



Analysis of DNA Expression of Mycobacterium Tuberculosis in Formalin-Fixed Paraffin-Embedded (FFPE) Granulomatous Mastitis

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Abstract. Granulomatous Mastitis (GM) is a chronic inflammatory breast disease characterized by sterile noncaseating granulomatous inflammation with etiopathogenesis that has not yet been fully evaluated. Tuberculous mastitis is a chronic infectious disease caused by bacillus *Mycobacterium tuberculosis* (MTB) and is also one of the differential diagnoses of GM. In developing countries such as Indonesia, the most common cause of granuloma is *Mycobacterium tuberculosis* infection, besides leprosy, foreign body granuloma, fungal infection, parasitic infection, and actinomycosis. The gold standard for diagnosing tuberculous mastitis is the detection of MTB in a specimen. This study aimed to analyze DNA expression of *Mycobacterium tuberculosis* using PCR technique on formalin-fixed and paraffin-embedded (FFPE) samples of granulomatous mastitis. A total of 45 FFPE samples were collected and subjected to histopathological examination (using Hematoxylin Eosin and Ziehl Neelsen staining) and DNA extraction (Invitrogen Purelink Genomic DNA Mini Kit). A conventional PCR was conducted using the IS6110 gene as target to detect MTB DNA. All GM patients in this study were women with an average age of 30.8 years. All FFPE samples of GM tested negative in the PCR, and no 123 bp band was observed. This suggests that Granulomatous mastitis can be caused by MTB or Mott, and conventional PCR is not sensitive to detect *Mycobacterium tuberculosis* in FFPE samples of GM.

Keywords: Granulomatous Mastitis · Tuberculous Mastitis · *Mycobacterium tuberculosis* · FFPE · PCR · MTB

1 Introduction

Mastitis is a prevalent breast condition that can occur in patients of any age but is most common in women during the breastfeeding period. It is characterized by inflammation of the breast tissue, which may or may not be accompanied by an infection. When

infection is present, mastitis can either be lactational (puerperal) or non-lactational (such as duct ectasia), and it is usually caused by the bacteria *Staphylococcus aureus*. Other causes of non-infectious mastitis include idiopathic granulomatous inflammation and other inflammatory conditions, such as a foreign body reaction [1].

Unspecified acute mastitis is most commonly found during the breastfeeding period and is caused by trauma, baby sucking, or poor hygiene. Mastitis examples include tuberculous mastitis, syphilitic mastitis, and mycotic mastitis, which typically run chronically with no inflammatory signs such as no pain, ulcers, and hard induration and are frequently used as a differential diagnosis for breast carcinoma. [1, 2].

Tuberculous mastitis is a condition caused by *Mycobacterium tuberculosis* infection. It is characterized pathologically by extensive involvement of the mammary lobules with epithelioid granulomas of varying degrees of caseation, consisting of Langhan's giant cells, epithelioid cells, mononuclear cell infiltration, surrounding fibrosis, and micro-abscess formation [2, 3].

Tuberculous mastitis is extremely uncommon in developed countries (0,6–1,6%). Yet, tuberculous mastitis is found in around 3–4,5% of cases in developing countries. Tuberculous mastitis is common in Indian and African women. In developed countries, the overall incidence of tuberculous mastitis is reported to be up to 0.1% of all breast lesions, whereas in developing countries, it is approximately 3% of all surgically treated breast diseases. Tuberculous mastitis primarily affects women of reproductive age (17–42 years), with an average age of 32 years [2].

According to other sources, tuberculous mastitis most often occurs during the sexually active period (20–40 years) and only very rarely occurs before the age of 10 years. Mammary glandular activity is at its peak during this period. Lactation is known to increase susceptibility to tuberculous mastitis, possibly due to parental stress and increased vascularity in the mother, which facilitates infection and bacilli spread. In addition, during this period of activity, mothers undergo more changes and are more susceptible to trauma and infection. Multiparity, trauma, a history of suppurative mastitis, and AIDS are all risk factors [2].

The incidence of bilateral tuberculous mastitis is rare, where the left breast is more often affected than the right breast. Primary tuberculous mastitis (only affecting the breast) is also uncommon, with an incidence ranging from 0.10% to 0.52%. The duration of tuberculous mastitis symptoms ranges from several months to several years but is usually less than a year [2].

