Isolation and Identification of Bacteria and Fungi Correlated with Mastitis in Breastfeeding Women in AI-Anbar Governorate/West of Iraq

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Abstract:

Mastitis is a common phenomenon experienced by a lot of breastfeeding women. Lactational mastitis, commonly occurs because of prolonged milk duct engorgement, which allows bacteria to enter through breaks in the skin, resulting in an infection. Mastitis can occur at various stages of lactation, although it is most observed within the second and third weeks following childbirth. For isolation and identification of bacteria associated with mastitis, Blood agar medium, MacConkey agar, and mannitol salt agar were used, whereas Sabouraud Dextrose Agar (SDA) was used for fungal isolation. Different biochemical test has been performed for bacterial isolates including gram stain, catalase, coagulase, and Vitec 2 compact. For fungal isolates, KOH treatment and lactophenol cotton blue test were performed. Fungal DNA was extracted then PCR was used for the detection ITS Gene of candida spp. Using gel electrophoresis of PCR product on 2% agarose. The study revealed Staphylococcus aureus, Klebsiella pneumoniae, and Candida spp. as significant bacteria that are commonly related to mastitis in women. The prevalence of Staphylococcus aureus was seen to be high, with a notable manifestation of lesions. Additionally, both Staphylococcus aureus and Klebsiella pneumoniae were shown to be associated with the presence of redness. Candida albicans had a notable
prominence, displaying a heightened prevalence in the regions of the nipple. Furthermore, it demonstrated a correlation with the presence of lesions and redness. The presence of redness was observed as a notable sign in cases linked with Candida tropicalis. This study provides insights into the microbial diversity linked to mastitis mainly in breastfeeding women, highlighting the prevalence of Staphylococcus aureus, Klebsiella pneumoniae, and Candida spp.

**Keywords:** Mastitis, Breastfeeding, Staphylococcus aureus, Candida spp.

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1. INTRODUCTION:

Breastfeeding is critical in maintaining the proper development of the brain and in preventing the simultaneous appearance of malnutrition, infectious illnesses, and mortality [1]. Furthermore, regardless of the country's economic situation, it provides long-term benefits by lowering the chance of obesity and chronic illnesses during maturity [2]. Breastfeeding, in addition to providing passive immunity, promotes active communication between the immune systems of mothers and newborns [3]. Furthermore, women pass on microbial elements to their offspring via breast milk [4].

When infants under the age of 12 months and young children between the ages of 12 and 36 months receive breast milk from their mothers through the act of breastfeeding, they have the best chance of survival, growth, and optimal development [5]. This is partly because breastfeeding is a dynamic intervention activity, and breast milk contains unique living qualities that contribute to these favorable results [6,7].

Breastfeeding women often encounter a condition known as lactational mastitis, which manifests as inflammation in the breast tissue [8]. This discomfort arises due to factors like nipple fissures and milk stasis, which can disrupt the otherwise harmonious experience of breastfeeding [9]. Due to a decline in their defense ability, bacteria through the milk ducts retrograde into the mammary gland, leading to infection [10].

In the initial stage, patients commonly exhibit symptoms such as breast redness, swelling, tenderness, and inadequate milk discharge [11]. As the condition advances, the formation of a lump becomes more pronounced, accompanied by additional symptoms including fever, chills, fatigue, headaches, and other manifestations [12]. Most often, S. aureus colonizes the skin and is one of the most important causes of locational mastitis [13,14]. Methicillin-resistant Staphylococcus aureus (MRSA) may also become a risk factor for this infection [15,16].

Mastitis is intimately associated with bacterial growth, a state where the mastitis-causing microbiota increases while the normal mammary microbiota diminishes [13]. Also, Klebsiella pneumonia has been reported as one of the organisms related to mastitis [16,17]. Fungal infections affecting the nipple and breast may be experienced by breastfeeding women. It is caused by Candida albicans, a prevalent fungal organism responsible for various thrush infections [18,19]. Inflammatory processes and pain may be sparked by the co-existence of Candida spp. in the lactating nipple and breast, including Staphylococcus aureus (S. aureus), Candida albicans, and another Candida spp.
Another possibility is that this syndrome could be caused by multiple organisms. Co-infection with Staphylococcus aureus, Candida albicans, and other Candida spp. in the lactating nipple and breast may lead to inflammation and pain [20].

