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Screening of middle ear effusion for the common sinus pathogen *Bipolaris*

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Abstract Recently, a third of middle ear effusions have been shown to harbor fungal DNA by polymerase chain reaction (PCR). This suggests that fungi, in addition to being an important sinus pathogen, may also play an important role in acute and serous otitis media. *Bipolaris* is an important sinus pathogen whose role in infections of the ear is unknown. In this study, we assessed if *Bipolaris* DNA was present in 19 middle ear effusions that were PCR positive for the presence of fungi. Primer pair specific for *Bipolaris spicifera* was tested against DNA from various bacterial and fungal species to demonstrate its specificity and was subsequently used on DNA isolated from effusions to determine if *Bipolaris*-specific DNA was present. None of the nineteen specimens tested positive for *Bipolaris* by PCR or standard culture technique. This suggests that while fungi may play an important role in otitis media, this study does not support a role for *Bipolaris* as a middle ear pathogen and may reflect regional differences in its prevalence.

Keywords Bipolaris · Polymerase chain reaction · Fungi · Otitis media

Introduction

Allergic fungal sinusitis (AFS) has received significant attention as a pathologic entity within the nasal cavity. AFS caused by a variety of fungi accounts for approximately 5–10% of chronic sinusitis cases requiring surgical intervention [2, 10]. The vast majority of AFS cases are due to dematiaceous fungi, *Bipolaris spicifera*. In fact, *Bipolaris* may play a role in more than 50% of the culture-proven cases of AFS [6, 9]. The greatest number of cases have been identified in the southwestern United States, suggesting that there is strong regional distribution of this fungi.

Because of similarities between the paranasal sinuses and middle ear space and the recognition of fungi as important pathogens in inflammation of the paranasal sinuses, we investigated the potential role of fungi in acute otitis media (AOM) and serous otitis media (SOM) using culture and PCR techniques [4]. Surprisingly, fungal DNA was present in 34% of middle ear effusion samples, an incidence similar to the paranasal sinuses [1]. While additional studies are necessary to establish a pathologic role, the presence of fungi in middle ear effusion suggests that it may play an etiologic role in SOM and AOM. While the aforementioned study identified fungi in middle ear effusion, they did not establish the prevalence of *Bipolaris* in middle ear effusion.

Traditional detection methods for fungi in general, and *Biopolaris* in particular, are difficult and unreliable. Microscopy alone, unfortunately, will not detect small fungal loads, nor aid in exact speciation. In addition, fungal cultures are often insensitive. We have demonstrated that amplification of target DNA through polymerase chain reaction (PCR) with sequence-specific primers is more sensitive and rapid than microbiologic techniques [1, 4, 5]. Unlike culture, PCR does not require the presence of viable organisms for success and may be performed even when sample volumes are small. In this study, we report the use of PCR assay for the detection of *Bipolaris* and determine its contribution to otitis media in northern California.

Material and methods

Clinical specimens

Middle ear effusions from patients with recurrent otitis media or persistent effusion were collected under sterile conditions using a middle ear suction trap, as previously described in Kim et al. [4]. In brief, collection of middle ear effusions was accomplished using Juhn Tym-Tap Middle Ear Fluid Aspirator/Collector (Medtronic/Xomed, Jacksonville, Fla.). The external auditory canal was first cleaned of cerumen, followed by disinfection with 70% ethyl alco-

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hol for 1 min. A myringotomy was performed followed by aspiration of the middle effusion. Nineteen patients who were PCR positive for the presence of fungal elements using universal fungal primers were further investigated in this study for the presence of *Bipolaris* DNA.

DNA isolation

Aliquots of *Streptococcus pneumonia*, *Staphylococcus aureus*, *Escherichia coli*, *Moraxella catarrhalis*, *Hemophilus influenza*, *Aspergillus*, *Alternatia*, *Bipolaris Cryptococcus* and *Candida* were obtained from the University of California at San Francisco Microbiology Laboratory. Extraction of DNA from middle ear effusions and microbiologic specimens was performed using standard DNA isolation techniques (PUREGENE DNA Isolation Kit, Gentra Systems, Minneapolis, Minn.) with some modifications [4]. Following the mixing of effusion in 300 µl of cell lysis solution, the samples were then incubated overnight at 65° C in a rotating incubator. Then 100 µl of protein precipitation solution was added to samples, vortexed for 20 s and centrifuged at 13,500 for 3 min, followed by a 5-min ice bath to precipitate out the proteins in the specimens and to increase the yield of precipitation, respectively. DNA was precipitated with 100% isopropanol followed by a 70% ethanol solution and rehydrated in 20 µl of DNA hydration solution. All DNA samples were stored at 4° C.

