Evaluation of extraction methods for the isolation of dust mites, bacterial, and fungal PCR-quality DNA from indoor environmental dust samples: A new scope for indoors research

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Abstract
Dust mites, bacteria, and fungi are ubiquitous organisms found in household dust and have been related to the development of three types of human diseases: hypersensitivity responses (allergic reactions), infections, and toxicosis of the respiratory system. Sometimes, the presence of these organisms cannot be detected by typical methods, which prevents an effective and accurate identification of these pathological sources.

An effective isolation of PCR-quality DNA from indoors environmental samples will allow employing the PCR technology as an indoor detection technique for these biological sources. Several DNA extraction methods have been employed with soil or sediment samples, but their DNA products usually contain organic contaminants, polysaccharides, proteins, and tannins that inhibit the PCR amplification.

We compared for the first time, seven DNA extraction methods to identify the most efficient protocol for obtaining dust mites, bacteria and fungi PCR-quality DNA from indoor settings. The SoilMasterTM DNA Extraction Kit showed a highly sensitive, reproducible and fast (less than an hour) method for obtaining PCR-quality DNA from dust mites, bacteria, and fungi when compared with the other methods studied. An added modification (sample homogenization) to this kit positively influenced the dust mites' mitochondrial DNA extraction by increasing its ratio over proteins. By using the DNA extraction and PCR approach in indoors-dust samples, non-cultivable indoor biological contaminant organisms can also be identified, contributing to an accurate diagnostic test.

Key Words: Bacteria, DNA extraction; dust mites, environmental health; fungi; indoor-dust.

Introduction
Several extraction methods for the isolation of DNA from various sources (e.g. cells, tissue, and plants) have been extensively described in past literature (Sharma, et al., 2003; Laird, et al., 1991; Tkach and Pawlowski, 1999). Among the most commonly employed methods are the CTAB (cetyltrimethylammonium) (Doyle and Doyle, 1987) and its modifications (Huang, et al., 2000), and the Phenol/Chloroform extraction using several lysis buffers (SDS, Triton X100). However, when these methods are employed on soil or sediment samples, the DNA product usually contains organic contaminants such as humic and fulvic acids (Tebbe and Vahjen, 1993). These components can act as inhibitors in the DNA amplification by polymerase chain reaction (PCR), and false negative results may be obtained (Morera, 2001). In addition, these extraction methods are not efficient in obtaining PCR-quality DNA from samples that are inherently rich in polysaccharides, proteins, and tannins (e.g. bacteria, arthropods and fungi) (Krause, et al., 2001). To achieve PCR-quality DNA from these sources, further purification protocols must be performed (Braid, et al., 2003). These procedures result in expensive processing time and a high cost per sample. Some methods have overcome the limitations inherent in these protocols and are capable of extracting PCR-quality DNA of bacterial and fungal origin from soil and sediment samples (Yeates, et al., 1998; Burgmann, et al., 2001; Knec and Wellington, 1999), as well as fungal DNA from indoor air settings (Haugland, et al., 2002). However, in the current literature, there is no description of an extraction method that can be used on indoor-collected dust samples for obtaining PCR-quality DNA from bacteria and/or domestic mites. These organisms (bacteria, fungi, and domestic mites) are consistently found world wide in households dust and have been related to the development of allergic diseases such as asthma, rhinitis, and atopic dermatitis, infections, and toxicosis at the respiratory system (Arlian and Platts-Mills, 2001; Nelson, 2001; Menetrez, et al., 2001; Meis, 2002). Certain species of these agents are also known to sensitize some individuals to other diseases (Foster, et al., 2003).

In this project, we compared for the first time, seven DNA extraction procedures (the Epicentre SoilMasterTM DNA Extraction Kit protocol, a modification to the Epicentre SoilMasterTM DNA Extraction Kit protocol, QIAmp DNA Mini Kit, CTAB, and three modifications of Phenol/Chloroform protocol) to obtain PCR-quality DNA of dust mites, bacteria, and fungi from indoor environmental samples. We intended to identify the most efficient, simple, and least time consuming protocol. The generation of PCR-quality DNA from indoor environmental samples will allow the use of PCR technology as an innovative detection tool of these organisms independently of their cultivable status.