The Human Immunodeficiency Virus (HIV) infection, the emergence of drug-resistant strains of *Mycobacterium tuberculosis*, and the global pandemic of Acquired Immunodeficiency Syndrome (AIDS) have all led to an increase in mammary tissue resistance to the survival and proliferation of tubercle bacilli (*Mycobacterium tuberculosis*), making the diagnosis of these cases more difficult. Tuberculous mastitis has also been recognized as a manifestation of AIDS [4, 5, 6].

Tuberculous mastitis can develop as a result of or in addition to lesions on other parts of the body. Primary tuberculous mastitis is a tuberculosis infection of the mammary gland. Primary tuberculous mammae can occur due to direct inoculation of tubercle bacilli through ducts in the nipple or through skin abrasion, which is a rare way of infection. In tuberculosis infections associated with pregnancy, direct inoculation of the

nipple through the lactiferous duct usually occurs. Tuberculous mastitis occurs when tubercular lesions are also discovered in location of the body other than the mammary gland [2].

Secondary tuberculous mastitis can occur in three ways. First, the lymphatic spread, particularly retrograde infection from axillary lymph nodes, occasionally from mediastinal, cervical, internal mammary, or other lymph nodes. This is a very common route of transmission, affecting 50–75% of patients. Another route is retrograde lymphatic spread from the focus in the lungs through the para-tracheal and internal mammary lymph nodes to the breast. Passage from tracheobronchial lymph nodes or internal mammary lymph nodes to the breast may also occur in some cases. Secondly, direct spread, or contiguous spread by contact of adjacent structures such as the rib, sternum, costochondral cartilage or costochondral junction, pleural space (including empyema necessitates), and infected lung, even from the rectus sheath from intra-abdominal sources and shoulder joint, can also occur and appears as a cold infra-mammary abscess. This is the second most common transmission mode, particularly direct infection spread from the chest wall. Lastly, hematogenous spread from miliary tuberculosis, a rare occurrence, can also result in mammary involvement, characterized by lesions in the breast as well as multiple lesions on other parts of the body. This has also been observed in AIDS patients with miliary breast disease. In most cases, bilateral mammary tissue involvement and enlarged axillary nodes are often present, where lymph nodes can cause caseosa [2, 3, 4, 5, 6, 7].

Mckeown and Wilkinson classified tuberculous mastitis into five different pathologic types. The first is Acute miliary tuberculous mastitis, which is associated with generalized miliary tuberculosis disease spread through the blood (blood-borne infection). The second type, Nodular tuberculous mastitis, is the most common and is characterized by a localized mass or lump with or without sinuses in one of the mammary quadrants and extensive caseosa. This type is often mistaken for fibroadenoma or carcinoma. The third type, Disseminated tuberculous mastitis, affects all mammary tissue and results in multiple caseation and sinus formation. The fourth type, Tuberculous mastitis of the sclerotic type, is characterized by minimal caseation and extensive hyalinization of the stroma, shrinkage of the breast tissue with initial skin retraction, and subsequent sinus formation. This type is clinically indistinguishable from carcinoma and is more common in older women with slow growth and no suppuration. The final type, Obliterated tuberculous mastitis, is a rare form caused by intra-ductal infection and results in marked epithelial fibrosis and obliteration of the ductal system; sinus formation is uncommon [2, 4, 5, 7].

Tuberculosis infection may be associated with cancer development; tuberculosis and malignancy may coexist in some cases; clinical and radiological similarities between tuberculosis infection and malignancy may lead to wrong diagnosis [8].

The diagnosis of granulomatous mastitis from other autoimmune and granulomatous conditions, including tuberculosis, sarcoidosis, and Wegener's granulomatosis, is difficult because, based on histopathology, the granulomatous appearance of autoimmune conditions is similar to that of the specific process. Histoplasmosis, actinomycosis, foreign body reaction, fat necrosis, IgG4-RD mastitis, and inflammatory breast cancer are all other possible diagnoses [9, 10, 11].

Therefore, accurate diagnosis is dependent on tissue biopsies, which are of higher quality and quantity when achieved by core needle biopsy rather than by FNA [12] pathologists use histological techniques such as paraffin block examination, hematoxylin and eosin staining, Gram staining, and Grocott's Methenamine silver to differentiate rapid stains of sarcoidosis or tuberculosis [10].

Lacambra et al. showed that tuberculous mastitis lesions had more fibrosis, eosinophils, and necrosis than granulomatous mastitis group, which had significantly more plasma cells.[10] Khan et al. investigated the features of tuberculous mastitis in Pakistan, discovering difficult differences between granulomatous mastitis and requiring further examination using the PCR technique on paraffin blocks [13]. Rindy et al. demonstrated that tuberculous mastitis lesions are still difficult to detect in granulomatous paraffin mastitis block samples [14].

