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Pathogens in patients with granulomatous lobular mastitis

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ABSTRACT

Objective: Granulomatous lobular mastitis (GLM) is a rare inflammatory disease of the breast that clinically mimics breast cancer. However, its etiology is not completely defined. The purpose of this study was to systematically study the bacteriology of GLM using advanced detection technology.
Methods: Paraffin-embedded tissue from patients with GLM was collected. DNA was extracted from the samples and analyzed using next-generation sequencing (NGS) technology, and the data were processed using bioinformatics analyses.
Results: A total of 40 patients were recruited into the study. A bioinformatics analysis revealed that a total of 17 genera or 19 species of pathogens were present in 39 of the GLM patients (97.5%). These included bacteria, fungi, and *Mycobacterium tuberculosis* complex group. Bacteria were found in 39 of the patient cases, while fungi were present in five. Only one case tested positive for *M. tuberculosis* complex. In addition, a single genus of pathogen was found in nine patients (23.1%), whereas 30 patients (76.9%) tested positive for multiple pathogens.
Conclusions: This study profiled the microbiota of patients with GLM using NGS technology, which provides more useful information for establishing patient treatment plans.
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Introduction

Granulomatous lobular mastitis (GLM) is a rare inflammatory disease of the breast that clinically mimics breast cancer. At present, the etiology and the pathogenesis of GLM remain unclear, and there are no standard treatment guidelines. The lack of successful standard treatment results in relapses or deformation of the mammary gland. Therefore, it is very important to unravel the etiology and the pathogenesis of GLM.

Previous studies have suggested that certain pathogens, including *Corynebacterium* species (Dobinson et al., 2015; Poojary et al., 2017), *Rhodococcus equi* (Nath et al., 2013), and *Mycobacterium abscessus* (Wang et al., 2017), contribute to GLM. Among these, *Corynebacterium kroppenstedtii* is considered to be the most common pathogen associated with GLM (Kutsuna et al., 2015; Wong et al., 2017; Johnstone et al., 2017). However, many potential pathogens for GLM remain to be identified.

Current methods for detecting pathogens that cause or are associated with GLM, including Gram staining, culture, real-time PCR, and Sanger sequencing, have many limitations, such as detection range, specificity, and sensitivity; they are also time-consuming. Hence, a new method is required to detect the potential pathogenic bacteria in mastitis.

Next-generation sequencing (NGS) is a novel approach to DNA/RNA sequencing. As an unbiased assay, NGS is theoretically capable of identifying all of the potential pathogens in a single assay, because it can amplify and sequence the entire DNA content of a sample without using any primers or probes. Recently, NGS technology has been applied in medical microbiology as an emerging technique, because of its high-throughput capabilities, rapid detection, and low cost. More and more successful cases of pathogen discovery using NGS technology have been reported (Fan et al., 2018a; Marks et al., 2018; Frémond et al., 2015). However, it appears that no studies reported in the

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Table 1
Baseline characteristics and clinical presentation of the cases.

Variable	Total (n = 40)	Percentage (%)
Age, years, median (range)	31.5 (21–41)	
<25	4	10
25–40	34	85
>40	2	5
Signs and symptoms		
Mass with abscess	35	87.5
Mass	2	5
Abscess	1	2.5
Ulcer	17	42.5
Nipple retraction	8	20
Lymph node enlargement	25	62.5
Lesion side		
Left	24	60
Right	8	20
Bilateral	5	12.5
Missing data	3	7.5
Location of lesions		
Upper-inner quadrant	12	30
Upper-outer quadrant	12	30
Lower-inner quadrant	8	20
Lower-outer quadrant	13	32.5
Laboratory testing		
High WBC count (neutrophils)	13	32.5
High estradiol	12	30
High prolactin	11	27.5
High progesterone	4	10

WBC, white blood cell.

literature have applied NGS for pathogen detection from GLM samples.

In this study, NGS was used to detect the potential pathogens in paraffin-embedded mastitis tissue. In addition, the infection rate of the potential pathogens in patients with GLM was analyzed.