Experimental procedures
Indoors sample collection Using a hand held vacuum cleaner (Medivac, England), dust was collected from 10 mattresses at a rate of 2 m2/min during three minutes. The vacuum cleaner’s nozzle was equipped with a 300mm stainless steel mesh and a 50mm diameter filter. Approximately 1g of dust was collected from 10 different locations and each sample was placed in sterile plastic bags. The 10 individual samples were mixed in a sterile plastic container in order to obtain a pooled dust sample. Eightyfour samples (12 per method, 100mg each and selected randomly) were weighed in 2ml Eppendorf sterile tubes and stored at -20°C until DNA extraction.

DNA Extraction
Total DNA from indoor dust samples (100mg each) was extracted using seven DNA extraction protocols and 12 repetitions per method:

1. Epicentre SoilMasterTM DNA Extraction Kit (EpiN): Protocol was followed as described in Meis and Chen (2002).
2. Epicentre SoilMaster™ DNA Extraction Kit with modification (EpiH): At step 2 according to the manufacturer’s manual, after the addition of the Soil DNA Extraction Buffer, a 30 seconds homogenization procedure was included and then the protocol was continued, by following the remaining steps as described by the manufacturer.

3. QIAAGEN®(QIamp DNA Mini Kit): DNA extraction was performed following the manufacturer’s instruction manual.

4. CTAB (cetyltrimethylammonium bromide): A volume of 500mL of 1% CTAB, 50mM Tris-HCl, pH 8.0, 10mM EDTA, 0.7 M NaCl, 0.1% 2-mercaptoethanol, was added to the sample and incubated at 60°C for one hour. Chloroform/isomyl alcohol (700 mL) was added; samples were mixed gently and thoroughly and spun at 14,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new tube and the process was repeated. Precipitation was done with 1X volume of isopropanol, followed by 2X ethanol and 1/10 3M Sodium Acetate (pH 5.2). The samples were stored at -80°C overnight followed by a 15 minutes maximum speed centrifugation at 4°C. Supernatant was removed.

5. Phenol/Chloroform: Samples were treated with 200mL of three different ysis buffers (B1, B2 and B3), homogenized for 30 seconds, and spun briefly. A total of 200mg of Proteinase K were added to the samples, followed by three hours incubation at 64°C. Saturated phenol (500mL) was added and mixed by inversion. A maximum speed centrifugation for 15 minutes at room temperature was performed. The upper aqueous phase was transferred to a new tube and chloroform (500mL) was added, followed by 15 minutes of centrifugation (RT). The upper phase was retained in a new tube and treated with 2.5X volume ethanol and 1/10 volume of 3M sodium acetate. The samples were stored at -80°C overnight followed by a 15 minutes maximum speed centrifugation at 4°C. Supernatant was removed. Components of the three lysis buffers were:
   a. Buffer 1 (B1): 10mM Tris-HCl, 320mM sucrose, 5mM MgCl2, 1% Triton X100
   b. Buffer 2 (B2): 100mM Tris-HCl, 100mM EDTA, 0.25% SDS
   c. Buffer 3 (B3): 50mM Tris, pH 8; 100mM EDTA, 100mM NaCl, 1% SDS

Table 1.0 Primer sets sequences and expected fragment size employed in PCR of DNA from indoor environmental samples

<table>
<thead>
<tr>
<th>Organism Group</th>
<th>Primer Pair</th>
<th>DNA sequence</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthropods</td>
<td>12SF, 12SR</td>
<td>5’-TACTATGTTAGCACTTA-3’</td>
<td>~400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-AAACTAGGATTAGTACCA-3’</td>
<td></td>
</tr>
<tr>
<td>Fungi, Protists, and Green Algae</td>
<td>NS1, NS2</td>
<td>5’-GTAGCTATGCTTGTGTTC-3’</td>
<td>555</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-GGCTGCTGGACGATTGC-3’</td>
<td></td>
</tr>
<tr>
<td>Bacteria consensus</td>
<td>P4, P5</td>
<td>5’-AACGCGAAGAAAACCTTA-3’</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-CGGTGTGTACAAGGCCGCCG-3’</td>
<td></td>
</tr>
</tbody>
</table>

The air-dried precipitated DNA from all seven protocols was re-suspended in 25mL TE (Epicentre SoilMaster™ DNA Extraction Kit). Further purification of 200ml from the CTAB, B1, B2 and B3 DNA extractions was performed using the QIAamp DNA Mini Kit (QIAGEN®) following the manufacturer’s instructions: CTABP, B1P, B2P, and B3P.

Absorbance ratios

Absorbance ratios (A260/A280) were determined by a GeneQuant Spectrophotometer (Pharmacia Biotech). Two readings per sample were performed, the mean value per tube was calculated (84) and then the mean values for each method (12) were used to construct a comparison table (Table 2.0).

