

Could fungi be detected in the fluid of persistent otitis media with effusion?

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Background

Otitis media with effusion (OME) often is considered a direct extension of the inflammatory process that occurs during long-lasting or recurrent episodes of acute otitis media. The observations above suggest that OME has an infectious etiology. Most bacterial and viral cultures of middle ear fluid that had been performed were often negative suggesting that other infectious agents may be involved such as fungi.

Materials and methods

Thirty patients (group A) suffering from chronic secretory otitis media (OME) were enrolled in this study. Three samples were collected and investigated using PCR assay with universal fungal primers and Sabouraud agar. The first sample was obtained from the fluid of the middle ear before insertion of the ventilation tube; the second sample was obtained from nasal secretions; and the third sample was obtained from the ipsilateral peritubal area of the nasopharynx. Thirty patients (group B) with comparable age group without history of ear diseases scheduled for tonsillectomy or adenotonsillectomy were added as a control group. Samples from peritubal area of the nasopharynx of patients and nasal secretion were tested using PCR assay with universal fungal primers and Sabouraud agar.

Results

PCR examination of the middle ear aspirate in group (A) cases was positive in 7 cases (23.3%), in nasal secretions samples 2 cases only (13.3%) were positive and no positive cases were detected in nasopharyngeal swab samples. In group (A), Sabouraud agar culture was positive for fungal culture of middle ear aspirates in 5 cases (16.6%) but in no cases for nasal secretion samples. Group A showed also negative (N_0) growth in 30 (100%) patients for nasopharyngeal swab on Sabouraud agar. In group B, the findings of nasopharyngeal swab were negative (N_0) growth in all examined samples on Sabouraud agar, and nasal secretions were also negative for fungal DNA detection using PCR assay.

Conclusion

In this study, fungal DNA could be detected in the middle ear fluid in seven (23.3%) of 30 patients with persistent OME using PCR assay, and fungi could be detected in five (16.6%) patients on Sabouraud agar. A significant relationship was found between detection of fungi in the middle ear fluid and the duration of the disease, associated adenoid, and history of asthma.

Keywords:

middle ear, otitis media, effusion-fungi, PCR

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Introduction

Otitis media is the second most common disease of childhood. In 10% of the cases, middle ear effusion after an episode of acute otitis media can persist for more than 3 months. Hearing loss associated with serous otitis media can potentially have a detrimental effect on speech and language development [1].

Various host factors and infectious agents have been implicated in the genesis of otitis media with effusion (OME). Bacterial and viral organisms are recognized as a common cause of otitis media [2].

Standard bacteriologic analysis of effusion from OME has shown the presence of various agents in some cases. Moreover, few studies have examined the presence of

fungi in middle ear effusion in patients with otitis media [3].

In 1999, fungi were reported to be isolated in the nasal secretions of nearly all healthy individuals as well as patients with chronic sinusitis [4].

Fungal routine diagnostic tests including culture and histopathological examinations have limited sensitivity and specificity. PCR showed to be useful molecular technique for detection of pathogens particularly for detection of those with slowly growing pattern [5].

If fungi are present in middle ear effusions, they may play important role as pathogenic microorganisms in the middle ear [6]. This study aimed to detect fungi in the middle ear fluid in patients with persistent OME

of 3 months duration or more using PCR assay and fungal culture techniques. If fungi could be detected in the middle ear effusion samples, this may highlight a role that fungi may play in the pathoetiology of persistent OME.

Materials and methods

Thirty patients suffering from chronic secretory otitis media (SOM) were enrolled in this study (group A). Patients were selected among those attending the ENT outpatient clinic of Fayoum University Hospital presenting with chronic OME during the period from February 2013 to November 2013. All patients had been informed about consent to participate in this study. In addition, approval from the ethical committee of ORL Department, Fayoum University was obtained. A preliminary thorough history of otolaryngologic symptoms, careful clinical examination, otoscopic examination, endoscopic nasal examination (not in all patients), tympanogram, and plain radiographic examination (nasopharynx soft tissue and lateral view) were performed.

