Primary invasive laryngeal mycosis in an immunocompetent patient: a case report and clinico-epidemiological update

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Abstract

Background: Laryngeal aspergillosis is uncommon and is usually secondary to pulmonary involvement in immunocompromised patients. Primary laryngeal aspergillosis in immunocompetent individuals is extremely rare, with a few cases documented over the last five decades.

Case presentation: We report a case of primary localised laryngeal aspergillosis in a 21-year-old apparently immunocompetent student. Septate hyphae were observed on histopathology of the laryngeal lesion, which was further confirmed as Aspergillus fumigatus after extraction of fungal DNA from formalin fixed paraffin embedded tissue (FFPET) and sequencing. The patient responded well to oral itraconazole therapy over a month.

Conclusions: Since last few decades, cases of primary laryngeal aspergillosis in immunocompetent individuals are on the rise, globally. This is the first case of invasive laryngeal aspergillosis reported in Nepal. The extraction of DNA from tissue and sequencing helps to identify the etiological agent, when culture fails to isolate the fungus.

Keywords: Primary laryngeal aspergillosis, Aspergillus fumigatus, FFPE-PCR

Background

Primary fungal laryngitis is commonly attributable to yeasts such as Candida, and Cryptococcus or fungi are known to cause endemic mycoses like Blastomyces, Paracoccidioides, and Coccidioides. The mold forms, such as Aspergillus and Mucor, may involve larynx as secondary pulmonary invasion [1, 2]. Immunocompromisation due to leukaemia, AIDS, severe aplastic anaemia, lymphoreticular neoplasms, or immunosuppressive therapy predispose person to invasive fungal infection [1, 2]. Primary laryngeal aspergillosis in immunocompetent individuals is extremely rare. It often mimics the pre-malignant and malignant conditions (squamous cell carcinoma) of larynx. Fungal laryngitis is usually characterized by sore throat, earache, hoarseness of voice, cough, odynophagia, formation of endolaryngeal and perilaryngeal white plaques, granulation tissue, ulcerations, erythema and edema [3].

 Diagnosis and prompt treatment are essential to prevent complications like scarring of the vocal folds, compromised airway due to glottic edema and dissemination of the pathogen. First case of aspergillosis of larynx was reported in 1969 from Pondicherry, South India [4]. Globally, less than 50 cases over the period of last 50 years have been documented. Herein, a case of primary laryngeal aspergillosis in an apparently immunocompetent young adult is reported. To the best of our knowledge, this is the first such case report from Nepal. In this endeavour, we conducted a comprehensive review of literature and analysed all previously reported cases.

Case presentation

A 21-year young male presented to Manipal Teaching Hospital, Pokhara, with progressive hoarseness of voice for two months and frequent cough with expectoration since one month. He had no history of phonotrauma, apparent immune deficiency, leukaemia, malignant disease, diabetes mellitus, broad-spectrum antibiotics or immunosuppressive therapy, including corticosteroids. He was not...
habituated to tobacco or alcohol. He did not have any previous history of laryngeal trauma, allergies or mycosis. A general physical examination did not reveal lymphadenopathy or organomegaly. There were no visible lesions or masses in the oral cavity, oropharynx or nasopharyngeal mucosa. His paranasal sinuses and chest X-rays were clear. Routine blood test report was within normal limits. Serological markers for Hepatitis B, C, and HIV were negative and VDRL test was non-reactive.

**Clinical examination and laboratory findings**

A direct laryngoscopic examination was performed under general anaesthesia. Videostroboscopy revealed a smooth, diffused whitish spheroid submucosal mass on the anterior surface of the left vocal cord. Vocal cord mobility was normal bilaterally, the airway was adequate, and both subglottis and supraglottis showed normal mucosa.

In order to exclude, glottic carcinoma the patient was subjected to punch biopsy from the lesion by the micro-laryngeal procedure under general anaesthesia. The histopathological examination showed conidia and broad septate hyphae, most of them showing acute angle branching without any evidence of malignant cells (Fig. 1). Repeat biopsy specimen processed for fungal culture did not yield any growth. For the identity of fungi, sections from paraffin-embedded tissue block were analysed by Polymerase Chain Reaction (PCR).