Materials and methods

Patients and sample selection

A systematic search of the electronic histopathology records of Shenzhen Traditional Chinese Medicine Hospital was conducted to identify all patients with GLM histology specimens collected between June 2016 and March 2018. The samples of patients who had a diagnosis of squamous metaplasia of the lactiferous ducts, duct ectasia, lactational abscess, fat necrosis, tuberculosis, and sarcoidosis were excluded. The study protocol was reviewed and approved by the local ethics committee.

Histochemical staining

Paraffin sections of 4 mm thickness were mounted onto 3-aminopropyltriethoxysilane-coated glass slides. Hematoxylin and eosin (HE) staining was performed to evaluate histopathological features. Gram staining, periodic acid–Schiff staining, and acid-fast staining were performed in all cases to detect bacteria, fungi, and *Mycobacterium tuberculosis*, respectively.

DNA isolation and sequencing

Sample DNA was extracted directly from the paraffin-embedded tissue using the MagPure FFPE DNA LQ Kit (Magen, China) as per the manufacturer's instructions. Briefly, 10 μm thick tissue sections were collected in 1.5 ml centrifuge tubes and immediately deparaffinized. The deparaffinized tissue was digested by proteinase K bonded with MagBind particles, adsorbed onto a spin column, washed twice, and eluted in 30 μl DNase-free water. Then, DNA libraries were constructed through DNA-fraGLMentation, end-repair, adapter-ligation, and PCR amplification. Agilent 2100 was used for quality control of the DNA libraries. Quality qualified libraries were sequenced by BGISEQ-50 platform (Jeon et al., 2014).

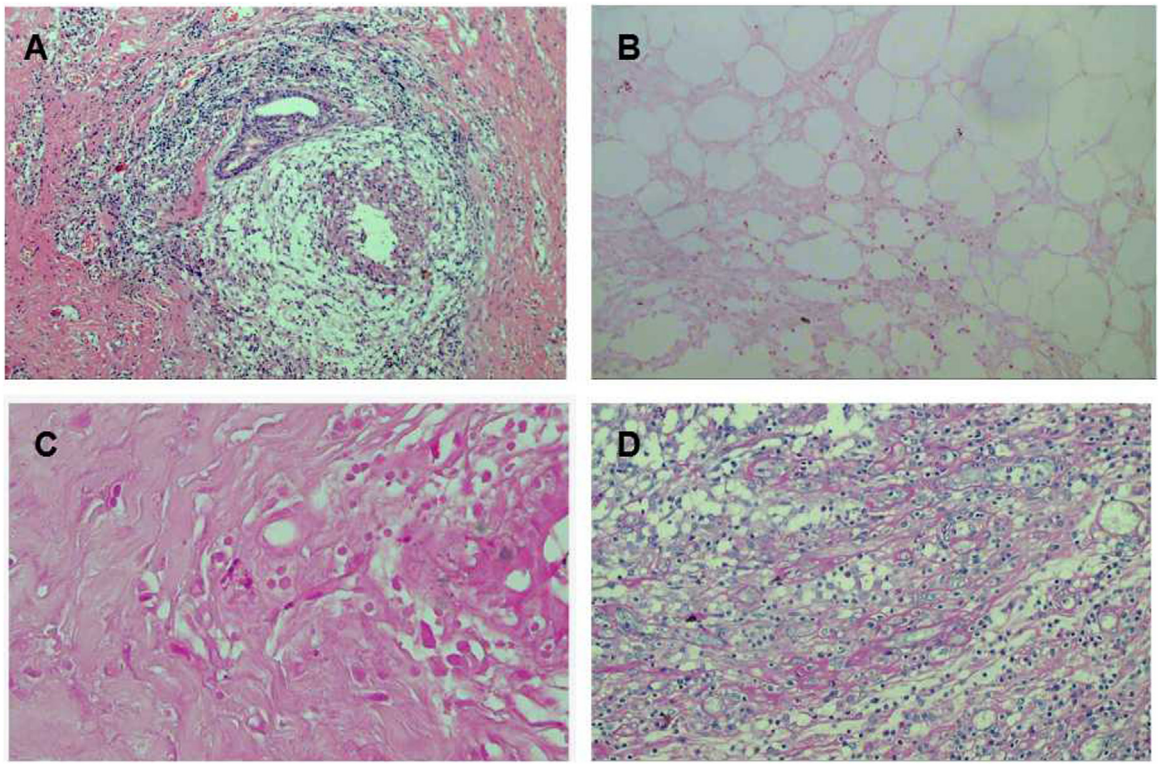

Figure 1. Histological staining: (A) hematoxylin–eosin stain (×100); (B) acid-fast stain (×200); (C) Gram stain (×200); (D) periodic acid–Schiff stain (×200).

