Nasal Mycology of Chronic Rhinosinusitis Revealed by Nanopore Sequencing

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Abstract: Background: Nanopore sequencing (NS) is a third-generation sequencing technology capable of generating reads of long sequences. In this study, we used NS to investigate nasal mycology in patients with chronic rhinosinusitis (CRS). Methods: Nasal cavities of 13 CRS patients were individually irrigated with 20 mL of distilled water. The irrigant was forcefully blown by the patient into a basin. The collected fluid was placed into a centrifuge tube and processed using the method of Ponikau et al. The collected specimens were used for traditional fungal culture and sequenced for total DNA using NS. Results: Traditional fungal culture successfully grew fungi in the specimens of 11 (84.6%) patients. Aspergillus sp. and Penicillium sp. were found in four (30.8%) patients, Cladosporium sp. in three (23.1%) patients, and Candida albicans, Mucor sp. and Chaetomium sp. in one patient. NS revealed fungi abundance ranged from 81 to 2226, with the Shannon species diversity ranging from 1.094 to 1.683 at the genus level. Malassezia sp. was sequenced in 13 patients, Aspergillus sp. in 12 (92.3%) patients, Candida albicans in 11 (84.6%) patients, and Penicillium sp. in 10 (76.9%) patients. Conclusion: Our results showed that NS was sensitive and fast in detecting nasal fungi in CRS patients.

Keywords: chronic rhinosinusitis; fungal culture; fungus; nanopore sequencing

1. Introduction

Chronic rhinosinusitis (CRS) is an inflammatory disorder of the paranasal sinuses and linings of the nasal passages, with the persistence of characteristic signs and symptoms lasting longer than 12 weeks [1]. The etiology of CRS is multifactorial, including infection, anatomic anomaly, allergy and genetic [2–4].

Over the last 20 years, it has also been suggested that fungi causes CRS by dysregulating the immune response, inducing the breakdown of the epithelial membrane, and exacerbating local inflammation of sinonasal mucosa [5–7]. The ubiquitous presence of fungi in CRS patients has been demonstrated in several studies [8–10]. These studies employed modified traditional culture techniques (e.g., method of Ponikau et al. [8]) to detect fungi. Although Ponikau et al.’s method [8] is more sensitive than traditional culture methods, culture-based methods are still limited by selective pressures of the nutrient
media. Moreover, only 1% to 10% of known microorganisms are presumed to be culturable in the laboratory [11].

Culture-independent techniques, like PCR assays, potentially detect all organisms in CRS patients [12]. However, PCR uses specific primers or probes, targeting species to detect a limited number of pathogens. Recently, metagenomic sequencing detected all DNA present in a sample, enabling analysis of the entire microbiome, as well as the human host genome [13]. Previous studies have used next-generation sequencing (NGS) techniques to detect the microbiota in CRS patients [14,15].

Nanopore sequencing (NS) (Oxford Nanopore Technologies, Oxford, UK) is a third generation (i.e., single-molecule) sequencing technology that generates long sequence read-lengths [16]. It is characterized by culture-free, fast, single-molecule sequencing and sequencing in real-time [17]. The long sequence read-lengths requires no PCR amplification of the template. It minimizes bias during the library construction, as in the case of PCR [17]. NS was reported to identify, in real-time, species of fungi in dogs [18]. Here, we aimed to use NS to characterize the fungal microbiome of the nasal cavity in CRS patients.

2. Materials and Methods
2.1. Patients

We included CRS patients who had failed medical treatment and underwent bilateral primary functional endoscopic sinus surgery (FESS) between September 2018 and September 2019. CRS was diagnosed according to the EPOS criteria based on history, and findings in endoscopy and CT imaging [19]. Sinonasal inflammation was defined as having characteristic symptoms that persisted longer than 12 weeks, and both endoscopic examinations and CT scans showed supporting evidence. We excluded patients aged < 20 years old, those that had a history of immunodeficiency, and those who had taken antibiotics within a week before FESS. Those who were diagnosed pathologically as having sinonasal tumors or fungal sinusitis were also excluded. All eligible patients underwent nasal irrigation to collect nasal secretion for the modified traditional fungal culture (Ponikau et al.’s method [8]) and NS on the day before surgery. This study was approved by the Institutional Review Board (I) of Taichung Veterans General Hospital (protocol code CF17328B). Written consent was obtained from each patient.

