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Fungal cultures of different parts of the upper and lower airways in chronic rhinosinusitis*

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SUMMARY

The relation between fungi, upper and lower airways in chronic rhinosinusitis (CRS) patients are not clear yet. So the aim of this study was to identify the different cultured fungi in various sub-sites of the nasal cavity and lower airways in adult (CRS) patients and to correlate the cultured fungi to the associated cellular inflammatory changes. In the outpatient clinic a control group of 10 normal subjects was subjected to total nasal lavages to validate our mycological culture technique. Twenty-five adult CRS patients were enrolled in this prospective study. Under general anaesthesia before functional endoscopic sinus surgery (FESS) operation 50 nasal vestibular swabs, 25 bronchoalveolar lavages (BALs), 50 middle meatal lavages (MMLs) and 50 nasal cavity lavages (NCLs) were obtained in the operating room. These samples were processed for fungal culture and eosinophilic cellular counts. The intra-operative pathological specimens were examined using Haematoxylin and Eosin (H&E) and Gomori methanamine silver (GMS) staining. In the normal control group total nasal lavages showed 100% positive fungal cultures. In the CRS patient group the BALs showed positive fungal cultures in 28%. Nasal vestibule cultures were positive in 8%. Positive middle meatal cultures were obtained in 44% of the 25 CRS patients. Two cases (8%) with maxillary fungal ball showed a positive maxillary sinus culture but a negative middle meatal culture. Nasal cavity lavages were positive in 36%. Middle meatal eosinophilia was identified in 33.6% of the positive middle meatal fungal culture. Following the deShazo's criteria of diagnosis of allergic fungal rhinosinusits (AFRS), only 16% of the subjects in this study fulfilled the criteria. No correlation existed between fungal culture, cellular and other clinical parameters. Also no correlation existed between upper and lower airway positive cultures. In conclusion fungi seemed to be present in different percentages and types in different sub sites of the airways but without associated eosinophilia. There were no significant correlations between the fungal culture and clinical parameters of CRS nor were there significant correlations between fungal culture and objective lower airway involvement.

Key words: AFS, chronic sinusitis, fungi, asthma, SAD.

INTRODUCTION

Fungi are ubiquitous saprophytes that reproduce by the formation of spores that are able to enter the respiratory tract by means of inhalation [1]. The process of retention and clearance of fungal spores that begin as soon as the spores make contact with the airway or alveolar wall must be differentiated from fungal related diseases of the paranasal sinuses. The pathophysiology depends on many factors including: physical and chemical properties of both the spores and the mucous surfaces, anatomic location of the deposition site and the nature of the structures with which the particles interact at the site of deposition (type of mucociliary layer at air fluid interface, the aqueous phase, free cells, epithelial cells or denteritic cells residing near the basal aspect of epithelium) [1].

Fungi can be pathogenic to the sinonasal passages via several different mechanisms and can cause diseases ranging from such non-invasive entities as allergic rhinitis to fungal antigens, fungal balls, allergic fungal sinusitis to the invasive chronic indolent sinusitis and the invasive acute fulminate fungal sinusitis [2]. The immune status of the host, the environmental load or mass of the fungi present, and local structural conditions of the sinuses or other tissues causing tissue hypoxia are all factors that predispose toward the development of a particular sinus disease [3].

The relation between fungal spores and sinonasal passages in allergic fungal rhinosinusits (AFRS) is not clear yet. Type 1 and Type III hypersensitivity are one form of this interaction, which was described as allergic fungal rhinosinusits by

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Manning et al. [4]. Another dominant role is eosinophilic interaction [5], which was described by Ponikau et al. [6] as eosinophilic fungal rhinosinusits. Another manner of interaction was shown in experimental studies and also in allergic bronchopulmonary aspergillosis where fungal spores impair mucosal defense not only because immunogenic interaction but because they are capable to alter the host immune response through macrophages and T cell suppression [7,8].
Recently multiple controversies rose concerning the relation of fungi to chronic rhinosinusits and its related lower airways involvement.
After validating our mycological culturing technique, with the highly proved culture rate technique of Ponikau et al. [6], the aim of this study was to identify in chronic rhinosinusits (CRS) the different cultured fungi in various sub sites of the nasal cavity and lower airways and to correlate the presence of these fungi with the cellular inflammatory changes.

