NATALIA SERON BRIZZOTTI MAZUCHI

Diversidade de espécies fúngicas na Água de Rede Pública de São José do Rio Preto.

São José do Rio Preto

2017

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Dissertação apresentada à Faculdade de Medicina de São José do Rio Preto para a obtenção do título de Mestre no curso de Pós-graduação em Ciências da Saúde. Eixo temático: Medicina e Ciências Correlatas.

Orientadora: Profa. Dra. Margarete Teresa Gottardo de Almeida

SÃO JOSÉ DO RIO PRETO
2017

Mazuchi, Natalia Seron Brizzotti
Diversidade de espécies fúngicas na Água de Rede Pública de São José do Rio Preto./ Natalia Seron Brizzotti Mazuchi
São José do Rio Preto, 2017
69 p.

Dissertação (Mestrado) – Faculdade de Medicina de São José do Rio Preto – FAMERP
Eixo Temático: Medicina e Ciências Correlatas

Orientadora: Profa. Dra. Margarete Teresa Gottardo de Almeida

1. Água de Rede Pública; 2. Fungos filamentosos; 3. Leveduras; 4. Sazonalidade; 5. Suscetibilidade antimicrobiana.

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Suplente: Profa. Dra. Crislene Barbosa de Almeida

São José do Rio Preto, 15/08/2017

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Dedicatória

À minha família, em especial à minha mãe Marilúcia que sempre me apoiou e incentivou a lutar por meus sonhos.

Agradecimentos

A Deus por me guiar em meus sonhos e me dar forças para sempre seguir em frente na busca de meus objetivos.

À minha família por todo o apoio, em especial a minha mãe Marilúcia, minha alma gêmea, pela sua força e determinação em conseguir e conquistar tudo aquilo que deseja, me mostrando que nada na vida é impossível, desde que feito com o coração. Espelhar-me nela, me fez ser a mulher que sou hoje, forte, objetiva e determinada a conseguir realizar todos meus sonhos.

À minha irmã Aline, por todo o companheirismo e por ser o meu elo único entre meu passado e meu futuro.

Ao meu marido Marcos, por toda sensibilidade diante das realizações dos meus sonhos, por dar sentido a minha vida e me fazer conhecer a pureza do amor na construção de uma família.

À Profa. Margarete Teresa Gottardo de Almeida pela orientação, por todo o conhecimento científico e pessoal que carregarei nessa e em outras vidas e pela amizade tão verdadeira. Nos momentos de desânimo, seus conselhos de mulher, amiga e mãe em conjunto com sua orientação espiritual trouxe conforto e sabedoria, se tornando uma fonte de inspiração na minha caminhada, me iluminado com a sua luz interior.

À Keith Cássia da Cunha, pela parceria profissional e amiga. Não mediu esforços em me ensinar tudo que sabia quando entrei no laboratório como estagiaria, me acolheu como irmã, sanando minhas dúvidas e me proporcionando, a cada dia, experiências profissionais que jamais esquecerei.

Aos meus tios e primos por todo o carinho e companheirismo durante esta vida, por constituírem a minha segunda família e sempre torcerem pela realização de meus sonhos.

Aos meus avós, em especial a vó Zaira, que sempre me colocou em suas orações, pedindo a Deus e aos Anjos que iluminasse meus caminhos e guiasse meus passos na busca do conhecimento.

Aos queridos amigos do Laboratório de Microbiologia da Faculdade de Medicina de São José do Rio Preto- FAMERP:

À Luceli Ferreira de Souza, pela disposição e por ser sempre tão prestativa no desenvolvimento deste trabalho. À Vanessa Beneton Pinto, Mayara Gambellini, Luis

Paulo Teixeira, Maira Gazzola Arroyo, João Paulo Zen, Mariela Ribeiro, Maicon Henrique Caetano, Thiago Henrique Lemes, Diego Maximiano, Maísa Guimarães Sartim, Bianca Gottardo Almeida, Eduardo José de Carvalho Reis, Emília Cristina Gianizella Amorin por todo o companheirismo e amizade.

À Profa. Elza Maria Castilho e a Profa. Cleuzenir por ter me acolhido em seu laboratório, pela amizade, companheirismo, disposição e atenção durante todos esses anos e que certamente se eternizarão.

À toda equipe do SeMAE, por me ajudarem na realização das coletas e das análises físico-químicas e bacteriológicas das amostras de água.

À Claudia, responsável técnica do laboratório de análises do SeMAE, pela sua prestatividade e paciência, me ajudando a entender o processo de tratamento da água, me mostrando cada etapa, auxiliando com as explicações e fornecendo todos os dados físico-químicos e bacteriológicos obtidos.

Aos meus queridos amigos e amigas de Tabapuã e Catanduva por serem uma fonte de confiança e de amizade verdadeira durante toda a minha vida, por sempre vibrarem com as minhas conquistas e pela compreensão nos momentos de ausência.

A todos que contribuíram direta e indiretamente para a realização desta dissertação Mestrado.

Meus Sinceros Agradecimentos!

Epígrafe

Seja quem você for, seja qual posição social que tenha na vida, a mais alta ou a mais baixa, tenha sempre como meta muita força, determinação e sempre faça tudo com muito amor e fé em Deus, que um dia você chega lá. De qualquer maneira você chega lá!

Ayrton Senna

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pH – Potencial hidrogeniônico

VMP – Valor Máximo Permitido

UH – Unidade de Hazen (MG Pt-Co/L)

UT – Unidades de Turbidez

SAD - Sabouraud Dextrose Agar

MOPS- 3-(N-morpholino) propanesulfonic acid

NaOH – sodium hydroxide

MIC- Minimal inhibition concentration

PAD – Potato Dextrose Agar

Resumo

Introdução: A capacidade de sobrevivência dos fungos na água é alta e sua veiculação pela água potável altera o padrão de potabilidade e causam danos à saúde publica quando ingerida ou em contato com a pele e mucosas, especialmente em hospitais. A presença de *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Rhodotorula* spp. e outros gêneros menos frequentes nos sistemas de distribuição de água juntamente com as condições físico-químicas presentes, pode ocorrer a formação de biofilme provocando alterações fenotípicas de virulência e resistência aos antimicrobianos, além da liberação de produtos de metabolismo que em contato com seres humanos e animais, tornam-se riscos potenciais para processos alérgicos e intoxicação. Diretrizes brasileiras e internacionais estabelecem normas de controle da qualidade da água, valorizando padrões físico-químicos e microbiológicos, sem inclusão dos fungos. **Objetivos:** Os objetivos deste estudo foram investigar a ocorrência e distribuição de espécies de fungos filamentosos e leveduras na água de rede pública; correlacionar a ocorrência de fungos em água com a sazonalidade e analisar o perfil fenotípico da suscetibilidade antimicrobiana de fungos leveduriformes. **Material e Métodos:** Amostras de água de 245 pontos distintos da rede pública, de um município do interior do Estado de São Paulo - Brasil - foram coletadas após 15 dias do início de cada estação do ano, 100 ml de água foram coletados e encaminhados ao Laboratório de Microbiologia da Faculdade de Medicina de São José do Rio Preto, SP – FAMERP para análise. Características morfológicas, fisiológicas e bioquímicas foram utilizadas para definição das espécies fúngicas e análise de susceptibilidade antimicrobiana. A análise estatística utilizou o teste Z para comparar proporções e o teste de Qui-Quadrado para avaliar a dependência ou associabilidade. **Resultados:** Um total de 26.3% das amostras foram positivas para fungos filamentosos com o gênero *Fusarium* spp. prevalente na primavera, verão, inverno e, *Penicillium* spp. no outono. Para leveduras, 11,4% das amostras foram positivas, com a espécie *Rhodotorula minuta* prevalente na primavera, outono e verão e, complexo *Candida parapsilosis* no inverno. O fenótipo de resistência apareceu em 66,5% das leveduras isoladas, sendo *Rhodotorula minuta* a espécie prevalente resistente encontrada no atual estudo. **Conclusões:** A presença de fungos filamentosos e leveduriformes na água, em especial os gêneros *Fusarium* spp. e *Rhodotorula* spp. é uma preocupação para a saúde pública por serem patógenos oportunistas comuns em infecções humanas e animais. Portanto, faz-se necessária a vigilância constante destes micro-organismos nos protocolos padrões de análises de controle de qualidade da água de rede pública.