There are many organisms involved in mastitis in breastfeeding women, so this study aimed to isolate and identification of microorganisms related to mastitis in breastfeeding women, determine the relation between mastitis symptoms and organisms, and assess between microorganisms and mastitis source.

2. Materials and Methods:

2.1. Study Design and Sampling: It is a cross-sectional study consisting of 100 patients with skin infections with mastitis who were recruited from AL-Ramadi Teaching Hospital in Anbar Governorate, Iraq. From the mother and her child, the sample was collected between November 2022 and March 2023. Samples were obtained from a mother with mastitis and her baby. The step in collecting samples is to use sterile cotton swabs, where a swab is taken from the mother from the site of lesion or redness located on the nipple and breast, and a swab is also taken from the mother’s milk and her baby’s mouth.

2.2. Identification of Bacterial Isolates: The blood Agar media used for isolation. 4.25-gram agar was dissolved in 100 ml dis.H2O and sterilized in an autoclave for 15 minutes at 121, cooled to 45 C then 10% V/V sterile defibrinated blood was added aseptically, plates were inoculated and incubated at 37 C for 48 hr [21].

Mannitol salt agar medium also was used to test the ability of the organisms to ferment mannitol, 11.1 gram of the media was dissolved in 100 ml dis H2O and sterilized in an autoclave for 15 minutes at 121 C. Cooled, poured in Petri dishes, and plated were inoculated then incubated for 48h at 37C [22].

Subculture on MacConkey agar was performed by dissolving 5.5 grams of medium in 100 ml dis. H2O was sterilized in an autoclave at 121C for 15 minutes poured onto plates, inoculum was spread on the surface of the plates, and incubated for 48h at 37 C. Gram stain test was carried out to characterize the isolates [23].

A 3% solution of hydrogen peroxide (H2O2) was created by diluting a stock solution (15%) in a dark bottle, then a few drops were added to culture on the slide and on blood agar to do catalase test [24] coagulase test was performed in a glass tube containing a few milliliters of citrated plasma, few drops of bacterial suspension were added, then the tube was incubated for 24 hours in the oven [25] and Vitek 2 were done by using (Gram-positive Vitek-2, Gram-negative Vitek-2) kits acquired from BioMerieux, France.
2.3 Identification of fungal isolates: Sabouraud Dextrose Agar (SDA) (Oxide, United Kingdom) was prepared and inoculated by swabs and then incubated for 24 hr at 37°C. For the KOH test, 10 grams of potassium hydroxide were dissolved in 80 mL dis.H2O. Drops of KOH solution were added to the specimen, a coverslip was placed over the specimen after 10 minutes [26]. Lactophenol cotton blue [27] and Germ tube tests were performed for fungal isolates [28].

2.4 Molecular study of fungal isolates: Fungal DNA was extracted by using the Fungi/Yeast Genomic DNA Isolation Kit Provided by Norgen Biotech, PCR was used for the detection ITS Gene of candida spp. The primers were designed based on the National Centre for Biotechnology Information (NCBI) and provided by the BioOneer Company Table (1). To detect the ITS gene of Candida spp., PCR protocol was employed in Table (2).

<table>
<thead>
<tr>
<th>Sequence of forward and reverse</th>
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</tr>
</thead>
<tbody>
<tr>
<td>F /TCCGTAGGTGAACCTGCGG</td>
<td></td>
</tr>
<tr>
<td>R /TCCTCCGCTTATTTGATATGC</td>
<td></td>
</tr>
</tbody>
</table>

After PCR amplification, gel electrophoresis of PCR product was done on 2% agarose. The investigation encompassed a statistical analysis of the data concerning the types of microorganisms found in each sample, together with the observed variances in symptoms linked with them. The analysis was properly carried out using SPSS Version 25.0 software. The data underwent extensive preprocessing and validation procedures to guarantee its quality and dependability.

<table>
<thead>
<tr>
<th>Step</th>
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<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94</td>
<td>5min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>45 S</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>52</td>
<td>1 min</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>7min</td>
<td>1</td>
</tr>
<tr>
<td>Hold Temperature</td>
<td>4</td>
<td>4min</td>
<td>----</td>
</tr>
</tbody>
</table>

3. Results and discussion:

3.1 Bacterial isolation and biochemical identification: For isolation of Staphylococcus aureus, the differential media blood agar was used, and the colonies appeared on the plate after 24 h (Figure 1. A). Gram stain test revealed that it is gram-positive bacteria which is a main character of Staphylococcus aureus. Also, it produced blurred edges and completely clear zones which indicates that beta-hemolysis took place. Figure 1. B
A) Growth of Staphylococcus aureus.

