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Airborne Fungi in Chronic Rhinosinusitis Patients Maxillary Sinus Lavage at Dr. Saiful Anwar Hospital Malang

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Background: Chronic rhinosinusitis has a significant impact on the quality of life and health of adult population. Role of airborne fungi remains a controversy and have become the source of discussion for decades. **Objective** to know the prevalence of airborne fungi in the chronic rhinosinusitis with or without polyps patients and to know the possible effect of airborne fungi on chronic rhinosinusitis inflammation. **Methods:** This is a cross sectional research in the Saiful Anwar Public Hospital Malang, there were 29 patients involved. We examine fungi culture, H&E staining and DNA fungi by using PCR from sinus lavage sample. From the blood serum we examine allergen specific IgE, IgG3, IL-13 and IL-5. **Results:** Fungi culture there were 31,03 % of sample growth but only matches the PCR result in 3 samples (10,34 %). From PCR examinations we found all sample were positive with 2-5 species fungi, *Alternaria alternata* was found positive in 24,13% samples. There was an increment of IgE allergen specific and IL-5, a decrement of IL-13 and IgG3 in all of our samples regardless presence of nasal polyps and species of fungi found in PCR. **Conclusions:** PCR is a more reliable method compare to fungal culture. The presence of fungi in all of our samples could indicate fungi contribution to the disease pathophysiology. The increased level of Il-5 was not followed by IL-13; it may happen through PRR pathway.

Key words chronic rhinosinusitis, airborne fungi, Il-5, IL-13, IgG3.

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INTRODUCTION

Chronic rhinosinusitis is a chronic inflammation of the nose and paranasal sinuses which clinically defined as a symptom of a continuous that include clogged nose or discharge of secretions from the nose more than 12 weeks confirmed by abnormalities on endoscopy (polyps, secretions mucopurulent or mucosal edema) or abnormalities in examination Computed Tomography scan (CT scan).[1]

Chronic rhinosinusitis has a significant impact on quality of life and health of the adult population.[1] It is estimated that nearly 31 million people per year in the United States (4 % of the adult population) experienced chronic rhinosinusitis.

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While in Dr. Saiful Anwar General Hospital, patients with chronic rhinosinusitis in 2012 reached 26 370 patients and included in the top 10 most prevalent diseases in ENT-Head and Neck Surgery Department.

Chronic rhinosinusitis has a multifactorial etiology, which includes the role of microorganisms, inflammation due to allergic and non-allergic as well as various non microorganisms and non-immunological causes.[2,3]

Hypothesis role of fungal in rhinosinusitis is still a controversy and has been a subject for debate for decades.[4] One hypothesis about how fungal can cause rhinosinusitis is that the colonization fungal of the mucus of the nose and paranasal sinuses will cause eosinophils gathered in the area around the fungal colony.[4,5]

Recent in vivo observations show that eosinophils are in the nasal mucosa in the intact condition, but in mucus, eosinophil cells flocking, undergo degranulation and release the MBP at concentrations well above the amount needed to damage the epithelium. This observation explains

the patterns of damage in chronic rhinosinusitis, in which the damage occurs only in the outer layer of mucous and starts imposing any damage of the sinus lumen. This makes the damaged sinus mucosa becomes more susceptible to secondary bacterial infections, resulting in acute exacerbation. Bacteria will cause neutrophilic inflammation, but eosinophilia inflammation which dominates the findings in chronic rhinosinusitis are less likely to be caused by a bacterial infection, suggesting the existence of non-bacterial etiology in chronic rhinosinusitis.[6]

Fungal spores that commonly found in free air, continuously inhaled during inspiration and deposited in the respiratory tract mucosa .[7] If the immune system in patients with chronic rhinosinusitis recognize fungi as a foreign object and use the eosinophils to attack fungi, we can surmise that eosinophils are recruited and activated through the production of cytokines that regulate the inflammatory eosinophils. Lymphocytes that are located in the body tissues is known as a major source of cytokines in patients with rhinosinusitis kronis.[6]

If fungus can trigger inflammatory cells to initiate complex eosinophilic reactions locally in susceptible individuals, it can be presumed that this process involves the T cells reacts against the fungal antigens by producing profile of cytokines produced by T helper 2 cells, including IL-5 and IL-13, IL-5 is a cytokine that is most important to induce eosinophils inflammation by stimulating the production, chemotaxis, survival and activation of eosinophils. IL-13 induces the expression of vascular cell adhesion molecule-1 (VCAM-1) involved in selective eosinophil migration from the blood vessels into the tissue.[7]