Palavras-chave: Água de rede pública; Fungos filamentosos; Leveduras; Sazonalidade; Suscetibilidade antimicrobiana.

Abstract

Introduction: The survival capacity of fungi in the water is high and their transmission through drinking water changes the drinking standard and causes damage to public health when ingested or in contact with the skin and mucous, especially in hospitals. In the presence of *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Rhodotorula* spp. and other fungi less frequent in the water distribution systems together with the Physical-chemical conditions present, biofilm formation may occur causing phenotypic changes in virulence and antimicrobial resistance, as well as the liberation of metabolism products that in contact with humans and animals, become potential risks for allergic processes and intoxication. Brazilian and international guidelines establish standards water quality control, streghtening physical-chemical and microbiological standards, for with no fungi's inclusion. **Objectives:** The objectives of this study were to investigate the occurrence and distribution of species of filamentous fungi and yeast in the water supply network; to correlate the occurrence of fungi in water with seasonality and to analyze the phenotypic profile of antimicrobial susceptibility of yeast. **Material and Methods:** Water samples from 245 different points of the water supply network of a municipality in the interior of the State of São Paulo - Brazil - were collected after 15 days of the beginning of each season, 100 ml of water were collected and sent to the Laboratory of Microbiology of Medical School of São José do Rio Preto - FAMERP for analysis. Morphological, physiological and biochemical characteristics were used for the definition of fungal species and analysis of antimicrobial susceptibility. Statistical analysis used the Z test to compare proportions and the Chi-Square test to evaluate dependency or associability. **Results:** A total of 26.3% of the samples was positive for filamentous fungi with the *Fusarium* spp. prevalent in spring, summer, winter and *Penicillium* spp. in the fall. For yeasts, 11.4% of the samples were positive, with the species *Rhodotorula minuta* prevalent in spring, fall, summer and the complex *Candida parapsilosis* in the winter. The resistance phenotype appeared in 66.5% of isolated yeast, being *Rhodotorula minuta* the prevalent resistant species found in the present study. **Conclusions:** The presence filamentous fungi and yeasts in the water, especially *Fusarium* spp. and *Rhodotorula* spp. is a public health concern because they are common opportunistic pathogens in human and animal infections. Therefore, frequent monitoring of these microorganisms in the standard protocols of water supply network for analyses of quality control important.

Keywords: Antimicrobial susceptibility; Filamentous fungi; Sazonality; Water supply network; Yeasts

INTRODUÇÃO

1. INTRODUÇÃO

Os fungos são micro-organismos de grande importância para as indústrias farmacêuticas e alimentícias, além da economia na ciclagem de nutrientes e decomposição de matéria orgânica em ambientes aquáticos e terrestres. A maioria das espécies fúngicas é constituída por organismos saprófitas e parasitas de: plantas, animais, algas e homem.[1,2]

Mais de 600 espécies de fungos foram encontradas na água, se proliferando, gerando perdas econômicas e causando danos à saúde, principalmente em idosos, debilitados e imunossuprimidos.[3,4] A prevalência de infecções fúngicas invasivas causadas por fungos oportunistas como *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., *Penicillium* spp., *Phialophora* spp., *Exophiala* spp., *Phoma* spp., *Acremonium* spp., *Cladosporium* spp., *Candida* spp., e *Rhodotorula* spp., em Unidades de Terapias Intensivas (UTI) é responsável pela morte de 1,4 milhões de pessoas anualmente devido a inalação de esporos fúngicos que podem ser dispersos pelo ar, solo ou rede de distribuição de água.[3–6]

Os fungos são considerados componentes aquáticos importantes, sobrevivem por longos períodos em formação colonial ou em biofilme prejudicando os sistemas de distribuição de água potável e, consequentemente alterando a qualidade da água.[7–10] Além dos perigos descritos, algumas espécies fúngicas são especialmente danosas por produzirem micotoxinas e/ou metabólitos secundários que podem contribuir com o aparecimento de efeitos hepatotóxicos, nefrotóxicos, imunotóxicos, genotóxicos, teratogênicos, mutagênicos, carcinogênicos e pulmonares em humanos. A hidropesia na cavidade pleural e defeitos de tubo neural também podem ser desencadeados por micotoxinas, em seres humanos e animais.[11]

A ocorrência de fungos na água tem recebido crescente atenção nas ultimas décadas, porém sua presença é pouco compreendida. Sabe-se que quando presentes na água potável modificam seu sabor e odor, e em concomitância com compostos orgânicos seu crescimento é favorecido.[1,4,12]

Devido a importância da água para a vida, o uso mundial estimado de água fresca é de 4 mil quilômetros cúbicos por ano, entretanto, mais de 1 milhão de pessoas ao redor do mundo não tem acesso à água potável segura para beber.[10,12–14]

Normatização

No Brasil, a qualidade da água é normatizada pelo Ministério da Saúde com destaque ao Decreto Federal nº 79.367 de 9 de março de 1977, em parceria com Conselho Nacional de Meio Ambiente (Conama), Organização Pan-Americana da Saúde (Opas) e da Asociación Interamericana de Ingenería Sanitária y Ambiental (Aidis). O DECRETO DEFINE: (1) valores máximos permissíveis para as características bacteriológicas, organolépticas, físicas e químicas da água potável; (2) a aprovação de normas relacionadas à proteção de mananciais, serviços de abastecimento público e instalações prediais.[15,16]

Em 12 de dezembro de 2011, surge a Portaria nº 2.914 que estabelece: (1) competências da União, do Estado e do Município na vigilância da qualidade da água; (2) normas para a construção dos sistemas de abastecimento, redes de distribuição e ligações prediais, com o propósito de que todos devam trabalhar em conjunto para que a água de consumo humano seja apropriada. Na portaria o fornecimento de água à população pode ser feita: pelo sistema de abastecimento de água (SAA) – conjunto de obras civis, materiais e equipamentos destinada a produção e distribuição canalizada de água potável para população, sob responsabilidade do poder público; a soluções

alternativas coletivas (SAC) – destinado a fornecer água potável providas ou desprovidas de rede de distribuição, com captação subterrânea ou superficial a instalações condominiais horizontal e vertical e, soluções alternativas individuais (SAI) que fornece água potável a um único domicilio.[17]

Padrões microbiológicos normatizados para a água

Nos padrões microbiológicos brasileiros da água para consumo humano *Escherichia coli* e coliformes totais em 100 ml de água devem estar ausentes; os padrões físico-químicos, pH – ácido ou básico, devem apresentar-se no intervalo de 6,0 a 9,5; a cor, resultante da dissolução de substâncias na água, principalmente de matéria orgânica, apresenta Valor Máximo Permitido (VMP) de 15UH (Unidade de Hazen: mg Pt-Co/L); a turbidez, relacionada à transparência com VMP de 5UT (Unidades de Turbidez); o nitrato (N) com VMP de 10mg/L e o cloro residual livre deve estar no intervalo de 0,2 mg/L a 2 mg/L.[17]

Entretanto, os padrões internacionais consistem numa investigação detalhada de determinados grupos microbianos: *E. coli*, coliformes totais, *Cryptosporidium* spp., *Giardia lamblia*, *Legionella* spp., Enterococcus e alguns grupos virais, de acordo com a Directiva 98/88/CE da Comunidade Européia e da Agência de Proteção Ambiental dos Estados Unidos (*United States Environmetal Protection Agency*).[18,19] Porém, nenhuma normatização visa parâmetros que analisam a presença de fungos filamentosos e leveduras na água de consumo humano.