High-quality sequencing data were generated by removing low-quality and short reads (length <35 bp), followed by computational subtraction of human host sequences mapped to the human reference genome (hg19) using Burrows–Wheeler alignment (Li and Durbin, 2009). The remaining data, after removal of low-complexity reads, were classified by simultaneously aligning to four microbial genome databases, consisting of viruses, bacteria, fungi, and parasites.

Bioinformatics analyses

The classification reference databases were downloaded from the National Center for Biotechnology Information (NCBI; ftp://ftp.ncbi.nlm.nih.gov/genomes/). RefSeq contains 1424 whole genome sequences of viral taxa, 2406 bacterial genomes or scaffolds, 199 fungi related to human infection, and 135 parasites associated with human diseases, and includes five species of tuberculosis pathogenic bacteria in *Mycobacterium tuberculosis* complex group and 41 species of *Mycoplasma*/*Chlamydia*.

Results

Characterization of the patient cohort

In total, 40 women diagnosed histologically with GLM were recruited into the study. The clinical data of the patients are summarized in Table 1. The patients ranged in age from 21 to 41 years (mean age 31.5 years). The clinical symptoms included breast lumps, abscesses, ulcers, and nipple retraction. Among the patients, 35 had a mass with abscess, two had a mass, one had an abscess, 17 had an ulcer, and eight had nipple retraction; five patients had bilateral mastitis (Table 1). Lymph node enlargement was present in 25 patients. Thirteen patients presented a high white blood cell count (neutrophils), 12 had high estradiol, 11 had high prolactin, and four had high progesterone.

Histological staining findings

The histology results of all patient cases were reviewed carefully. All showed granulomatous inflammation centered on vacuolated spaces surrounded by neutrophils in a background of acute-on-chronic inflammation including neutrophils, plasma cells, lymphocytes, and eosinophils (Figure 1A). No pathogens were detected on acid-fast, Gram, or periodic acid–Schiff staining (Figure 1B–D), indicating that paraffin-embedded tissues were not suitable for the detection of pathogens by traditional histochemical staining, and the sensitivity and specificity were very low.

Detection and characterization of pathogens

To detect the pathogens of infection involved in patients with GLM, NGS was performed and data analyzed. A bioinformatics analysis revealed that a total of 17 genera of pathogens were present in 39 GLM patients (97.5%) and 19 species of pathogens were present in 38 GLM patients (95%) (Figure 2). The pathogens