In patients with rhinosinusitis, the nasal mucosa contains eosinophils, Charcot-Leyden crystals, IgG antibody towards fungi, without any helper T lymphocytes, and without cell antigen presenters. In the peripheral blood and nasal mucosa contains many fungal specific antigen IgG. This antigen activates T helper lymphocytes in the blood, producing cytokines (IL-5 and IL-13) which recruit eosinophils and it is not found in normal people, that is the basic hypothesis of the existence of fungi in chronic rhinosinusitis.[9,10]

Chronic rhinosinusitis occur in patients who have or do not have IgE antibodies against fungi and other inhalant allergens. In contrast, IgE-mediated allergy and exposure to a specific allergen can cause allergic rhinitis. Thus patients with chronic rhinosinusitis can have IgE-mediated hypersensitivity, but the underlying inflammatory eosinophils seem to be regulated by mechanisms that are not dependent on IgE. VCAM-1 identified in vascular endothelium of chronic rhinosinusitis patients. This expression occurs in people without allergies and explains the discovery of eosinophils in patients with chronic rhinosinusitis regardless the presence of allergies.[6]

Shin et al, as quoted by Ponikau JU, Sherris DA, Kita H [6] indicates that the isolation of mononuclear cells peripheral blood of patients with chronic rhinosinusitis, containing lymphocytes and other cells that may act as antigen presenting cells (APC), producing IL-13 in large quantities when exposed to *Alternaria*. Expression of IL-13 increased this can increase the expression of VCAM-1 on vascular endothelium sinus.[6]

Interleukin 13 (IL-13) will result in the exchange of immunoglobulin isotip (eg IgE - IgG1) and IgG produced by the B lymphocytes of immature whereas interleukin 5 (IL-5) triggers the activation of eosinophils and B cells to differentiate into cells IgG secretion. Eosinophils and IgG ready to migrate through the mucosal membrane and attack the fungi in the nasal mucosa, this is a classic reaction of hypersensitivity type 2.[8,9]

The mean levels of *Alternaria* specific IgG in the serum were fivefold in 18 patients with chronic rhinosinusitis compared to 15 controls. Levels of IgG in patients with chronic rhinosinusitis is directly correlated with the levels of IL-5 produced when peripheral blood mononuclear cells were incubated with *Alternaria*. IgG immunologically indicates the amount of exposure. These results indicate a direct link between exposure to antigens of *Alternaria* and severity of immune response is determined by the amount of production of IL-5.[6]

The objective of this study is to find a correlation between chronic maxillary rhinosinusitis with / without polyps with fungi present in the air (airborne fungi) in Dr. Saiful Anwar Malang General Hospital.

MATERIALS AND METHODS

Sample selection

This is a cross sectional study, taken place in dr. Saiful anwar General Hospital from March-October 2014. In this study, all patients with chronic maxillary sinusitis confirmed by nasoendoscopy or CT scan sinus irrigation that met the inclusion criteria (age ≥ 18 and willing to participate in the study) and exclusion criteria (patients who have previously been treated with corticosteroids or systemic or topical antifungal for chronic rhinosinusitis, patients with dentogenic chronic rhinosinusitis, immunocompromised patients (patients with diabetes mellitus, ASA intolerance, Cystic Fibrosis), patients with a history of recurrent maxillary sinus irrigation and a history of previous sinus surgery / FESS, patients with severe heart defects, osteomyelitis) underwent an maxillary sinus irrigation Then from the sinus lavage fluid fungal culture and H&E staining was performed, RNA PCR assay for airborne fungi. Samples from the patient's peripheral blood was taken to check for

IgG3 levels, allergen-specific IgE, IL-13 and IL-5 by ELISA.

