Molecular characterization of mycobiota in four different drinking water sources in Jeddah City (Saudi Arabia)

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Running title (Drinking water mycobiota …..)

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Abstract

The study included collection of water samples (150 samples) from different sources of treated water (30 samples) and tap water in some hospitals (30 samples from cold water tap and 30 samples from hot water tap) and private houses (60 samples) belonging to each water network in Jeddah City (Saudi Arabia). From these sources, fungi were isolated and identified using the traditional methods. Moreover, the molecular genetic techniques including PCR amplification and sequencing of ITSs of unculturable fungi were also considered. All of sequenced strains were identified by similarity searches with ITS sequences in EMBL/GenBank. All strains that are defined in water samples of the distribution stations and private homes have been defined by traditional methods and has not been appreciated strains unable to grow (unculturable) on synthetic media. *Acremonium strictum* was detected as unculturable strain in hospital cold water. In hospital hot water, three fungal species have been identified by the traditional methods, which were also defined using the methods of molecular diagnostics (partial sequencing of ITS gene). In addition, 6 other species including *Acremonium strictum*, *Cladosporium cladosporioides*, *Rhizopus azygosporus*, *Penicillium citrinum*, *Penicillium oxalicum* and *Trichoderma viride* were identified using the molecular method. It can be concluded that, drinking water in Jeddah city contain many different fungi species that do not affect the quality of drinking water. However, some of these strains cause disease in humans, produce mycotoxins, or may cause allergic diseases. The study also emphasized the need for application of molecular diagnostic techniques as rapid and accurate methods for detecting the presence of fungi and to ensure the quality and safety of the water.

**Keywords:** DGGE, Hospital, ITS, PCR
Introduction

Limited attention has been given to the presence of fungi in the aquatic environment (Standard Method 9610, 1995; Dogget, 2000; Arvanitidou et al., 2002; Hageskal et al., 2006; Hageskal et al., 2007; Pereira et al., 2009). Further research is therefore needed in this area since waterborne fungi could also be associated with a variety of health related effects, taste and odour problems and contamination in food and beverages (Dogget, 2000). A study of fungal diversity and significance in the two USA distribution systems has also recently been conducted (Kelley et al., 2003), involving samples taken from several points throughout the systems. This included an examination of the ability of selected fungi to grow in water and to produce metabolites of concern to human health, and also looked at the effectiveness of different control measures. Kinsey et al. (1999) have summarized many of the main considerations in sampling fungi from water. Hageskal et al. (2006) reported a wide occurrence of fungi in Norwegian drinking water and concluded that the mycobiota of water should be considered when the microbiological safety and quality of drinking water are assessed. Several studies have focused on analysing hospital water systems with respect to the presence of fungi (Arvanitidou et al., 2000, Anaissie et al., 2001, 2003, Warris et al., 2001, 2003; Panagopoulou et al., 2002, Hapcioglu et al., 2005, Kanzler et al., 2007, Kennedy and Williams, 2007, Pires-Gonçalves et al., 2008,). The results indicate that hospital and private home water may contain a wide diversity of fungi, including potential pathogens. The hypothesis is that fungi in water are aerosolized into air when water passes installations, such as taps and showers, and thus introduced to severely immunocompromised patients with a high risk of fungal infections. Several microorganisms can cause taste and odor problems in drinking water, and such sensoric problems have been connected to the presence of fungi. Several fungal species, such as, e.g. Chaetomium globosum (Kikuchi et al. 1981), have been found to produce geosmin, a compound often associated with earthy odor and taste in drinking water (Paterson et al. 2007). In addition, fungi may produce a variety of other

With the continued development of genomic techniques, it is now possible to detect and analyse fungal DNA in environmental samples (Bridge et al., 1998). These techniques are based on the polymerase chain reaction (PCR) (Edel, 1998). A widely used approach is to conduct PCR using oligonucleotide primers specific for the conserved flanking regions of the internally transcriber spacers (ITS) of the fungal rRNA gene (White et al., 1990). The ITS primers are regarded as universal for fungi because rRNA genes appear to be present in most fungi (Gardes and Bruns, 1993). The technique has the potential for very sensitive detection of fungi in environmental samples. However, with environmental samples there is a need for caution as a range of factors can bias the DNA amplification, such as achieving initial DNA extractions for only a subset of the total species present, the presence of compounds inhibitory to amplification, obtaining primerprimer annealment, or the formation of chimeric co-amplified sequences (Wang and Wang, 1997; Hastings, 1999; Head, 1999).

