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Opportunistic fungi in lake water and fungal infections in associated human population in Dal Lake, Kashmir

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ABSTRACT

Natural habitats of opportunistic fungal pathogens are outside of the host; therefore, it is critically important to understand their ecology and routes of transmission. In this study, we investigated the presence of human pathogenic opportunistic fungi in lake water and incidence of fungal infections in associated population in Kashmir, India. Six hundred forty water samples were taken on seasonal basis from a wide network of sampling stations of the lake for an extended period of two years for screening their occurrence. The samples were inoculated onto rose bengal agar, malt extract agar, potato dextrose agar and other specified culture media supplemented with Chloramphenicol and Streptomycin followed by incubation at 37 °C. All the samples were positive for fungi, which were later identified by sequencing the rDNA internal transcribed spacer region aided by classical morphological culture techniques and physiological profiling. The whole process led to the isolation of sixteen species of opportunistic fungal pathogens belonging to genus *Aspergillus*, *Candida*, *Penicillium*, *Cryptococcus*, *Fusarium*, *Rhizopus* and *Mucor* in decreasing order of prevalence. Furthermore, 20% population (n = 384) of Dal inhabitants was examined for possible fungal infections and it was observed that only 8.07% individuals were positive for fungal infections with 4.68% skin infection cases, 2.34% onychomycosis cases and 1.04% candidiasis cases. Scrapings from onychomycosis and candidiasis patients showed the presence of *Aversicolor* and *Calbicans* respectively, resembling exactly the strains isolated from the lake water. However, the skin infection was because of a dermatophyte not isolated for the lake water. Higher prevalence of infection (6.77%) was seen in people using lake water followed by a positive prevalence of 1.30% using tap water. The results of present study suggest that the lake inhabitants are at a greater risk of getting life threatening fungal diseases which may lead to various morbidities.

1. Introduction

It is thought that, of the 50,000 to 250,000 known species of fungi a limited number of species are responsible for diseases [72,73]. The most problematic species are *Candida* spp. (especially *Calbicans*), *Aspergillus* spp. (especially *Afumigatus*) and *Cryptococcus* sp. [51] and [48]. Acute or chronic Aspergillar infections account for a large number of mycoses due to environmental fungi of the *Aspergillus* genus [24,25]. Immuno-competent individuals with no underlying health condition may experience superficial or localized infections but with fewer complications and a much smaller risk of disseminated or invasive diseases and death [2,20,67]. It is worth mentioning that not all species of the same genus have same degree of toxicity, pathogenicity or allergenicity [31]. However, the genetic boundaries between species are not well defined and can be misleading [49]. Fungi are ubiquitous organisms that are widely distributed in nature. Several fungal genera such as *Aspergillus*, *Alternaria* and *Cladosporium* [16,17,33] have been found to be allergenic. Several studies have suggested an important role for waterborne fungi to endanger human health [3,4,59]. Some of these studies have linked a genetic relationship between waterborne fungi and fungi isolated from clinical samples [3,4]. Sammon et al. [62] demonstrated that numerous microfungal genera, including those that contain species which are opportunistic human pathogens, populate typically treated municipal water supply and thus contaminate the lakes and other water bodies.

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The consumption of fungi-contaminated drinking water has, as far as is known, not caused acute disease, at least in immunocompetent individuals [31]. However, there is a risk of superficial or localized infection in healthy individuals and more severe and invasive infection in immuno-compromised patients. Some species also have the potential to cause allergic reactions and diseases. There are a number of reasons to suggest that water should be considered as a potential transmission route for pathogenic or allergenic fungi. However, a number of other environmental sources exist. Aerosolisation of spores or fragments of hyphae from water has been particularly investigated as a pathway of exposure. There a number of infections like candidiasis, invasive mycosis, invasive aspergillosis, onychomycosis, and ocular infection [7,50,51] known to be caused by fungi, which can be classified according to the site of initial infection [58]. It is in this back drop the present study was taken up to search out the diversity of opportunistic fungal pathogens in the lake water that is still being used by the people living within and outside the lake and then to check out the rate and relevance of fungal infections in the population actively engaged with various activities related to the lake water. The study was carried out in Dal Lake (N 34°07′, E 74°52′, 1583–1600 m) – a Himalayan lake in Srinagar, representing a unique ecosystem with respect to its species diversity and their interaction with people living within it. As it is a multi-basined lake with many inlets and outlets, an extensive network of sixteen sites with eight (8) sites from four basins, four (4) sites from two inlets and four (4) more from two outlets were selected for the exploration of human pathogenic opportunistic fungi.

2. Materials and methods

2.1. Sample collection

Water samples from different sites of Dal Lake (N 34°07′, E 74°52′) for exploring human pathogenic opportunistic fungi were collected seasonally (spring, summer, autumn and winter) for a period of two years from June 2011 to June 2013 in Polyethylene (PET) bottles, which were previously carefully cleaned and rinsed three to four times with distilled water and sterilized with 70% alcohol. The water samples were collected in the months of April, July, October and January respectively in the four seasons. In total, 640 water samples were taken from a wide network of sampling stations of the lake and were screened for occurrence of opportunistic fungal pathogens.