Sampling method

1. Patients with chronic maxillary rhinosinusitis maxillary who will undergo the sinus irrigation procedure are being given explanation about the purpose of research and management plan that will be carried out. If the patient is willing to participate in research, then they will have to signed a letter of consent to participate in research.
2. Peripheral blood sampling was done as preparation for sinus irrigation and to measure the levels of IgG3, allergen-specific IgE, IL-13 and IL-5 from the peripheral blood of patients. Examination using *Elabscience* Human IgG3 IgG3 (Immunoglobulin G3) ELISA Kit, examination of allergen-specific IgE using *AccuDiag*™ Human Allergen Specific IgE ELISA Kit, examination of IL-5 by ELISA MAX ™ Deluxe Set Human IL-5, IL-13 using the examination Human *RayBio*® IL-13 ELISA Kit. Examination results IgG3 levels, IL-13 and IL-5 from peripheral blood will be compared with normal levels in adult humans, whereas of allergen-specific IgE would be obtained the data the tendency of complaints caused by hypersensitivity reaction or not.
3. Maxillary sinus irrigation is done under local anesthesia in the operating room local ENT Dr. Saiful Anwar General Hospital in accordance with the standard operating procedures of the maxillary sinus irrigation SMF ENT Dr. Saiful Anwar General Hospital. Conducted antrostomy intranasal through the inferior meatus, then put 10 mL of NaCl 0.9 percent in the maxillary sinus, aspirated and put it back then accommodated, labeled separated into two parts. The first part of specimens was sent to the Laboratory of Physiology Sciences at Brawijaya University for fungal RNA isolation and identification of fungi (*Candida albicans*, *Trichophyton* sp, *Alternaria alternata*, *Aspergillus flavus*, *Candida parapsilosis*, *Cladosporium* sp, *Penicillium* sp.) to be conducted on the same day. The second part of specimens were sent to the Laboratory of Microbiology, Faculty of Medicine, Brawijaya University on the same day. In the Laboratory of Microbiology, Faculty of Medicine, Brawijaya University direct smear examination were done in some part of the specimen and then stained with Lactophenol Cotton Blue and examined under a microscope with a magnification of 400 x in the search for hyphae. Another part of the specimen was planted in two media of Sabouroud Dextrose Agar (SDA), one of which was incubated in 37 ° C for one week to see the growth of yeast (*Candida* sp and others) and the other specimens were incubated in 37 ° C for one week to see the growth of mold (*Penicillium* sp, *Trichophyton* sp, etc.). If there is growth, the cell structures will be seen with magnification 1000 x. Hematoxylin and eosin staining (H & E) were performed to search for fungal hyphae surrounded by eosinophil cells with 1000x magnification microscope.

RESULT

In this study, we obtained 29 patients with chronic rhinosinusitis with or without nasal polyps as our sample during 8 months of sampling (March 2014 to October 2014) as described in Table 1. In all the samples examined IgE, IgG3, IL-13 and Il-5 from serum; fungal culture, Hematoxylin & Eosin staining of eosinophils and PCR to search for fungus from maxillary sinus lavage fluid. IgE test results, IgG3, IL-13 and Il-5 from the serum will compared to the results from level of normal adult human. In this study, the results obtained are as follows:

Table 1 Profile of patients included in the study

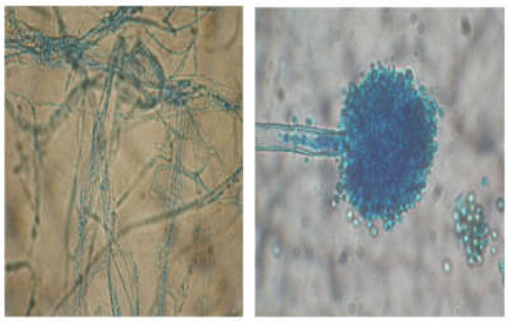
Characteristics	P	%
Gender		
Male	**18**	**62,07 %**
Female	**11**	**37,93 %**
Age		
18-20 years old	**2**	**6,89 %**
21-30 years old	**11**	**37,94 %**
31-40 years old	**7**	**24,14 %**
41-50 years old	**5**	**17,25 %**
51-60 years old	**2**	**6,89 %**
61-70 years old	**2**	**6,89 %**
Diagnosis		
Chronic rhinosinusitis without nasal polyps	**17**	**58,62 %**
Chronic rhinosinusitis with nasal polyps	**12**	**41,38 %**
Skin fork test results		
Positive	**10**	**34,48 %**
Negative	**9**	**31,04 %**
Not performed	**10**	**34,48 %**
Results of X-rays Waters		
Paranasal sinus cloaking	**21**	**72,42 %**
Paranasal sinus mucosal thickening	**4**	**13,79 %**
Water fluid level in the paranasal sinuses	**4**	**13,79 %**

In this research there were 18 male patients (62.07%) and 11 female patients (37.93%) involved, ages ranged from 18 years to 70 years. Highest number of patients in the study were at the age of 21 to 30 years, as much as 11 patients (37.94%).