Patients included in this group fulfilled the following criteria.

Selection criteria

- (1) Age range from 4 to 15 years.
- (2) Unilateral or bilateral OME of at least 3 months duration.
- (3) Craniofacial anomalies such as cleft palate.
- (4) History of asthma or chronic respiratory tract infection.

Exclusion criteria

- (1) No history of ear discharge.
- (2) No history of middle ear surgery before enrollment of the patient in the study.
- (3) No active middle ear inflammation at the time of middle ear fluid aspiration.

Three samples were taken from the patients of this group. The first sample was obtained from the fluid of the middle ear before insertion of the ventilation tube. The second sample was obtained from nasal secretions. The third sample was obtained from the peritubal area of the nasopharynx. All samples collected from the middle ear fluid were examined for fungal DNA using PCR assay, whereas only 15 samples were cultured on Sabouraud agar. Half of the total number of the second and third samples were examined using fungal culture and PCR assay.

Another 30 patients (group B) with comparable age group scheduled for tonsillectomy or adenotonsillectomy, without history of previous ear diseases, were added as a control group. Samples were collected as in group A, but for ethical reasons middle ear samples in this control group were replaced by samples from peritubal area of the nasopharynx for fungal culture (15 patients) as well as nasal secretions for PCR assay (15 patients).

Patients and control subjects were subjected to informed consent for routine clinical management and an extra laboratory samples for the research purpose.

Sample collection

Collection of middle ear effusions was accomplished using tap middle ear fluid aspirator/collector. The external auditory canal was first cleaned of cerumen followed by disinfection with 70% ethyl alcohol for 1 min. A myringotomy was performed, followed by aspiration of the middle ear effusion before ventilation tube insertion. A portion of the effusion was sent for fungal culture and the remainder was used for DNA isolation. In addition, the nasopharyngeal swab was obtained from the eustachian tube orifice of diseased ear.

Fungal culture

Standard mycological culture assay was performed on the samples. The samples were cultured on Sabouraud glucose agar (S) for almost 21 days at 37°C. The cultures then were investigated every day for detection of fungal growth.

Fungal detection using DNA analysis

Collection of DNA from the middle ear effusion was performed using standard DNA isolation techniques (DNA isolation kit) with some modifications. Each effusion sample was mixed with 300 µl of cell lysis solution. The samples were then incubated overnight at 65°C in a rotating incubator. All samples were added to 100 µl of protein precipitation solution, vortexed for 20 s, and centrifuged at 13 500 for 3 min to precipitate out the proteins in the specimens. A 5 min ice bath was used to increase the yield of precipitation. DNA precipitation was accomplished using 100% isopropanol followed by a 70% ethanol solution.

DNA hydration was performed by adding 20 µl DNA hydration solution to the dehydrated samples and incubating at 65°C for 60 min and overnight at room temperature. All DNA samples were stored at 4°C.

PCR analysis was performed using specific pan-fungal primers (universal fungal primers) on the isolated DNA molecules from middle ear effusion samples.

PCR analysis of genomic DNA obtained from middle ear effusion samples was performed using synthetic oligonucleotide pan-fungal primers including PAN-S1, 5'-tcc gta ggt gaa cct gcg g-3', as forward and PAN-As1, 5'-tcc tcc gct tat tga tat gc-3', as reverse primers.

The PCR reaction was performed in a volume of 50 μ l containing 20 mmol/l of each mentioned primers, 20 mmol/l (NH₄)₂ SO₄, 75 mmol/l Tris-HCl (pH 8.8), 1 mmol/l MgCl₂, 0.2 mmol/l dNTP mix, 1.2 U of thermostable DNA polymerase, and 1 μ l of template (genomic DNA). The PCR reaction parameters were 30 s at 95°C, followed by 35 cycles of denaturation at 48°C for 60 s and annealing at 72°C for 90 s. PCR products were analyzed by ethidium bromide staining after electrophoresis on 1% agarose gel. Samples were dried for 2 h and exposed overnight, then visualized under ultraviolet light.