2.2. Fungal Culture Using Ponikau et al’s Method

Patients were instructed to inspire deeply and to hold that position. One nasal cavity was irrigated by a syringe containing 20 mL of sterile distilled water. The irrigant was forcefully exhaled by the patient into a sterile pan. The irrigant in the pan was poured into a centrifuge tube and transferred to the microbiology laboratory. Under a laminar flow hood, an equal volume of diluted dithiothreitol (1.055 mg/mL) was added to the centrifuge tube. The tube was vortexed for 30 s and placed at room temperature for 15 m to allow the dithiothreitol to break down the disulfide bonds in the mucus. Then, the tube was centrifuged at 3000 \( \times g \) for 10 m and the supernatant was discarded. The sediment in the tube was vortexed for 30 s and the sediment was divided into two parts. One part was inoculated onto a Sabouraud dextrose agar plate and a Sabouraud dextrose agar plate containing chloramphenicol and cycloheximide. The agar plates were incubated at 30 °C and examined for 30 days on a daily basis. All isolates were identified. The other part was transferred for NS.

2.3. DNA Extraction from the Nasal Irrigant

Using sterile tips, the sediment of the nasal irrigant was transferred into a tube containing 100–200 \( \mu L \) of sterile water, which was centrifuged for 5 s at high speed to pellet cells. The cell suspension and lytic enzyme solution were mixed by inverting 25 times and incubated for 30 m at 37 °C. The remaining procedure of the extraction was in accordance with instructions of the Puregene yeast/bacteria kit B (Qiagen cat. 1042607). The extracted DNA was stored at \(-80 \) °C.
2.4. PCR-Free Library Preparation and NS

In the laboratory, 0.1–0.2 µg of the extracted DNA was packaged into the library for the NS system. DNA libraries were prepared according to the manufacturer’s instructions, using the ligation sequencing kit (SQK-LSK109) and the native barcoding kit (EXP-NBD104), including the optimization DNA sequence of the KAPA Hyper Prep Kit. The MinION (Oxford Nanopore Technologies, Oxford, UK) flow cell preparation and sample loading were in accordance with the 75 µL DNA library of the SQK-LSK109 protocol for the sequencing. The sequencing mixture was added into the R9.4.1 or R10 flow cell for 48–72 h.

2.5. Bioinformatic Analysis

NS from Oxford Nanopore Technologies included a real-time analysis with the EP2ME platform ‘what’s In My Pot’ (WIMP). For further in-depth analyses, the fast5 or fastq files with the sequencing reads were basecalled. Barcodes and adapters were removed using the porechop (https://github.com/rrwick/Porechop (accessed on 19 October 2018)). Taxonomy was assigned with the cloud-based analysis WIMP software application from the EPI2ME platform (Oxford Nanopore Technologies Ltd., UK) based on the Centrifuge (https://ccb.jhu.edu/software/centrifuge/manual.shtml (accessed on 5 June 2018)). The R9.4.1 flow cells (Oxford Nanopore Technologies Ltd., UK) were loaded with 75 µL of DNA library. The 18s rRNA and ITS gene sequence libraries were prepared with the kit according to the standard procedures, described by Oxford Nanopore Technologies Ltd., UK. The complete 18s rRNA gene was amplified using LongAmp®Taq 2X Master Mix (New England Biolabs, Ipswich, MA, USA) with the barcoded nanopore sequence primers (27F 5′-AGA GTT TGA TCM TGG CTC AG-3′ and 149R 5′-CGG TTA CCT TGT TAC GAC TT-3′). DNA amplification was performed on a T100 Thermal Cycler (Biorad, Lunteren, The Netherlands) using the following procedure: 1 m denaturation at 95 °C, 25 cycles (95 °C—20 s, 55 °C—30 s, 68 °C—2 m) and a final extension step of 5 m at 65 °C. The 16S rRNA gene amplicons were quantified using Quant-IT™ PicoGreen™ (Thermo Fisher Scientific, Breda, The Netherlands). Equal amounts of amplicons per sample were pooled and the library was further processed following manufacturer’s instructions. Next, the library was incubated with Library Loading Beads (Oxford Nanopore Technologies, Oxford, UK) and the mixture was added to the MinIon/GridIon flow cell (version R9.2 or R.9.4, Oxford Nanopore Technologies, Oxford, UK) (Figure 1).