Selection of primers

A *Bipolaris spicifera* primer (Bipol) was designed in our lab using the Primer3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi) and used to test for the presence of *Bipolaris* [1]. These were Bipol A73: TCA TGC TGA CAA CGC TCC AG and Bipol B572: TAC CGA TGG CCA TGC ACC T. This primer amplifies a sequence of the BRN-1 gene found in all *Bipolaris* species. In addition to the *Bipolaris*-specific primers, all samples underwent PCR amplification with primers specific for a housekeeping gene, alpha-1 anitrypsin-specific primers, to establish the integrity of genomic DNA. These were ATT f: CCC ACC TTC CCC TCT CTC CAG GCA AAT GGG and ATT r: GGG CCT CAG TCC CAA ACA TGG CTA AGA GGT.

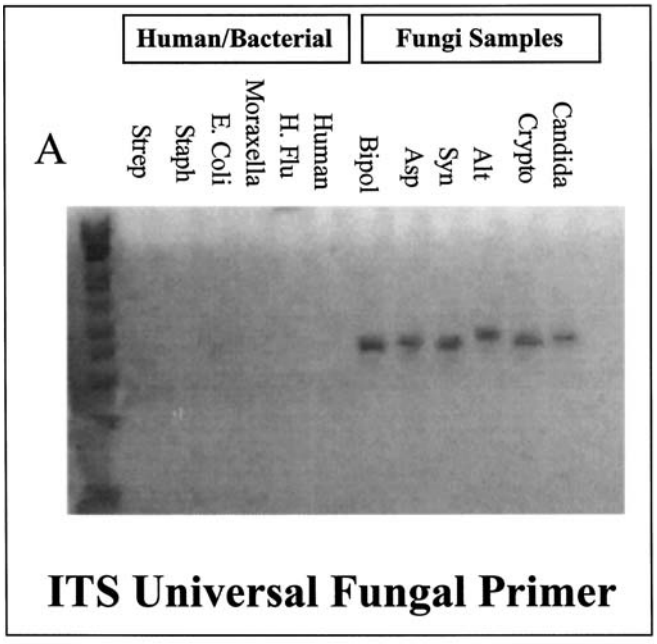
DNA amplification

PCR primers were end-labeled with [α-^{32}P] d ATP. PCR reactions were performed in 20 µL contianing 200 ng of genomic DNA, 200 µ M 4 dNP mix, 1X PCR buffer (Perkin Elmer Cetus, Norwalk, Conn.), 0.5 U of AmpliTaq DNA polymersase (Perkin Elmer Cetus, Norwalk, Conn.) and 100 nM of each primer. The PCR reaction parameters were 5 min at 95° C, followed by 35 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 1 min and extension at 72° C for 7 min, for all primers. The PCR products were analyzed by ethidium bromide staining after electrophoresis in on Nu-Sieve 2% denaturing acrylamide gel. Samples were dried for 2 h and exposed overnight using a phosphoimager.

Results

Specificity of PCR

Each of the clinical isolates of *Bipolaris* tested showed the same signal upon amplification with a band of the expected size (518 bp). In contrast, this band was not observed with human DNA or DNA from *Aspergillus* or other fungal or bacterial species. Thus, the primers and the parameters of the PCR allowed us to amplify *Bipolaris* (Fig. 1).