**Molecular identification by PCR**

**Extraction of DNA from formalin fixed paraffin embedded (FFPE) tissue**

To avoid environmental fungal DNA (or) amplicon contamination, all steps were performed inside laminar air flow cabinets in separate closed cabins equipped with a dedicated set of micropipettes and instruments. A 50 μm thick FFPE tissue section was cut using a clean blade by microtomy and transferred to a 1.5 ml microcentrifuge tube. Deparaffinization and DNA extraction was performed as per Anna Lau et al. [5] with minor modifications where phenol-CHCl₃-isooamyl alcohol extraction protocol was performed. The pellet was dissolved in 50 μl of nuclease-free water and stored at −20°C until further testing.

**Amplification of 28 s region of rDNA**

PCR was performed in a 45 μl mixture consisting of 1× PCR buffer without MgCl₂ (Genei, Bangalore), 2.5 mM MgCl₂, 0.25 mM deoxyribonucleotide blend (Fermentas), 0.4 μM primers 12F 5’GTTGATAGAAAYAATGATAAGGA3’ and 13R 5’GACAGTAGATTCCCTTG3’ (Eurofins), 1.2 U of Taq Polymerase (Bangalore Genei) and 5 μl (~ 80 ng) of template DNA. Thermal cycling was performed in an Eppendorf Mastercycler Gradient thermal cycler (Eppendorf AG, Hamburg) with the following conditions: denaturation at 95 °C for 10 mins followed by 60 cycles of 94 °C for 15 secs, annealing at 53 °C for 20 secs, and extension 72 °C for 25 secs and finally once at 72 °C for 5 mins. Positive and negative controls were included. Amplification was confirmed by electrophoresis on 2% agarose gel with ethidium bromide, and the amplicon was purified using Qiagen gel extraction kit according to manufacturer’s instructions.

**Sequencing**

Bidirectional Sanger sequencing of purified amplicons was performed with the primers mentioned above, using the BigDye Terminator sequencing ready reaction kit (v 3.1) and the products was capillary electrophoresed in an ABI Prism 3130 genetic analyser (Applied Biosystems). Sequences were analysed using Bionumerics software version 7.1 (Applied Maths, Ghent, Belgium and identified through BLASTn (https://blast.ncbi.nlm.nih.gov/BlastSearch). On the basis of comparing the

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**Fig. 1 a** Histopathological features of biopsy sample showing numerous septate hyphae, “spaghetti-like” fungal filaments branching at an angle of approximate 45°, interspersed with shreds of vocal cord squamous epithelium (Haematoxylin and Eosin stained, magnification X400. **b** Calcofluor white staining of tissue section observed under fluorescent microscopy showing numerous branched septate hyphal forms X1000.
sequences from the specimen with those in the GenBank databases, the fungus in the biopsy was identified as *Aspergillus fumigatus*. Our isolate had 100% matches with the standard strain of ATCC 1022. The sequence data have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) with the accession number MH465665.

**Treatment and follow-up of the patient**

According to the clinical features and the mycological data, diagnosis of vocal cord aspergillosis was made and the patient was treated with oral itraconazole 200 mg BD for four weeks. Clinical improvement was noticed after a month of antifungal therapy. At the third months’ follow up, his voice had returned to normal, and no residual lesion was seen on laryngoscopy. At the end of 6 months follow up, there was no recurrence.

**Literature review**

Extensive search in PubMed/MEDLINE and Google Scholar by two investigators independently, revealed only 38 peer-reviewed reported cases of primary laryngeal aspergillosis in immunocompetent subjects (Table 1).

**Discussion**

**Aetiology of laryngeal aspergillosis**

Primary invasion of the larynx by *Aspergillus* is uncommon and is very rare in immunocompetent individuals. As per the literature review, till date, 38 cases of primary laryngeal aspergillosis in immunocompetent patients have been documented over 50 years. *Aspergillus fumigatus* was reported to be the underlying causative mould in the majority of cases, documented so far; except for two cases. *A. niger* infection reported by Gangopadhyay et al. from India [6] and Gallo and colleagues from Italy reported *Aspergillus flavus* as the etiologic agent in a patient with Felty’s syndrome [7]. In the present case a immunocompetent student had *Aspergillus fumigatus* responsible for the laryngeal pathology.