![Figure 1](image-url)

Figure 1. Schematic representation of the culture-based method and oxford nanopore sequencing process. The clinical culture-based system needs more than one month for fungal growth. The nanopore sequencing system only needs 24 h to identify fungi.

3. Results

3.1. Clinical Characteristics of Patients

In this study, we collected 13 CRS patients. Their demographic data are shown in Table 1. There were nine males and four females, with a mean age of 48.8 years old (range:
21 to 80 years). There was one smoker, but none suffered from asthma. Three patients had atopic dermatitis, and three used nasal steroids before enrollment. The severity of rhinosinusitis on the irrigated side of the nasal cavity was evaluated using the Lund-Kennedy endoscopic scoring system [20] and the Lund-Mackay CT scoring system [21]. Their endoscopic scores ranged from 2 to 5 at a mean of 3.4, and the CT scores ranged from 5 to 11 at a mean of 7.7. Among these CRS patients, six had nasal polyps, and seven had no nasal polyp. The surgical specimens of FESS showed that eight patients were eosinophilic CRS (tissue eosinophils > 10 cells per high power field) and five were non-eosinophilic CRS [22].

3.2. Isolation of Fungi Using Ponikau et al’s Method

Using Ponikau et al.’s method [8], 11 of 13 (84.6%) specimens grew fungi of one to three species. The most common fungi were *Aspergillus* sp. and *Penicillium* sp. cultured from four (30.8%) patients. *Cladosporium* sp. was isolated from three (23.1%) patients. *Candida albicans*, *Mucor* sp., *Chaetomium* sp., and an unidentified mold grew in one (7.7%) patient (Table 1).

3.3. Identification of Fungi by Nanopore Sequencing

The nanopore output total sequence reads were 91,476 to 1,598,608, and human read counts accounted for 28.35% to 96.68% (Table 2). However, the nanopore output sequence had fungal read counts that only accounted for 0.02 to 0.47% (Table 2). In this study, we only focused and analyzed fungal DNA. Nanopore sequence reads of fungi were from 54 to 2219 (Table 2). In 13 CRS patients, 36 to 2226 operational taxonomic units (OUTs) were identified, and the Shannon species diversity was from 1.094 to 1.683 at the genus level (Table 3). Ten most abundant fungal genera accounted for 56.15% to 89.13% of the nasal microbiota (Table 3). At the genus level, NS-identified major fungi were *Malassezia*, *Verticillium*, *Phycomyces*, and *Lobosporangium* et al (Figure 2).

![Figure 2](image-url)

**Figure 2.** A Pie chart of the proportion abundance of fungi identified through nanopore sequencing at the genus level.

Using NS, *Malassezia* sp. was identified in 13 (100%) of the specimens, *Aspergillus* sp. in 12 (92.3%), *Candida albicans* in 11, and *Penicillium* sp., *Chaetomium* sp. in 10 (Table 4). Thus, NS was more sensitive in detecting fungal species. However, *Cladosporium* sp. and *Mucor* sp. were not detected by NS (Table 4). We detected one to five pathogenic fungi in CRS patients using NS, such as *Malassezia*, *Aspergillus*, *Penicillium*, *Candida albicans* and *Chaetomium* (Table 5). The relative abundance of *Malassezia* sp. was from 12.34 to 52.08% and *Aspergillus* sp. from 0.8 to 4.26% (Table 3).
### Table 1. Demographic data of chronic rhinosinusitis patients.