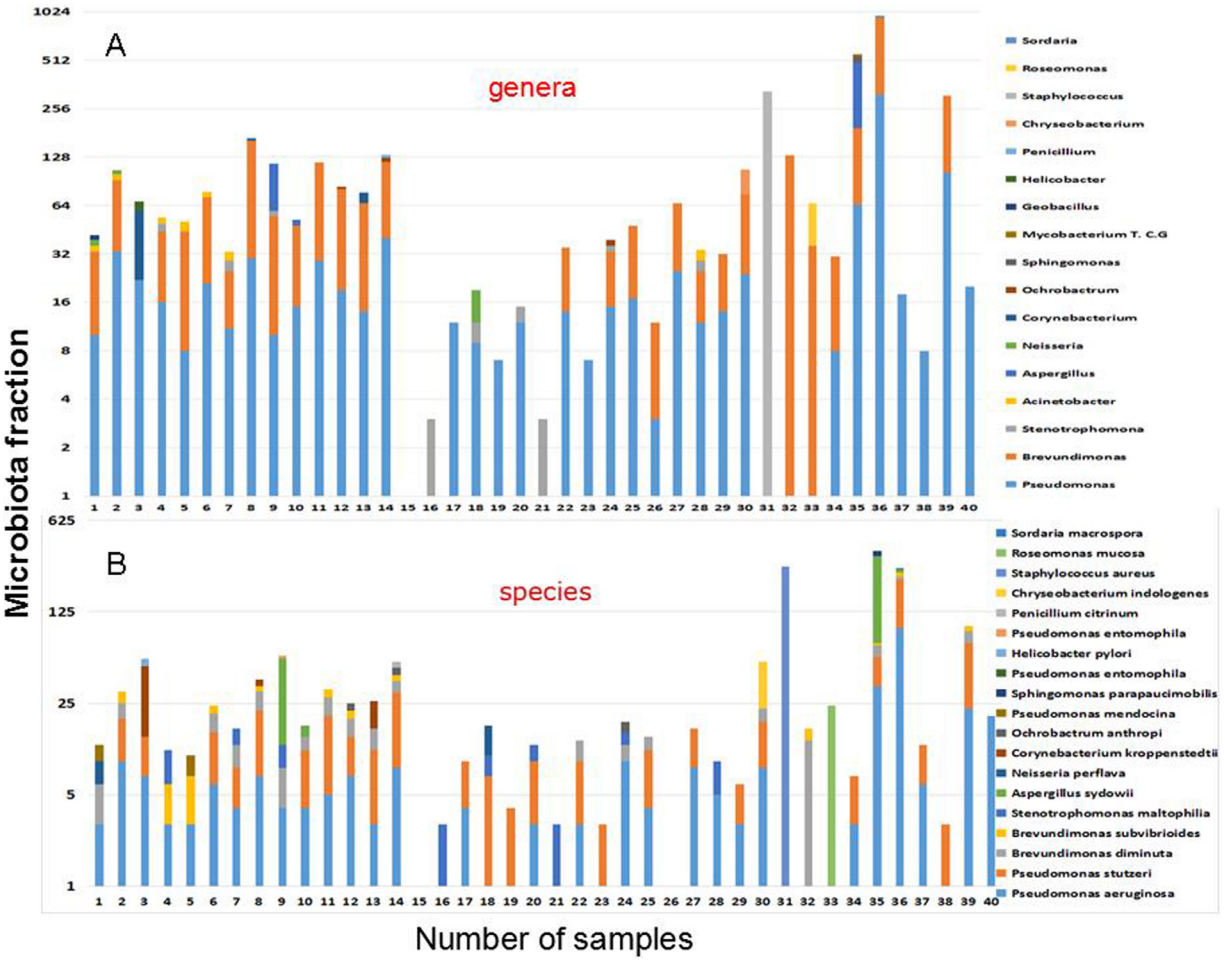

Figure 2. Taxonomic profiles of the granulomatous lobular mastitis microbiota at the genera and the species level. A bioinformatics analysis compared the relative abundances of bacterial taxa in 40 patients with granulomatous mastitis at the genera level (A) and the species level (B). Each bar represents a subject. The taxa names are shown on the right.

