Fungal Ecology 20 (2016) 30–39

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Yeasts and yeast-like fungi in tap water and groundwater, and their transmission to household appliances

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ARTICLE INFO

Article history:
Received 13 July 2015
Revision received 21 September 2015
Accepted 1 October 2015
Available online xxx

Corresponding editor: Felix Bärlocher

Keywords:
Black yeasts
DGGE
NGS sequencing
Drinking water
Exophiala spp.
Household appliances
Inorganic ions
Opportunistic human pathogens
Water quality

ABSTRACT

In the present study we describe the occurrence of fungi in 100 tap water and 16 groundwater samples from Slovenia. We used culture-dependent and culture-independent techniques. 28 fungal species belonging to 16 genera were isolated with selected culturing conditions, targeting human opportunistic yeasts and yeast-like fungi. Of special concern was the detection of *Aureobasidium melanogenum*, *Exophiala dermatitidis*, *Rhinocladiella similis*, *Candida parapsilosis* and *Rhodotorula mucilaginosa*. The DGGE analysis of ITS1 rDNA revealed from 6 to 16 bands hypothetically corresponding to different taxa, while pyrosequencing showed the presence of *Aspergillus* and *Exophiala*. According to the statistic machine learning methodology, the profile of fungi in water is determined by the concentration of calcium and magnesium ions and the presence of nitrate. *Exophiala* spp., *C. parapsilosis* and *R. mucilaginosa* are known as dominant contaminants of household appliances. It appears that they are transferred with water to dishwashers and washing machines, where they subsequently proliferate.

1. Introduction

Over the last 30 yr, fungi in indoor environments, in particular the clinical environments have increasingly been recognized as a health problem, linked to a growing immunocompromised population. Over the last 30 yr more than one billion people around the world have suffered from different fungal infections. These were also reported from more than 10% of autopsied patients (Lehrnbecher et al., 2010; Vos et al., 2010). Fungi can cause infections of skin, hair, nails, urinary and respiratory tract, catheter related and systemic infections. People not only come across pathogenic fungi in nature, but also in public places and households. Thus, fungi are present in indoor air, they can invade indoor damp walls (Adan and Samson, 2011), household wet cells, such as bathrooms and kitchens (Matos et al., 2002; Adams et al., 2013), and even extreme indoor habitats such as household appliances, for example dishwashers (Zalar et al., 2011) and washing machines (Gattlen et al., 2010; Novak Babič et al., 2015). Conditions inside household appliances used to be considered hostile to microbial growth. However, increased consumer awareness toward sustainable use of resources and hazardous chemicals, and novel technologies led to the development of household appliances operating at lower temperatures, with reduced amounts of water, and increased use of biodegradable detergents. These conditions are selective for thermotolerant, oxidative-stress resistant, and stress-tolerant fungi generally recognized as polyextremotolerant fungi, many of which are

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http://dx.doi.org/10.1016/j.funeco.2015.10.001
1754-5048/

opportunistic human pathogens (Gostinčar et al., 2009; Zalar et al., 2011). Thus, dishwashers around the world are consistently colonized with polyextremotolerant yeasts, black-pigmented *Exophiala dermatitidis* and *Exophiala phaeomuriformis,* white *Candida parapsilosis*, red-pigmented *Rhodotorula mucilaginosa*, and filamentous *Fusarium dimerum*, *Fusarium oxysporum* and *Fusarium solani* species complexes (Zalar et al., 2011; Gümral et al., 2015). Surprisingly, mycobiota of washing machines showed an overlap with the mycobiota of dishwashers in the occurrence of *C. parapsilosis, R. mucilaginosa* and *E. phaeomuriformis*, whereas members of the *F. oxysporum* species complex, recovered from dishwashers with very low frequency, have been isolated with the highest frequency from washing machines (Novak Babič et al., 2015).

The majority of *Exophiala* species are opportunistic pathogens that can cause cutaneous and subcutaneous infections, lung and neurotropic infections, mainly of immunocompromised but also of immunocompetent individuals (de Hoog et al., 2009; Machouart et al., 2011). Both *R. mucilaginosa* and *C. parapsilosis* have been reported as newly emerging pathogens, causing primarily catheter-related infections and opportunistic nosocomial fungemias in immunocompromised patients (Neofytos et al., 2007; Pfaller et al., 2007; Van Asbeck et al., 2009; Miceli et al., 2011). Various *Fusarium* species are causative agents of approximately 80% of human fungal infections. They produce mycotoxins in water (Kelley et al., 2003), cause localised subcutaneous infections, sinusitis, and onychomycosis (O'Donnell et al., 2010; Sutton and Brandt, 2011; Garnica and Nucci, 2013).

