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Molecular characterization of Klebsiella oxytoca isolated from breast cancer tissues with evaluation of some breast cancer risk factors in Salaheddin Province – Iraq

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ABSTRACT

Introduction: Cancer comprises a heterogeneous group of diseases that can originate in almost any organ or tissue of the body. It is characterized by the uncontrolled proliferation of abnormal cells that extend beyond their usual boundaries, invading adjacent tissues and potentially spreading to distant organs. This dissemination, known as metastasis, represents the leading cause of cancer-related mortality. The terms neoplasm and malignant tumor are commonly used interchangeably with cancer.

Objective: The present thesis aims to conduct a microbiological, molecular and serological investigation of breast cancer patients in Salah Al-Din Governorate-Iraq, with a focus on identifying bacterial isolates and assessing associated risk factors.

Materials and Methods: This study was carried out between July 1, 2024, and August 31, 2025, in Salah Al-Din Governorate, Iraq. A total of 60 breast cancer tissue samples were collected, along with 40 blood samples for serological testing(progesterone and cholesterol). The patients enrolled in this study were between 45 and 70 years of age.

Results: Microbiological culture of breast cancer tissue swabs revealed that 60% of samples exhibited bacterial growth, whereas 40% showed no growth. Among the culture-positive cases, Gram-positive bacteria (55.6%) were more prevalent than Gram-negative bacteria (44.4%). Of the 36 positive cultures, 28 isolates were successfully identified at the species level, while 8 represented mixed cultures. The most frequently isolated bacterium was *Klebsiella oxytoca* (4/28; 14.3%), *Klebsiella oxytoca* was isolated from breast tissue of breast cancer patients for the first time in Iraq and for the second time worldwide, as it had previously been isolated in the United States. Other isolates included *Cedecea lapagei* (2/28; 7.1%), *Serratia fonticola* (2/28; 7.1%), and *Sphingobacterium thalpophilum* (1/28; 3.6%). Antibiotic susceptibility testing of K. oxytoca isolates demonstrated complete resistance to ampicillin, nalidixic acid, gentamicin, and levofloxacin, with variable resistance to other antibiotics. Moderate susceptibility (50%) was observed for imipenem and meropenem. Molecular analysis revealed the presence of the resistance gene blaNDM in approximately 50% of K. oxytoca isolates. In contrast, the virulence-associated genes entB, luxS, and mrkA were not detected in any of the tested isolates, indicating their absence.

Progesterone levels among the participants were within the normal range in 90% of cases, while 10% showed levels above the normal range. Cholesterol levels were within the normal range in 90% of cases, with 10% above the normal range.

Conclusion: Bacteriological culture of breast tissue samples showed 60% positivity (36/60), with 55.6% Gram-positive and 44.4% Gram-negative isolates. Klebsiella oxytoca was the predominant isolate (14.3%) and was recovered from breast cancer tissue for the first time in Iraq and the second time worldwide. The isolates exhibited multidrug resistance, moderate sensitivity to imipenem and meropenem, and carried the blaNDM gene (420 bp), while virulence genes entB, luxS, and mrkA were absent. Progesterone and cholesterol at normal range in about 90 % of cases

Keywords: *breast cancer, breast tissue, Klebsiella oxytoca, PCR, bla-NDM, progesterone, and cholesterol*

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1. INTRODUCTION

Cancer (CA) refers to diseases arising in almost any tissue when abnormal cells proliferate uncontrollably and spread (metastasis), a major cause of cancer-related death. The terms malignant tumor and neoplasm are also used [(1,2)] . All cancer cells share two fundamental properties: First abnormal cell growth and division (proliferation), and second defects in the normal restraints that keep cells from spreading and colonizing other parts of the body (metastasis) [(3)]. Breast cancer (BC)

is the second most common cancer after lung cancer, influenced by genetic and environmental factors. Despite advances in therapy, recurrence remains a major challenge [4] . Breast cancer is the most common cancer worldwide. The occurrence of breast cancer is associated with many risk factors**,** including genetic and hereditary pre-disposition [5]. Breast cancer stage is the most important prognostic variable. Early-stage breast cancer BC has 10-year survival rates of over 90%. In contrast, metastatic BC, which accounts for approximately 6–7% of de-novo presentations and develops in ~30% of women with early-stage BC at diagnosis, is associated with 5-year relative survival rates of ~25%, and a median overall survival of ~2 years [6].

