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Comparison of six simple methods for extracting ribosomal and mitochondrial DNA from *Toxocara* and *Toxascaris* nematodes

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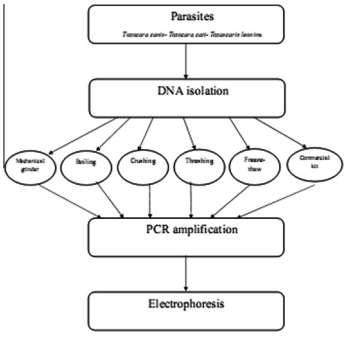
HIGHLIGHTS

- Six different methods were compared for DNA extraction of *T. canis*, *T. cati* and *T. leonina*.
- The beating method proved particularly useful for extraction of mitochondrial DNA.
- The commercial kit method appeared especially useful for extraction of ribosomal DNA.
- We therefore recommend the beating method for studies where costs need to be kept at low levels.

GRAPHICAL ABSTRACT



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ABSTRACT

Six simple methods for extraction of ribosomal and mitochondrial DNA from *Toxocara canis*, *Toxocara cati* and *Toxascaris leonina* were compared by evaluating the presence, appearance and intensity of PCR products visualized on agarose gels and amplified from DNA extracted by each of the methods. For each species, two isolates were obtained from the intestines of their respective hosts: *T. canis* and *T. leonina* from dogs, and *T. cati* from cats. For all isolates, total DNA was extracted using six different methods, including grinding, boiling, crushing, beating, freeze–thawing and the use of a commercial kit. To evaluate the efficacy of each method, the internal transcribed spacer (ITS) region and the cytochrome c oxidase subunit 1 (cox1) gene were chosen as representative markers for ribosomal and mitochondrial DNA, respectively. Among the six DNA extraction methods, the beating method was the most cost effective for all three species, followed by the commercial kit. Both methods produced high intensity bands on agarose gels and were characterized by no or minimal smear formation, depending on gene target; however, beating was less expensive. We therefore recommend the beating method for studies where costs need to be kept at low levels.

1. Introduction

Toxocara canis, *Toxocara cati* and *Toxascaris leonina* are ascaridoid nematodes of dogs and cats. Adult nematodes lodge within the lumen of the small intestine of definitive hosts. Humans infected by larval stages of *T. canis* and *T. cati* may develop clinical toxocariasis, including one or more of a variety of very different

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symptoms (Stensvold et al., 2009). When *Toxocara* eggs containing infective larvae are accidentally ingested by humans, the larvae hatch in the small intestine and migrate through somatic organs, preferably liver and eye. Larvae of *T. leonina* can also invade the tissues of laboratory animals and has the potential to cause human disease (Despommier, 2003).

The polymerase chain reaction (PCR) has broad applicability mainly because its sensitivity permits the amplification of genes or gene fragments from minute amounts of parasite material (Gasser, 1999). DNA techniques targeting genetic markers in ribosomal DNA (rDNA) have been employed to resolve taxonomic issues pertinent to various parasitic groups, including cestodes (Bowles et al., 1995), trematodes (Blair et al., 1996) and nematodes (Chilton et al., 1995; Hoste et al., 1995; Stevenson et al., 1995). The second internal transcribed spacer (ITS-2) rDNA region has proven to be particularly valuable in this context for studies of parasitic nematodes (Gasser et al., 1994; Stevenson et al., 1996). However, there is a paucity of information on the genetic variation in populations of some important parasite groups, such as ascaridoid nematodes of human and animal health significance (Gasser, 1999).

Isolation of high purity and intact nucleic acids is essential for techniques such as PCR, Southern blotting and genomic DNA library construction. It can be difficult to obtain sufficient and pure DNA template from some parasites and stages, for example from parasitic helminths, because of their robust cuticle (Dawkins and Spencer, 1989) and flocculate substance(s) found to co-precipitate with nucleic acids during isolation which inhibit subsequent enzymatic amplification (McManus et al., 1985).

In most molecular studies commercial kits are used for the extraction of total genomic DNA. However, such kits are still relatively expensive and not always readily available in some countries. Identifying and optimizing the best protocol that produces the highest quality and quantity of nucleic acids is therefore essential. A few comparative studies of different DNA extraction methods are available for organisms, such as fungi (Noor Adila et al., 2007; Yamada et al., 2002; Fredricks et al., 2005; Griffiths et al., 2006; Van Burik et al., 1998), bacteria (Dauphin et al., 2010 Mygind et al., 2003), viruses (Dokanehiifard and Bidmeshkipour, 2010) and parasites (Shayan et al., 2007; Yu et al., 2009; Sharbatkhori et al., 2009; Lachaud et al., 2001; Alfonso et al., 2008; Babaei et al., 2011; Adamska et al., 2010; Capuano et al., 2007); however, to our knowledge, no study has addressed the efficiency of different methods for DNA extraction from *Toxocara* and *Toxascaris* nematodes.