Statistical analysis

- (1) Data were collected, coded, and double entered into Microsoft Access and data analysis was performed using SPSS (IBM® incorporation, version 18, USA) software, version 18 under Windows 7.
- (2) Simple descriptive analysis in the form of numbers and percentages for qualitative data, and arithmetic means as central tendency measurement, standard deviations as measure of dispersion for quantitative parametric data, and inferential statistic test.

For quantitative parametric data:

- (a) The independent Student's *t*-test was used to compare measures of two independent groups of quantitative data.

For qualitative data:

- (b) The χ^2 -test was used to compare two or more than two qualitative groups.
- (3) The level *P* less than or equal to 0.05 was considered the cutoff value for significance.

Results

This study included two groups of patients; the first group (group A) included 30 patients with persistent SOM. There were 15 (50%) male patients and 15 (50%) female patients. Their ages ranged from 5 to 14 years with a mean age of 7.9 years. Three samples were taken from every case. The first sample was obtained from the fluid of the middle ear before insertion of

the ventilation tube. The second sample was obtained from nasal secretions. The third sample was obtained from the peritubal area of the nasopharynx. The second group (group B) included another 30 patients as a control group who were scheduled for tonsillectomy or adenotonsillectomy without middle ear disease. There were 15 (50%) male patients and 15 (50%) female patients. Their ages ranged between 5 and 14 years with a mean age of 8.2 years. For ethical reasons, middle ear samples of this control group were replaced by samples from peritubal area of the nasopharynx.

The review of the clinical presentations in group A showed that 22 (73.33%) patients presented with adenoid, three (10%) patients presented with asthma, two (6.66%) patients presented with only chronic SOM, two (6.66%) patients presented with cleft palate, and one (3.33%) patient presented with chronic bacterial rhinosinusitis (Table 1).

Group A middle ear fluid samples showed positive detection of fungal DNA in seven (23.3%) patients on PCR examination (Photo 1) and positive

Table 1 Description of clinical presentations among the study and control groups

Clinical presentation	Study groups (n = 60) [n (%)]
Cases (n = 30)	
Adenoid	22 (73.3)
Asthma	3 (10)
SOM	2 (6.7)
Craniofacial anomaly	2 (6.7)
Chronic sinusitis	1 (3.3)
Controls (n = 30)	
Adenoid	12 (40)
Chronic tonsillitis	11 (36.7)
Adenoid and chronic tonsillitis	7 (23.3)

SOM, secretory otitis media.

Photo 1



Positive fungal culture on Sabouraud agar

growth in five (16.6%) patients on Sabouraud agar (Photo 2).

The fungal culture results for nasopharyngeal swab in group A showed positive growth in one (6.7%) patient on Sabouraud agar and negative (N_0) growth in 14 (93.3%) patients.

The fungal culture results for nasal secretions of group A showed positive growth in two (13.3%) patients on Sabouraud agar and negative (N_0) growth in 13 (86.7%) patients. In addition, these results were confirmed by PCR assay (Table 2).

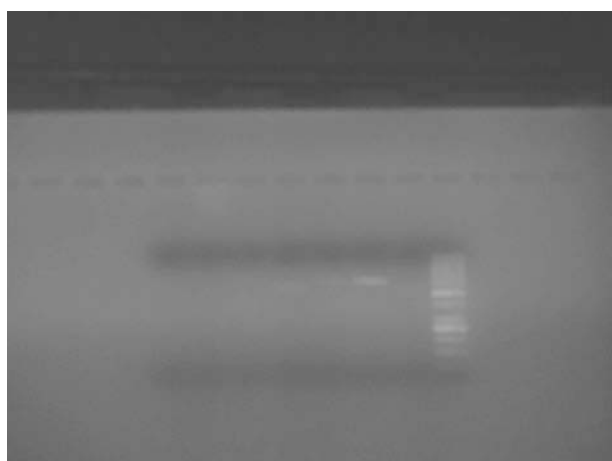
All positive middle ear samples detected by Sabouraud agar culture were also positive by PCR assay. In addition, all positive results detected by Sabouraud agar culture from nasal secretions and nasopharyngeal swabs were positive middle ear samples by PCR assay but with no statistical significance (Table 3).