The human breast harbors a distinct and complex microbial community that differs from those found at other body sites, independent of sampling location within the breast, pregnancy history, presence or absence of breast cancer, age, country of origin, or sequencing method used [7]. This community is referred to here as the "breast microbiome" to distinguish it from microbiomes in other regions of the body. Among the dominant phyla detected in breast tissue of women aged 18 to 90, both in tumor-adjacent and non-cancerous tissue, Proteobacteria is most prevalent, followed by Firmicutes [8].

The breast was initially thought to be sterile; however, several recent studies have revealed that microorganisms reside in the breast tissue. Microbiota in the breast may actually originate from the skin, intestine, mammary gland, and breast milk during the Early stages of life [8].

Klebsiella oxytoca

Klebsiella oxytoca is a non-motile, Gram-negative bacillus of the Enterobacteriaceae family that is part of the normal human microbiota, being detected in the gut of 8–10% of healthy adults and also found on the skin and in the oropharynx [10] . Although it can exist as a commensal, *K. oxytoca* is an important opportunistic pathogen capable of causing infections ranging from mild diarrhea to severe conditions such as bacteremia and meningitis, and has been implicated in healthcare-associated outbreaks [11] . It has emerged as a notable cause of both community-acquired and hospital-acquired urinary tract infections and can acquire antibiotic resistance through the production of β-lactamases and carbapenemases. Furthermore, *K. oxytoca* forms biofilms and adheres to host cells, which contributes to infection persistence and resistance, with several adhesin genes being associated with biofilm formation and antimicrobial resistance [12] .

2. MATERIALS & METHODS

Collection of Swab From Tissue of Breast Cancer :

A swab was taken from the breast tissue affected by cancer during the mastectomy procedure for the purpose of culture and diagnosis of bacteria present in the tissue .

Collection of Blood Sample:

By using a sterile disposable syringe, 5 ml of venous blood sample was drawn from the antecubital vein of each woman breast cancer was collected in a gel tube and allowed to clot at room temperature, then each sample was centrifuged at 4000 rpm for 5 minutes to obtain serum. The serum was aspirated then divided into aliquots in plain tubes and stored at -20 C until the time of estimation. Serum of the patients had assayed for: Progesterone hormone, Cholesterol level .

Culture media preparation

Media are prepared and sterilized according to the recommended instructions by manufacturing Company, preparing of media is depending on the installed information on the packaging by the supplier and then the media were sterilized using an autoclave at 121 °C for 15 minutes. To confirm the absence of contamination, the plates were incubated in an inverted position at 37 °C for 24 hours, after which they were stored in a refrigerator at 4 °C [13] .

Microbial diagnosis using Vitek 2 compact system

In this study, bacterial isolates were first identified using conventional methods, and further confirmed with the Vitek 2 system (BioMérieux, France) following the manufacturer's guidelines for accurate bacterial detection [14] .

Susceptible Antibiotics test :

Bacterial isolates were cultured on Mueller–Hinton agar and tested for antimicrobial susceptibility using the Kirby–Bauer disk diffusion method, according to CLSI guidelines . Fresh colonies (18–24 h) were suspended in sterile saline and adjusted to 0.5 McFarland standard. The inoculum was spread evenly over Mueller–Hinton agar plates using a sterile cotton swab. Antibiotic disks were placed on the surface of the agar within 15 minutes, and plates were incubated at 35 ± 2 °C for 16–18 h. After incubation, the diameters of inhibition zones were measured (in mm) and interpreted as Sensitive, Intermediate, or Resistant according to CLSI breakpoints [15].

DNA Extraction of Bacteria:

The extraction of genomic DNA from bacterial isolates was performed using a commercial DNA extraction kit provided

by MEBEP BIOSCIENCE (Country of manufacturer). All procedures were carried out in strict accordance with the manufacturer's protocol to ensure the recovery of high-quality and intact DNA. The obtained DNA was subsequently stored at –20 °C until further molecular analysis.