In this study, a comparative assessment of six methods was conducted for DNA extraction based on the mitochondrial cytochrome c oxidase subunit 1 (cox1) and nuclear ribosomal (ITS region) genes from two *Toxocara* species and *T. leonina*. The aim of the study was to compare the ability of different protocols for DNA extraction to produce the most useful yield of ribosomal and mitochondrial DNA suitable for molecular genetics analyses such as PCR.

2. Material and methods

2.1. Parasites

Adult stages of *T. canis* and *T. leonina* were collected from the intestinal tracts of infected dogs, and *T. cati* from cats, two isolates for each species. Individual helminths were washed extensively in physiological saline, identified to species level according to their morphological features using existing keys and descriptions (Muller, 2002) and fixed in 70% (v/v) ethanol until extraction of genomic DNA.

2.2. DNA isolation

Step 1 included DNA extraction. Equal fragments (approximately 0.5 cm) of each adult worm isolate were washed twice with sterile distilled water. Subsequently, six different easy-to-use methods were applied for DNA extraction:

(1) Mechanical grinding: Sample material was mixed with 50 μL lysis buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl, 2% Triton X100, and 0.5% SDS) and crushed for 3 min using a mechanical grinder (Micro Multi Mixer, Ieda Trading Corporation, Tokyo, Japan).
(2) Boiling: 50μl lysis buffer was added to the sample and incubated in boiling water for 10 min.
(3) Crushing: The sample was crushed between 2 microscope glass slides for about 2–3 min and the product was transferred to a tube for further processing.
(4) Beating with steel pellet: A steel pellet was added to a 2 mL Eppendorf tube containing the sample, and the tube was kept in liquid nitrogen for 1.5 min and shaken 1 min to convert the sample to powder.
(5) Freeze–thawing method: 50μl lysis buffer was added to the sample and frozen in liquid nitrogen and thawed in boiling water three times, each time for 1.5 min.
(6) Commercial kit: Samples were grinded for 3 min using a mechanical grinder and was subsequently purified using the commercially available QIAamp DNA mini Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Step 2 included DNA purification. Except for the QIAGEN method, a conventional phenol chloroform DNA purification protocol was used. Briefly, 300 μL lysis buffer and 30 μL of proteinase K were added, and the mixture was incubated at 56 °C overnight. 300 μL phenol chloroform was added to the sample and centrifuged at 5000 rpm for 5 min. The supernatant was extracted by chloroform once again and DNA in the supernatant was precipitated by an equal volume of Isopropanol (Merck, Germany) and 0.1 × volume of 3 M sodium acetate pH 5.2 (Merck, Germany). After 10 min of incubation at −20 °C, tubes were centrifuged at 12,000 rpm for 12 min. The pellet was washed using 300 μL of 70% ethanol (v/v), diluted in 50 μL deionized water, and stored at −20 °C until use.

2.3. PCR amplification

For PCR amplification, two primer sets were used: The forward primer (JB3: 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and the reverse primer (JB4.5: 5′- TAAAGAAAGAACATAATGAAAATG-3′) were used to amplify *cox1* (Li et al., 2008); and the forward primer (NC5: 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and the reverse primer (NC2: 5′-TTAGTTTCTTTTCCTCCGCT-3′) were used for amplification of the nuclear ITS region (Li et al., 2007). All PCRs were carried out in a final reaction volume of 25 μL, including 12.5 μL of PCR mix containing 1.25 U *Taq* DNA polymerase, 200 μM of dNTPs and 1.5 mM $MgCl_2$ (2x Master Mix RED Ampliqon, Denmark); 25 pmol of each primer and 5 μL of template DNA. The temperature profile was one cycle of 95 °C for 5 min (primary denaturation), followed by 35 cycles of 94 °C for 45 s (denaturation), 55 °C (for *cox1*) or 60 °C (for ITS) for 1 min (annealing), and 72 °C for 1 min (extension), and a final extension 72 °C for 7 min. A sample containing water instead of template DNA was included in each run as a negative control. PCR products were analyzed using a 1.5% TBE (Tris 0.09 M, Borate 0.09 M, EDTA 0.02 M) agarose gel. Gels were stained with 0.5 μg/mL ethidium bromide (Roche, Germany). Electrophoresis was carried out at 80 V for 1 h. The bands were visualized using a UV Transilluminator and digitally photographed.

3. Results

In this study, we compared six different methods for DNA extraction from adult *T. canis*, *T. cati* and *T. leonina*, by evaluating their potential for amplification of individual extracts by PCR and gel visualization. Amplicons of ITS and cox1 genes (1000–1100 and 450 bp, respectively) were amplified individually and subjected to agarose gel electrophoresis.