MATERIAL AND METHODS

Subjects

In this prospective study two groups of subjects were included:
- A control group of 10 normal subjects was subjected to total nasal lavages in the outpatient clinic in order to validate our culture methodology. No BALs were performed in this group, as we judged it was not ethical to perform BALs in completely normal persons.
- A patient group of twenty-five adult CRS patients was enrolled in this study. All patients were scheduled for functional endoscopic sinus surgery (FESS) after failure of medical treatment. The study population included 18 males and 7 females with ages ranging between 25-66 y and a mean age of 34.1 y. Chronic rhinosinusits was diagnosed in these patients based on the definition of the consensus report of the rhinosinusits task force group [9]. Patients with cystic fibrosis, primary ciliary dyskinesia, recognized immunodeficiency or other systemic diseases that can affect the upper and lower airways were excluded. In addition, no medications were allowed one month before the study except for asthma medications. Chronic rhinosinusits (CRS) was assessed using visual analogue scale for the symptoms, Lund - Mackay scoring system for CT scan and Lund-Kennedy score for endoscopic assessment of nasal polyposis as published [10]. The lower airways were assessed subjectively and objectively [10]. Three subgroups of lower airways involvement were identified: asthma subgroup (24%), small airways disease (SAD) subgroup (36%) and normal lower airways (NLA) subgroup (40%) (Table 1) [10].
The study was approved by the Ethical Committee of the hospital (Academic Hospital, Free University Brussels), and all patients gave an informed consent

Table 1. Age and sex characteristics of different chronic rhinosinusitis subgroups.

	CRS total no (25 patients)		
	Asthma	SAD	NLA
No of patient	6	9	10
Age (mean)	(35-66) 46	(25-54) 44,8	(31-52) 38,7
Sex M	4	6	8
Sex F	2	3	2

Specimens collection

1. Control group samples: The total nasal lavages were performed in the same way as described by Ponikau et al. [6]. In the outpatient clinic, while the test subjects were in sitting position, they were subjected to nasal lavages for each side separately. Each side was irrigated by 20 cc saline without disinfecting the nasal vestibule and was collected in sterile containers.
2. CRS patient group: The specimens were collected in the operating room under general anaesthesia before FESS.
2.1 Nasal vestibule samples: Before sterilization of the face and vestibule of the nose a swab of the nasal vestibule for mycological culture was obtained. This was followed by sterilization of the face and vestibule of the nose before draping the patient.
2.2 Bronchoalveolar lavage (BAL) samples: Under general anaesthesia, a sterilised flexible bronchoscope was passed into the airways through the endotracheal ventilation tube and gently impacted or wedged into a subsegmental bronchus (right middle lobe). A standardised 5x20 ml aliquot regimen was used. The 1st aliquot was sent directly for mycological examination. The remaining specimens were placed in a special container surrounded by ice and sent directly for cytological examination.
2.3 Middle meatal samples: Before the operation nasal endoscopy was performed with a 30-degree wide angle, 4.00 mm rigid endoscope (Wolf, Tuttingen, Germany) attached to a video camera. To avoid any effects of local anaesthetics and vasoconstrictors over the microbiological , cellular count and morphology, sampling procedures were obtained without these medications.
A sterilized irrigation double ways suction tip was used. It was connected to one side to a 20-ml syringe full of sterile buffered physiological saline at body temperature. To the other side it was connected to a special double way bottle attached to an aspiration machine. The saline was injected under minimal hand pressure over 30 seconds into the middle meatus with the guidance of the nasal endoscopes. This allowed for perfect sampling of mucus from the sinuses and its related mucous membrane. The collected fluids are divided for mycological and cellular examination. The procedures were done similarly in both nasal passages.
2.4 Nasal cavity lavage samples: After steps 2.1 till 2.3 irrigation suction of the left and right nasal cavities were obtained with the same technique using 20 ml saline under guidance of the nasal endoscopes and send directly for mycological examination.
2.5 Histopathological samples: All the tissue samples obtained during FESS intra-operatively were collected on a saline moist-

ened non-stick sheet (Telfa) and send for histopathological staining and examination.