Design primers

The primers for the study were designed by Prof. Dr. Ahmed Abdul Jabbar Al-Fahdawi at Anbar University and the primers were obtained from Korea (Macrogen : Korea) in lyophilized form .

Primer Design And Target Gene of *Klebsiella oxytoca*

Table-1: Primer Design And Target Gene of *Klebsiella oxytoca*

OX16s	**F: CCTGGACAAAGACTGACGCT 20**
	R: CCTTTGAGTTCCCGACCGAA 20
Annealing 59 product 417	
entB	F: TATTACCACGCTGACCCTGC 20
	R : AATAGCCACAAACGAGCCGA 20
annealing 59 product 346	
luxS	F: GGTATTCACACCCTTGGGCA 20
	R: GTAAACGTTCAGCTCCGGGA 20
annealing 59 product 225	
bla- NDM	F: GATACCGCCTGGACCGATG 19
	R: GTAGTGCTCAGTGTCGGCAT 20
Annealing 59 product 420	
mrkA	F: GGCAGCGATGCAAACGTTTA 20
	R: CTTTAGGCTGGGTGGCATCA 20
annealing 59 product 325	

Polymerase chain reaction (PCR)

The molecular analysis was performed using the 2×pfu PCR Super Master Mix, with blue dye kit in combination with the Applied Biosystems (**Veriti** 96 Well Thermal Cycler) device, following the manufacturer's protocols to ensure accuracy and reliability of the results.

Electrophoretic Separation of Genomic DNA Using Agarose Gel

Electrophoretic separation of genomic DNA was performed using a horizontal Agarose gel electrophoresis system (Cleaver, UK). A 2% Agarose gel was prepared in 1× TBE buffer, and samples were loaded alongside a molecular weight DNA ladder. Electrophoresis was conducted at a constant voltage for 75 minutes to allow adequate separation of DNA fragments according to their molecular sizes. Following electrophoresis, the gel was stained with Safday (0.5 µg/mL) and visualized under a UV trans-illuminator (Tinzyme / China) to evaluate DNA integrity and fragment size distribution.

Serological testing

Advanced laboratory instruments were used to measure the levels of progesterone and cholesterol in the serum of breast cancer patients according to the manufacturer's instructions.

3. RESULTS

Frequency of Bacterial Growth in Breast Tissue of Cancer Patients

Sixty samples were collected from the breast tissue of patients suffering from breast cancer. Bacteriological culture was positive in 60% (36/60) of cases and negative in 40% (24/60)

Distribution of isolates according to gram stain

The bacterial identification of all 36 isolates obtained from breast tissue specimens was performed. Among these, 20 isolates (55.6%) were Gram-positive, while 16 isolates (44.4%) were Gram-negative, representing the distribution of bacteria in the 36 specimens (60%) with positive culture

Distribution of Gram-Positive and Gram-Negative Bacteria Identified in Breast Tissue

A total of 60 samples were collected from breast cancer patients, of which 36 (60%) showed bacterial growth. Among these 36 positive cultures, 28 samples were successfully identified at the species level and 8 were mixed. The most frequently isolated organism was *Klebsiella oxytoca*, an isolate from the Enterobacteriaceae family (Gram-negative) (4/28; 14.3%). Klebsiella oxytoca was isolated from breast tissue of breast cancer patients for the first time in Iraq and for the

second time worldwide, as it had previously been isolated in the United States.

Distribution of Bacterial Isolates According to Antibiotic Sensitivity

The *Klebsiella oxytoca* isolates exhibited complete resistance to ampicillin, nalidixic acid, gentamicin, and levofloxacin. In contrast, varying levels of resistance were observed against ceftazidime, cefixime, cefotaxime, ciprofloxacin, and fosfomycin, at 50%, 75%, 75%, 25%, and 50%, respectively. Moreover, the isolates showed moderate susceptibility, with 50% sensitivity reported for both imipenem and meropenem.

Molecular Identification of Klebsiella oxytoca Clinical Isolates Using 16S rRNA Gene

The *16S rRNA* gene of the *Klebsiella oxytoca* isolates was amplified using PCR, and the analysis showed complete concordance when compared with the band size reference guide, confirming the precise molecular identification of the bacterium.