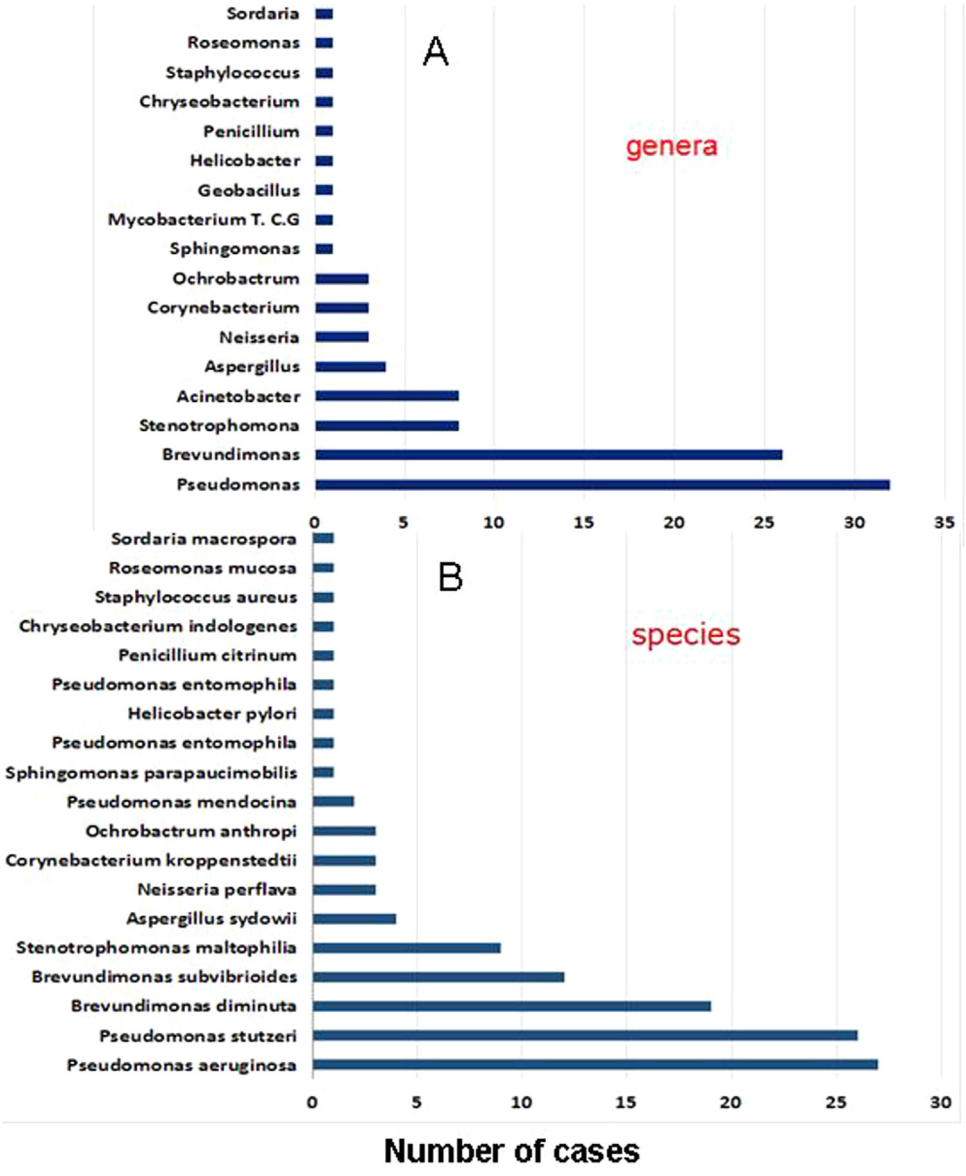

Figure 3. The number of cases detected by each pathogen. The number of cases detected in 40 patients with granulomatous mastitis at the genera level (A) and the species level (B). The taxa names are shown on the right.

included bacteria, fungi, and *M. tuberculosis* complex group. In this sample, bacteria were present in 39 patients and fungi in five patients, while only one patient tested positive for *M. tuberculosis* complex species. In addition, single pathogens were found in nine patients (23.1%), and the remaining 30 patients (76.9%) had multiple pathogens detected in their samples.

The top five genera of pathogens that were detected in the current study were *Pseudomonas* (32/40), *Brevundimonas* (26/40), *Stenotrophomonas* (8/40), *Acinetobacter* (8/40), and *Aspergillus* (4/40). Using species classification, the following were found to be the top pathogens in this sample: *Pseudomonas aeruginosa* (27/40), *Pseudomonas stutzeri* (26/40), *Brevundimonas diminuta* (19/40), *Brevundimonas subvibrioides* (12/40), and *Stenotrophomonas maltophilia* (9/40) (Figure 3).