Poleple et al. in Zambia found that the internal PCR test in non-lymphatic tissue had a sensitivity of 82% (95% CI: 56%-95%), which was significantly higher than the Xpert MTB/RIF test ($P = 0.004$). This indicates that in cases of non-lymph mastitis, it is possible to confirm the presence of mycobacterium strain that causes granulomatous mastitis. However, Rindy et al. encountered difficulties obtaining tuberculosis mastitis samples for examination with the Genexpert technique, particularly in granulomatous paraffin mastitis block samples [15].

The purpose of this study was to analyze the expression of Mycobacterium tuberculosis DNA using the Polymerase Chain Reaction (PCR) technique in formalin-fixed paraffin-embedded tissue (FFPE) samples of granulomatous mastitis. The analysis results are expected to assist in diagnosing granulomatous mastitis, especially in difficult histopathological cases.

2 Method

We conducted a retrospective laboratory-based study to analyze confirmed granulomatous mastitis samples that were fixed in formalin and embedded in paraffin (FFPE). The samples were analyzed using HE and Ziehl Nielsen staining. This study excluded invalid samples, such as samples that were moldy, damaged by animals such as rats, or underwent improper maturation during the paraffin embedding process.

2.1 Deparaffinization

The basic procedure for tissue deparaffinization involved several steps. Firstly, three parts of paraffin block (15 mm each) were added to 1 ml of xylene, then vortexed and incubated at 65 °C for 20 min. Subsequently, the mixture was centrifuged at 13,000 rpm for 5 min, and the supernatant was discarded. Lastly, to remove the remaining xylene, 1 ml of 100% ethanol was added to the pellet and centrifuged at 13,000 rpm for 5 min. The procedure was repeated twice.

2.2 DNA Extraction

The procedure for DNA isolation involved using the Invitrogen Purelink Genomic DNA Mini Kit and following the kit's instructions. The deparaffinized tissue and isolation

Table 1. Detection of mycobacterium of tuberculosis using PCR

Target Gen	Primet Set	Nucleotides Description	Product Length
<i>IS6110</i>	Forward	5'-CCTGCGAGCGTAGGCGTCGG-3	123 bp
	Reverse	5'-CTCGTCCAGCGCCGCTTCGG-3	

liquid were combined in an Eppendorf tube and centrifuged at 13,000 RPM for 5 min. To lyse the tissue, 250 μ l of lysis buffer and 10 μ l (10 mg/ml) of proteinase K were added to the tube, which was then placed in a water bath at 55 °C for 1 h, with occasional shaking. Afterward, the mixture was left at room temperature for 5 min, and 100 μ l of protein precipitation solution was added. The mixture was then vortexed at high speed for 20 s and centrifuged for 3 min at 6,000 RPM. The supernatant was transferred to a new Eppendorf tube containing 300 μ l of isopropanol, where the tube was inverted until a precipitate formed. The mixture was then centrifuged for 3 min at 13,000 RPM, and the precipitate was washed with 300 μ l of 70% ethanol solution, followed by another centrifugation for 1 min at 6,000 RPM. The supernatant was discarded, and the pellet was dried on paper towels. The DNA was dissolved in 20 μ l of water, mixed, and then heated in a water bath at 65°C for 30 min. The tube was covered with parafilm with enough holes to allow for evaporation.

2.3 IS6110 Real-Time Polymerase Chain Reaction

The *IS6110* gene from *Mycobacterium tuberculosis* was detected using the PCR method. The gene sequences used are as follows (Table 1):

The *IS6110* gene was detected using amplification that included predenaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C in 30 s, annealing at 60 °C for 50 s, extension at 72 °C for 60 s, and final extension at 72 °C for 5 min. PCR amplicons were electrophoresed on 2% agarose gel with 1X Tris-buffer EDTA at 100 V for 60 min and stained with 10 μ g/mL ethidium bromide. Furthermore, the electrophoresis results were recorded using gel documentation. The length of the marker used was a 50 bp marker, and the length of the *IS6110* gene PCR product was 123 bp.

3 Result

In this study, DNA was extracted from 45 formalin-fixed and paraffin-embedded (FFPE) samples of granulomatous mastitis (histopathological examination with Hematoxylin Eosin, and Ziehl Neelsen staining) (Invitrogen Purelink Genomic DNA Mini Kit). A conventional PCR was used to detect *Mycobacterium tuberculosis* DNA using a specific primer target gene *IS 6110*. All of the GM patients were women, with an average age of 30,8 years.