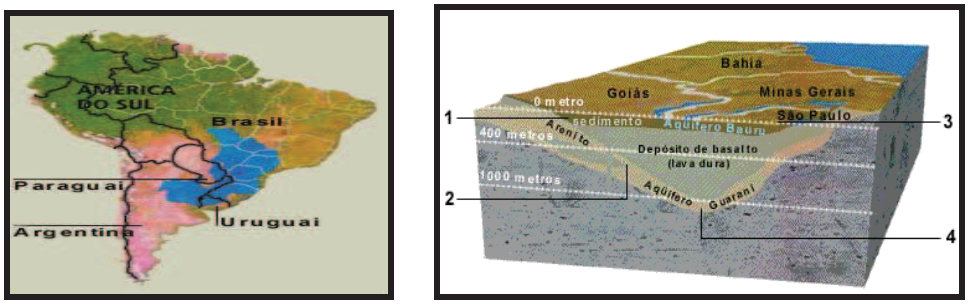
Aquíferos

No Brasil, a água subterrânea é utilizada como fonte de abastecimento público, sendo o consumo das cidades do Estado de São Paulo dependente em 80% de aquíferos.[20] A preocupação com a contaminação desta fonte hídrica esta diretamente

relacionada à qualidade da água dos Aquíferos, que proporcionam este recurso: (1) Aquífero Guarani (figura 1), situado na região centro-leste da América do Sul, ocupando uma área de 1,2 milhões de Km², que se estende pelo Brasil através dos Estados do Rio Grande do Sul, Santa Catarina, Paraná, São Paulo, Minhas Gerais, Mato Grosso, Mato Grosso do Sul e Goiás; Paraguai, Uruguai e Argentina, sendo considerado o maior manancial de água doce subterrânea do mundo.(21) (2) Aquifero Bauru (figura 2), estende-se por uma área de 96.900 Km^2. Estudos hidrogeológicos que consideram (a) os Planos de Bacias Hidrográficas, (b) Relatórios de Situação dos Recursos Hídricos e (c) Programas Estaduais de Monitoramento de Qualidade e Atendimento à Potabilidade, selecionaram o Sistema Aquífero Bauru como importante fonte de abastecimento para região oeste do Estado de São Paulo, principalmente, o município de São José do Rio Preto. É composto por sedimentos arenosos, areno-argilosos e siltosos e sua espessura pode atingir valores superiores a 300 metros, com vazões de até 22 litros/segundo em cada poço.(22,23)

Cada cidade do Estado São Paulo possui um serviço de distribuição de água e esgoto. A cidade de São José do Rio Preto é representada pelo Serviço Municipal Autônomo de Água e Esgoto – SeMAE, na qual afirma que 70% do volume distribuído é proveniente do sistema Aquífero Bauru.(22)

Figura 1 – Localização e disposição do Aquífero Guarani



Fonte: CETESB, 2011

Figura 2 – Localização e disposição do Aquífero Bauru

Fonte: EMDAEP, 2014

De acordo com levantamentos do SeMAE, da Companhia de Pesquisas de Recursos Minerais – CPRM e o Instituto de Pesquisas Tecnológicas – IPT, o Departamento de Água e Energia Elétrica – DAEE, possui pouco mais de 360 poços com outorga (legalizados) em São José do Rio Preto e a existência de outros 1700 sem outorga (clandestinos), totalizando 2000 poços tubulares na área. Estudos hidrogeológicos podem avaliar os efeitos negativos e da contaminação do Aquífero Bauru, em conformidade com a Deliberação nº 52 de 2005 do Conselho Estadual de Recursos Hídricos – CRH que institui diretrizes e procedimentos para controle de captação e uso de água subterrâneas.[23] Neste sentido, os processos de coleta de água, filtração, armazenamento, transporte e processamento para análise devem cumprir todas as normas sanitárias para que não haja alteração na composição original.[10]

Considerando que fungos patogênicos oportunistas podem causar riscos a saúde humana, nos diversos nichos ecológicos, inclusive nas fontes hídricas, o presente estudo avaliou a diversidade desses microrganismos na água que esta disponível em todos os locais de sobrevivência humana e animal e não há normatização nacional ou mundial sobre essa questão.

OBJETIVOS

2. OBJETIVOS

2.1. Objetivo Geral

O presente trabalho teve por objetivo avaliar a diversidade e suscetibilidade antimicrobiana de fungos presentes em água de rede pública na cidade de São José do Rio Preto, SP.

2.2. Objetivos específicos

- Investigar a ocorrência e distribuição de espécies de fungos filamentosos e leveduriformes na água de rede pública;
- Correlacionar a ocorrência de fungos em água com a sazonalidade;
- Analisar o perfil fenotípico da suscetibilidade antimicrobiana de fungos leveduriformes.

ARTIGOS CIENTÍFICOS

3. ARTIGOS CIENTÍFICOS

Nesta seção estão demonstrados dois artigos científicos originais, os respectivos periódicos e classificação Qualis.

Artigo 1.

Título: Diversity and antifungal susceptibility of yeasts in the public drinking water supply

Periódico de submissão: Water Research

Qualis: A1

Artigo 2.

Título: Epidemiology of filamentous fungi in a treated public water supply

Periódico de submissão: Water Science and Technology

Qualis: B1

ARTIGO 1

ARTIGO 1

Diversity and antifungal susceptibility of yeasts in the public drinking water supply

Natalia Seron Brizzotti Mazuchi[a], Keith Cássia da Cunha[b], João Paulo Zen Siqueira[c], Bianca Gottardo de Almeida[c], Thiago Henrique Lemes[c], Diego Maximiano da Conceição[a], Maicon Henrique Caetano[c], Mariela Domiciano Ribeiro[a], Cláudia Regina Rodrigues[d], Elza Maria Castilho[a], Margarete Teresa Gottardo de Almeida[a*].

[a] *Department of Infectious Diseases, Faculty of Medicine of São José do Rio Preto, São José do Rio Preto, São Paulo, Brazil.*

[b] *Dermatology Laboratory, University Hospital of Geneva, Street Gabrielle Perret-Gentil 4, 1205, Geneva, Switzerland.*

[c] *Post-Graduate Program in Microbiology,"São Paulo State University (Unesp), Institute of Biosciences, Humanities and Exact Sciences (Ibilce), Campus São José do Rio Preto", São Paulo, Brazil.*

[d] *Capture/Quality Division, SeMAE- Autonomous Municipal Water and Sewage Service, Street Antônio de Godoy, 2181, Jd Seixas, 15061-020, São José do Rio Preto, São Paulo, Brazil.*

***Correspondence and reprint requests:**

Margarete Teresa Gottardo de Almeida (Ph.D.)

Department of Infectious Diseases, School of Medicine of São José do Rio Preto.

Av. Brig. Faria Lima, 5416, 15090-000, São José do Rio Preto, São Paulo, Brazil.