As PCR techniques continue to become more widely used, further development will undoubtedly resolve many of these potential problems (Viaud et al., 2000). It is usual to incorporate further analysis of the PCR product, and this essentially involves either sequence analysis, restriction fragment analysis, or the use of taxon specific probes or primers (Bridge et al., 1998). In many cases the techniques target specific fungal taxa but some, although originally designed for bacteriological studies (examples in Hurst et al., 1997; Edwards, 1999), are targeted much more broadly and are being adapted for analyzing community structures in mixed fungal populations. Denaturing gradient gel electrophoresis (DGGE) involves running PCR product (using ITS primers) through a gel containing a gradient of denaturants; the sequence of the bases in the DNA determines at what point on the gel’s gradient the strands denature and hinder further migration, so that on visualization the resulting bands reflect the diversity of fungi present in the original sample. DGGE is
now beginning to be used for fungal populations (Kowalchuk et al., 1997; Vainio and Hantula, 2000).

This study was designed to achieve the following objectives: Applying new rapid molecular technique for detecting the fungal contaminants in water supply sources in Jeddah city, recognize the ability of these modern procedures for detecting the unculturable fungi in water sources.

**Materials and Methods**

**Water samples**

The sampling protocol of Hageskal *et al.* (2006) was applied in this study. Briefly, about 150 water samples were collected from 10 water supplies. Each water supply was sampled three times. Sample points were included treated water at the treatment plants and hot- and cold-water taps in hospitals and private homes attached to each supply network.

**Isolation of fungi**

The isolation procedure of Hageskal *et al.* (2006) was used in the present study. Briefly, 100-ml water samples were filtered through membrane filters. These filters were incubated on dichloran-18% glycerol agar (Hocking and Pitt 1980) in darkness at 20°C ± 1°C for up to 2 weeks. The number of colonies was determined and expressed as the number of CFU per 100-ml water sample.

The isolation frequency (percent) for each species was calculated by dividing the number of positive samples by the total number of samples.

Concentrations of each species were expressed as minimum and maximum numbers of CFU per 100 ml.

For isolation of pure single colonies, positive cultures were subcultured on potato dextrose agar (Oxoid, Basingstoke, United Kingdom) or malt extract agar (Oxoid) and incubated at 25°C ± 1°C for 7 days. For further work, representative isolates were stored on potato dextrose agar slants at 4°C.
Identification of fungi

Classic identification


To ensure correct identification of closely related species, some *Penicillium* species, in addition to morphological identification, were confirmed by detection of indole metabolites by a filter paper method (Lund, 1995.). Briefly, a piece of filter paper dipped in Ehrlich reagent (Samson, et al. 2004) was placed on an agar plug with mycelia. In the case of indole metabolite production, a violet ring will appeared within 10 min.

Rapid molecular identification

DNA extraction

The genomic DNA of culturable fungi isolates was extracted using the CTAB-method described by Ausubel et al. (1987). For direct extraction of DNA from water samples, 1 liter of water sample filtered through a Nuclepore polycarbonate filter, 47 mm in diameter, with a pore size of 0.45 µm (Whatman, Clifton, NJ). With sterile forceps, the membrane was carefully separated from the pad/base and placed into an empty, sterile Petri dish. Using flame-sterilized scissors, the membrane was cut into small pieces (about 1 cm²). DNA extraction was achieved using the Watermaster-DNAPurification Kit (Epicenter-Biotechnologies, WI, USA), which is referenced for DNA extraction from water matrices since it reduces possible PCR inhibition by natural organic matter (Pereira et al., 2010).
**Determine fungi population profiles using denaturing gradient gel electrophoresis**

Amplification of the internal transcribed spacer (ITS) gene was used for the comparison of different culture media in terms of diversity coverage and population dynamics in time (Pereira et al., 2010). Nested-PCR was used to improve the amplification of fungi with low number of representatives in the sample. The first step was carried out using primers ITS1 and ITS4 (White et al., 1990), without GC-clamp to facilitate amplification from whole genomic DNA. The resulting almost complete ITS fragments were then used as template in the second step, where a 40 bp GC-clamp (Muyzer et al., 1993) was incorporated to the ITS5 primer (White et al., 1990). The PCR mixture consisted of 1X ThermoPol buffer, 0.5 μM of each primer, 0.2 mM of dNTPs and 1.25 U of Taq DNA polymerase. The PCR protocol included a 7 min initial denaturation at 95 °C, 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by 5 min of final extension.