Skin prick tests conducted on 19 patients with 10 patients with a positive result against one or more allergens, while 9 patients had negative skin prick test results. A total of 10 patients skin prick test was not carried out because they do not have

allergic rhinitis symptoms (paroxysmal sneezing, itching of the nose / throat / eye). In Dr. Saiful Anwar Malang General Hospital, skin prick tests were performed using 5 inhalant allergens and 28 allergen ingestion. Among the inhalant allergens that there are no fungi, so there is no data regarding sensitivity test in vivo against fungi.

Samples for fungal culture and PCR RNA fungi was taken from maxillary sinus by washing with sterile fluid. Grossly we did not found the characteristic of eosinophilic mucin or allergic fungal mucin as described by Bent and Kuhn, which is this thick, highly viscous, variably colored mucin has been described as being similar to peanut butter or axle grease.[10] On direct smears for sinus lavage liquid with Lactophenol Cotton Blue staining does not show any fungal hyphae, as well as in Hematoxylin and Eosin staining does not show fungal hyphae surrounded by eosinophil cells in all of our samples. On examination using fungal culture media Sabouroud Dextrose Agar (SDA), there were 9 samples (31.03%) obtained positive growth. Growth of fungal species observed was *Trichophyton* sp. in 5 samples, *Penicillium* sp. in one sample, Aspergillus sp. in 2 samples, Candida sp. in one sample.



Pic. 1. a. Shows Trichophyton sp. on Lactophenol Cotton Blue stain under microscope with 1000 x magnification. Pic 1.b shows Aspergillus sp. under 1000 x magnification

On examination using PCR we found fungi DNA on all of the samples, each sample is positive for 2-5 types of airborne fungi (as shown in table 4.2). Fungi species that were examined are *Alternaria alternata*, *Aspergillus flavus*, *Candida albicans*, *Candida parapsilosis*, *Penicillium* sp., *Cladosporium* sp. and *Trichophyton* sp. There is a discrepancy between the results of fungal cultures with fungal DNA testing on 6 samples (66.67%), whereas there was fungus growth in cultures but the on fungal DNA PCR the same species were not found.

Table 2 shows the cross-tabulation between fungal species obtained from PCR with allergen-specific IgE levels of peripheral blood of research subjects. Fischer exact test results showed that there was no relationship between allergen specific IgE levels with all fungal species found in the study subjects.

Table 2. The relationship between the classification of allergen-specific IgE levels and fungal species on PCR

Fungi Species	Level of Allergens Specific IgE		P value
	Medium	High	
Alternaria alternata	1	6	**0.367**
Aspergillus flavus	8	13	**1.000**
Candida albicans	5	6	**0.432**
Candida parapsilosis	2	4	**1.000**
Penicilium sp.	9	17	**1.000**
Cladosporium sp.	7	12	**1.000**
Tricopyton sp.	5	7	**0.694**

Table 3 Cross-tabulation of allergen-specific IgE levels and diagnosis of patients.

Chronic Rhinosinusitis	IgE Allergen Specific Level	
	Moderate	High
Without nasal polyps	7	10
With nasal polyps	4	8
Total	11	18

Table 3 shows the cross-tabulation between semi-quantitative levels of allergen-specific IgE in patient diagnosis. There were 7 chronic rhinosinusitis without nasal polyps patients with moderate allergen-specific IgE level and 10 patients with high levels of allergen-specific IgE. The existence of nasal polyps is not associated with fungi species found in the PCR (Table 4). It is said that chronic rhinosinusitis with nasal polyps is characterized by Th2 inflammation and characterized by tissue eosinophilia and one of the possible cause is allergies.[4]

Table 4 Cross-tabulation between the diagnosis of chronic rhinosinusitis with or without polyps with fungal species