Figure 1: Staphylococcus aureus on blood agar medium.

Small yellow colonies appeared on mannitol salt agar due to mannitol fermentation by producing an acid that causes the red color of phenol to turn yellow and this is a character of Staphylococcus aureus. Figure (2).

Figure 2: positive mannitol fermenter Staphylococcus aureus.

Staphylococcus aureus is characterized by being catalase positive as it transforms hydrogen peroxide (H2O2) to water and oxygen this test was done on both blood agar and on the slide. Figure (3).

Figure 3: Catalase test positive of staphylococcus aureus

Staphylococcus aureus cells clumped together within 10 seconds in comparison to the control because of staphylococcus aureus being coagulase positive. Figure (4).
Vitek 2 was done and with all results biochemical tests showed that the isolate was Staphylococcus aureus.

The identification of Klebsiella pneumoniae using MacConkey agar is based on the ability of this organism to ferment lactose as Klebsiella pneumoniae is a lactose-fermenting bacterium so pink mucoid colonies are present on the MacConkey agar plate. Figure (5). Gram stain test of the isolate showed that it is a gram-negative bacterium, which is consistent with Klebsiella pneumoniae. The Vitek 2 compact system confirmed the identification of the isolate as Klebsiella pneumoniae.

3.2. Biochemical identification of fungi: Candida spp. grew on SDA medium which is differential media for pathogenic fungi including different species of Candida. Figure (6).
Upon microscopic examination of isolates treated with potassium hydroxide (KOH), the result may exhibit a combination of hyphae, pseudohyphae, and budding yeast cells Figure (7).

Morphology of Candida spp. become obvious after LPCB staining. LPCB staining is a useful method for the visualization and identification of fungal structures and spores, including those of Candida spp Figure (8).

Germ tubes appeared after 3, 6, and 9 hours indicating that the Candida spp. isolates are likely Candida albicans Figure (9).
3.3. **Molecular study of fungi:** Gel electrophoresis has been performed on genomic DNA extracted from Candida spp and the presence of DNA bands on the gel indicates the presence of genomic DNA in the sample **Figure (10).**

![Figure 10 Gel electrophoresis of genomic DNA extraction, 1% agarose gel.](image)

PCR was used for the detection of the ITS Gene of candida spp. After PCR amplification, gel electrophoresis of PCR product was done on 2% agarose. The target gene was found, and its band size is 550 bp **Figure (11).**

![Figure 11 Gel electrophoresis showing PCR product with a band size of 550 bp. N: DNA ladder (1000 plus)](image)

3.4. **The association between the type of organism and the mastitis occurrence and symptoms in women:** Percentage of various organisms (Staphylococcus aureus, Klebsiella pneumonia, and Candida albicans) found in various sources (nipples, breast, and kid oral cavity) among women with mastitis. The presence of Candida albicans in different sources with a higher percentage than Staphylococcus aureus & Klebsiella pneumonia **Table (4).**

<table>
<thead>
<tr>
<th></th>
<th>Staphylococcus aureus</th>
<th>Klebsiella pneumonia</th>
<th>Candida albicans</th>
<th>$\chi^2$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nipples</td>
<td>38 %</td>
<td>20 %</td>
<td>90%</td>
<td>3.324</td>
<td>0.1897</td>
</tr>
<tr>
<td>Breast</td>
<td>50%</td>
<td>20%</td>
<td>73%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nipples</td>
<td>38 %</td>
<td>20%</td>
<td>90%</td>
<td>0.009</td>
<td>0.995</td>
</tr>
<tr>
<td>Child oral cavity</td>
<td>37%</td>
<td>20%</td>
<td>90%</td>
<td>3.661</td>
<td>0.16</td>
</tr>
<tr>
<td>Breast</td>
<td>50%</td>
<td>20%</td>
<td>73%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child Oral Cavity</td>
<td>37%</td>
<td>20%</td>
<td>90%</td>
<td></td>
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</tbody>
</table>

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Different microorganisms are linked to distinct mastitis symptoms. Staphylococcus aureus and Candida albicans appear to be linked to the symptom of lesions, whereas Staphylococcus aureus, Klebsiella pneumonia, and Candida albicans appear to be linked to redness. Cases involving Candida albicans alone, on the other hand, have a higher prevalence of the combination of redness and lesions. Finally, Candida tropicalis is associated with redness as a symptom Table (5).

