Case Report

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Aetiological factors contributing to the development of primary laryngeal aspergillosis in immunocompetent patients

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Primary laryngeal aspergillosis is extremely rare, especially in an immunocompetent host. It is commonly found as part of a systemic infection involving the respiratory system in immunocompromised people. Two cases of laryngeal aspergillosis with no systemic extension and no generalized immune deficiency are presented here. We report what is to the best of our knowledge only the second case of *Aspergillus* infection in a vocal cord cyst. *Aspergillus* species were identified in tissue sections and confirmed by PCR studies. We present a literature review of laryngeal aspergillosis cases and discuss predisposing factors, clinical presentation, histopathology, PCR, diagnosis and treatment of *Aspergillus* laryngitis. The known aetiological causes of the disease are increasing and include iatrogenic factors, vocal abuse, vocal-fold cysts and occupational factors, and immunocompetent patients are susceptible to these predisposing factors.

Introduction

Aspergillus is an inherently non-pathogenic or very weakly pathogenic fungus that produces a group of opportunistic infections (Agarwal *et al.*, 2001; McAlester *et al.*, 2008). In the field of otorhinolaryngology, aspergillosis most frequently occurs in the external auditory canal or the nasal sinus, but is very rarely localized in the larynx (Athanassiadou *et al.*, 2005). Laryngeal aspergillosis is most often found in immunocompromised people, such as patients with leukaemia, AIDS or severe aplastic anaemia (Athanassiadou *et al.*, 2005; Nagasawa *et al.*, 2002; Sriskandabalan & Roy, 1996), but infections in non-immunocompromised hosts are rare. Previous to the present study, we were aware of only 19 cases of primary laryngeal aspergillosis in non-immunocompromised patients. We report two additional cases in healthy individuals, and review and analyse the aetiological factors that may underlie this condition.

Case reports

Case 1

A 30-year-old woman presented to our department for evaluation of chronic hoarseness that had worsened over 2 months. She was a businesswoman who had a history of vocal abuse and no history of any generalized immune deficiency, leukaemia, malignant disease, diabetes mellitus or use of immunosuppressive drugs, including corticosteroids. She denied alcohol and tobacco abuse or any previous

laryngeal trauma. A general physical examination revealed nothing remarkable. Her chest X-ray and human immunodeficiency virus test were both negative. Videostroboscopy revealed a smooth, white spheroid submucosal mass on the anterior surface of the left vocal cord (Fig. 1). Vocal cord mobility was normal bilaterally. Excision of the cystic lesion was performed under general anaesthesia using a suspension laryngoscope. Pathological examination revealed that the cystic wall consisted of squamous epithelium containing septate hyphae at a 45° angle and a dichotomous branching pattern that was consistent with *Aspergillus*. Paraffin-embedded tissue sections were sent for analysis by *Aspergillus* PCR to our laboratory (State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University), and this confirmed the presence of *Aspergillus fumigatus*. The patient's post-operative course was unremarkable and her voice returned to normal. The patient received no further antifungal therapy. The patient was given instruction about good vocal hygiene, and had no lesions 24 months after the therapy.

Case 2

A 32-year-old female with no generalized immune deficiency sought treatment for a 3 month history of progressively worsening hoarseness. She was a teacher, and had a history of vocal abuse and therapy with broad-spectrum antibiotics. Videostroboscopy revealed white or grey plaques on both vocal cords, the left ventricular fold and part of the subglottic region with normal mobility (Fig. 2). The remainder of the head and neck examination was unremarkable. Her chest X-ray showed no

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

Fig. 1. Videostroboscopy image from case 1 showing a submucosal cystic mass on the left vocal cord.

abnormalities and a test for human immunodeficiency virus was negative.

The patient had no history of immune deficiency, leukaemia, malignant diseases, diabetes mellitus or steroid use. Direct laryngoscopy was used to take a biopsy. The histopathological examination showed *Aspergillus* (Fig. 3), and PCR identified that the aetiological agent was *A. fumigatus*. We cleaned the lesions using a fibrolaryngoscope, and the patient was treated with a 4 week course of oral itraconazole (Sporanox; Xian-Janssen Pharmaceutical), 200 mg twice a day with full fat milk, and given instruction about good vocal hygiene. Her hoarseness decreased 3 weeks later. Videostroboscopy showed the lesions had completely disappeared. After the 4 week course of oral itraconazole, the patient was cured, and there was no evidence of recurrence 24 months after surgery.