The PCR products were analyzed by DGGE that was performed using DCode System DGGE Apparatus (Bio-Rad, Hercules, USA), following the manufacturer’s instructions (Novinscak et al., 2008). The denaturing gradient was performed 25%–75% in a gel of 6% Acrylamide/Bis 40% after 16 h at 80 V in 1X TAE buffer. Gels were then stained for 10 min in ethidium bromide and destained in distilled water for 10 min. Bands of interest were excised from the gels and placed in 30 μl of 10 mM Tris overnight at 4 ºC. 9 μl of the Tris/band solution was used for sequencing with the Big Dye terminator sequencing kit v. 3.1 (Applied Biosystems, Foster City, USA) and primer R1378 on a 3130xl ABI Analyser (Applied Biosystems). All of sequenced strains were identified by similarity searches with ITS sequences in EMBL/GenBank.

**Results and Discussion**

In the present study, 150 water samples were collected from 50 points in the drinking water distribution system of Jeddah city, kingdom of Saudi Arabia. Samples were tested for
the presence of fungi. The highest isolation frequency of fungi (90%) was recorded for water samples collected from the private home (Table 1). The isolation frequency in cold and hot water obtained from hospitals were 70 and 76.7%, respectively. On the other hand, the lowest isolation frequency of fungi (40%) was obtained by the water samples collected from drinking water treatment plants. These results indicated that, the best quality of drinking water, from the mycobiologist point of view, was recorded in treatment plants. This quality was deteriorated from the starting point to the end points of distribution system. This finding was in agreement with the finding of Hageskal et al., (2006). They demonstrated that, a wide variety of fungi species is present in all parts of the water distribution systems in Norway. Moreover, piped drinking water can offer a transmission route for fungi.

Fungi numbers were also determined in all positive samples. Averages of fungi numbers in the positive samples of each tested points were statistically analyzed and illustrated in Figure 1. The lowest obtained numbers of fungi were found in the treatment water (TW). These numbers were significantly ($p<0.5$) increased in hospital cold water (HCW). More significant increase was also recorded when the private home water (PHW) was investigated. These results demonstrated that, not only isolation frequency (previously discussed) but also the mycobiota load was increased through the drinking water distribution system of Jeddah city. On the other hand, no significant ($p>0.05$) difference in the mycobiota load was obtained between TW and the hospital hot water (HHW). Comparing with PHW, HCW and HHW had low fungi number. This may be due to some additional treatments such as UV-treatment or ozonation that might be process to improve water quality. Also, heating of water might be lead to reduce the total number of fungi.

Occurrence of fungi species in the different four tested-points were tested in the positive samples (Table 2). In the treated water (TW), six species were identified by the classic methods. These species were *Alternaria alternate*, *Acremonium strictum*, *Mucor plumbeus*, *Penicillium montanense*, *Penicillium melinii* and. The most frequently fungi
species occurred in TW were *Trichoderma viride* (66.6 %) followed by *Alternaria alternate* and *Acremonium strictum* (50 %), whereas the lowest frequently occurred species was *Penicillium melinii* (25 %). Pereira et al., 2009 detected genera *Aspergillus, Cladosporium, Penicillium*, and *Candida* in three different drinking water sources in Portugal.

In the hospital cold water (HCW), 13 different fungal strains were identified by the classical methods. By applying the molecular method an addition species, *Acremonium strictum*, was identified. This means that, *Acremonium strictum* could be characterized as an unculturable strain. In general, *Penicillium montanense* was the dominant fungi strain in HCW followed by *Aspergillus fumigates* (Table 2).

In the hospital hot water (HHW), only three different fungi species were classically identified (Table 2). In addition to these three species, six additional species were also identified by the molecular method. In addition to *Acremonium strictum*, the species *Cladosporium cladosporioides, Rhizopus azygosporus, Penicillium citrinum, Penicillium oxalicum* and *Trichoderma viride* were also identified as unculturable strains in HHW. In a recent study of Pereira et al. (2010) *Acremonium* sp. was identified as unculturable strain in spring water. The highest occurrence percentage (19 %) was recorded by *Alternaria alternate* and *Penicillium montanense* in HHW. These two species were identified by both classic and molecular method (Table 2).

In private home water (PHW), 16 different fungi species were identified by the two examined procedure (classic and molecular methods). This means that, unculturable fungi species were not detected by the molecular method applied in this work (Table 2). *Trichoderma viride* was the dominant fungi (35 %) in PHW followed by *Alternaria alternate* and *Aspergillus niger* (27.8 %).

