epidermidis* isolates. All isolates except one (Figure 2) have mecA and hence were methicillin-resistant. But this does not exclude the ability of this bacterium that lacks mecA from being pathogenic. It has been demonstrated that the pathogenicity of *S. epidermidis* relies in part on the presence or absence of the virulence genes ica and/or mecA [57]. Here, we suggest checking the presence of ica gene in *S. epidermidis* isolates especially those lacking mecA to determine their pathogenicity.

Unlike our study, different breast microbiota has been identified in breast tissues from women with malignant tumor compared to those with benign breast disease, and these findings were declared to be intriguing [3]. Our data show consistency in the predominant bacteria isolated from malignant and benign specimens.

CONCLUSION

To conclude, *S. epidermidis* isolated from tumor samples were mostly multi-drug resistant, and they might have an essential role in breast tissue disease status. It would be necessary to perform further studies with a larger sample size of healthy and cancerous women to determine further differences in microbiota that could be seen between tumor and normal adjacent tissue, especially in Iraq where antibiotics are usually taken without a prescription. An association may be present between breast cancer and antibiotics used in humans, which needs to be profoundly investigated. It is still ambiguous whether the presence of a virulent strain or absence of beneficial one could take part in carcinogenesis stimulation. Therefore, additional work is required to study the influence that might be caused by *S. epidermidis* and its virulence factors on breast tissue and its association with other endogenous or exogenous factors. In addition, studying the bacterial load in breast tumors and healthy breast tissue would be valuable to determine whether bacterial

load could be an additional indicator of diagnosis, staging or progression of breast cancer. Importantly, more attention should be paid to the detection of anaerobic bacteria associated with breast tumors.

DECLARATIONS

Conflict of Interest

The authors have disclosed no conflict of interest, financial or otherwise.

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