Statistical analysis of the results showed that there is a significant relationship between positive cases on PCR and more prolonged disease duration, asthmatics and nonasthmatics, and among those with adenoid and nonadenoid cases with *P*-value less than 0.05 (Table 4).

In group B, radiographic findings among this group showed that 11 (36.7%) patients presented with adenoid and 19 (63.3%) patients presented without adenoid.

The fungal culture results for nasopharyngeal swabs and nasal secretions showed negative (N_0) fungal growth in all examined samples as well as negative fungal DNA detection by PCR assessment for the nasal secretion samples.

Photo 2



PCR analysis of the middle ear fluid

Discussion

OME is an inflammation of the middle ear in which fluid accumulates behind the eardrum, without any signs or symptoms of acute infection and with an intact tympanic membrane. It is considered a direct extension of the inflammatory process that occurs during long-lasting or recurrent episodes of acute otitis media, which is confirmed by the fact that almost all cases of OME follow episodes of acute otitis media, suggesting that OME has an infectious etiology.

Table 2 Description of the results of laboratory investigations among cases (group A)

Investigations	Cases (group A) (<i>n</i> = 30) [<i>n</i> (%)]
Middle ear effusion culture (<i>n</i> = 15)	
Negative	10 (66.7)
Positive	5 (33.3)
Middle ear effusion PCR (<i>n</i> = 30)	
Negative	23 (76.7)
Positive	7 (23.3)
Nasal secretion culture (<i>n</i> = 15)	
Negative	13 (86.7)
Positive	2 (13.3)
Nasal secretion PCR (<i>n</i> = 15)	
Negative	13 (86.7)
Positive	2 (13.3)
Nasopharyngeal swap culture (<i>n</i> = 15)	
Negative	14 (93.3)
Positive	1 (6.7)

Table 3 Comparisons of different investigation results among patients with middle ear effusion according to PCR results

Investigations	Middle ear effusion PCR		<i>P</i> -value	Significance
	Negative (<i>n</i> = 23)	Positive (<i>n</i> = 7)		
Middle ear effusion culture (<i>n</i> = 15)				
Negative	8	2	0.007	HS
Positive	0	5		
Nasal secretion culture (<i>n</i> = 15)				
Negative	10	3	0.09	NS
Positive	0	2		
Nasal secretion PCR (<i>n</i> = 15)				
Negative	10	3	0.09	NS
Positive	0	2		
Nasopharyngeal swap culture (<i>n</i> = 15)				
Negative	10	4	0.3	NS
Positive	0	1		

HS, highly significant; NS, not significant.

Table 4 Comparisons of different clinical presentation among group A patients with middle ear effusion according to PCR results

Clinical presentation	Middle ear effusion PCR		P-value	Significance
	Negative (n = 23)	Positive (n = 7)		
Adenoid patients (n = 22)				
Yes	20	2	0.007	HS
No	3	5		
Asthmatic patients (n = 3)				
Yes	0	3	0.009	HS
No	23	4		
SOM patients (n = 2)				
Yes	1	1	0.4	NS
No	22	6		
Craniofacial anomaly (n = 2)				
Yes	2	0	0.9	NS
No	21	7		
Chronic sinusitis (n = 1)				
Yes	0	1	0.2	NS
No	23	6		

HS, highly significant; NS, not significant; SOM, secretory otitis media.