Generally, most filamentous fungi identified are known to be widely distributed in nature and could therefore present no harmful impact in terms of water quality. However, among the isolated fungi, there are potentially pathogenic, allergenic, and toxigenic species.
For instance, *Aspergillus fumigates* was recovered from 19% of the positive tested samples in general. This species was recovered from 26.1% and 24.1% from HCW and PHW, respectively (Table 2). The occurrence of potentially pathogenic species, such as *A. fumigatus*, in drinking water has lead to speculations whether hospital water systems may serve as a transmission route for fungal infections. Several studies have focused on analyzing hospital water systems with respect to the presence of fungi (Anaissie et al., 2003; Kanzler et al. 2007; Kennedy & Williams 2007; Pires-Goncalves et al. 2008). The results indicate that hospital water may contain a wide diversity of fungi, including potential pathogens. The hypothesis is that fungi in water are aerosolized into air when water passes installations, such as taps and showers, and thus introduced to severely immunocompromised patients with a high risk of fungal infections.

*Penicillium* species have been frequently recovered from water in the various studies performed. Several of the species in both genus *Penicillium* and *Aspergillus* are known to produce mycotoxins in other substrates, such as food and beverages (Pitt & Hocking 1999). However, mycotoxins produced in water will of course be extremely diluted, and are perhaps of minor concern (Hageskal et al., 2009).

On the other hand, several microorganisms can cause taste and odor troubles in drinking water. These sensory problems have been connected to the presence of fungi. In this respect, *Chaetomium globosum*, have been found in PHW in the present study. This fungus is known as a geosmin producer, a compound often associated with earthy odor and taste in drinking water (Paterson et al. 2007).

Generally, the total positive samples were 110 from total of 150 examined water samples from the different four sampling points. It can be concluded that, there are 18 different fungi species distributed in the drinking water network. From these species 6, 14 and 16 species were found in TW, HCW and HHW, respectively. This result indicated that number of species The dominant fungi species in the drinking water distribution systems in
general was *Trichoderma viride* (29.1 %) followed by *Penicillium montanense* (25.5 %) and *Alternaria alternate* (25.5 %) (Fig. 2). These species were found in all sampling points by different recovery percentage within each water type. This finding is in agreement with those mentioned by Hageskal et al. (2006). They stated that *Trichoderma viride* was found to be the most dominant species in Norwegian drinking water.

**Conclusions**

The general result of the present study demonstrated that, a wide diversity of fungi species has been isolated from drinking water. Also, the molecular techniques used in this study established an alternative quick procedure for determine the mycobiota in water supply sources.

**Acknowledgment**

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Amsterdam, The Netherlands.
Company, New York, N.Y.


Table 1. Distribution and isolation frequency of fungi at sample points of drinking water network in Jeddah city

<table>
<thead>
<tr>
<th>Sample point</th>
<th>No. of tested points</th>
<th>No. of samples</th>
<th>No. of Positive samples</th>
<th>Isolation frequency</th>
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<tr>
<td>Treatment Plants</td>
<td>10</td>
<td>30</td>
<td>12</td>
<td>40</td>
</tr>
<tr>
<td>Hospital</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold-water tap</td>
<td>10</td>
<td>30</td>
<td>23</td>
<td>76.7</td>
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<tr>
<td>Hot-water tap</td>
<td>10</td>
<td>30</td>
<td>21</td>
<td>70</td>
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<tr>
<td>Private home</td>
<td>20</td>
<td>60</td>
<td>54</td>
<td>90</td>
</tr>
</tbody>
</table>
Table 2. Occurrence of fungi species in the positive samples collected from different points in the drinking water distribution system of Jeddah city.