Following the DNA extraction from the blocks of each sample, PCR analysis was carried out on each of the preparations. Using the *IS6110* specific primer, PCR analysis results showed no band of 123 bp, indicating the absence of the *IS 6110* gene in the

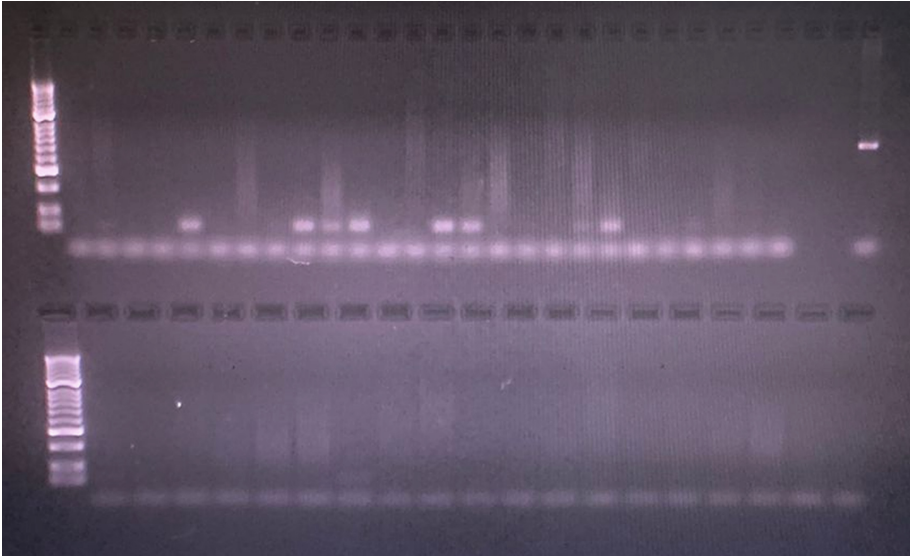


Fig. 1. MTBC PCR electrophoresis results with a histopathological diagnosis of granulomatous mastitis found that seven samples showed DNA bands of around 50 bp. (M: Marker 50 bp, 1–45: sample, K-: Negative control, K +: Positive control)

samples tested. The PCR results revealed that seven patients with granulomatous mastitis had DNA bands of about 50 bp in size (Fig. 1).

Of seven subjects diagnosed with granulomatous mastitis, all of whom had PCR results showing a band of approximately 50 bp, meaning that all demonstrated histopathological features of giant cell Langhans. One subject also presented with necrosis (Fig. 2).

4 Discussion

Granulomatous inflammation is a type of chronic inflammation that has a distinct pattern and is frequently seen in infectious conditions. The histopathological pattern of granulomatous inflammation and determining the etiology in a biopsy specimen are very important in providing the best treatment. Based on their etiology, granulomatous inflammatory disorders are classified as infections, vasculitis, immunological disorders, leukocyte oxidase deficiency, hypersensitivity, chemicals, and neoplasia. The most common cause of granulomas is tuberculosis, followed by leprosy, foreign body granulomas, fungal infections, rhinoscleroma, parasites, tumor granulomas, and actinomycosis, but infection with *Mycobacterium tuberculosis* is the most common. *Mycobacterium leprae*, *Treponema pallidum*, and some gram-negative bacilli are among the other germs that can cause granulomatosis. Granulomatous mastitis can also be caused by hypersensitivity to lactation products extravasating into the breast tissue, local trauma, and autoimmune diseases, such as Crohn's Disease and Duct Ectasia [16].

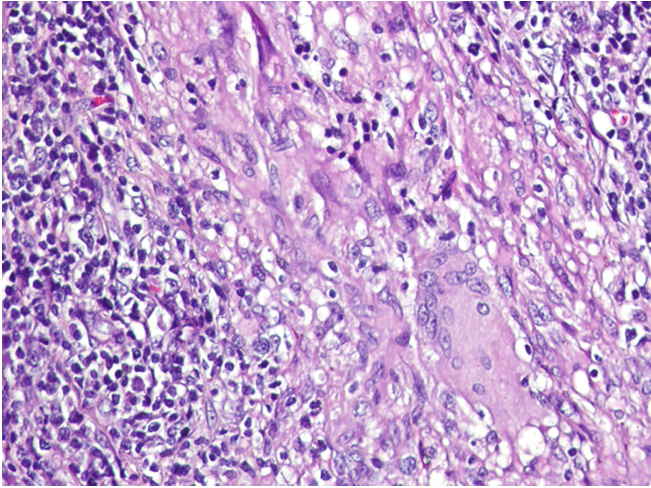


Fig. 2. Microscopic view of granulomatous mastitis. Langhans data cell structure is shown. Hematoxylin and Eosin, magnification at 400x