Mycological examination

The collected lavage fluids were sent directly to the mycology laboratory where the specimens were processed under a laminar flow hood to prevent contamination. One vial 3 ml of sterile 20% Mensa (UCB Pharmaceutics Company, Brussels, Belgium) was added to 3 ml of sterile water. The collected specimens were suspended with an equal volume of this diluted Mensa and vortexed for 30 seconds. The mixture was allowed to stand at room temperature for 15 minutes to allow the Mensa to liquefy the mucus. The mixture was then centrifuged at 3000 g in a 50 ml tube for 10 minutes. The prepared sediments and supernatants were vortexed separately for 30 seconds. Afterwards they were inoculated on Malt agar plates containing 0.05 % chloramphenicol. The plates were incubated at 25°C and at 30°C and allowed to grow for 30 days. The plates were examined at 2-days intervals. Identification at the species level was performed.

Eosinophilic examination

In this study middle meatal secretion and BAL were processed directly after their collection for a cytospin. The cytocentrifuge prepared smears were rapidly air-dried and stained using May-Grünwald-Giemsa. Eosinophilia was determined in middle meatal samples at ≥ 4% according to Miller [11].

Table 2. Number and percentage of the normal control group with positive cultured fungi in nasal cavity lavages (total number =10). N.B. Some normal control subjects may have positive fungal culture identified for more than one species of the same genus.

Fungal genus	Fungal species	n	%
Aspergillus		7	70
Aspergillus	*flavus*	1	10
Aspergillus	*fumigatus*	3	30
Aspergillus	*glaucus*	2	20
Aspergillus	*niger*	2	20
Aspergillus	*versicolor*	2	20
Penicillium		10	100
Penicillium	*spp.*	7	70
Pencillium	*auratiogriseum*	2	20
Pencillium	*chrysogenum*	1	10
Pencillium	*citreonigrum*	1	10
Pencillium	*spinulosum*	1	10
Cladosporium		2	20
Cladosporium	*spp.*	1	10
Cladosporium	*cladosporioides*	1	10
Apiospora	*montagnei*	1	10
Acremonium	*terricola*	1	10
Paecilomyces	*variotii*	1	10
Mucor		1	10
Mucor	*sp.*	1	10
Mucor	*hiemalis*	1	10
Sterile Mycelium		4	40
Yeast		1	10

Histopathological examination

Multiple serial sections of different nasal tissue specimens from each patient were stained with Haematoxylin & Eosin for cellular examination, and PAS and Gomori-Methenamine-Silver staining for fungal detection.

Statistical analysis

The analysis was performed using SPSS version 7.5 (SPSS, Inc, Chicago, IL). Using Wilcoxon signed rank tests, the authors compared different positive culture fungal types in different lower airways involvement subgroups. Statistical significance was set at a p value ≤ 0.05.

RESULTS

Fungal cultures

1. Normal control group:

Positive nasal cultures were obtained in all the cases (100%). *Penicillium* was cultured in 100% and *Aspergillus* in 70% (Table 2).

2. Patient group:

2.1 Nasal vestibule samples

These samples were positive in only 2 cases. The types of fungi were *A. niger* and a yeast.

2.2 Bronchoalveolar lavage (BAL)

The positive culture rates of BAL for fungus were 28% in all CRS patients. There was no significant difference between different lower airways involvement subgroups ($p>0.05$): asthma 1/6 (16%), SAD 2/9 (22%) and NLA 4/10 (40%). Also the positive culture results did not correlate to the lower airways functional changes ($p>0.05$)

Different genus of fungi was identified. *Aspergillus* was cultured in 42.5% and *Penicillium* in 71.4% of CRS patients with positive BAL fungal culture results (Table 3).

2.3 Middle meatal lavage (MML)

From Table 3 in all CRS patients the culture rate was 11/25 (44%). There existed positive culture rate in 3/6 (50%) of asthma subgroup, in 2/9 (22%) of SAD subgroup and in 11/25 (60%) of NLA.

Fungal balls were found in two patients (8%). Both cases were identified in the SAD subgroup. One case presented with a unilateral involvement of the left maxillary sinus. The other case showed bilateral involvement, left maxillary sinus and right maxillary and ethmoidal sinuses. Both cases were associated with negative middle meatal cultures.