Phone: +55 17 3201 5843

Email: margarete@famerp.br

Abstract

The conveyance of microorganisms in potable water alters the quality of the water and can affect public health when ingested or after contact with skin and mucous membranes on drinking and washing, especially in hospitals. In Brazil, physical chemistry and microbiological standards do not include investigations for fungi, especially yeasts, which are common in aquatic environments. In water supply systems, the concentration of microorganisms may vary and occur in biofilms causing phenotypic changes in virulence as well as resistance to antimicrobial agents. In this study, 100 mL of water was collected from each of 245 distinct reservoirs of the public water system of one municipality in the state of São Paulo 15 days after the beginning of each climatic season to evaluate the diversity of the yeasts in the water and their susceptibility to antifungal agents. The results show that 11.4% of the water samples were positive for yeasts, with *Rhodotorula minuta* the most prevalent in spring, autumn and summer, and *Candida parapsilosis* complex in winter. Resistant phenotypes appeared in 66.5% of the isolated yeasts, with *Rhodotorula minuta* being the most prevalent resistant species found in the current study. The presence of yeasts in water, especially the genus *Rhodotorula* spp. is a public health concern because it is a common opportunistic pathogen in human and animal infections. The results of this study suggest that water quality control protocols require changes.

Key words: Public water system; Resistance to antifungal agents; Seasonality; Yeast diversity

1. Introduction

Recently studies showing the conveyance of microorganisms in potable water are receiving special attention because the quality of the water is altered and contaminated water can affect public health when ingested or even after contact with skin and mucous membranes, especially in hospitals (Carneiro et al., 2015; Oliveira et al., 2016).

In Brazil, the norms described in Ordinance No. 2.914 of December 2011 determine quality control constituted by the physical chemistry and microbiological analysis of water. The physical chemistry analysis follows the parameters of maximum allowed values: pH between 6.0 and 9.5, color of 15 HU (Hazen Unit: mg Pt-Co/L), 5 turbidity units (TU), 10 mg/L nitrate (N), 1.5 mg/L of fluorine and free residual chlorine between 0.2 mg/L and 2 mg/L. The microbiological norms include the absence of pathogenic microorganisms and fecal bacteria (BRASIL, 2011). For the European Community and the US Environmental Protection Agency, the physical chemistry criteria of water follow the same guidelines as the Brazilian norms but other groups of microorganisms, such as *Cryptosporidium* spp., *Giardia lamblia*, *Legionella* spp., *Enterococcus* spp. and some viral groups, are included (APHA/AWWA/WEF, 2012; Directive, 1998). It is noteworthy that fungi are not included as possible agents of water contamination in any of these documents.

Human domestic, hospital and food industry activities depend on water for routine sanitation and cleaning practices. Water for human consumption, regardless of its origin, whether from wells or the public supply systems, is analyzed by official bodies for quality standards. However, the final consumer is responsible for preserving the quality of the domestic supply (BRASIL, 2011).

Several researchers have found a wide diversity of fungal species in the surface waters of lakes and rivers, domestic sources, storage tanks and hospitals with the following fungi being prevalent: *Aspergillus* spp*., Fusarium* spp*., Scedosporium* spp*., Penicillium* spp*., Phialophora* spp., *Exophiala* spp., *Phoma* spp., *Acremonium* spp., *Cladosporium* spp*., Candida* spp*.,* e *Rhodotorula* spp. (Al-gabr et al., 2014; Ma et al., 2015; Medeiros et al., 2012; Sisti et al., 2012).

Studies in Health Institutions have confirmed the correlation of pathogens present in water and diseases in humans, with or without immunological impairment (Moghadam et al., 2015; Oliveira et al., 2016; Sisti et al., 2012). Water in hospitals with these conditions of can enter in contact with patients through faucets and showers resulting becoming a risk for infectious diseases. It is considered that the concentration of microorganisms can vary according to the physical chemistry conditions of water supply systems and the formation of biofilm can often occur. The biological and chemical environment generated by the biofilm itself leads to phenotypic alterations of virulence and antimicrobial resistance, resulting in new communities of microorganisms (Altun et al., 2014; Brilhante et al., 2011; Nunes et al., 2013; Posteraro et al., 2015). When present in the pipes of the water supply network (Al-gabr et al., 2014; Fish et al., 2015; Nett et al., 2015; Oliveira et al., 2016), corroded and retentive areas are created by the products of the biofilm that contribute to the formation of complex microbial systems.

Yeasts are common inhabitants in aquatic environments. Some authors suggest that yeasts should be used as bioindicators of water pollution in association with fecal bacteria (Carneiro et al., 2015; Medeiros et al., 2012; Monapathi et al., 2017; Silva-Bedoya et al., 2014). However, these microorganisms when present in domestic and

hospital water supplies are considered etiological agents of opportunistic fungal diseases, as demonstrated by several studies (Nunes et al., 2013; Pontara et al., 2011). Additionally, reports on the occurrence of yeasts resistant to antifungal agents have an impact on public health due to the inexistence of specific treatment (Mata et al., 2015; Monapathi et al., 2017; Wirth, 2011).

The association of yeasts of the genus Candida in mono and multispecific biofilm favors the permanence and dissemination of microorganisms. The resulting metabolic products may alter the chemical properties of water and, in contact with humans and animals, become potential hazards for allergic processes and intoxication (Hirota et al., 2016; Mata et al., 2015; Nett et al., 2015; Seneviratne et al., 2016).

The objective of this study was to evaluate the occurrence and genetic diversity of yeast species in the public water supply as well as to evaluate antifungal susceptibility patterns.

2. Materials and Methods

2.1 Samples

Samples were collected from the public water supply of a municipality in the state of São Paulo, Brazil during the four seasons of the year. The collection points were located in two distinct geographic regions of the city, the north (n = 113) and south (n = 130), as well as for two points in the water treatment plant reservoir.

The 1923 samples were collected 15 days after the beginning of each season of the year, as follows: 605 in the spring; 497 in summer; 456 in the fall and 365 in the winter.

Sterile glass vials containing a 10% sodium thiosulfate pellet were used to

collect 100 mL of water after opening the faucet for two minutes with a continuous water flow (BRASIL, 2011). The samples were placed in isothermal boxes and transported to the microbiology laboratory for analysis. The entire volume of collected water was filtered through a 0.2 μm cellulose membrane (Millipore, CA, USA) which was subsequently upturned on Sabouraud Dextrose Agar medium (SDA) (Difco Laboratories, Detroit, MI) in a petri dish and incubated for 30 days at 30°C.

2.2 Identification of the yeasts

Yeast colonies isolated from the Sabouraud Dextrose Agar (Difco Laboratories, Detroit, MI) were inoculated in CHROMagar Candida medium (CHROMagar Microbiology, Paris, France) and ID32C - Ref 32 200 panel (BioMérieux SA, Marcy l'Etoile , France) following the instructions of the manufacturer. The results obtained using the panel were input into the apiweb^{TM} program for species identification.

2.3 Antifungal Sensitivity Test

Antifungal susceptibility assays were performed according to M27-S4 published by the Clinical and Laboratory Standards Institute (CLSI, 2012). The test utilized RPMI-1640 liquid medium (Sigma Chemical Co., St. Louis, MO) buffered with morphopropylene sulfonic acid (MOPS - Sigma Chemical Co., St. Louis, MO) plus 2% glucose and the pH was adjusted to 7.0 with sodium hydroxide (NaOH). Yeast were previously subcultured on Sabouraud Dextrose Agar (Difco Laboratories, Detroit, MI) and incubated for 24 hours at 35°C. Standardization of the inoculum used the 0.5 scale of MacFarland adjusted to between 85% and 95% transmittance in a spectrophotometer (Biospectro) at a wavelength of 530 nm. The antifungal agents used, Amphotericin B

(Sigma Chemical Co., St Louis, MO), Ketoconazole (Janssen Pharmaceutica, Belgium), Fluconazole (Pfizer, Inc., USA) and Itraconazole (Janssen Pharmaceutica, Belgium), were added to 100 µL standard inoculum in 96-well sterile microplates (Cralplast). Plates were incubated in a bacteriological oven at 35°C for 24 hours for determining the Minimum Inhibitory Concentration (MIC).

2.4 Statistical analysis

The Z test was used to compare the proportions of positive yeast samples between the northern and southern water sample collection points. The chi-square test was applied to evaluate the dependence of resistant yeasts in the different seasons of the year and collection points.