Fig. 2. Videostroboscopy image from case 2 showing white lesions, involving both true vocal cords, left false vocal folds and some subglottic structures.

Fig. 3. Histopathological features of case 2 biopsy sample showing numerous septate fungal hyphae, branching at an angle of 45° (haematoxylin and eosin stained, magnification $\times 400$).

PCR

We utilized formalin-fixed paraffin-embedded tissue sections from two cases for analysis by *Aspergillus* PCR. Genomic DNA was extracted using a QIAamp kit (Qiagen), according to the manufacturer's instructions. In brief, the tissue was lysed in a buffer in a 60 °C water bath for 30 min, followed by a simple purification procedure, and extracted based on the selectivity of the membrane. PCR amplification of the D1/D2 region of the large-subunit (LSU) rRNA gene and internal transcribed spacer (ITS) regions in the rRNA gene was then carried out. The PCR used fungi-conserved primer pairs NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG) and NL4 (5'-GGT CCG TGT TTC AAG ACG G), and ITS1 (5'-TCC GTA GGT GAA CCT GCG G) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC), respectively. The PCR amplification mixture was 25 µl total volume, including 20 mmol Tris/HCl l⁻¹, 20 mmol KCl l⁻¹, 1.5 mmol MgCl₂ l⁻¹, 0.2 mmol dNTP l⁻¹, 1 U DNA *Taq* polymerase, 1 µmol each primer l⁻¹, and the DNA sample.

For NL1/NL4, PCR was performed using a denaturation step at 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s, and a final extension step at 72 °C for 10 min. For ITS1/ITS4, PCR was performed using a denaturation step of 95 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 45 s and 72 °C for 60 s, with a final extension step at 72 °C for 10 min.

The amplicon DNA was subjected to agarose gel electrophoresis in TAE buffer and stained with ethidium bromide. All of the PCR products were purified before DNA sequence analysis. Purified amplicons were sequenced using the same primers as described for the ABI 3730 DNA sequencer. Purification and sequence analysis was

conducted by Invitrogen Life Technologies. The primers successfully amplified the target part of the LSU rRNA gene and the ITS1–5.8S–ITS2 ribosomal region. The sizes of the LSU and ITS1–ITS2 DNA sequence samples were 605 and 610 bp, respectively. The query sequences of LSU and ITS1–ITS2 were submitted to the BLAST system and aligned using ClustalW analysis. According to the results of BLAST, the closest match was considered to be the most likely correct identification. The DNA sequence of the 28 rRNA gene regions were 100 % (604/604) identical with the *A. fumigatus* strain ATCC 16907 28S rRNA gene (GenBank accession no. AY216670.1) and the ITS1–ITS2 regions were also 100 % (556/556) identical with *A. fumigatus* strain SRRRC (GenBank accession no. AY373851). The comparisons between our submitted sequences and the GenBank entries identified our isolate as *A. fumigatus*.

Discussion

Aspergillus laryngitis is most commonly seen as part of a broader infection involving the respiratory system in immunocompromised people (Sambatakou *et al.*, 2006), but *Aspergillus* can also infect the larynx of healthy people (Klein *et al.*, 2005). Thirty-eight cases of primary laryngeal aspergillosis have been reported in the English language literature; of those, twenty cases involve immunocompetent patients (Table 1).

In general, *Aspergillus* fungi are non-pathogenic, or very weakly pathogenic, and cause opportunistic infections. Aspergillosis is thought to be due to a deficiency in the host's defence mechanism rather than fungal pathogenicity (Ogawa *et al.*, 2002). Thus the question arises as to how healthy people become infected by *Aspergillus*. At present, the answer to this question is unclear because few cases have been reported. Several factors may be associated with

the development of primary laryngeal aspergillosis in immunocompetent patients.