PCR amplification and Gel electrophoresis

PCR from the DNA of dust mites, fungi, and bacteria was performed using a Triple Master Kit (Eppendorf Co.). A PCR reaction volume of 20ml: 5.0ml dust DNA, 0.5ml of each 10mM primer (Table 1.0), 3.2ml of 10X High Fidelity Buffer with Mg2+, 0.4ml of dntp’s mix (10mM each), 0.3ml of Triple Master Enzyme Mix (1.5u), and 1.0ml of molecular grade distilled water. PCR conditions were set in an Eppendorf Gradient Mastercycler as follows: denaturation for 5 min at 94°C and 30 cycles of amplification using a step program of 20 sec at 94°C, 20 sec at 38.4°C (12S) or 52.0°C (P4/P5 and NS1/NS2), and 5 min at 72°C. This was followed by a final extension of 10 min at 72°C and held at 4°C. PCR products were stored at -20°C until electrophoresis was performed in a 3% agarose gel. PCR products were visualized under UV light by using ethidium bromide.

Results

The quality of the seven DNA extraction methods and purifications were evaluated by comparing their absorbance ratios (A260/A280) (Table 2.0). The obtained A260/A280 ranged from 0.5770 (CTAB) to 2.1545 (EpiH). The lysis buffers B1, B2, and B3, and CTAB experienced an increase in their ratio after the purification was performed: B1 from 1.1885 to 1.3695; B2 from 0.9305 to 1.6065; B3 from 1.1675 to 1.5305; and CTAB from 0.5770 to 1.6300 (Table 2.0). A successful DNA amplification of dust mites, bacteria, and fungi was observed only with the use of the Epicentre SoilMaster™ DNA Extraction Kit: EpiN and EpiH (Figures 1.0, 2.0, and 3.0). The homogenization step, included as a modification of the Epicentre SoilMaster™ DNA Extraction Kit, had a positive influence in the DNA yield of dust mites (Figure 3.0) but no changes were observed in the other organisms (Figures 1.0 and 2.0).

Discussion

Frequently, there are remaining proteins present in the DNA solution, when it is isolated. These proteins are tightly bound to DNA and may interfere with the PCR. To determine the concentration and purity of a DNA solution, the absorbance of UV light is measured in a spectrophotometer. Both proteins and DNA absorb UV light, but they have different absorbance curves. The peak of light absorption is at 260nm for DNA and at 280nm for protein. By dividing these two absorbance values, one can calculate the purity of the DNA solution or the absorbance ratio (A260/A280). Accordingly, a pure sample of DNA substantially free of protein will have a high A260/A280 (between 1.9-2.1).

We noticed an increase in the A260/A280 ratio when the purification was performed on the DNA obtained from the three phenol/chloroform (B1-B3) and the CTAB extractions. Although this finding suggests higher purity of the DNA after the purification step in these extractions, the Epicentre SoilMaster™ DNA Extraction Kit (EpiN and EpiH) processed samples showed not only higher A260/A280 when compared with all other methods, but also values closer to optimal range: EpiN: 2.0575 and EpiH: 2.1545.
Table 2.0 Average absorbance ratios (A260/A280) from DNA samples after seven DNA extraction treatments and purification treatments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>B1</th>
<th>B1P</th>
<th>B2</th>
<th>B2P</th>
<th>B3</th>
<th>B3P</th>
<th>CTAB</th>
<th>CTABP</th>
<th>QIAGEN</th>
<th>EpiN</th>
<th>EpiH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azol A20</td>
<td>1.1885</td>
<td>1.3695</td>
<td>0.9305</td>
<td>1.6065</td>
<td>1.1675</td>
<td>1.5305</td>
<td>0.5770</td>
<td>1.6300</td>
<td>1.1705</td>
<td>2.0575</td>
<td>2.1545</td>
</tr>
</tbody>
</table>

B1: Buffer 1 of Phenol Chloroform extraction; B1P: Buffer 1 of Phenol Chloroform extraction purified; B2: Buffer 2 of Phenol Chloroform extraction; B2P: Buffer 2 of Phenol Chloroform extraction purified; B3: Buffer 3 of Phenol Chloroform extraction; B3P: Buffer 3 of Phenol Chloroform extraction purified; CTAB: cetyltrimethylammonium bromide protocol for PCR; CTABP: cetyltrimethylammonium bromide protocol for PCR purified; QIAGEN: QIAamp DNA Mini Kit; EpiN: Epicentre SoilMaster™ DNA Extraction Kit; EpiH: Epicentre SoilMaster™ DNA Extraction Kit with homogenization step.