Both cases showed mixed cultures; in one case a combination of *Aspergillus niger* and *Alternaria alternata* and in the other case *Aspergillus niger* and *Rhizopus microsporus.*

2.4 Nasal cavity lavage (NCL) results

A positive culture rate was present in 36% of the cases (Table 3). There was no significant difference between different lower airways involvement subgroups ($p>0.05$) with a culture rate of

Table 3. Numbers and percentages of CRS patients with positive cultured fungal species in different parts of the respiratory tract; MML (middle meatal lavage); NCL (nasal cavity lavage); BAL (bronchoalveolar lavage).
N.B. Some patients may have positive fungal culture identified for more than one species of the same genus.

Genus	Species	MML	NCL	BAL
		Total positive cultures	Total positive cultures	Total positive cultures
	n (%)	11 (44)	9 (36)	7 (28)
Aspergillus	*fumigatus*	5 (45.4)	3 (33.3)	2 (28.5)
Aspergillus	*niger*	3 (27.2)	3 (33.3)	1 (14.2)
Aspergillus	*sydowii*	0	0	1 (14.2)
Penicillium	*chrysogenum*	3 (27.2)	2 (22.2)	2 (28.5)
Penicillium	*brevicompactum*	1 (8.1)	0 (0)	0
Penicillium	*melinii*	1 (8.1)	0	0
Penicillium	*verrucosum*	0	1 (11.1)	0
Penicillium	*spinulosum*	0	1 (11.1)	0
Penicillium	*purpurogenum*	0	0	1 (14.2)
Penicillium	*aurantiogrisium*	0	0	1 (14.2)
Penicillium	*glabrum*	0	0	1 (14.2)
Cladosporium	*sphaerospermum*	0	2 (22.2)	0
Cladosporium	*herparum*	0	2 (22.2)	0
Alternaria	*alternata*	0	1 (11.1)	0
Chaetomium	*globosum*	0	1 (11.1)	0
Sterile mycelium		0	1 (11.1)	0
Monilia	*sitophilla*	1 (9.1)	0	0
Candida	*albicans*	1 (11.1)	1 (11.1)	0

2/9 (22%) in SAD, 2/6 (33%) in the asthma and 5/10 (50%) in the NLA subgroups.

Different fungal genera were identified with *Aspergillus* in 76.3%, *Penicillium* in 44.4%, *Cladosporium* in (22.2%) and other genus (55.5%). The same types of fungi that were cultured from NCL were cultured in 40% of the positive middle meatal culture results.

Eosinophilic examination

Middle meatal lavage eosinophilia was found in 33.6% of the positive fungal middle meatal cases and in 40% of the negative middle meatal cultures.

Histopathological examination

Fungal hyphae were only seen in the histopathological examination of both cases of fungal balls (8%). In one case *Aspergillus* (with characteristic hyphae) together with *Dematiacious* fungi (melanin pigment) were identified (Figure 1). In the other case *Aspergillus* and *Rhizopus* microsporus could be cultivated.

In the other cases of chronic rhinosinusits using the previous staining techniques, no fungi can be detected in the histopathological examination of the tissue samples.

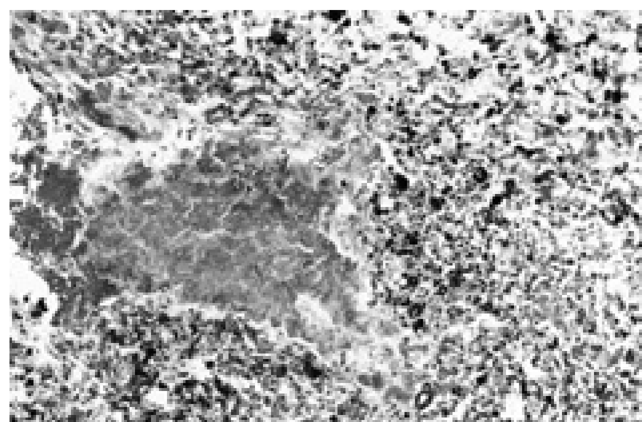

Figure 1. Histopathology section of a fungal ball showing Aspergillus sp. in the center, and Dematiaceous species (Alternaria alternata by culture) in brown colour. They appear brown because their cell wall contains melanin and possesses a brown to black pigment (H&E staining, Original magnification x100).

DISCUSSION

Chronic rhinosinusitis is an inflammatory disease of the nose and paranasal sinuses mucosa that persists and causes symptoms for longer than 3 months [9]. Recently many discussions arise concerning the role of fungi in CRS. This study is the first study to our knowledge that looks for the fungi in different sub-units of the nose and lower airways in CRS patients.