Fungi Species	Chronic Rhinosinusitis		P value
	Without nasal polyps	With nasal polyps	
Alternaria alternata	5	2	0.410
Aspergillus flavus	11	10	0.697
Candida albicans	8	3	0.249
Candida parapsilosis	3	3	1.000
Penicilium sp.	15	11	0.573
Cladosporium sp.	11	8	1.000
Tricopyton sp.	7	5	1.000

IgG3 normal values in the peripheral blood of a healthy adult is 0.2 to 1.1 g / l or 200000-1100000 ng / ml. [11] While the value of normal levels of IL-13 in a healthy adult is 12-17 pg / ml and normal levels of IL-5 adults is <0.7 pg / ml. [12] In this

study, elevated levels of IL-5serum (29,61- 53.12 pg / ml) and decreased levels of IgG3 serum (6074 -23 424 ng / ml) in all samples (100%) compared to normal levels in healthy adult individuals. While the levels of IL-13 in the normal range (3.77 to 11.46 pg / ml) in all samples.

DISCUSSIONS

The fungal hypothesis proposes that patients with CRS mount an eosinophilic response to fungi, with initial evidence showing some degree of fungi and eosinophilic mucin in all patients with CRS. The mechanistic implications of the fungal hypothesis are twofold: first, *Alternaria* proteins are apparently recognized by antigen-presenting cells (APCs) and presented to T cells with a TH1/TH2 cytokine response that attracts and activates eosinophils. Second, *Alternaria* is hypothesized to trigger the intraluminal targeting and degranulation of eosinophils by a protease-dependent mechanism.[5]

In 1994, Bent and Kuhn published their diagnostic criteria centered on the histologic, radiographic, and immunologic characteristics of the disease. Patients must meet all the major criteria for diagnosis, while the minor criteria serve to support the diagnosis and describe individual patients but are not used to make a diagnosis allergic fungal mucin itself, and not the surrounding mucosa, is the most reliable indicator of disease. Grossly, this thick, highly viscous, variably colored mucin has been described as being similar to peanut butter or axle grease. Microscopically, the mucin often takes on a chondroid appearance with sheets of eosinophils, frequently with the presence of eosinophilic breakdown products or Charcot-Leyden crystals.[10]

IL-5 produced by Th2 and mast cells are key in the development, recruitment and activation of eosinophils.[13] Even though on direct smear examination of the sinus lavage we do not find any cluster of fungi or eosinophil but this could be due to the dilution of the mucus layer during irrigation. And we did not give any treatment to break the mucus layer such as disulfide or mucolytic. It is best to obtain the sample for culture from scrapping a substantial area of the sinus mucosa surface or from the sinus biopsy.

Chronic rhinosinusitis without nasal polyps predominantly involves the response of neutrophils mediated by Th1 response characterized by IL-12 and IFN-γ, while chronic rhinosinusitis with nasal polyps is characterized by increased Th2 cytokines such as IL-5, IL-4 and IL-13. Eosinophils and inflammation-related products are considered as the main marker of inflammatory polypoid.[4] In our study found an increase in IL-5 levels in all of our samples, even though there are 16 samples of chronic rhinosinusitis without nasal polyps. The increment we found was around 50 times the normal adult level.

In a study conducted by Riechelmann H, Deutschle T, Rozsasi A, Keck T, D and Burner H.[14] Polzehl found in nasal secretions there is an increase in IL-4, IL-5, IL-10, IL-12 and IL-13. Increased IL-12 is involved in immune system activation by Antigen Presenting Cells (APC), distinguish between acute or chronic rhinosinusitis. It also correlates positively with IL-4, IL-10 and IL-13, which plays a role in the resolution of infection. It distinguishes between rhinosinusitis without nasal polyps and rhinosinusitis with nasal polyps is the presence of IL-5 and IgE nasal and concluded that increased nasal IgE and IL-5 levels specific for nasal polyps.[14] While Shin SH, Ponikau JU, Sherris DA, et al., As cited by Mor N, Sherris DA, Kita H, Ponikau[15], found that 90% of patients with chronic rhinosinusitis (CRS) produced IL-5 in large quantities in response to *Alternaria*, while all patients CRS produce large amounts of IL-13. This immune reaction such as CRS obtained only in patients and not in the control group. What is interesting in this study we get an increase in IL-5 expression but it is not followed by an increase in IL-13. It is possible that the higher expression of IL-5 was not produced by T helper 2 cells. To answer exactly why a such high level of expression of IL-5 in CRS is not followed by IL-13 require further research.