Figure 1.0 PCR amplification of indoors-dust sample DNA: ~555 bp fragment, amplified with the NS1/NS2 Fungi, algae and protists universal primers.

Figure 2.0 PCR amplification of indoors-dust sample DNA: ~450-500 bp fragment, amplified with the P4/P5 Bacteria consensus primers.

Figure 3.0 PCR amplification with the 12S arthropods universal primers.
At this stage, the results pointed to the Epicentre SoilMasterTM DNA Extraction Kit as being the more efficient method for extracting PCR-quality DNA from indoor environmental samples. However, the measurement of the A260/A280 has been argued by some studies, which stated that lower ratios do not always translate to non PCR-quality DNA (Glace, 1985).

To determine the verisimilitude of that statement, we performed PCR testing on all of the DNA that resulted from the extractions methods. Our analysis showed that the obtained A260/A280 ratio was directly related to the resultant success in the amplification of dust mites, fungal, and bacterial DNA. Only the samples processed with the Epicentre SoilMasterTM DNA Extraction Kit: EpiN (with a ratio of 2.0575) and EpiH (with a ratio of 2.1545) showed positive PCR results when compared with the other tested methods (Figures 1.0, 2.0 and 3.0).

Although the CTAB A260/A280 showed a significant increase when the purification was performed, and it was the method that obtained an absorbance ratio closest to 2 after the Epicentre’s (Table 2.0), no amplification was detected for any of the tested organisms (dust mites, fungi, and bacteria). These results contrasted with those of previous studies, which identified the CTAB method as the protocol of choice for fungal DNA extraction from clinical samples (Velegrai, et al., 1999) and indoor air samples (Haugland, et al., 2002). Similarly, bacterial DNA extractions have frequently been obtained from clinical samples and cultures using the phenol/ chloroform extraction and the QIamp DNA Mini Kit, but these techniques didn’t work on our indoor samples. These results may be explained by the presence of PCR inhibitors (Tebbe and Yahnjen, 1993; Moreira, 2001) in the dust, which were not efficiently removed by the employed purification method. It can also be the result of the limited amount of organisms present at indoor settings when compared to outdoors soil.

The slight augment in the A260/A280 ratio between the EpiN and EpiH (Table 2.0) can be attributed to an increase in dust mite DNA (Figure 3.0). This is supported by the fact that no significant differences were observed in the PCR amplification of fungal and bacterial DNA when the homogenization was performed (Figures 1.0 and 2.0). We suspect that the homogenization step (EpiH) contributed to a physical breakage of the dust mites' chitin exoskeleton. This breakage allowed more inside cells to be exposed to the lytic reagents, increasing the availability of DNA and thereby its ratio over proteins.

Conclusions
- The obtained ratios (A260/A280) concurred with the PCR results. Only the dust mite, fungal, and bacterial DNA with ratio values close to 2 were successfully amplified. Although an increase in the ratio was observed in the purified DNA samples, PCR-quality DNA was not achieved.
- We discovered in our study that except for in the case of the Epicentre, all other protocols (even after purification) failed in the amplification of dust mite, fungal, and bacterial DNA from indoor environmental samples. This failure may be explained by the presence of PCR inhibitors in the dust, which were not efficiently removed by the employed purification method or due to the limited amount of organisms. However, the Epicentre SoilMasterTM DNA extraction Kit seems to overcome this limitation.
- In this study, we identified for the first time an efficient, rapid (less than an hour), and sensitive method for the extraction of PCR-quality DNA from the dust mites, fungi, and bacteria that are found in indoor environmental samples: the Epicentre SoilMasterTM DNA Extraction Kit.
- Furthermore, this is the first report that describes DNA isolation of dust mites from indoor dust samples.
- We were also able to demonstrate an increase in the dust mite DNA yield when our homogenization modification was included to the standard Epicentre SoilMasterTM DNA Extraction Kit protocol.
- The generation of PCR-quality DNA from indoor environmental samples of dust mites, bacteria, and fungi, will allow us to employ the PCR technology as an innovative detection tool.
- This new approach may contribute to the detection and quantification, as well as to the identification of different populations of these ubiquitous organisms that cannot be differentiated by typical methods such as microscopy, culturing, and antigen-antibody tests.

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