**Disease pathogenesis**

*Aspergillus* is a well-known opportunistic fungus causing allergic and invasive disease in immunocompromised hosts [8]. The pathogenesis of laryngeal aspergillosis in an immunocompetent host is not well understood. The *Aspergillus* conidia are ubiquitous in nature as the fungus grows in a saprophytic environment (soil and decaying matter), it could well be possible that exposure of heavy fungal load in air may allow the fungus colonise the dark airway cavities [3] which could favour their slow germination without any symptoms. Such colonisation of the paranasal sinuses leads to fungal ball formation [9]. Hoarseness of voice was the only symptom in this case without any other predisposing conditions like corticosteroid or systemic antibiotic therapy. This is in contrast with other reports where patients developed symptoms after being treated with corticosteroid or systemic antibiotic therapy or after vocal abuse for many years (Table 1).

**Epidemiology and risk factors for developing primary laryngeal aspergillosis**

Amongst the reviewed 38 reported cases 16/37 (44%) were males, and 21/37 (57%) were females. Age group ranged from 12 to 74 years. Dutta M et al. [3] reported in 2015 that 50% of immunocompetent subjects with primary laryngeal aspergillosis had no identifiable contributory factors, but 14.29% had vocal abuse and steroid intake. Smoking, broad-spectrum antibiotics and exposure to radiation was detected in 10.7% of cases. Of the cases, 7.1% had vocal fold cyst, whereas 3.6% had a history of COPD, oral sex and diabetes. Six cases have been reported between 2015 and 2017 (Table 1); three of these cases were without any identifiable risk factors. Remaining three cases (and a few documented prior to 2015) had a history of using corticosteroid inhalers for bronchial asthma, which could have led to abrogation of the local immunity in the throat or could have altered the flora of the laryngeal mucosa, allowing the overgrowth of *Aspergillus* [10]. The exact predisposing conditions contributing towards the disease progression in the present case remains obscure but could be multifactorial with a complex interplay between host and the environment.

**Time trend and geographical distribution of primary laryngeal aspergillosis**

Laryngeal aspergillosis in the immunocompetent individual, though infrequently reported, seems to be an emerging condition. Lack of definite guidelines for clinical diagnosis due to the rarity of the disease might have resulted in under-reporting in the past. As shown in Fig. 2, the incidence of primary laryngeal aspergillosis in immunocompetent patients has been steadily rising over the past ten years. It seems that there has been a higher rate of reporting of the cases, especially after the 1990s, with a steady rise in the number of cases in the past seven years (Fig. 2). As depicted in the map, (Fig. 2) most of the new cases reported (11/20,) between 2010 and 2017, were from the Indian subcontinent, followed by China (4/20). This emphasizes that possibility of primary laryngeal aspergillosis must be entertained in all cases, presenting with typical features of laryngeal inflammation along with hoarseness of voice.