![Table 5: Type of microorganisms and symptoms of mastitis correlation](image)

4. Discussion:

The bacterial isolate was Staphylococcus aureus as resulted from the biochemical tests performed which included growth on blood agar and making beta-hemolysis, and mannitol fermentation as these are characteristics of Staphylococcus aureus as reported [31]. Also gram-positive, catalase-positive [32], and the positive result of the coagulase test [31] support that it is Staphylococcus aureus. Vitek 2 results align with other biochemical tests and the demonstrated characteristics of Staphylococcus aureus [33].

For Klebsiella pneumoniae, the identification tests conducted are going with characters of it which had mentioned in previous studies. Vitek2 [33], lactose fermentation on MacConkey agar [34], being gram-negative [35]. According to the Clinical and Laboratory Standards Institute (CLSI), gram stain testing is a rapid and reliable method for the preliminary identification of bacterial isolates [36].

In the context of fungi, Candida spp. characters found in previous studies are the same results obtained from the biochemical tests. Growing on Sabouraud Dextrose Agar (SDA) which contains antibiotics such as chloramphenicol and cycloheximide that inhibit the growth of bacteria and other fungi, thereby allowing for the selective growth of Candida spp. [37]. The presence of a combination of hyphae, pseudohyphae, and budding yeast cells after KOH treatment [38] and Morphology of Candida spp. after LPCB staining [39, 40] are recognized as characteristic attributes frequently linked to Candida spp. Germ tube is also used for the
Identification of a Candida as albicans or non-albicans species depending on the presence or absence of germ tubes [41]. A molecular study was also done to detect the ITS gene of Candida using PCR and a single band of 550 bp was found [42]. The ITS region (Internal Transcribed Spacer) is a highly conserved region found in the rDNA gene of fungi, including Candida spp. This region has been widely used as a target for molecular identification of fungal species using PCR-based techniques, including PCR amplification and agarose gel electrophoresis [43].

Many studies have studied the relation between type of microorganisms and mastitis. One study reported that Staphylococcus aureus, Streptococcus agalactiae, and Mycoplasma spp. are the most common pathogens that cause mastitis [44]. A study in 2022 demonstrated that Staphylococcus aureus and Klebsiella pneumonia were found at lower proportions in mastitis cases. Staphylococcus aureus is a common pathogen in cases of mastitis, but it may not be as common as Candida albicans [45]. In a case-control study, women with mastitis were more likely than women in the control group to have Staphylococcus aureus in their breast milk [46]. According to research performed in Germany, the pathogens most found in mastitis instances can differ. Coliform infections like E. coli were discovered to be the most common pathogens in severe episodes of mastitis [47].

A lot of studies investigated the relation between the mastitis symptoms’ and microorganism’s type. Staphylococcus aureus and Candida albicans: an investigation reported that these germs are linked to lesions. Women with Staphylococcus aureus plus Candida albicans in their breast milk may have more lesions than those with Candida albicans [46]. Both bacteria have been linked to the symptoms of breast tissue lesions. These bacteria and their link to the development of breast lesions emphasize the necessity of appropriate cleanliness and efficient preventive measures during breastfeeding [48].

Staphylococcus aureus, Klebsiella pneumonia, and Candida albicans: This combination of bacteria is linked to redness as a mastitis symptom according to a study performed in China [49]. Redness is associated with Staphylococcus aureus, Klebsiella pneumoniae, and Candida albicans [50].

**Candida albicans**: Cases involving Candida albicans alone had a higher frequency of redness and lesions due to an investigation [51,52]. When Candida albicans causes an infection without the presence of other microbes, it may generate a more prominent inflammatory response, including redness [50].

**Candida tropicalis**: This bacterium has been related to redness as a mastitis symptom as mentioned [53]. When Candida tropicalis infects breast tissue, it can cause mastitis or breast
inflammation. The affected area may exhibit redness, swelling, and discomfort as part of the inflammatory response [54].

5 Conclusion:

This study utilized a range of microbiological and molecular methodologies to identify the microorganisms accountable for mastitis in women. The investigation also analyzed the correlation between bacteria and the incidence and manifestations of mastitis. Candida albicans had a significant prevalence, namely in the nipple. The findings of the research demonstrated that various microorganisms exhibit unique symptom profiles.

6 References:


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