| Patient | Sex | Age | Smoking History | Nasal Steroid | Atopic Dermatitis | Nasal Polyps | Eosinophilic CRS | Endoscopic Score | CT Score | Culture Result          |
|---------|-----|-----|-----------------|---------------|-------------------|--------------|------------------|-----------------|----------|-------------------------|
| 1       | F   | 44  | N               | N             | N                 | Y            | N                | 4               | 9        | Mucor species            |
| 2       | F   | 73  | N               | N             | N                 | N            | N                | 3               | 7        | Aspergillus fumigatus    |
| 3       | M   | 39  | N               | N             | Y                 | N            | Y                | 3               | 7        | Cladosporium species     |
| 4       | M   | 51  | Y               | N             | N                 | N            | Y                | 4               | 11       | Aspergillus niger        |
| 5       | M   | 27  | N               | N             | Y                 | N            | N                | 3               | 5        | Penicillium species      |
| 6       | M   | 61  | N               | Y             | N                 | Y            | Y                | 3               | 8        | Candida albicans          |
| 7       | M   | 53  | N               | Y             | N                 | Y            | Y                | 3               | 7        | Aspergillus niger        |
| 8       | M   | 45  | N               | Y             | N                 | N            | Y                | 4               | 6        | Penicillium species      |
| 9       | M   | 21  | N               | N             | N                 | Y            | Y                | 3               | 6        | Cladosporium species     |
| 10      | M   | 54  | N               | N             | N                 | N            | N                | 2               | 8        | Penicillium species      |
| 11      | M   | 45  | N               | N             | N                 | Y            | 2                | 6               | 5        | Chaetomium species       |
| 12      | F   | 41  | N               | N             | N                 | N            | Y                | 4               | 9        | Penicillium species      |

CRS, chronic rhinosinusitis; CT, computed tomography; F, Female; M, Male; Y, yes; N, No.

### Table 2. Summary nanopore output total sequence reads and percentage of species form the nasal irrigant in patients of chronic rhinosinusitis.