3. Results

3.1 Isolation, identification and seasonal distribution of samples

Of the 1923 water samples, yeasts were found in 11.4% (n = 221) of the samples fifteen of these presented more than one species, totaling 236 yeasts. Of these isolates, 37.3% (n = 88) were obtained in the fall, 30.5% (n = 72) in the spring, 22.9% (n = 54) in summer and 9.3% (n = 22) in winter.

The northern region had the highest number of positive samples with 49.1% (n = 116) (p-value <0.05) compared to the southern region with 33.9% (n = 80) and the treatment plant reservoirs with 17% (n = 40).

Considering the species of yeasts found, 66.5% (n = 157) were *Rhodotorula* spp., 29.2% (n = 69) *Candida* spp., 1.7% (n = 4) *Trichosporon* spp. and 1.3% (n = 3) *Aureobasidium* spp. *Cryptococcus neoformans*, *Geotrichum candidum* and *Kodamaea*

homeri were rare with a frequency of 1.3% for each species.

In the seasonal distribution of prevalent species (Fig. 1), *Rhodotorula minuta* occurred predominantly in the hot seasons with 48.6% (n = 35) in the spring, 46.3% (n = 25) in the summer and 35.2% (n = 31) in the fall. The *C. parapsilosis* complex (*C. orthopsilosis*, *C. metapsilosis* and *C. parapsilosis*) occurred in all seasons, especially *C. parapsilosis stricto sensu* in autumn (n = 22) and *C. orthopsilosis* in winter (n = 4). Only one isolate of *C. metapsilosis* occurred in winter.

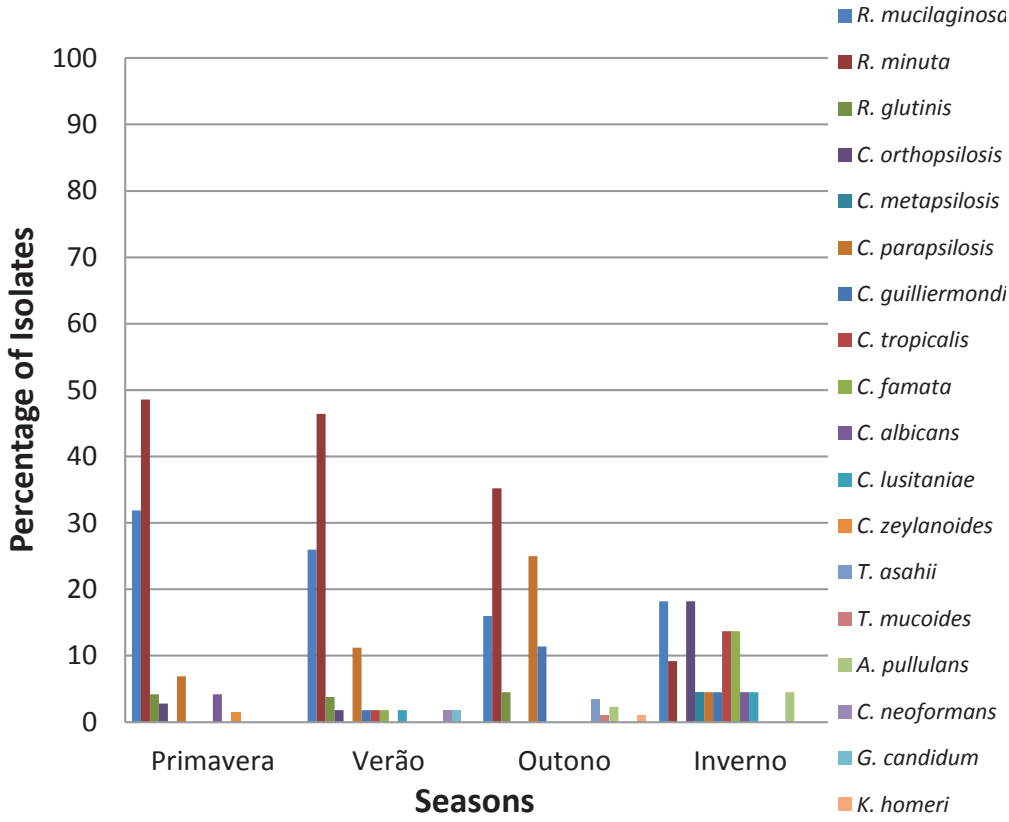


Figure 1 - Seasonal distribution of yeast species in the public water supply

With reference to physical chemistry characteristics, only the color was within the normal permitted range (15 HU) for all the samples. The following values are not allowed: chlorine 1% (n = 19), pH 0.05% (n = 1), fluorine 0.1% (n = 2) and turbidity

0.1% (n = 2). Fungi were found in all the samples that the chlorine concentration was below the permitted level.

3.2 Antifungal susceptibility

Of the 236 yeasts isolated from water, 66.5% (n = 157) presented resistance to the antifungal agents tested: 86% (n = 135) were resistant to Itraconazole, 74.5% (n = 117) to Fluconazole, 17.9% (n = 28) to Amphotericin B and 7.6% (n = 12) to Ketoconazole.

Forty-five (28.7%) were resistant to just one antifungal agent, 89 (56.7%) to two, 21 (13.4%) to three and two (1.2%) were resistant to all four antifungal agents.

Regarding the seasons, the highest rate of resistant yeasts was found in summer with 72.2% (n = 39) although this was statistically insignificant (p-value >0.05). In the other seasons, the percentages of occurrence varied between 36.4% (n = 8) in the winter, 57.9% (n = 51) in the fall and 69.4% (n = 59) in the spring.

The yeast resistance phenotype was not associated with the geographical origin, with 52.2% (n = 82) found in the northern region and 31.2% (n = 49) in the southern region, (p-value >0.05). At the two collection points of the water treatment plant reservoirs, resistant yeasts were found in 16.6% (n = 26) of the samples.

Rhodotorula minuta was the most common resistant species found in the present study with 54.8% (n = 86) of the isolates, followed by *Rhodotorula mucilaginosa* with 33.1% (n = 52). The remaining 12.1% (n = 19) were distributed among *R. gutinis* (n = 8), *C. guilliermondii* (n = 3), *C. albicans* (n = 3), C. *tropicalis* (n = 1), *C. famata* (n = 1), *C. parapsilosis* (n = 1), *K. ohmeri* (n = 1) and *T. mucoides* (n = 1).

4. Discussion

4.1 Isolated yeasts, seasonal distribution and pathogenic potential

The presence of yeast in the public water supply in this study was low (12.2%). However, considering the various fungal species detected at 221 water sample collection points, the potential risk for superficial or deep fungal diseases exists, especially in patients with immunological impairment. In addition, the possibility of proliferation in domestic water tanks cannot be excluded, which increases the risk of infections. Superficial, subcutaneous and deep mycoses can be caused by direct skin contact, trauma, in the digestive tract or simply by inhalation. In this sense, when the fungus is present in water, the potential of transmission is high, either as a primary or opportunistic pathogen (Monapathi et al., 2017). Studies in Australia, Brazil and Egypt report yeasts in public water supplies and in water sold for human consumption, with high incidence rates of between 18% and 75% (Mohamed et al., 2014; Pontara et al., 2011; Sammon et al., 2010; Sessegolo et al., 2011). Great variations in fungi contamination rates in different water sources such as the sea, lakes and domestic water supplies was reported by Al-gabr et al., (2014), Medeiros et al., (2012) and Oliveira et al., (2011). It was found that salinity, pH and temperature outside the allowed range, and organic and inorganic compounds may favor the appearance of some fungi, but may control other more sensitive species thereby explaining the differences found (Pontara et al., 2011; Sessegolo et al., 2011; Silva-Bedoya et al., 2014). In the current research, these parameters were not evaluated except for the pH, which prevents investigations of the correlation between fungi and other physical chemistry conditions.