2.2. Isolation and identification of fungi

Water samples obtained from the lake were serially diluted five folds. Then 0.1 ml inoculum from the serial dilution tubes was spread on Petri dishes containing Rose-Bengal Streptomycin Agar and Potato Dextrose Agar (MERCK, Germany) using direct plating method [5]. Plates were incubated at 25–37 °C for one week in dark. For yeasts, YM Broth (Acumedia, USA) was used for culture enrichment followed by YM Agar (Acumedia, USA) for their isolation. Identification of fungal species was done on the basis of classical morphological and culture approach, physiological profiling and molecular techniques.

2.3. Classical morphological and culture approach

Identification of fungi was done on the basis of micro and macro-morphological features of fungal colonies grown on differential media. Fungi were identified to genus level using [13]. Cultures were identified to species level using various mycological texts like *Penicillium* LINK [56], and PITT's monograph (2000). The species were first grown on Rose-Bengal Streptomycin Agar and Potato Dextrose Agar (MERCK, Germany) and then transferred to Petri dishes containing different culture media like Malt Extract Agar (MEA) (Acumedia, USA), Czapek's dox Agar (CZ) (MERCK, Germany) and Czapek's Yeast Agar (CYA) (MERCK, Germany), and 25% Glycerol nitrate Agar (G25A) all prepared according to the recipes of [53] for identification. Each filamentous fungal culture was inoculated in triplicate on each medium and incubated at three different temperatures (15 °C, 25 °C and 37 °C) for a period of 7 days in dark. Additionally the morphological characteristics were studied by making slide cultures obtained by inoculating microfungi directly on small square blocks of potato dextrose agar, corn meal agar, oatmeal agar, and sabouraud agar medium [32]. For the identification of *Candida* species CHROM Agar *Candida* was used. Morphology was characterized by using a semiautomatic image analysis system consisting of an Olympus microscope (Olympus, New Hyde Park, NY, U.S.A.) operated as phase contrast, a charge coupled device (CCD) camera (Sony, Cambridge, U.K.) a PC with a frame-grabber, and the image analysis software (SIS, Olympus, Germany). Sample preparation and measurement was done as described by [46,47]. A magnification of 100× was applied for measurement of mycelial particles to estimate the individual mycelia and other micro-morphological features.

2.4. Physiological profiling using microplate technology

Physiological profiling of yeasts was carried out by biochemical tests including physiological tests (germ tube test, hydrolysis, growth on Cycloheximide medium, growth at 37°C), fermentation tests (glucose, trehalose, lactose, galactose, maltose, sucrose) and assimilation tests (glucose, trehalose, lactose, galactose, maltose, sucrose, raffinose, D-manitol, soluble starch, glycerol, lactic acid, potassium nitrate, citric acid, succinic acid). Basal medium for carbohydrates assimilation tests (BMC) {demineralized water 100 ml, yeast nitrogen base (Difco) 1.0 g}, basal medium for nitrogen compounds assimilation tests (BMN) {demineralized water 100 ml, yeast carbon base (Difco) 1.77 g} and basal medium for growth testing without some vitamin compounds (BMV) {demineralized water 100 ml, vitamin free yeast base (Difco) 2.52 g} were prepared as per the protocol of Ref. [71]. 50 μl inoculum (MacFarland standard No. 2 diluted by a factor of 10) was inoculated into each well using a multi-channel pipette incubated at 25 °C for 3–10 days. The inoculated microplates were properly shaken (with a microplate shaker) just before automatic reading using a microplate reader. Absorbance values at 405 nm were transferred by cable (RS-232 through a serial port) to the computer and transformed by the BioloMICS software into negative, weak or positive results.

2.5. DNA extraction, PCR, and molecular analysis

The fungal strains were grown in potato dextrose broth and sabouraud dextrose broth containing 10 μl of Tween-80 before autoclaving to keeps the cells dispersed. DNA extraction was performed using a HiPur A™ SP Fungal DNA Mini kit (Himedia) following the manufacturer's instructions. After DNA extraction rDNA regions spanning the internal transcribed spacer (ITS) were amplified using universal fungal primers ITS1 and ITS4 [69]. Amplification was performed in 50 μl PCR reaction tubes containing 6 μl genomic DNA, 5 μl 10× thermophilic buffer, 2 μl $MgCl_2$ (2.5 mM), 1.4 μl dNTP (10 mM), 1.2 μl (10 μM) ITS1, 1.2 μl (10 μM) ITS4, 5 μl (10u/μl) *Taq* (Promega) and 28.2 μl ddH2O in an Eppendorf Thermal Cycler with the following program for 40 cycles: initial denaturation temperature 94 °C for 5 min, melt temperature 94 °C for 50 s, annealing temperature 54.5 °C for 45 s, extending