Most bacterial and viral cultures of middle ear fluid that had been performed were often negative suggesting that other infectious agents may be involved [7].

Fungi are omnipresent in nature and are found as normal flora in the oral and nasal cavities. Their role in the pathogenesis of sinus disease has only recently been appreciated [8].

In the present study, PCR analysis and fungal culture have been performed for detection of fungal agents in middle ear effusion patients (group A), and fungal culture has been performed for detection of fungal agents in nasopharyngeal swab of the control group (group B).

Detection of fungal DNA in middle ear samples was positive in seven (23.3%) patients by PCR analysis and in five patients by Sabouraud agar culture, and negative (N_0) growth was seen in 23 (76.7%) patients among group (A), whereas in the control group (B), the findings were negative (N_0) growth on Sabouraud agar and by PCR assay.

In this study, PCR analysis of OME demonstrated that fungal DNA was detected in 23.3% of samples, which was nearly comparable with the results of other studies.

Jalali *et al.* [9] showed that 23 (29.1%) samples were positive for fungal DNA sample using PCR of 62 children with proven OME.

Kim *et al.* [5] reported that the presence of fungi in middle ear effusion could not be demonstrated by culture, whereas fungal DNA was detected in 34% of the tested effusion samples by PCR.

PCR examination of nasal secretions among the patients group (group A) could detect fungal DNA in two (13.3%) patients and negative in 13 (86.7%) patients.

According to Sabouraud agar culture of nasopharyngeal swab results, group A showed positive fungal growth in one (6.7%) patient, whereas group B showed negative (N_0) growth in all examined samples.

Regarding the clinical data of the patients, there was high statistically significant correlation between positive middle ear effusion fungal detection by PCR and patients presented clinically with history of asthma. The asthmatic patients represented about 14.8% of our entire positive middle ear fungal assay by PCR.

Ponikau *et al.* [4] described that fungi in nasal secretions induce the migration of eosinophils in the secretion, resulting in eosinophilic inflammation of the paranasal sinuses, and they proposed to call this disease entity eosinophilic fungal rhinosinusitis. If this was correct, fungi passing into the middle ear cavity would cause eosinophilic inflammation in the middle ear cleft.

Atsushi *et al.* [6] detected fungal hyphae in the middle ear fluid in all patients with eosinophilic otitis media. Charcot-Leyden crystals could be detected in six of the seven patients of their study suggesting that many eosinophils have degenerated.

According to the duration of the disease, all cases were 3 months (17 patients) or more (13 patients) to fulfill the criteria of the chronicity of the disease with mean duration 7 months. The results showed that most of middle ear effusion samples positive for fungal DNA were of more than 3 months duration. This may be explained by that the more the duration of the disease, the more the multiplication of the fungi to be detected by PCR, or the more the duration of the disease, the more the chance for passage of the fungi to middle ear cleft from nasal cavity and nasopharynx. However, no previous studies made attention to role of the duration of the disease.

This study included 22 patients with adenoid, 73.3% of all cases. Positive fungal DNA by PCR was detected in two patients with adenoid as 9.1%

of all adenoid cases and 28.5% of all positive cases. It was suggested that adenoid tissue could serve as a reservoir for bacterial infection, the eustachian tube being the migration route for bacteria into the middle ear cavity. This could also be applied for the fungal theory.

Conclusion

In this study, fungal DNA could be detected in the middle ear fluid in seven (23.3%) patients of 30 patients with persistent OME using PCR assay, and fungi could be detected in five (16.6%) patients on Sabouraud agar.

The results of this study according to statistical analysis demonstrated significant relationship between detection of fungi in the middle ear fluid and the duration of the disease, associated adenoid, and history of asthma. This study highlighted the possibility that fungi detected in the fluid of the middle ear can be considered as one of the etiological factors in the pathogenesis of chronic OME.

Acknowledgements

Conflicts of interest

None declared.

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