In general, fungal epidemiology studies in environmental settings report high incidences in periods of high temperatures and low humidity (Carneiro et al., 2015;

Shahbazy et al., 2015), data that corroborate the current research.

The geographical characteristics of the collection points, north and south in the current research, are different. In the northern region, the wells are deeper (1000m) than the southern region, as they draw water from the Guarani aquifer, with high temperatures (43º C). However, the water in the southern region is predominantly from the Bauru aquifer, which is closer to the surface (200-300m deep). The higher occurrence of yeasts in the northern region can be explained by the better physical conditions for cell multiplication because of the high temperature and alkaline pH (8.5). Data from the literature show that waters that are more superficial usually contain a higher amount of microorganisms (Hageskal et al., 2007, 2006), contrary to what was found in this research.

The presence of fungi in water samples with insufficient chlorination occurred as expected. In the water supply system, the use of automatic pumps to introduce chlorine may possibly not add the product due to equipment failure, favoring microbial contamination. The presence of fungi in chlorinated water was unexpected and points to the inefficiency of the chemical in microbial control, a fact previously reported by Doggett (2000).

Fluoride and turbidity levels found in this study did not affect the finding of yeasts in water.

Rhodotorula spp. and *Candida parapsilosis* were the most common yeasts, thus corroborating the studies of Medeiros et al. (2012), Monapathi et al. (2017) and Silva-Bedoya et al. (2014). Although in the present investigation no analysis of biofilm formation by yeasts was performed, this event may justify the wide distribution of these species in water samples and have a direct impact on public health. Infections caused by

these fungi range from localized patches involving the skin, paranasal sinuses and lungs to disseminated infections and, given the low frequency of occurrence, few clinical, therapeutic and epidemiological data are known (Wirth, 2011).

4.2 Analysis of antifungal susceptibility

As expected and consistent with the literature, 93% (n = 146) of the *Rhodotorula* species found in the current study were resistant to azole antifungal agents (Altun et al., 2014; Nunes et al., 2013; Posteraro et al., 2015). However, only one isolate of *C. parapsilosis* presented a resistance phenotype to Itraconazole. In relation to Amphotericin B, resistance phenotypes were detected in 15.9% (n = 25) of the *Rhodotorula* species and in the *Kodamaea ohmeri* isolate. The induction of antifungal resistance in the public water supply is rare since potential inducer molecules in this niche are diluted or rarely present. Thus, patterns of antifungal susceptibility are constant. The northern region had the highest number of isolates with resistance phenotypes 52.2% (n = 82). This fact may be explained by the proximity to rural areas and the use of antifungal agents in agricultural, which are absorbed by the soil, and reach the water table with probable contamination of the water.

5. Conclusion

The results obtained in this study demonstrate that it is important to include investigations of fungi in water quality protocols, as the dissemination of these agents poses a risk to public health. The common presence of *Rhodotorula* spp. in water increases the concern for public health since it is frequently an opportunistic pathogen in human and animal infections. Adaptations of public water supply quality control

protocols are necessary with the inclusion of investigations for fungi.

Acknowledgements

The authors wish to thank the Serviço Municipal Autônomo de Água e Esgoto - SeMAE for the water samples and physicochemical data, Prof. Dr. Fernando Ferrari of the Department of Computer Science and Statistics of the Paulista State University "Júlio de Mesquita Filho" - UNESP for the statistical analyzes of the study, and David Hewitt for support in the translation of the article.

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ARTIGO 2

ARTIGO 2

Epidemiology of filamentous fungi in a treated public water supply

Natalia Seron Brizzotti Mazuchi[1], Keith Cássia da Cunha[2], João Paulo Zen Siqueira[1], Bianca Gottardo de Almeida[3], Thiago Henrique Lemes[3], Diego Maximiano da Conceição[1], Maicon Henrique Caetano[3], Mariela Domiciano Ribeiro[1], Cláudia Regina Rodrigues[4], Elza Maria Castilho[1], Margarete Teresa Gottardo de Almeida[1*].

[1] *Department of Infectious Diseases, Faculty of Medicine of São José do Rio Preto, Av. Brigadeiro Faria Lima, 5416, Vila São Pedro, 15090-000, São José do Rio Preto, São Paulo, Brazil.*

[2] *Dermatology Laboratory, University Hospital of Geneva, Street Gabrielle Perret-Gentil 4, 1205, Geneva, Switzerland.*

[3] *Post-Graduate Program in Microbiology, São Paulo State University (Unesp), Institute of Biosciences, Humanities and Exact Sciences (Ibilce), Campus São José do Rio Preto, Street Cristóvão Colombo, 2265, Jd. Nazareth, 15054-000, São José do Rio Preto, São Paulo, Brazil.*

[4] *Capture/Quality Division, SeMAE- Autonomous Municipal Water and Sewage Service, Street Antônio de Godoy, 2181, Jd Seixas, 15061-020, São José do Rio Preto, São Paulo, Brazil.*

***Correspondence and reprint requests:**

Margarete Teresa Gottardo de Almeida (Ph.D.)

Department of Infectious Diseases, School of Medicine of São José do Rio Preto.

Av. Brig. Faria Lima, 5416, 15090-000, São José do Rio Preto, São Paulo, Brazil.

Phone: +55 17 3201 5843

E-mail: margarete@famerp.br

Abstract

The survival capacity of fungi in water is generally high. The presence of *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. and other less common genera in water supplies changes the taste, odor and appearance of the water. Pathogenic or non-pathogenic waterborne species can cause disease in healthy and immunocompromised individuals through the inhalation of fungal spores and thus constitute a risk factor for respiratory and systemic diseases. Brazilian and international guidelines establish water quality control standards in respect to physicochemical and microbiological standards but without considering fungi. In this study, 100 mL samples were collected from 245 distinct points of the public water supply of a municipality in the state of São Paulo 15 days after the beginning of each climatic season, in order to evaluate the diversity and composition of fungal communities. The results of this work revealed that 26.3% of the samples were positive for filamentous fungi with the genus *Fusarium* spp. prevalent in the spring, summer and winter and *Penicillium* spp. prevalent in the fall. The presence of filamentous fungi in the water highlights the importance of constant monitoring for these microorganisms in standard potable water analysis protocols.

Key words: Filamentous Fungi, Public Water Supply, Seasonality

Introduction

The presence of microorganisms in water has been reported in recent studies, with descriptions of health problems caused by primary or opportunistic pathogens (Pereira et al. 2013; Al-gabr et al. 2014; Ma et al. 2015).

Although the fungi have a ubiquitous character, their significance in water has been explored little (Oliveira et al. 2011; Bonnal et al. 2015; Oliveira et al. 2016; Scala et al. 2016). However, it is known that the survival capacity of fungi, especially filamentous fungi, is high in the aquatic environment (Oliveira et al. 2011).

In a study by Ma et al. (2015), the application of monochloramine in a hospital hot water distribution system did not change the fungal community. Pereira et al. (2013) tested the concentration of free residual chlorine in water that was capable of inhibiting fungal growth and found that a concentration of 3 mg/L is efficient even though the maximum legal limit is 2 mg/L. In a study by Oliveira et al. (2013), common genera such as *Penicillium* spp. and *Aspergillus* spp. were subjected to temperatures of between 30°C and 42°C to verify their growth capacity; the authors revealed that these fungi could develop normally at high temperatures.

In water supply systems, fungi can cause changes in taste, odor and appearance, thereby affecting the quality of drinking water. Furthermore, pathogenic or non-pathogenic waterborne species can cause disease in healthy and immunocompromised individuals (Ma et al. 2015; Oliveira et al. 2016).