**Challenges in the diagnosis of laryngeal aspergillosis and utility of molecular diagnostics methods**

As per the literature review, most of the laryngeal aspergillosis cases were diagnosed by the characteristic morphological features of the fungus in the biopsied material.
| Case | Reference | Age/gender | Geographical area | Clinical presentation | Initial diagnosis | Associated factors | Diagnosis method | Fungal culture | Aspergillus species involved | Treatment | Follow up period and Outcome |
|------|-----------|------------|------------------|----------------------|------------------|-------------------|-----------------|--------------|-----------------------------|-----------|-----------------------------|
| 1    | Rao PB. 1969 | 48/M       | Pondicherry, S. India | Hoarseness of voice  | NA               | None              | HE              | No           | NA                          | No treatment | 2 M- asymptomatic          |
| 2    | Ferlito A. et al. 1974 | 76/M       | Verona, Italy     | Hoarseness of voice  | NA               | None              | HE              | No           | NA                          | No treatment | 2 M- asymptomatic          |
| 3    | Kheir SM et al. 1983 | 50/M       | Birmingham, UK    | Hoarseness of voice  | Malignancy       | COPD              | HE and Immunofluorescence studies | No | NA                          | Topical nystatin powder | 24 M- asymptomatic |
| 4    | Benson-Mitchell R et al. 1994 | 62/M       | London, UK        | Hoarseness of voice  | Malignancy       | None              | HE              | No           | NA                          | No treatment | 2 M- asymptomatic          |
| 5    | Nong D, et al. 1997 | 30-40/4 M+ 4F | China             | Hoarseness of voice  | Leading to aphonia, sore throat, | NA               | NA              | NA           | NA                          | NA        |
| 6    | Beust L, et al. 1998 | 53/M       | Rennes, France    | Hoarseness of voice, respiratory distress  | None              | None              | HE              | No           | NA                          | Laryngectomy | 3 M- asymptomatic          |
| 7    | Fairfax AJ, et al. 1999 | 75/M       | Stafford, UK      | Hoarseness of voice, aphonía  | None              | None              | HE and culture | Yes | A. fumigatus | AMP-lozenges, 10 mg-4 W | 1 M- Improved |
| 8    | Dean CM, et al. 2001 | 17/F       | Philadelphia, USA. | Hoarseness of voice, vocal fatigue  | None              | NG                | NA              | NA           | NA                          | NA        |
| 9    | Ogawa Y, et al. 2002 | 73/M       | Tokyo, Japan      | Hoarseness of voice  | (History of Radiotherapy and DB) | Malignancy       | HE, surgery    | NO | NA                          | Oral ITCZ- 8 W and AMP-B gargle | 2 M- No recurrence |
| 10   | Wittkopf J, et al. 2006 | 62/F       | Iowa, USA         | Fluctuating hoarseness | True vocal fold cyst (? aspergilloma) | Type II DB (well-controlled), hypertension, and GERD | HE | No | NA                          | Surgery | NA- No recurrence          |
| 11   | Ran Y, et al. 2008 | 36/F       | China             | Hoarseness of voice, vocal fatigue  | None              | Dexamethasone therapy for rhinitis and asthma | HE, KOH, SEM and Culture | Yes | A. fumigatus | Oral ITCZ (200 mg bd-4 W) | 1 M- asymptomatic |
| 12   | Liu YC, et al. 2010 | 30/F       | Hangzhou, China   | Hoarseness of voice  | True vocal cord cyst | Vocal abuse, broad spectrum antibiotic therapy | HE and Culture and FFPE-PCR | Yes (no growth) | A. fumigatus | Oral ITCZ (200 mg bd-4 W) | 1 M- asymptomatic |
| 13   | Ran Y, et al. 2011 | 30/F       | Chengdu, China    | Hoarseness of voice, vocal fatigue, expectoration, and occasional vomiting | Laryngitis | Vocal abuse, oral Antibiotics and dexamethasone use | HE, KOH, SEM and Culture | Yes | A. fumigatus | Oral ITCZ (200 mg bd-4 W, 200 mg qd next 2 W) | 1 M- asymptomatic |
| 14   | Sundaramay C et al. 2011 | NA         | Cuttack, India    | NA                  | NA               | NA                | NA              | NA           | NA                          | NA        |
| 15   | Ran Y, et al. 2013 | 23/F       | China             | Hoarseness of voice, severe paroxysmal cough, tachypnea  | None              | Oral sex | HE, SEM and Culture | Yes | A. fumigatus | Oral ITCZ (200 mg bd-4 W) | 1 M- asymptomatic |
| 16   | Dolo PK, et al. 2014 | 35/F       | Assam, India      | Hoarseness of voice, cough | None              | Keratois of the larynx | HE, KOH and Culture | Yes | A. fumigatus | Oral ITCZ (100 mg qd-3 W) | 3 W- asymptomatic |
| Case | Reference | Age/ gender | Geographical area | Clinical presentation | Initial diagnosis | Associated factors | Diagnosis method | Fungal culture | Aspergillus species involved | treatment | Follow up period and Outcome |
|------|-----------|-------------|-------------------|-----------------------|------------------|-------------------|-----------------|----------------|-----------------------------|------------|-------------------------------|
| 17   | Al-Ogaili Z, et al. 2014 | 77/F | Australia | Dysphagia and hoarseness | Lymphoma | Smoking, inhaled corticosteroids | HE and Culture | Yes | Not speciated | NA | NA |
| 18   | Gangopadhyay M, et al. 2014 | 42/M | West Bengal, India. | Hoarseness, fever, cough with expectoration | Malignancy | Smoking, vocal abuse | HE, and Culture | Yes | A. niger | Oral ITCZ | 18 M- asymptomatic |
| 19   | Ravikumar et al. 2014 | 34/F | Tamil Nadu, India | Hoarseness, cough, Dysphagia, vocal fatigue | None | NA | GERD | HE, KOH mount | No | NA | Oral ITCZ (100 mg bd- 3 W) | 3 W- asymptomatic |
| 20   | David et al. 2014 | 59/F | Sydney, Australia | Hoarseness of voice | None | Asthma- fluticasone therapy | HE | No | NA | Oral ITCZ | NA: No recurrence |
| 21   | M Dutta, et al. 2014 | 45/F | WB, India | Hoarseness of voice | Malignancy | None | HE, KOH and Culture | Yes | A. fumigatus | Oral ITCZ- (300 mg qd- 3 W) | 6 M- asymptomatic |
| 22   | JCR Villanueva, et al. 2015 | 28/F | Philippines | Hoarseness of voice | Antibiotics and steroids | None | HE | No | No | Oral VCZ (400 mg qd- 4 W) | 1 M- asymptomatic |
| 23   | Arpita Saha, et al. 2015 | 28/F | Odisha, India | Severe dysphonia | None | Asthma, long-term steroid inhaler, vocal abuse, broadspectrum antibiotics | HE, and Culture | Yes | A. fumigatus | VCZ (200 mg bd-8 days) | 2 W- asymptomatic |
| 24   | Santosh Kumar Swain et al. 2016 | 35/M | Orissa, India | Hoarseness of voice | Flute player | Malignancy | HE, KOH and Culture | Yes | A. fumigatus | Oral ITCZ-100 mg bd- 3 W | 6 M- asymptomatic |
| 25   | Richard H. et al. 2016 | 73/F | USA | Persistent hoarseness | None | Inhaled and oral corticosteroids, and nebulized tobramycin | HE | NO | NA | Oral ITCZ-20 W | 5 M- asymptomatic |
| 26   | Santosh Kumar et al. 2017 | 12/M | India | Hoarseness of voice | None | Asthma, inhaled corticosteroids, microlaryngeal surgery with stripping of the vocal cords | HE, and Culture | Yes | A. fumigatus | Oral ITCZ-50 mg bd- 3 W | 3 W- asymptomatic |
| 27   | Soumen Chatterjee et al. 2017 | 43/F | India West Bengal | Hoarseness of voice | None | None | HE, KOH and Culture | Yes | A. fumigatus | Oral ITC -100 mg bd- 8 W | 1 M- asymptomatic |
| 28   | Present study | 22/M | Nepal | Hoarseness and frequent expectoration | None | None | HE, KOH, Culture and FFPET-PCR assay | Yes | A. fumigatus | Oral ITCZ- 4 W | 1 M- asymptomatic |