Discussion

In this study, the potential pathogens of GLM were detected by NGS. This study is novel in using NGS technology to detect pathogens in patients with GLM. One of the major findings of this study was that up to 95% of GLM specimens had associated pathogens. Another key finding is that the pathogens in GLM were from bacteria, fungi, and *M. tuberculosis* complex groups, and these were further identified as belonging to 17 genera and 19 species of pathogens by bioinformatics analysis. In addition, this study found that most GLM patients (about 76.9%) had multiple pathogens as detected by NGS technology. The results indicate that in almost all GLM cases, pathogens can be detected, and that each case of GLM may involve multiple pathogenic infections. Although it remains uncertain which of these pathogens can cause this disease, the pathogens detected should have a certain guiding effect on clinical treatment.

The pathogens in patients with GLM have not yet been fully clarified. Previous studies have suggested that *C. kroppenstedtii* is the main pathogen of GLM (Yu et al., 2016; Fernández-Natal et al., 2016; Johnstone et al., 2017). In the present study, the initial data showed the presence of the *C. kroppenstedtii* sequence in 14 of the 40 patients (35%). After removing the low-quality reads, only three cases (7.5%) remained. The detection rate of *C. kroppenstedtii* in this study differs from those of most previous reports. The reason for the difference is that the test samples are different. The samples in

the present study were formalin-fixed, paraffin-embedded (FFPE) specimens, while those in most previous studies were fresh biopsy samples (Mihalcea et al., 2017). Using fresh biopsy samples for DNA extraction would undoubtedly improve sensitivity of detection, however, FFPE specimens are more readily available at Shenzhen Traditional Chinese Medicine Hospital. In terms of the detection rate of *C. kroppenstedtii*, the study results are consistent with those of Fujii et al. (35% vs. 38.9%) (Fujii et al., 2018), who detected *C. kroppenstedtii* in FFPE specimens of granulomatous mastitis using real-time PCR. This indicates that the detection method currently being used at Shenzhen Traditional Chinese Medicine Hospital is reliable.

In contrast to previous studies, *C. kroppenstedtii* was not the main pathogen of GLM identified in the present study. *P. aeruginosa*, *P. stutzeri*, *B. diminuta*, *B. subvibrioides*, and *S. maltophilia* were the top five species found in the patient sample. *P. aeruginosa* is one of the more common conditional pathogens in the clinic and is also a common bacterial infection in wounds. A literature search showed that *P. aeruginosa* has been isolated from mastitis tissues of bovines and caprines (Banerjee et al., 2017; Scaccabarozzi et al., 2015), but there are few reports of this pathogen infection in human mastitis. *P. stutzeri* is a Gram-negative, rod-shaped, aerobic, catalase- and oxidase-positive bacterium that is ubiquitously present in the environment. A few studies have indicated that *P. stutzeri* can cause valve endocarditis (Halabi et al., 2018; Héquette-Ruz et al., 2018), but there are no reports suggesting that this bacterium can lead to mastitis. Similarly, *B. diminuta*, *B. subvibrioides*, and *Stenotrophomonas* have not been reported in human mastitis. Thus, the present study appears to be the first to report these major pathogens in GLM. However, whether these major pathogens can cause GLM, or secondary infections, requires further investigation.

The NGS method used in this study is a relatively new technology, and several studies have demonstrated the use of NGS as a diagnostic tool for infectious diseases (O'Flaherty et al., 2018; Long et al., 2016; Fan et al., 2018b). Taking advantage of unbiased sequencing, NGS can detect multiple pathogenic microorganisms, and this strength was confirmed by the results of this study. Another advantage of NGS is its ability to detect potentially unknown pathogens in specimens, a function not available in conventional assays. Most of the pathogens detected in this study have not been reported previously in GLM specimens.

A limitation of this study is that it was not possible to identify the pathogen of the initial infection or distinguish this from the pathogen of the secondary infection. However, the results of this study are still of great value in guiding clinical treatment for GLM, as these cases all involved patients with GLM, and the pathogens detected may cause the onset and progression of GLM.

In summary, this study used NGS technology to detect a variety of potential pathogens in GLM. NGS technology can be considered a potentially powerful diagnostic tool for the analysis of bacterial and other agents of infections that currently place a heavy burden on health care systems worldwide.

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Ethical considerations

We have read and complied with the policy of the journal on ethical consent.

Conflict of interest

There are no potential conflicts of interest for the participating authors.

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