Granulomatous mastitis appears clinically as a slow-growing, unilateral mass, sometimes accompanied by abscess and sinus formation. A hard mass and a retracted nipple can resemble breast carcinoma. The anatomical pathology examination performed in this study was a biopsy sample. The histopathologic features are caseous necrosis, tubercles composed of epithelioid cells, lymphocytes, and giant cell Langhans. However, this examination cannot differentiate the etiology of granulomatous mastitis, so more research is required [9, 10].

The gold standard examination for diagnosing tuberculous mastitis is the finding of AFB on anatomic pathological examination. However, the sensitivity of this examination is very low, which is around 25%, or in other words, as many as 75% of undiagnosed cases will be found if only rely on AFB [9, 16].

In this study, 45 subjects with granulomatous mastitis underwent IS6110 gene PCR testing. The subjects had an average age of 30 years, which is categorized into the productive age group. Tuberculous mastitis is known to affect women in this age group [2] with a prevalence rate of 15%. PCR examination has a high sensitivity and specificity value for tuberculosis. The PCR test showed negative results for all the samples tested; however, seven subjects had a band of around 50 bp. Further research is necessary to explore the possible causes of granulomatous mastitis in these patients and understand the significance of the 50 bp band.

In the Mycobacterium tuberculosis complex (MTBC), the *IS6110* gene is a special element insertion that repeats more than once. The *IS6110* gene is an important diagnostic tool for differentiating MTBC from other Mycobacteria and has been used extensively for epidemiological studies [17]. According to Hillemann (2011), examining tissue preparations using real-time PCR is more sensitive for detecting MTBC DNA than conventional *IS6110* gene PCR [18]. Differences in PCR positive rates observed

during tissue preparations are related to chemical and physical changes during fixation and tissue preparation [19].

Samples exhibiting granulomatous pathology can also be caused by Non-Tuberculous Mycobacteria (NTM) or other atypical mycobacterial species. NTM refers to all species within the Mycobacterium family that can cause disease, excluding the Mycobacterium tuberculosis complex (*M. tuberculosis*, *M. africanum*, *M. bovis*, *M. canettii*, *M. microti*, *M. caprae*, *M. orygis*, and *M. pinnipedii*) and *M. leprae*. NTM are opportunistic bacteria that often infect patients with pre-existing lung conditions, immunodeficiencies, or other chronic diseases. A misdiagnosis of NTM can lead to incorrect therapy regimens, high medical costs, and societal stigma that can negatively impact the socioeconomic status of patients [17, 20].

NTM infection can result in chronic bronchopulmonary, lymphadenitis, and skin and other soft tissue infections. Symptoms of NTM infection include fever, chills, night sweats, weight loss, abdominal pain, fatigue, diarrhea, enlarged lymph nodes, and anemia [20]. NTM has clinical manifestations similar to tuberculosis, but NTM therapy is different from tuberculosis. Standard NTM therapy consists of 3–4 types of antibiotics (rifampicin, ethambutol, and macrolides such as azithromycin or clarithromycin) and is given over a long period of time, usually 18 to 24 months. NTM is diagnosed based on the patient's complaints and clinical symptoms, radiological images from chest X-rays and CT scans, and detection of NTM species through culture and PCR methods [20]. The MPCR-ULFA (Multiplex PCR-Ultra Lateral Flow Assay) test detects the presence of MTB as well as NTM in patients with suspected pulmonary TB using a molecular-based examination with amplification of three target genes at once (*IS1660*, *mtp40*, and *rpoB*) [21].

Several possibilities for negative PCR results in this study include the use of paraffin block tissue, which can cause physical and chemical changes in MTBC DNA during the fixation and preparation of tissue blocks, or the small amount of MTBC DNA in the paraffin blocks. Secondly, the conventional PCR method used has a lower sensitivity than real-time PCR. Third, PCR with the *IS6110* gene-specific primer yielded a negative result, indicating that no MTBC was found.

5 Conclusion

The Polymerase Chain Reaction (PCR) examination can be a useful tool for clinicians in diagnosing the cause of granulomatous mastitis. However, it is important to note that this examination has limitations, and further research using appropriate methods is necessary to improve its accuracy.

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