Legend: *Species identified via sequencing; HE Histopathological examination, COPD Chronic obstructive pulmonary disease, ITCZ Itraconazole, W Weeks, M Month, AMP-B Amphotericin B, DM Diabetes mellitus, GERD Gastroesophageal reflux disease, SEM scanning electron microscopy, VCZ Voriconazole
However, result on species identification was lacking in majority of reported cases. Detection of hyphae, simulating those of *Aspergillus* in a biopsy specimen can be suggestive of fungal invasion but, is not necessarily pathognomonic of aspergillosis. Therefore, it becomes mandatory that the organism be isolated in pure culture and accurately identified. Few authors identified *Aspergillus* up to species level based on morphology and a couple of reports provided evidences of identification based on molecular methods [11, 12]. Moreover it is well known that fungal identification with conventional culture technique has its own limitations. As per the studies [13] conducted earlier, as well as in the present case, we could not successfully culture the fungus from the laryngeal biopsy. In these situations, etiological identification directly from clinical specimen via extraction of DNA and sequencing is advantageous. In this study, PCR on DNA extracted from paraffin-embedded tissue confirmed the aetiology. The extended region of the gene encoding the large ribosomal subunit (28S) of fungi was used for PCR amplification and sequencing. This region was previously explored for designing of broad range PCR primers and showed generation of successful amplicons and sequences from yeasts and filamentous fungi [14]. Because of the paucity of sequences of this extended region of fungal 28S rDNA in the public databases, the non D1/D2 region was rarely utilized for sequence-based detection and identification of fungi directly from clinical specimens. A recent study [15] showed the utility of the non D1/D2 region as a favorable target for the genus, and to a limited extent, species-level identification of pathogenic fungi in various fresh and FFPE samples. In the present study, attempt to amplify the internal transcribed spacer 1 (ITS1) region from the DNA extracted from the sample was not successful. One possible explanation might be due to the relatively larger size of the ITS1 region (~250–350 bp) than this non D1/D2 region (198 + 25 bp). Although accurate species identification required sequencing of at least a partial ITS region such as ITS 1 or ITS2, the non D1/D2 multicopy gene could give a satisfying genus level
identification. In our study, this region could identify the genus and species of the pathogen with clear discrimination from other species of *Aspergillus* (with less % similarity scores) as evidenced from the BLAST hits. Therefore, this non D1/D2 region must be considered for PCR-sequencing from direct clinical specimens in those cases where partial ITS genes fail to amplify.