| Patient | Total Reads | Reads Classified | Reads Unclassified | Human Read Counts | % of Human Read | Fungal Read Counts | % of Fungal Read | Bacterial Read Counts | % of Bacterial Read | Archaeal Reads | Viral Reads |
|---------|-------------|------------------|--------------------|-------------------|-----------------|--------------------|-------------------|----------------------|-------------------|----------------|-------------|
| 1       | 91,476      | 86,209           | 5267               | 85,768            | 93.76%          | 105                | 0.11%             | 246                  | 0.27%             | 1              | 2           |
| 2       | 260,652     | 247,711          | 12,941             | 246,976           | 94.75%          | 276                | 0.10%             | 257                  | 0.11%             | 3              | 5           |
| 3       | 124,092     | 119,782          | 4310               | 119,206           | 96.06%          | 125                | 0.10%             | 378                  | 0.30%             | 0              | 2           |
| 4       | 461,270     | 438,200          | 23,570             | 437,483           | 94.74%          | 309                | 0.07%             | 121                  | 0.03%             | 0              | 4           |
| 5       | 473,888     | 454,052          | 19,836             | 449,829           | 94.92%          | 2219               | 0.47%             | 689                  | 0.15%             | 4              | 191         |
| 6       | 587,767     | 553,744          | 34,023             | 548,601           | 93.34%          | 675                | 0.11%             | 3763                 | 0.64%             | 9              | 12          |
| 7       | 673,783     | 655,678          | 18,105             | 651,395           | 96.68%          | 347                | 0.05%             | 2499                 | 0.37%             | 4              | 34          |
| 8       | 944,779     | 919,864          | 24,915             | 827,692           | 87.61%          | 912                | 0.10%             | 64,239               | 6.80%             | 4              | 244         |
| 9       | 1,598,608   | 1,537,782        | 60,826             | 1,389,765         | 86.94%          | 1458               | 0.09%             | 101,373              | 6.34%             | 5              | 379         |
| 10      | 598,282     | 577,110          | 21,172             | 530,416           | 88.66%          | 634                | 0.11%             | 34,257               | 5.73%             | 2              | 156         |
| 11      | 339,267     | 328,838          | 10,429             | 226,793           | 66.85%          | 273                | 0.08%             | 69,760               | 20.56%            | 2              | 314         |
| 12      | 228,665     | 226,967          | 1698               | 64,832            | 28.35%          | 54                 | 0.02%             | 128,244              | 56.08%            | 0              | 348         |
| 13      | 516,000     | 485,958          | 32,042             | 314,131           | 60.88%          | 470                | 0.09%             | 107,473              | 20.83%            | 5              | 783         |
| Patient | Shannon Species Diversity | Number of Fungi Genus Identified (OTU) | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 |
|---------|---------------------------|--------------------------------------|----|----|----|----|----|----|----|----|----|----|
| 1       | 1.672                     | 81                                   | Malassezia | Phycomyces | Lobosporangium | Aspergillus | Coccidioides | Marssonina | Sclerotinia | Capronia | Penicilliosis | Paracoccidioides |
|         |                           |                                      |              | 17.2% | 8.64% | 4.94% | 3.70% | 3.70% | 3.70% | 3.70% | 2.47% | 1.65% |
| 2       | 1.297                     | 242                                  | Malassezia | Phycomyces | Lobosporangium | Verticillium | Aspergillus | Bipolaris | Mucoromycota | Laccaria | Lobosporangium | Trichoderma |
|         |                           |                                      |              | 7.02% | 4.96% | 3.72% | 3.31% | 2.07% | 2.07% | 1.65% | 1.65% | 1.65% |