<table>
<thead>
<tr>
<th>Fungi species</th>
<th>TW n=12</th>
<th>HCW n=23</th>
<th>HHW n=21</th>
<th>PHW n=54</th>
<th>Total positive samples n=110</th>
<th>Species concentration Min-Max range (CFU/100mL)</th>
<th>Accession Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria alternate</td>
<td>6 (50)</td>
<td>3 (13)</td>
<td>4 (19)</td>
<td>15 (27.8)</td>
<td>28 (25.5)</td>
<td>4 – 15</td>
<td>FR717899</td>
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<tr>
<td>Aspergillus niger</td>
<td>----</td>
<td>5 (21.7)</td>
<td>2 (9.5)</td>
<td>15 (27.8)</td>
<td>22 (20)</td>
<td>2-18</td>
<td>FR717900</td>
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<tr>
<td>Aspergillus fumigates</td>
<td>----</td>
<td>6 (26.1)</td>
<td>----</td>
<td>13 (24.1)</td>
<td>19 (17.3)</td>
<td>1-8</td>
<td>FR717901</td>
</tr>
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<td>Acremonium strictum</td>
<td>6 (50)</td>
<td>1 (4.3)*</td>
<td>1 (4.8)*</td>
<td>11 (20.4)</td>
<td>19 (17.3)</td>
<td>4 – 32</td>
<td>FR717902</td>
</tr>
<tr>
<td>Chaetomium globosum</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>12 (22.2)</td>
<td>12 (10.9)</td>
<td>3 – 12</td>
<td>FR717903</td>
</tr>
<tr>
<td>Cladosporium cladosporoides</td>
<td>----</td>
<td>3 (13)</td>
<td>1 (4.8)*</td>
<td>12 (22.2)</td>
<td>16 (14.5)</td>
<td>6 – 21</td>
<td>FR717904</td>
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<td>Cladosporium herbarum</td>
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<td>4 (17.4)</td>
<td>----</td>
<td>15 (27.8)</td>
<td>19 (17.3)</td>
<td>2 – 4</td>
<td>FR717905</td>
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<td>Fusarium dimerum</td>
<td>----</td>
<td>1 (4.3)</td>
<td>----</td>
<td>11 (20.4)</td>
<td>12 (10.9)</td>
<td>8 – 43</td>
<td>FR717906</td>
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<td>Rhizopus azygosporus</td>
<td>2 (8.7)</td>
<td>1 (4.8)*</td>
<td>----</td>
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<td>14 (12.7)</td>
<td>1 – 3</td>
<td>FR717915</td>
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<tr>
<td>Mucor plumbeus</td>
<td>2 (16.7)</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>2 (1.8)</td>
<td>2 – 5</td>
<td>FR717907</td>
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<tr>
<td>Penicillium brevicompactum</td>
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<td>2 (8.7)</td>
<td>----</td>
<td>12 (22.2)</td>
<td>14 (12.7)</td>
<td>3 – 8</td>
<td>FR717908</td>
</tr>
<tr>
<td>Penicillium citrinum</td>
<td>----</td>
<td>3 (13)</td>
<td>2 (9.5)*</td>
<td>----</td>
<td>5 (4.5)</td>
<td>1 – 4</td>
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<tr>
<td>Penicillium glabrum</td>
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<td>----</td>
<td>----</td>
<td>11 (20.4)</td>
<td>11 (10)</td>
<td>0 – 2</td>
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<tr>
<td>Penicillium montanense</td>
<td>4 (33.3)</td>
<td>7 (30.4)</td>
<td>4 (19)</td>
<td>13 (24.1)</td>
<td>28 (25.5)</td>
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<tr>
<td>Penicillium melinii</td>
<td>3 (25)</td>
<td>----</td>
<td>----</td>
<td>13 (24.1)</td>
<td>16 (14.5)</td>
<td>0 – 2</td>
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<tr>
<td>Penicilliumolsonii</td>
<td>----</td>
<td>1 (4.3)</td>
<td>----</td>
<td>11 (20.4)</td>
<td>12 (10.9)</td>
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<td>Penicillium oxalicum</td>
<td>----</td>
<td>1 (4.3)</td>
<td>1 (4.8)*</td>
<td>12 (22.2)</td>
<td>14 (12.7)</td>
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<tr>
<td>Trichoderma viride</td>
<td>8 (66.6)</td>
<td>3 (13)</td>
<td>2 (9.5)*</td>
<td>19 (35.2)</td>
<td>32 (29.1)</td>
<td>6 – 22</td>
<td>FR717914</td>
</tr>
</tbody>
</table>

* species which did not able to phenotypically identify but could be identified by partial sequence of ITS gene as a rapid molecular technique.
**Figure 1:** Average of total fungi number in the positive samples of treatment water (TW, \( n = 12\)), hospital cold water (HCW \( n = 23\)), hospital hot water (HHW \( n = 21\)) and private home water (PHW \( n = 54\)) in drinking water distribution system of Jeddah city. Error bars represent the standard deviations. Columns with the same letter are not significantly different.
Figure 2: Occurrence percentage of the aquatic fungi species in drinking water distribution system of Jeddah city.