To avoid any contaminations from other possible sites the study samples were obtained under complete aseptic precautions. Therefore the nasal vestibule and surrounding skin was disinfected. To avoid contaminations from surrounding air, samples were obtained in the operating theater. As nasal swabs with absorbent tips are not a reliable method for culturing fungus [12], the authors used the irrigation suction technique of the middle meatus for obtaining adequate mucus sampling. Furthermore a sequential order of collection from different

parts of the nose was used starting by the vestibule, its sterilization, then lavage of the middle meatus, and finally a lavage of the remaining nasal cavity. The specimens obtained were sent directly to the mycology unit and processed under laminar flow [6].

Geiser [1] found in his experimental study that fungal spores after inhalation are submerged in the aqueous lining layers of the airways. So during respiration many spores will stick more to the mucus gel layer than to the skin of the vestibule that contains long chain fatty acids that inhibit fungal colonization [13]. This was demonstrated by the higher fungal culture rate from the nasal cavity (36%) and BAL (28%) compared to the nasal vestibule (8%). Also as the nose represents the first mucus lined station of the respiratory tract, more fungi were recovered from the nasal lining than from the lower airways.
Validating our mycological culturing technique using the same technique of collection as Ponikau et al. [6], the authors found a 100% culture rate from the total nasal lavages of the healthy subjects. Several authors [6,14] used the same method of collection. The total nasal lavages retrieve fungi not only from different parts of the nose, but also from the surrounding parts including the skin, the nasopharynx and the orophaynx. In CRS patients they found a positive culture rate in 96% [6] and 91.3% [14], respectively.
Middle meatal cultures correlated highly with antral punctures and appear to be an effective non-invasive diagnostic tool for diagnosis of the microbiology of CRS [15]. Because of this high correlation rate, the middle meatal endoscopic guided culture was used in this study with a positive fungal culture rate of 44%. The more precise lavage of a well determined site without contamination from other areas explains the difference between this study and the previous last two studies. Scheuller et al. [16] used another sampling technique by scraping the nasal mucosa of the middle meatus with a cytology brush. Using the polymerase chain reaction, they reported fungal DNA elements in 21.1% of CRS cases. Only in 40% of our positive MML, the same types of fungi were cultured from both MML and NCL. Additionally, 2 cases (8%) of maxillary fungal ball showed negative middle meatal cultures.
Aspergillus, *Pencillium* and *Cladosporium* were the most common cultured fungi in this study. This corresponds well with the types of fungal biodiversity in Belgium homes as studied by Nolard et al. [17]. Also in Europe, Braun et al. [14] had a higher culture rate of the same genera (*Aspergillus* 83.8%, *Penicillium* 56.6% and *Cladosporium* 41.4%) and lower rate of *Alternaria* (22.9%). In Ponikau`s study [6] that was conducted in the USA, a higher culture rate for *Alternaria* was found (44%) than the other genera (*Aspergillus* 29.6%, *Penicillium* 43% and *Cladosporium* 39%) (Table 4).
Reviewing the literature the most common identified fungi in AFRS were *Dematiacious* (87%) and *Aspergillus* (13%) [18]. Also *Alternaria*, but not *Penicillium* or *Cladosporium* has recently been reported to be a potent, in vitro peripheral blood lymphocyte stimulant for IL-5 and IL-13 production from patients with CRS [19]. This study, as well as the Ponikau et al. [6] and Braun et al. [14] studies had a higher culture rate for *Penicillium* and *Cladosporium*.

Many cases of CRS showed an associated increase of the number of eosinophils in nasal secretions [19]. The histological markers for AFS are the striking numbers of tissue eosinophils in the sinuses in contrast to an absence of eosinophils in the sinuses of healthy controls [4]. Also Khan et al. [5] showed the presence of numerous intact or degenerated eosinophils (eosinophils concretion) in the sinus contents (mucin) of AFS. In this study eosinophilia was identified in only 33.6% of the positive fungal middle meatal culture cases. Watanabe et al. [21] showed in a transmission electron microscopic study of the nasal discharge from 5 patients with AFS, that fungal hyphae surrounded by eosinophils were observed in only one patient although fungal hyphae were detected in the mucus of all 5 patients. This is in agreement with the absence of eosinophilia in 66.4% of positive MML fungal culture in this study. Watanabe et al. [21] showed only in some cases of CRS, eosinophils phagocytosing the cuticle substance of the hyphae into a sheet like invaginated space and releasing granular protein into that space in the presence of fungal hyphae [21]. This process of eosinophilic reaction mostly depends on the immunopathological signals in a genetically susceptible host [22]. Eosinophil counts can be elevated with other causes other than fungal etiology e.g. allergic as well as non-allergic syndromes, most notably, non-allergic rhinitis with eosinophilia and ASA triad syndrome [23] or as a result of superantigenic stimulation [24,25]. In this study, eosinophilia was present in 40% of negative MML culture cases.