It is said that the IgG subclass deficiency was found in 7-57 % of patients with chronic infection in the ear nose throat and lungs, the most common is deficiency IgG3.[16] Armenaka M, Grizzanti J, Rosenstreich DL[16], found that among a group of patients with chronic rhinitis, chronic rhinosinusitis and control there was no significant difference in serum levels of IgG, IgA, IgM, IgG1, IgG2, and IgG4. However, the mean serum IgG3 levels were significantly lower in the group of chronic rhinosinusitis than chronic rhinitis and control groups, as well as a lower level of IgG3 was obtained more frequently.[17] This is consistent with the results obtained in our study, where all the samples have decreased levels of serum IgG3. Buckley, as quoted by Ocampo CJ, Peters AJ[18], describes IgG3 deficiency, observed in some cases of refractory CRS, as a secondary phenomenon rather than a cause of disease. IgG3 has a half-life of only 9 days in serum and is the most susceptible to degradation. Therefore, IgG3 deficiency may be secondary to the disease process itself. [18]

Pant, H., Kette, FE, Smith, WB, Wormald PJ and Macardle, PJ[19] IgG fungi specific level (*Alternaria alternata* P = .0002; Aspergillus fumigatus P = .004), and the levels of IgA (*Alternaria alternata* P = .0016; Aspergillus fumigatus P = .002) was found higher levels in patients with chronic rhinosinusitis with eosinophilic mucin compared to healthy controls but not with groups of other rhinosinusitis. Fungal-specific IgG3 levels were significantly increased in

all subgroups of chronic rhinosinusitis with eosinophilic mucin compared to the levels in the control group. Fungal-specific IgE levels are not higher in the group with mucin eosinophilic chronic rhinosinusitis who have allergic fungal rhinosinusitis compared to other groups.[19]

Mike, S., A. S. McWillam, and H. Kita, as quoted by Inoue Y, Matsuwaki Y, Shin SH, Ponikau JU, Kita H[20;] demonstrated that human eosinophils express functional protease-activated receptor 2 (PAR-2), and that serine proteases, such as trypsin, activate effector functions of human eosinophils through this receptor. *Alternaria* induced degranulation of eosinophils isolated from normal individuals, suggesting that this response to *Alternaria* is not limited to sensitive patients. [20] PARS expressed in epithelial cells, airway cells, leukocytes and blood vessels that activate intracellular signaling pathways that lead to a variety of responses, including the production of cytokines, chemokines, eicosanoids and metalloproteinases, which in turn causes damage to the bond between the cells and the basement membrane. Compared proteases from Aspergillus fumigatus and Cladosporium herbarum, *Alternaria alternata* protease is most potent to stimulate the production of inflammatory cytokines (IL-6, IL-8) of the primary nasal epithelial cells. [7]

In our research we found the most frequent airborne fungi is *Penicillium* (93,1 %) followed by Aspergilus fumigatus (72,41 %) and Cladosporium (65,51 %), while *Alternaria alternata* positive in 24,13% of our samples. There seem to be no specific pattern between the species of fungi found with PCR and the level of IgG3, Il-5, IL-13 and allergen-specific IgE levels. We found all samples have the same level of IgG3, Il-5, IL-13 and allergen-specific IgE regardless the prescence or absesence of *Alternaria alternata*. It is believed that products of certain environmental fungi, such as *Alternaria* and *Penicillium*, may directly induce exocytosis release of granule proteins from eosinophils in the absence of other immune cells or Igs. [20] In summary, current data supporting the fungal hypothesis of CRS suggests that high levels of

Alternaria can trigger effects on PBMCs and eosinophils obtained from patients with CRS, although it is not clear that this is a disease-specific response. Intranasal fungi in a patient with CRS would probably exacerbate the disease process through protease effects on nasal epithelial cells as well as activated eosinophils and lymphocytes present in the nose. It is unclear whether *Alternaria* has any relevance to the establishment of CRS in the first place, however. [5]

CONCLUSIONS

PCR is a more reliable method to detect fungi compare to culture. We do not know where is the exact role of fungi but its presence in all of our samples could contribute to the disease pathophysiology. The increased level of Il-5 was not followed by IL-13, this unusual finding requires more reach in the future and the most possible option for an alternative therapy is anti-IL 5.

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