Molecular detection of specific genes in Klebsiella oxytoca

The PCR results revealed successful amplification of the *16S rRNA* gene in all four bacterial isolates, with distinct bands of approximately 417 bp, confirming the identity of the isolates. The resistance gene *blaNDM* (420 bp) was detected in isolates 1 and 2 only, while no amplification was observed in isolates 3 and 4. In contrast, the virulence genes *entB*, *luxS*, and *mrkA* were not detected in any of the studied isolates, indicating their absence in the tested samples.

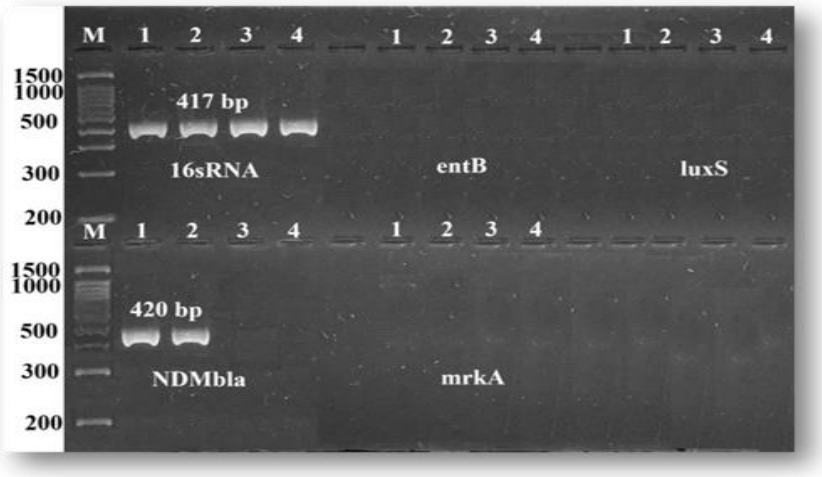

Figure 1 : Molecular detection of specific genes in *Klebsiella oxytoca*

Measurement of progesterone Levels in the Blood of Women with Breast Cancer

Progesterone levels among the participants were within the normal range in 90% of cases, while 10% showed levels above the normal range.

Measurement of cholesterol Levels in the Blood of Women with Breast Cancer Cholesterol levels were within the normal range in 90% of cases, with 10% above the normal range.

4. DISCUSSION

Frequency of Bacterial Growth in Breast Tissue of Cancer Patients

Bacteriological culture was positive in 60% (36/60) of cases and negative in 40% (24/60). The substantial presence of bacteria in malignant breast tissue underscores a potential link between the breast microbiome and cancer development. However, the absence of bacterial growth in a significant portion of samples indicates that this relationship is not universal and may depend on factors such as tumor type, disease stage, or host immunity. These findings emphasize the complexity of tumor–microbiome interactions. The other study done in Kirkuk city [16], [17] through his study revealed that breast tissue harbors bacterial communities whose relative abundance differs significantly from those detected in breast cancer tissue. Furthermore, the microbial composition varies among patients, which may account for the absence of bacterial growth in some samples or the presence of distinct microbial profiles.

Distribution of isolates according to Gram stain

The bacterial identification of all 36 isolates obtained from breast tissue specimens was performed. Among these, 20 isolates (55.6%) were Gram-positive, while 16 isolates (44.4%) were Gram-negative, representing the distribution of bacteria in the 36 specimens (60%) with positive culture. The other study done in Babylon [18] through his study revealed

that the isolation and diagnostic of bacteria from women with breast tumors (50 biopsies), bacteria were found growth (88.57%) detected as Gram-positive (G+ve) and Gram-negative (G-ve) ,distribution between (54.8%) as G+ve bacteria, while (45.20%) were detected as negative (G-ve) bacteria.

Distribution of Gram-Positive and Gram-Negative Bacteria Identified in Breast Tissue

According to Vitek 2 compact diagnostic system bacterial isolates were identified as following: *Klebsiella oxytoca* was the bacterial isolated in breast cancer patients with percentage reached 14.29 % at the study . Other study done in USA [(19)] found that the percentage of isolated bacteria from breast tumor biopsy is *Klebsiella oxytoca* was 1%.