Preliminary studies analyzing water samples from the beach, surface water, groundwater and drinking water found high frequencies of fungi in the different sources of water. *Aspergillus* spp., *Fusarium* spp. and Pen*icillium* spp. were among the prevalent genera with the Aspergillus spp. species being the common denominator

(Oliveira et al. 2011; Oliveira et al. 2013; Al-gabr et al. 2014; Shahbazy et al. 2015; Oliveira et al. 2016). Opportunistic fungi were also found in the niches of these studies including *Acremonium* spp., *Cladosporium* spp., *Curvularia* spp., *Exophiala* spp.; *Mucor* spp., *Phialophora* spp., *Phoma* spp., *Rhizopus* spp., *Scedosporium* spp., *Trichoderma* spp., *Candida* spp. and *Rhodotorula* spp. (Oliveira et al. 2011; Oliveira et al. 2013; Al-gabr et al. 2014; Ma et al. 2015; Oliveira et al. 2016; Prest et al. 2016).

The inhalation of fungal spores present in water is common as the primary contact of the host and fungus, instituting a risk factor for respiratory and systemic diseases (Al-gabr et al. 2014; Ma et al. 2015; Shahbazy et al. 2015; Prest et al. 2016).

In the hospital environment, fungal diseases - bronchopulmonary or systemic diseases - are responsible for the death of 1.4 million people/year, particularly in intensive care units (Shor & Perlin 2015). In this scenario, the fungus *Aspergillus* spp., a prevalent micro-organism that can cause liver disease and cancer, is of great importance (Oliveira et al. 2013; Pereira et al. 2013; Al-gabr et al. 2014; Bonnal et al. 2015).

Brazilian Directives (Ordinance No. 2914 of December 2011) and international such as those of the United States Environmental Protection Agency (APHA, 2012) and the European Union (EU) (Directive 98/83 EC of 3 November 1998) were set up to control water quality using physicochemical and microbiological standards as well as standard analysis techniques (ISO5667- 5: 2006). However, these documents only outline limits for bacteria, algae, protozoa and viruses without any mention of fungi.

According to the Ministry of Health, the cost of treating diseases transmitted or caused by contaminated water in Brazil is 2.7 billion dollars a year (Yamaguchi et al. 2013).

As the analysis or monitoring of water quality is essential for human health, the objective of the present study was to evaluate the diversity and composition of filamentous fungus communities in public water supplies in the different seasons of the year.

Methods

Over one year, 1923 water samples were collected from a public water supply at 245 distinct reservoirs (northern region: n = 113; southern region: n = 130; internal reservoir of water treatment plants: n = 2) in a municipality in the state of São Paulo. The samples were collected 15 days after the start of each season: spring (n = 605), summer (n = 497), fall (n = 456) and winter (n = 365).

After opening the faucet for two minutes with a continuous water flow, samples were collected in sterile glass vials (100 mL) containing a 10% sodium thiosulfate pellet (BRASIL 2011), packed in Styrofoam boxes, and sent to the laboratory for analysis. Initially, 100 mL of each water sample was filtered through a 0.2 μm cellulose membrane (Millipore, CA, USA), which was then turned upside down and placed in contact with Sabouraud Dextrose Agar (SDA) culture medium (Difco Laboratories, Detroit, USA) in a Petri dish. The Petri dish was incubated at 30°C for 30 days with visual analysis every day. Identification of the genus and species of filamentous fungi was performed by morphological analysis of the giant colony and microscopy of cellular structures, as well as using the microculture technique in Potato Agar Dextrose (PAD) (Larone 1995; Lacaz et al., 1998).

Statistical analysis used the chi-square technique to calculate percentages of fungi found in the different water samples.

Results and Discussion

Several studies carried out in Europe, and North and South America describe the occurrence of filamentous fungus in different water sources including in residences, hospitals, lakes and sea water (Oliveira et al. 2011; Pereira et al. 2013; Ma et al. 2015; Oliveira et al. 2016). However, few studies have evaluated treated water from the public water supply.

In the present study, filamentous fungi, in particular *Fusarium* spp. (n = 162), *Chaetomium perpulchrum* (n = 66), *Aspergillus* spp. (n = 52) and *Penicillium* spp. (n = 40) in addition to Sterile Mycelium fungi (n = 107) were isolated in 543 water samples (26.3% of the total). Additional species were isolated at frequencies ranging from 0.2 to 3.3% as shown in Table 1. Other authors have identified these fungi in residential, hospital, lake, and marine waters (Oliveira et al. 2011; Al-gabr et al. 2014; Ma et al. 2015; Oliveira et al. 2016).

The second most prevalent species was *Chaetomium* spp., which had been found in soil, plant and air samples (Abbott et al. 1995; Wang, X. et al. 2016). However, to the best of our knowledge, this is the first report of the presence of this fungal species in treated water. The occurrence of this species in water is of concern, since it is the most common opportunistic fungi responsible for infections in hospitalized patients, whether immunocompromised or not. In fact, several pathologies are related to this fungi including respiratory tract infections, fungemia, sinusitis, keratitis (Schwartz et al. 2015; Bonnal et al. 2015). In this scenario, the species *Penicillium* spp., commonly identified in systemic human infections (Chan et al. 2016), is of note, while the association of *Chaetomium* spp. and *Aspergillus fumigatus* has recently been described as the etiology of pneumonia (Wang H. et al. 2016).

147 The other species listed in Table 1 are less common and have equal importance
148 as pathogenic microorganisms of human, animal and plant mycoses (Oliveira et al.
149 2013; Shahbazy et al. 2015).

150 In the present study, considering the seasonality and distribution of filamentous
151 fungi in the public water supply, winter was the most important season, with 42.4% (n =
152 155) occurrences, followed by fall (30.7%; n = 140), spring (26.1%; n = 158), and
153 summer (18.1%; n = 90). This association between the presence of filamentous fungi
154 and the season was significant (p-value <0.0001). On evaluating the ubiquity of fungi, it
155 is known that their occurrence and distribution are affected by several factors, such as:
156 spore size, density, temperature, relative air humidity and human activity (Jones &
157 Harrison 2004; Fang et al. 2007).

158 According to Scala et al. (2016), the spread of fungal diseases caused by
159 *Fusarium* spp. such as *Fusarium head blight* (*FEB*) during the flowering of wheat,
160 infects the heads of the crop, reducing grain yield. Nine species of *Fusarium* (n = 162)
161 were found over the four seasons of the year in this study; however, the number of cases
162 in summer was higher (n = 50), possibly due to the high rainfall with consequent
163 dissemination into the public water supply. This result was highly significant (p-value
164 <0.0001) indicating a strong association between *Fusarium* species and the different
165 seasons.

166 Detection of *F. sacchari* in winter corroborates the study by Scala et al. (2016)
167 regarding seasonality, even though the origins were different: wheat flowering and
168 water. The description of this species in recent studies indicates that this fungus is an
169 emerging human pathogen isolated in cases of keratitis and onychomycosis (Bansal et
170 al. 2016; Gupta et al. 2016).

The tropical regions favor the development of fungus due to the pattern of rainfall and temperatures between 25ºC and 37ºC. In this sense, the dissemination of *Fusarium incarnatum* is highlighted by the water activity on sorghum grains, as investigated by Lahouar et al. (2017), and in the current study on the public water supply.

The low occurrence of other *Fusarium* species in the present investigation does not exclude the possibility of clinical findings as primary or opportunistic agents of infection as reported in the studies of Collado et al. (2013) and Sidhu et al. (2013).

Penicillium species, described as microorganisms of clinical importance in intoxication and infection, originate from different environmental niches including air, soil and water (Oliveira et al. 2011; Oliveira et al. 2013; Al-gabr et al. 2014; Ma et al. 2015; Oliveira et al. 2016). The persistence of this fungus in water treated using chemical compounds was investigated by Le et al. (2011) and Pereira et al. (2013), who proved its high adaptability and resistance.