There are a number of potential predisposing factors. (i) Iatrogenic factors – local factors, such as radiotherapy, steroid inhaler use (Fairfax *et al.*, 1999) and laser treatment, are more likely to be associated with the onset of localized aspergillosis than decreased immunity (Beust *et al.*, 1998; Ogawa *et al.*, 2002). A substantial proportion of corticosteroid inhaled through dry powder devices is deposited in the larynx. Colonization of the larynx by *A. fumigatus* may be a direct consequence of the deposition of topical corticosteroid on the superior surface of the vocal cords (Fairfax *et al.*, 1999). Beust *et al.* (1998) suggested that local factors were associated more with isolated laryngeal aspergillosis than immunodepression. Furthermore, systemic immunodeficiency may not contribute to the development of isolated laryngeal aspergillosis (Wittkopf *et al.*, 2006). Systemic factors include previous prolonged antibiotic therapy (Nong *et al.*, 1997). Antibiotic abuse alters the local bacterial flora and disturbs the ecological balance between bacteria and fungi, thus allowing the overgrowth of *Aspergillus*. Several cases of invasive aspergillosis have been observed in patients receiving multiple antibiotics (Nong *et al.*, 1997). (ii) Vocal abuse (Nong *et al.*, 1997) – both of our patients had a history of vocal abuse as a result of their occupation. A healthy mucosal covering provides a physical barrier to potential pathogens and protection from infection, but vocal abuse may impair this local protective barrier and allow colonization and subsequent invasion by *Aspergillus* (Mehanna *et al.*, 2004). (iii) Laryngeal aspergillosis can occur in a true vocal-fold cyst. Our second patient had a vocal cord cyst showing an *Aspergillus* infection. The only other case of this was reported by Wittkopf *et al.* (2006). Wittkopf *et al.* (2006) suggested that their patient had the vocal cord cyst before the *Aspergillus* infection, and proposed that the *Aspergillus*

Table 1. Details of 21 cases of primary laryngeal aspergillosis in immunocompetent patients

Case	Reference	Sex/age (years)	Complaint	Aetiological factor
1	Present study – case 1	F/30	Hoarseness	Vocal abuse
2	Present study – case 2	F/32	Hoarseness	Broad-spectrum antibiotics and vocal abuse
3	Wittkopf <i>et al.</i> (2006)	F/62	Hoarseness	True vocal-fold cyst
4–11	Nong <i>et al.</i> (1997)	4 M and 4 F/30–40	Hoarseness	All were misdiagnosed and treated incorrectly on the first visit; antibiotics and steroids were administered later
12	Fairfax <i>et al.</i> (1999)	M/75	Hoarseness	Long-term inhaled corticosteroid
13	Ogawa <i>et al.</i> (2002)	M/73	Hoarseness	Local radiotherapy for laryngeal cancer
14–15	Beust <i>et al.</i> (1998)	M/53, M/64	Hoarseness and dyspnoea	Local radiotherapy for laryngeal cancer
16	Dean <i>et al.</i> (2001)	F/17	Hoarseness and vocal fatigue	No noticeable events
17	Benson-Mitchell <i>et al.</i> (1994)	M/62	Hoarseness	No noticeable events
18	Kheir <i>et al.</i> (1983)	M/50	Hoarseness	No noticeable events
19	Ferlito (1974)	M/76	Hoarseness	No noticeable events
20	Rao (1969)	M/48	Hoarseness	No noticeable events

F, Female; M, male.

infection was a submucosal aspergilloma rather than aspergillosis. The nature of the relationship between the cyst and *Aspergillus* in the larynx is not clear. (iv) The cause of the majority of the cases was not a marked event. *Aspergillus* lives in the soil as a saprophyte, drawing nutrients from organic substances (Morelli *et al.*, 2000); thus, occupation may be an aetiological factor for healthy patients; for example, farmers or carpenters may be at higher risk (Nong *et al.*, 1997; Kheir *et al.*, 1983; Rao, 1969). All otherwise healthy patients who present with primary laryngeal aspergillosis are likely to have been exposed to one or more of the above aetiological factors.

In conclusion, laryngeal aspergillosis in immunocompetent patients is rare; however, we suggest that the increasing occurrence of primary laryngeal aspergillosis in immunocompetent patients is an emerging trend. The aetiological causes of the disease are increasing and include iatrogenic factors, vocal abuse, vocal-fold cysts and occupational factors, and immunocompetent patients are susceptible to these predisposing factors.

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