Fungi might enter household appliances via air or water, food and waste, with influence of humans and their pets. Colonization of both dishwashers and washing machines with largely overlapping fungal species points at the water supply system as the main vector. Although several studies investigated the presence of fungi in water, their primary focus was on fungal genera that can be dispersed from water to air due to sporulation (Anaissie et al., 2002). Therefore, genera such as *Acremonium*, *Alternaria, Aspergillus*, *Aureobasidium, Cladosporium*, *Fusarium, Kloeckera, Paecilomyces, Penicillium, Phialophora, Phoma, Scopulariopsis, Stachybotrys* and *Trichoderma* were mostly isolated. Among yeasts, the presence of the genera *Candida, Cryptococcus* and *Rhodotorula* has been reported (Kinsey et al., 1999; Göttlich et al., 2002; Paterson and Lima, 2005; Hageskal et al., 2006; Grabinska-Loniewska et al., 2007; Pereira et al., 2010). No study has so far focused on the potential presence of human opportunistic pathogenic yeast and yeast-like fungi in public tap water systems as an entry point to household appliances, where selection and enrichment of selected species occurs.

In the present study we focused on the diversity of human opportunistic pathogenic yeasts and yeast-like fungi in groundwater and tap water, based on culturing techniques. In parallel, we analysed fungal communities in raw water sources (rivers, groundwater), selected tap water samples, as well as water after waste water cleaning treatment, by the analysis of ITS1 rDNA amplicons from total DNA by Denaturing Gradient Gel Electrophoresis (DGGE). Using next generation sequencing (NGS) technology, we have analysed a single tap water sample by pyrosequencing of ITS2 rDNA. We investigated the potential correlations between the appearance of yeasts and yeast-like fungi, detected by culture-dependent techniques, and water characteristics, using machine learning methodology. The overall aim was to determine whether tap water acts as the main vector for the inoculation of fungi in household appliances, where extreme abiotic conditions promote settlement and proliferation of selected human opportunistic fungal pathogens.

2. Materials and methods

2.1. Isolation and cultivation of fungi from water

Slovenia can be divided into 5 geographical regions: Alpine and Subalpine, Littoral, Pannonian and Dinaric Karst regions, which differ in geology, and thus also in water characteristics. The study mainly focused on water sampling in the Ljubljana valley, which is partly Subalpine and Dinaric Karstic, while the remaining samples originated from 9 Slovenian cities, representing all geographical regions mentioned above. Samples of tap water were collected from regularly used water pipes (running tap water) of 100 private homes in different locations in Slovenia. Out of these, 50 samples were obtained in the capital city of Ljubljana, and originated from the 8 main water supply systems, while 50 samples originated from the following cities and sub-urban areas: Bohinj, Celje, Mislinja, Laško, Litija, Logatec, Ljutomer, Ormož, Portorož, Postojna, Ravne na Koroškem, Radomerje, Rodica, Ruše, Sečovlje, Sežana, Trebnje, Trebče and Velenje (Fig. 1). Additionally, 16 samples of groundwater used for tap water were obtained from Ljubljana. In the case of running tap water, 5 l of cold water were collected according to the standard SIST ISO 5667-5:2007. The groundwater samples were collected at main water supplies in sterile containers by employees of Waterworks and Sewage Company, according to the standard SIST ISO 5667-5:2007 (VO-KA, Ljubljana). An aliquot of 1 L of each sample was filtered twice using 0.45 μm membrane filters (Merck, Millipore), which were placed on Dichloran Rose Bengal Agar (DRBC; Oxoid Ltd., England) (Pereira et al., 2010), and were each incubated at 30 and 37 °C for 5–7 d. Pure cultures of fungi were transferred to malt extract agar (MEA) and deposited in the Ex Culture Collection of the Infrastructural Centre Mycosmo, MRIC UL, Slovenia: http://www.ex-genebank.com/, at the Department of Biology, Biotechnical Faculty, University of Ljubljana.

2.2. Genomic DNA extraction from pure cultures and from water samples

DNA from 3 d old yeast cultures grown on malt extract medium (MEA; Biolife, Italy) was extracted using PrepMan Ultra reagent (Applied Biosystems) following the manufacturer's instructions. DNA of filamentous fungi was extracted from 7 d old cultures grown on MEA using mechanical lysis of 1 cm^2 of mycelium, following instructions of Van den Ende and de Hoog (1999). Genomic DNA from water samples was obtained from 3 l of water, filtered through 0.45 μm membrane filters (Merck, Millipore) and extracted using PowerWater DNA Isolation Kit (MO BIO Laboratories Inc.) according to the manufacturer's instructions. DNA samples ready for downstream applications were stored at −20 °C.

2.3. Identification of pure cultures

Fungi were identified according to their morphological characters, but their identification was complemented with rDNA nucleotide sequence analyses of internal transcribed spacer region 1, 5.8S rDNA and ITS 2 (ITS). For amplification and sequencing primers ITS5 and ITS4 were used (White et al., 1990). Yeasts were identified by sequencing D1/D2 domains of 28S rDNA, (large subunit of ribosomal DNA; LSU) using primer set NL1 and NL4 (O'Donnell, 1993). All sequences were obtained at Microsynth AG, Switzerland using an ABI Prism 3700 Big Dye Sequencer (Applied Biosystems). Sequences were assembled by FinchTV 1.4 (Geospiza, PerkinElmer, Inc.). Fungi were identified with the BLAST algorithm at NCBI web page (Altschul et al., 1990) and by use of other taxonomically important databases (Centraalbureau voor Schimelcultures, Utrecht, the Netherlands CBS). Software Molecular Evolutionary