Table 4. Percentage of different cultured fungal genera in the nasal lavages of CRS patients in different studies.

The study	*Aspergillus*	*Pencillium*	*Cladosporium*	*Alternaria*	Percentage of positive cultures
Ponikau (1999)	29.6	43	39	44	96
Braun (2003)	83.8	56.6	41.4	22.9	91.3
The present study					
NCL	24	16	16	4	36
MML	28	12	0	0	44
Nasal vestibule	4	0	0	0	8

The incidence and prevalence of AFRS are uncertain. If we apply the criteria for diagnosis of allergic fungal rhinosinusits (AFRS) according to DeShazo [26], 16% of the CRS patients in this study had AFRS. This rate was in the same range of the last survey in 20 medical units in USA with their incidence being between 0% to 23% [27]. Seventy five percent of the AFRS patients in our study had asthma, which is in agreement with the Cody [28] and Manning [4] studies who reported an asthma rate of 50% of the cases.

There were no significant correlations between the fungal cultures and the clinical parameters of CRS used in the same patients as published recently [10]. Also there were no significant correlations between the fungal culture and objective lower airways involvement.

In this study the absence of identified fungi in the intraoperative tissue specimens can be explained by the use of irrigation suction with the disadvantage of removing the mucus layer.

Fungal ball is a special form of non-invasive fungal rhinosinusits. Fungal balls were found in 2 cases of our patients. Upon CT examination these cases showed radiodensities in the central part of soft tissue mass. Histopathological examinations showed extra-mucosal fungal infestations without invasion. Although a lack of viability of these fungi [29] by culturing these fungal balls, two types of fungi were identified in both cases. This proves the sensitivity of our culture method. Limitations of a fungal ball to certain sinuses can be explained because of the lack of viability and sporulation of the fungi (Figure 2) together with possible sinus ostium occlusion.

In this study no invasive fungal sinusitis was found, as all our study patients were immunocompetent.

In conclusion one can state that the fungal culture of the respiratory tract is highly dependent on the cultured subunits. The highest culture rate was in the nasal cavity (MML and NCL) followed by the lower airways and the lowest was in the vestibule of the nose. Positive middle meatal cultures were obtained in 44% of all CRS cases. No correlation existed between upper and lower airways positive cultures. Fungi seemed to be present in different subunits of the airways but without associated eosinophilia. In a selected group of CRS patients fungi could play a role, when a positive middle meatal culture was associated with mucus eosinophilia.

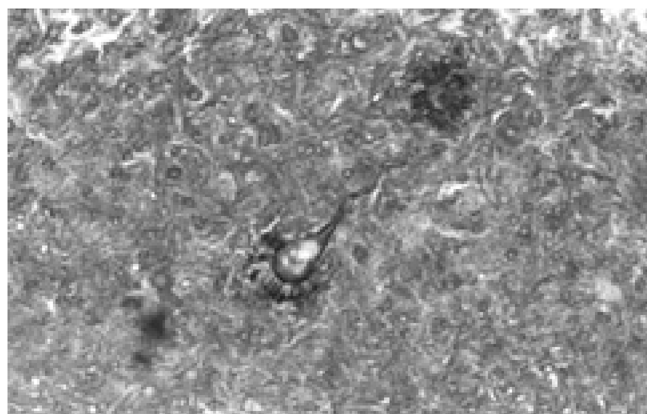

Figure 2. Histopathology section of a fungal ball of the maxillary sinus showing spore formation within maxillary sinus by Aspergillus niger. The vesicle can be seen, the spore forming cells on one side and the supporting stalk conidiophore on the other side of the vesicle. (H&E staining, original magnification x300).

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