Several authors have reported a wide diversity of species of the *Penicillium* genus in water samples from rivers and the sea including *Penicillium citrinum*, *Penicillium griseofulvum*, *Penicillium glabrum*, *Penicillium janczewskii*, *Penicillium oxalicum* and *Penicillium waksmanii* (Pereira et al. 2013; Al-gabr et al. 2014; Oliveira et al. 2016). Discordant with previous studies, the current study found that *Penicillium decumbens* was the most prevalent member of this species (n = 16) in the fall. There are few reports in the literature that describe water as the source of this species. Kadaifciler et al. (2013) isolated this species in high-speed drill water in dental clinics in Istanbul, Turkey. The occurrence of opportunistic diseases by this fungal species has been

194 reported by some studies (Tomsíková et al., 1973; Alvarez 1990) thereby highlighting
195 the importance of further research.

196 As reported in this work, the occurrence of fungal species in water that have not
197 been reported in other studies warns of the spread of potentially pathogenic fungi in
198 drinking water supplies, exposing the population to opportunistic diseases and causing a
199 great impact on public health.

200

201 **Table 1 -** Distribution of filamentous fungi isolated in the different seasons of the year

Genus/Species	**Spring**	**Summer**	**Fall**	**Winter**	**Total Isolates**	**Isolates (%)**
Ascomycota						
Chaetomium perpulchrum	30	-	1	35	66	12.2
Monascus ruber	-	-	1	-	1	0.2
Coelomycetes						
Colletotrichum coccodes	1	-	1	-	2	0.4
Chaetophoma	1	-	-	-	1	0.2
Phoma cava	1	-	-	2	3	0.5
Phoma hibernica	4	-	-	-	4	0.7
Hyphomycetes						
Aspergillus alliaceus	-	1	3	-	4	0.7
Aspergillus caesiellus	-	-	2	-	2	0.4
Aspergillus carneus	3	-	-	-	3	0.5
Aspergillus clavatus	-	-	1	-	1	0.2
Aspergillus flavus	-	1	1	-	2	0.4
Aspergillus fumigatus	-	-	1	-	1	0.2
Aspergillus janus	1	-	-	-	1	0.2
Aspergillus japonicus	-	1	-	-	1	0.2
Aspergillus oryzae	-	-	2	-	2	0.4
Aspergillus penicillioides	-	-	8	-	8	1.5

Aspergillus potronii	-	-	-	1	1	0.2
Aspergillus restrictus	1	-	-	-	1	0.2
Aspergillus sclerotiorum	-	-	-	1	1	0.2
Aspergillus tamarii	6	2	1	15	24	4.5
Acremonium cuvulum	-	1	1	-	2	0.4
Acremonium hyalinulum	-	3	1	-	4	0.7
Acremonium potronii	-	-	2	-	2	0.4
Acremonium recifei	1	-	-	-	1	0.2
Acremonium roseogriseum	-	-	1	-	1	0.2
Alternaria alternata	-	-	1	2	3	0.5
Cladosporium bantianum	2	-	1	-	3	0.5
Cladosporium cladosporioides	1	-	-	3	4	0.7
Cladosporium carrionii	-	-	-	1	1	0.2
Curvularia geniculata	-	-	5	5	10	1.8
Curvularia lunata	1	-	2	5	8	1.5
Drechlera bisseptada	1	-	-	-	1	0.2
Exserohilum rostratum	2	-	2	6	10	1.8
Fusarium aquaeductuum	-	-	2	-	2	0.4
Fusarium chlamydosporum	-	9	1	5	15	2.9
Fusarium dimerum	2	5	4	2	13	2.4
Fusarium incarnatum	13	13	5	5	36	6.7
Fusarium oxysporum	1	3	2	-	6	1.1
Fusarium sacchari	17	17	10	24	68	12.6
Fusarium solani	10	-	-	3	13	2.4
Fusarium tabacinum	1	1	2	2	6	1.1
Fusarium verticillioides	-	2	1	-	3	0.5
Feococcus	1	-	-	-	1	0.2
Histoplasma capsulatum	-	-	-	1	1	0.2

Lecythophora mutabilis	1	-	-	-	1	0.2
Micelio esteril	40	15	38	14	107	19.8
Nigrospora painici	1	-	-	-	1	0.2
Penicillium citrinum	-	-	1	2	3	0.5
Penicillium decumbens	3	3	16	1	23	4.3
Penicillium expansum	1	-	-	-	1	0.2
Penicillium griseofulvum	-	-	1	-	1	0.2
Penicillium lilacinum	1	-	3	2	6	1.1
Penicillium marneffei	-	-	-	1	1	0.2
Penicillium purpurogenum	-	-	1	-	1	0.2
Penicillium rugulosum	-	-	1	-	1	0.2
Penicillium spinulosum	-	-	2	-	2	0.4
Penicilium verruculosum	-	-	1	-	1	0.2
Paecilomyces javanicus	4	-	-	2	6	1.1
Paecilomyces variotii	-	-	-	1	1	0.2
Phaeoacremonium	1	-	-	-	1	0.2
Scedosporium apiospermum	2	4	4	2	12	2.2
Scedosporium prolificans	1	-	-	-	1	0.2
Scopulariopsis brevicaulis	-	1	1	2	4	0.7
Scytalidium dimitiatum	-	1	1	1	3	0.5
Scytalidium hialinum	-	-	3	1	4	0.7
Scytalidium lignicola	1	-	-	-	1	0.2
Sporotrichum pruinosum	-	-	1	-	1	0.2
Sporothrix schenckii	-	-	1	-	1	0.2
Trichocladium asperum	-	1	-	-	1	0.2
Trichoderma viride	-	-	-	4	4	0.7
Zygomycota						
Mucor racemosus	1	2	-	4	7	1.4

Conclusion

Filamentous fungi are found in the water in all seasons of the year. Studies that analyze the presence of fungi in drinking water are increasingly important since some are human pathogens and others are opportunistic.

The presence of species not reported in the literature but found in the present study highlights the need for further research in order to prevent opportunistic infections and to encourage inspections for fungi being included in standard potable water analysis protocols in order to set limits and improve the mycological quality of water provided to the population.

This study calls for a review of the regulatory norms of water quality control, which currently only investigates bacteria, algae, viruses and some protozoa.

Acknowledgements

The authors wish to thank the Serviço Municipal Autônomo de Água e Esgoto - SeMAE for the water samples and physicochemical data, Prof. Dr. Fernando Ferrari of the Department of Computer Science and Statistics of the Paulista State University "Júlio de Mesquita Filho" - UNESP for the statistical analyzes of the study, and David Hewitt for support in the translation of the article.

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CONCLUSÕES

4. CONCLUSÕES

Os resultados obtidos com o estudo demonstram que a inclusão de fungos nos protocolos de investigação da Qualidade da Água é importante, uma vez que, a disseminação destes agentes coloca em risco a saúde pública. A presença de *Rhodotorula* spp., como a levedura frequentemente isolada em água, aumenta a preocupação para área de saúde pública; uma vez que é um patógeno oportunista comum em infecções humanas e em animais. A presença de fungos filamentosos na água é vista em todas as estações do ano.

Estudos que analisem a presença de fungos na água potável são cada vez mais necessários; uma vez, que a presença de espécies pouco relatadas na literatura, como encontrado no presente estudo, aumenta a importância de novas pesquisas na área. Devem abordar o propósito para prevenir doenças oportunistas e também, incluir a vigilância frequente para fungos nos protocolos padrões de análises de água potável, fixando limites e melhorando a qualidade micológica da água distribuída para a população.

Este estudo alerta para uma revisão das normas regulamentadoras do controle de qualidade da água, que atualmente apenas destacam bactérias e alguns protozoários.

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