**Treatment of cases**

In majority of the reported cases, including the present one, itraconazole was used as an empiric treatment, though voriconazole is the treatment of choice against invasive aspergillosis [16]. Possibly cost of the antifungal agent is an important limiting factor during treatment of fungal infections in developing countries. The critical condition of the patient, arising out of the acute laryngeal pathology may be a compelling reason for the empiric treatment on an emergency basis, yielding invariably positive outcome following therapy. Recent reports of the global emergence ofazole resistance in *A. fumigatus* [17] may be of concern in the management of such patients in future. Prompt species identification and detection of resistance are of paramount importance in the management of laryngeal mycosis.

**Therapeutic outcome and relapse**

In all 38 cases reviewed (Table 1), there was complete resolution of symptoms without any relapse, irrespective of the therapeutic modality adopted. There was not much difference in the time period between administration of antifungal drugs and relief of symptoms, regardless of whether the drug administered was itraconazole or voriconazole. Thus, considering the toxicity of conventional amphotericin B, and the cost of liposomal amphotericin B; empiric therapy with either itraconazole or voriconazole may be strongly advocated as better therapeutic options.

**Conclusion**

Since last few decades cases of primary laryngeal aspergillosis in immunocompetent individuals are on the rise, globally. Patients responded to azoles with good prognosis. This is the first case of invasive laryngeal mycosis reported in Nepal. The extraction of DNA from tissue and sequencing helps to identify the etiological agent, when culture fails to isolate the fungus.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article. The sequence data have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) with the accession number MH465665.

**Authors’ contributions**

SHS: observed the incidence and case, performed the laboratory tests, followed the case, literature review and wrote the manuscript; KR: observed the incidence and case, collected specimens, case follow-up, and treatment, J.J: molecular studies and manuscript preparation, SMR: molecular analysis and manuscript drafting, NN & AC: Contributed toward providing clinical relevance, manuscript drafting, and critically reviewed the manuscript, AG: contributed toward histopathological diagnosis. All authors read and approved the final manuscript.

**Consent for publication**

Authors would like to acknowledge the patient, who gave written, informed consent for the publication of this case report.

**Competing interests**

The authors declare that they have no competing interests.

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