Distribution of Bacterial Isolates According to Antibiotic Sensitivity

The isolates *Klebsiella oxytoca* exhibited complete resistance to ampicillin, Nalidixic acid, gentamicin, and levofloxacin. In contrast, varying levels of resistance were observed against Ceftazidime, Cefixime, Cefotaxime, ciprofloxacin, and Fosfomycin, 50%, 75%, 75%, 25%, 50%, respectively, with different resistance rates across the isolates. Moreover, the isolates showed a moderate susceptibility pattern, with 50% sensitivity reported for both Imipenem and Meropenem. These findings highlight a potential clinical challenge posed by this emerging opportunistic pathogen. Other study done in Kirkuk [(20)] reported the highest resistance pattern of K. oxytoca was 100% against Ampicillin, and 62.50%, 59.37%, 53.12%, against Ceftazidime, Cefixime, Cefotaxime. The highest sensitivity was observed against Amikacin and Imipenem (9.37%) and it was 21.87%, 21.87%, 25%, 25%, 28.12%, 28.12%, and 28.12% against Meropenem, Chloramphenicol, Nalidixic acid, Ciprofloxacin, Tobramycin, Gentamicin, and Doxycycline, respectively.

Molecular Identification of Klebsiella oxytoca Clinical Isolates Using 16S rRNA Gene.

The *16S rRNA gene* of *Klebsiella oxytoca* isolates was amplified by PCR, showing full concordance with the band size reference and confirming precise identification. Vitek 2 compact system results were identical to PCR, demonstrating its reliability as a rapid diagnostic tool. These findings align with previous reports from the USA [(21)] , supporting the credibility of phenotypic methods when validated against molecular approaches.

Molecular detection of specific genes in Klebsiella oxytoca

PCR successfully amplified the *16S rRNA gene* (~417 bp) in all four isolates, confirming accurate molecular identification. The blaNDM resistance gene (420 bp) was detected in 50% of the isolates, consistent with a study from Pakistan [(22)] , indicating that resistance is not universal and may reflect selective pressures or prior antibiotic exposure. The virulence genes entB, luxS, and mrkA were absent in all isolates, similar to findings from China [(23)] , suggesting reduced pathogenic potential, although their absence does not exclude other virulence mechanisms.

Measurement of progesterone Levels in the Blood of Women with Breast Cancer

Progesterone levels among the participants were within the normal range in 90% of cases, while 10% showed levels above the normal range. The results showed that breast cancer patients had progesterone levels below the reference value (3.2 nmol/L), indicating that low progesterone is common among breast cancer patients in this sample . There are numerous studies reporting about the progesterone hormone and breast cancer such as the study done in Indian [(24)] this study reported Progesterone, there was no statistically significant difference in its level between breast cancer patients and healthy women, both in premenopausal and postmenopausal stages .

Measurement of cholesterol Levels in the Blood of Women with Breast Cancer Cholesterol levels were within the normal range in 90% of cases, with 10% above the normal range. indicating that the majority of the cohort maintained cholesterol levels considered normal. There are numerous studies reporting about the cholesterol and breast cancer such as the study done in Samarra [(25)] the results of this study showed that total cholesterol (TC) levels in the serum were significantly lower in breast cancer patients compared to healthy women, both in patients before chemotherapy and after treatment.

5. CONCLUSIONS

Bacteriological culture of breast tissue samples showed 60% positivity (36/60), with 55.6% Gram-positive and 44.4% Gram-negative isolates. *Klebsiella oxytoca* was the predominant isolate (14.3%) among Enterobacteriaceae Klebsiella oxytoca was isolated from breast tissue of breast cancer patients for the first time in Iraq and for the second time worldwide, as it had previously been isolated in the United States., while several Gram-positive species were also recovered. *K. oxytoca* isolates demonstrated multidrug resistance, with moderate sensitivity to imipenem and meropenem. PCR amplification confirmed species identity through *16S rRNA* (~417 bp), verified DNA integrity, and detected the *blaNDM* resistance gene (420 bp) in some isolates, whereas the virulence genes *entB, luxS*, and *mrkA* were absent

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