| 3       | 1.445                     | 92                                   | Malassezia | Phycomyces | ParaCoccidioides | Tubera | Marssonina | Verticillium | Lodderomyces | Penicilliosis | Penicilliosis | Coccidioides |
|         |                           |                                      |              | 7.61% | 4.35% | 3.26% | 3.26% | 3.26% | 3.26% | 3.26% | 3.26% | 3.26% |
| 4       | 1.321                     | 276                                  | Malassezia | Phycomyces | Colletotrichum | Lobosporangium | Aspergillus | Lodderomyces | Penicilliosis | Metarhizium | Tetragnosphora | Mucoromycota |
|         |                           |                                      |              | 6.88% | 4.71% | 3.62% | 3.26% | 2.90% | 1.81% | 1.81% | 1.81% | 1.81% |
| 5       | 1.516                     | 2226                                 | Verticillium | Malassezia | Lobosporangium | Phycomyces | Pestalotiopsis | Penicillium | Beauveria | Penicilliosis | Metarhizium | Candida |
|         |                           |                                      |              | 13.88% | 4.67% | 3.86% | 3.28% | 3.05% | 2.53% | 2.38% | 2.38% | 2.38% |
| 6       | 1.23                      | 644                                  | Malassezia | Phycomyces | Lobosporangium | Aspergillus | Verticillium | Trichoderma | Lodderomyces | Penicilliosis | Penicilliosis | Candida |
|         |                           |                                      |              | 4.72% | 4.81% | 3.26% | 2.80% | 2.64% | 2.02% | 1.55% | 1.55% | 1.55% |
| 7       | 1.283                     | 312                                  | Malassezia | Phycomyces | Lobosporangium | Aspergillus | Blastomyces | Verticillium | Trichoderma | Lodderomyces | Penicilliosis | Candida |
|         |                           |                                      |              | 4.97% | 5.13% | 3.55% | 2.24% | 2.24% | 2.24% | 2.24% | 2.24% | 2.24% |
| 8       | 1.128                     | 888                                  | Malassezia | Phycomyces | Verticillium | Phycomyces | Lobosporangium | Paracoccidioides | Aspergillus | Candida | Penicilliosis | Candida |
|         |                           |                                      |              | 5.05% | 5.13% | 3.55% | 2.24% | 2.24% | 2.24% | 2.24% | 2.24% | 2.24% |
| 9       | 1.094                     | 1439                                 | Malassezia | Phycomyces | Lobosporangium | Verticillium | Aspergillus | Paracoccidioides | Lobosporangium | Trichoderma | Trichoderma | Candida |
|         |                           |                                      |              | 4.59% | 2.78% | 2.43% | 2.15% | 1.88% | 1.67% | 1.39% | 1.39% | 1.39% |
| 10      | 1.683                     | 608                                  | Malassezia | Candida | Colletotrichum | Phycomyces | Lobosporangium | Isaria | Penicilliosis | Aspergillus | Penicilliosis | Mucoromycota |
|         |                           |                                      |              | 18.7% | 9.38% | 6.58% | 5.92% | 3.62% | 2.80% | 2.63% | 2.63% | 2.63% |
| 11      | 1.441                     | 259                                  | Malassezia | Candida | Colletotrichum | Phycomyces | Lobosporangium | Isaria | Anthracocystis | Marssonina | Lobosporangium | Setosphaeria |
|         |                           |                                      |              | 36.29% | 8.49% | 8.11% | 4.25% | 3.86% | 2.70% | 1.93% | 1.54% | 1.54% |
| 12      | 1.358                     | 55                                   | Malassezia | Phycomyces | Anthracocystis | Lobosporangium | Schizosaccharomyceae | Exophiala | Penicilliosis | Nannizzia | Mucoromycota | Trichophyton |
|         |                           |                                      |              | 50.91% | 7.27% | 3.64% | 3.64% | 1.82% | 1.82% | 1.82% | 1.82% | 1.82% |
| 13      | 1.52                      | 447                                  | Malassezia | Lobosporangium | Phycomyces | Candida | Aspergillus | Colletotrichum | Lodderomyces | Metarhizium | Histoplasma | Isaria |
|         |                           |                                      |              | 12.98% | 8.95% | 7.83% | 7.38% | 4.47% | 4.47% | 3.13% | 2.46% | 2.24% |