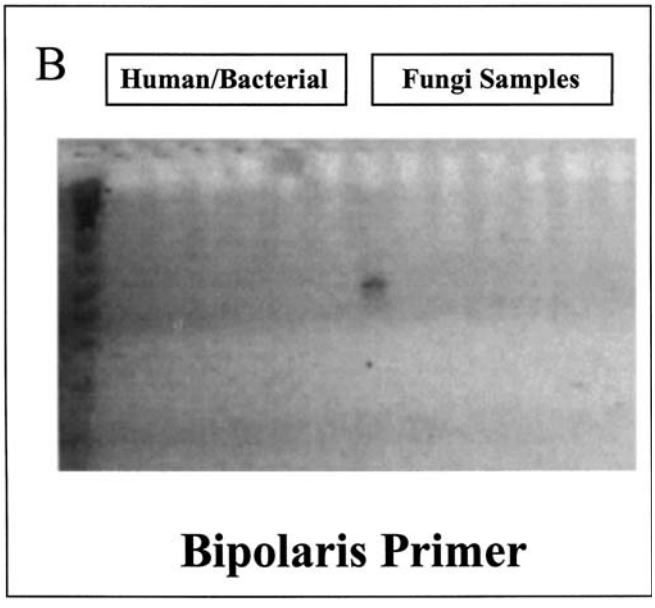


Fig. 1 PCR analysis of bacterial and fungal DNA using the universal fungal primer and the *Bipolaris*-specific primers. **A** PCR amplification with ITS universal fungal primers demonstrates a PCR product in the presence of fungal DNA, but not human or bacterial DNA. As expected, different species of fungi generate a different size PCR product. **B** PCR amplification using *Bipolaris*-specific primers demonstrates a product only in the lane containing *Bipolaris* DNA with the expected size of 518 base pairs

Clinical sample testing

Of the 19 patients with middle ear effusions tested, none had *Bipolaris* detected by PCR amplification and were classified as PCR negative for *Bipolaris*; the housekeeping gene primers amplified a PCR fragment in all cases, suggesting that the DNA was intact. Likewise, all 19 samples were culture negative and did not grow any fungi on potato flake agar.

Discussion

PCR has been shown to be a highly sensitive diagnostic tool for the detection of fungi from specimens of diverse locations [3, 7, 11]. Because of the small volume of middle ear effusions, PCR may be the ideal assay for the investigation of infectious agents responsible for otitis media. In this report, we utilized a rapid, sensitive and reliable PCR method for the detection of *Bipolaris*. The *Bipolaris* primers were demonstrated to be specific and sensitive. However, the false negative rate of fungal PCR, the failure to detect the presence of fungi when present by PCR, is unknown. The detection threshold of the PCR assay described here was between 10–20 copies of template DNA [1, 4]. This sensitivity may allow PCR assay to detect low burdens of fungi, and, therefore, PCR can be a crucial tool in the diagnosis of fungal disease.

Using PCR, fungal elements have been demonstrated to be present in 34% of chronic middle ear effusions [4]. Interestingly, none of the effusions showing fungi by PCR grew fungi on potato flake agar, suggesting that cultures are insensitive for detecting fungi. Traditional culturing methods have been shown to be inadequate in detecting viable organisms present in OM, possibly because pathogens are not free-floating in a middle-ear effusion that would be required for successful cultures. This has recently been shown by Post, who demonstrated the presence of bacterial aggregates attached to the middle-ear mucosa as a bacterial biofilm [8]. The findings of his study may explain why standard culture techniques fail to detect pathogens and why "culture negative" effusions may be resistant to treatment by antibiotics. Therefore, negative fungal cultures may either reflect the true absence of viable organisms, the failure of the fungal culture itself or the unavailability of fungi because of fungal biofilms.

There are extensive similarities between the paranasal sinuses and the middle ear cleft to give credence to the hypothesis that fungi may also play a role in AOM and SOM. *Bipolaris* is an important nasal pathogen in AFS and therefore is an excellent candidate for causing middle ear disease. However, this study did not demonstrate the presence of *Bipolaris* in middle ear effusion. Absence of *Bipolaris* in the patient population from northern California is likely due to regional differences in the prevalence of *Bipolaris*. Catten et al. had previously shown that only 3% (2/67) of normal patients and patients with chronic rhinosinusitis from northern California harbored *Bipolaris*. Therefore, additional studies will be required in regions of high prevalence of *Bipolaris* to determine its role in middle ear disease.

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