OUT, Operational taxonomic unit.
Table 4. Comparison of the genus level of fungi between Ponikau et al.’s method [8] and nanopore sequencing.

| Genus        | Ponikau et al.’s Method [8] (N, %) | Nanopore Sequencing (N, %) |
|--------------|------------------------------------|----------------------------|
| Malassezia   | -                                  | 13 (100%)                  |
| Aspergillus  | 4 (30.8%)                          | 12 (92.3%)                 |
| Penicillium  | 4 (30.8%)                          | 10 (76.9%)                 |
| Cladosporium | 3 (23.1%)                          | -                          |
| Candida albicans | 1 (7.7%)                   | 11 (84.6%)                 |
| Mucor        | 1 (7.7%)                           | -                          |
| Chaetomium   | 1 (7.7%)                           | 10 (76.9%)                 |
| Total        | 11 (84.6%)                         | 13 (100%)                  |

Table 5. The pathogenic fungi identified by nanopore sequencing.

| Patient | Pathogenic Fungi | Read Counts | Relative Abundance of Fungi (%) |
|---------|------------------|-------------|---------------------------------|
| 1       | Malassezia sp.   | 29          | 27.62%                          |
|         | Aspergillus sp.  | 4           | 3.81%                           |
|         | Chaetomium sp.   | 1           | 0.95%                           |
|         | Penicillium sp.  | 1           | 0.95%                           |
| 2       | Aspergillus sp.  | 8           | 2.90%                           |
|         | Chaetomium sp.   | 2           | 0.722%                          |
|         | Malassezia sp.   | 105         | 38.05%                          |
|         | Penicillium sp.  | 4           | 1.45%                           |
| 3       | Aspergillus sp.  | 1           | 0.8%                            |
|         | Chaetomium sp.   | 1           | 0.8%                            |
|         | Malassezia sp.   | 50          | 40.0%                           |
| 4       | Aspergillus sp.  | 9           | 2.91%                           |
|         | Candida albicans | 2           | 0.65%                           |
|         | Malassezia sp.   | 100         | 32.36%                          |
|         | Penicillium sp.  | 1           | 0.32%                           |
| 5       | Aspergillus sp.  | 56          | 2.52%                           |
|         | Candida albicans | 1           | 0.05%                           |
|         | Chaetomium sp.   | 1           | 0.05%                           |
|         | Malassezia sp.   | 309         | 13.93%                          |
|         | Penicillium sp.  | 73          | 3.29%                           |
| 6       | Aspergillus sp.  | 20          | 2.96%                           |
|         | Candida albicans | 3           | 0.44%                           |
|         | Chaetomium sp.   | 3           | 0.44%                           |
|         | Malassezia sp.   | 304         | 45.04%                          |
|         | Penicillium sp.  | 6           | 0.89%                           |
| 7       | Aspergillus sp.  | 11          | 3.17%                           |
|         | Chaetomium sp.   | 1           | 0.29%                           |
|         | Candida albicans | 1           | 0.29%                           |
|         | Malassezia sp.   | 137         | 39.48%                          |
| 8       | Aspergillus sp.  | 13          | 1.43%                           |
|         | Candida albicans | 4           | 0.44%                           |
|         | Chaetomium sp.   | 3           | 0.33%                           |
|         | Malassezia sp.   | 475         | 52.08%                          |
|         | Penicillium sp.  | 11          | 1.21%                           |
| 9       | Aspergillus sp.  | 31          | 2.13%                           |
|         | Candida albicans | 6           | 0.41%                           |
|         | Chaetomium sp.   | 5           | 0.34%                           |
|         | Malassezia sp.   | 678         | 46.5%                           |
|         | Penicillium sp.  | 11          | 0.75%                           |
| 10      | Malassezia sp.   | 112         | 17.67%                          |
|         | Aspergillus sp.  | 16          | 2.52%                           |
|         | Candida albicans | 1           | 0.16%                           |
|         | Chaetomium sp.   | 2           | 0.32%                           |
|         | Penicillium sp.  | 17          | 2.68%                           |
| 11      | Malassezia sp.   | 94          | 34.43%                          |
|         | Aspergillus sp.  | 3           | 1.10%                           |
|         | Penicillium sp.  | 1           | 0.37%                           |
| 12      | Malassezia sp.   | 28          | 51.85%                          |
| 13      | Aspergillus sp.  | 20          | 4.26%                           |
|         | Candida albicans | 7           | 1.49%                           |
|         | Chaetomium sp.   | 2           | 0.43%                           |
|         | Malassezia sp.   | 58          | 12.34%                          |
|         | Penicillium sp.  | 5           | 1.06%                           |
4. Discussion

Fungi have been reported to be ubiquitous in the sinuses of healthy subjects and CRS patients [8–10]. However, fungi are difficult to grow on the culture plate. Culture-negativity does not mean absence of fungi in the clinical samples [23]. We have applied third generation sequencing to evaluate fungi communities in 13 CRS patients with fungal rhinosinusitis. With NS, we found in all 13 samples of nasal irrigant the presence of three major pathogen species including Malassezia sp., Aspergillus sp. and Candida albicans. In contrast, the traditional culture method yielded only one or two species, and the growth process took several weeks. Therefore, we concluded that NS was more sensitive and faster in detecting fungi compared to the traditional culture method [24].

The sequencing techniques (NGS and third-generation sequence) are culture-independent. They have contributed to the knowledge of the comprehensive microbial communities in the human body. Modern technological advances in NGS allow the cost-effective assessment of microbial communities (microbiota) from environmental samples. Specifically, targeted sequencing of taxonomically informative regions of the genome (such as the 16S rDNA gene and internal transcribed spacer regions) reliably identifies most bacteria and fungi, down to the genus level [25].

Recently, metagenomic sequencing has been used to characterize the CRS mycobiome. One study on CRS patients, using NGS, reported nasal fungi as identified through sequencing the internal transcribed spacer region [26]. The most frequently detected fungi were Aspergillus sp., Schizophyllum sp., Curvularia sp., and Malassezia sp. Our study is the first to use NS to detect nasal fungi in CRS patients. We found the most frequently detected fungi were Malassezia sp., Aspergillus sp., Candida albicans, Penicillium sp. and Chaetomium sp. in nasal samples of 13 CRS patients. Our data showed that the NS was real-time and faster than the NGS in detecting microbiota of CRS. While NGS needs to collect a panel of specimens before sequencing, NS needs only one specimen to do the sequencing.

5. Conclusions

NS was found to be faster than the culture method in detecting fungi in nasal specimens of CRS patients, yielding more species of pathogenic fungi.

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