Fig. 1 shows PCR products of the ITS region amplified using genomic DNA extracted by the six different methods. The sizes of PCR products amplified from DNA samples using the primer set NC5–NC2 varied in size from ~1000 to 1100 bp. No amplification was seen in negative controls. Fig. 2 shows cox1 gene PCR products amplified using mitochondrial DNA extracted by the same six different methods. As seen, target DNA was successfully amplified from all samples except for negative controls, producing an expected band size (~450 bp); band intensity however was variable.

Smears were present on the agaorse gel for all ITS PCR products (Fig. 1), but cox1 PCR amplicons gave no smears. The beating method as well as the commercial method enabled DNA extraction sufficient to yield high intensity bands in electrophoresis of PCR products of both ribosomal and mitochondrial DNA for the majority of samples. However, while the beating method proved particularly useful for extraction of mitochondrial DNA, the commercial kit method appeared especially useful for extraction of ribosomal DNA (Figs. 1 and 2). The freeze–thawing method yielded high intensity bands in electrophoresis of PCR products of ribosomal and mitochondrial DNA for *T. canis.*

The processing time for each DNA extraction method was determined. For the crushing, boiling and beating methods, step 1 took 15, 10 and 15 min, respectively, for six samples, while it took 20 min with the grinding, freeze–thawing and commercial kit methods. Step 2 was similar for all methods, and took about one and half hours on average. Hence, boiling was the fastest method for the DNA extraction**.**

4. Discussion

Genetic variation is widespread in parasite populations, and accurate analysis of genetic variation in parasites has important implications for studies of taxonomy, population biology, epidemiology, genetic structure, pathogenesis and diagnosis of parasites (Gasser, 1999; Stensvold et al., 2011). Isolation of high quality nucleic acids is essential for molecular analyses since any DNA-based analysis requires DNA extraction as the first step and significantly influences downstream analyses. A suitable method for extraction of nucleic acids should be efficient, sensitive, rapid, simple and cost effective. The important critical point for the DNA extraction from adult nematodes is to ensure that the worm is well disrupted, homogenized, and the cells completely lysed.

In the present study, six different DNA extraction protocols, including grinding, boiling, crushing, beating, freeze–thawing and application of a commercial kit, were evaluated to identify how to cost-effectively produce the highest yield of ribosomal and mitochondrial DNA for molecular genetics analyses such as PCR.

In this study, DNA extraction by the QIAGEN kit showed bright and distinct bands in electrophoresis of PCR products of ribosomal DNA of both *Toxocara* and *Toxascaris*, but yielded high intensity bands in electrophoresis of PCR products of mitochondrial DNA for only *T. leonina*. In most molecular studies on *T. canis, T. cati* and *T. leonina* commercial DNA extraction kits, such as Wizard Genomic DNA Purification Kit (Li et al., 2007, 2008; Jacobs et al., 1997), Easy DNA kit (Wickramasinghe et al., 2009) and QIAamp DNA mini Kit (QIAGEN, Hilden, Germany) (Epe et al., 1999) have been used for DNA extraction. The application of commercial kits is quick and easy, especially when a large numbers of samples are processed and usually yields high quality nucleic acids. However, such kits are relatively expensive and are not always readily available in some countries.

The beating method yielded sufficient mitochondrial DNA and showed sharp bands in electrophoresis of PCR products of cox1 gene in all three nematode species. However, it showed some

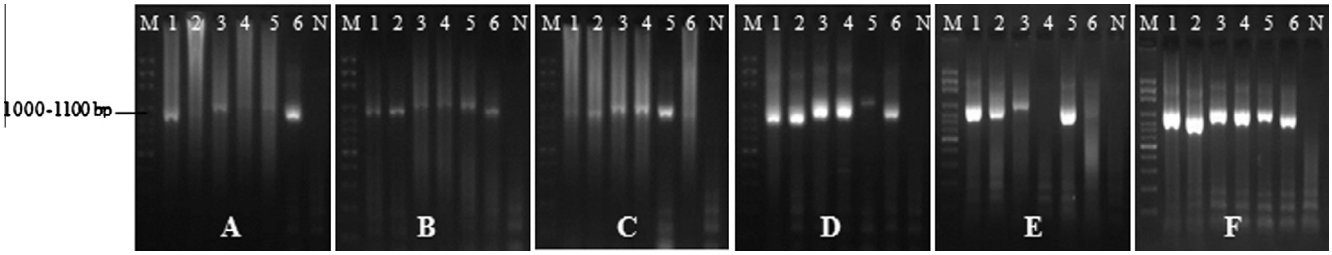

Fig. 1. PCR products of ITS region amplified to extract ribosomal DNA of *Toxocara canis*, *Toxocara cati* and *Toxascaris leonina* by six different extraction methods including: (A). Crushing, (B). Grinding, (C). Boiling, (D). Beating, (E). Freezing-thawing, and (F). Commercial kit. 1, 5 *T. canis*, 3, 4 *T. cati,* 2, 6 *T. leonina*, M: 100 bp DNA marker N: negative control.

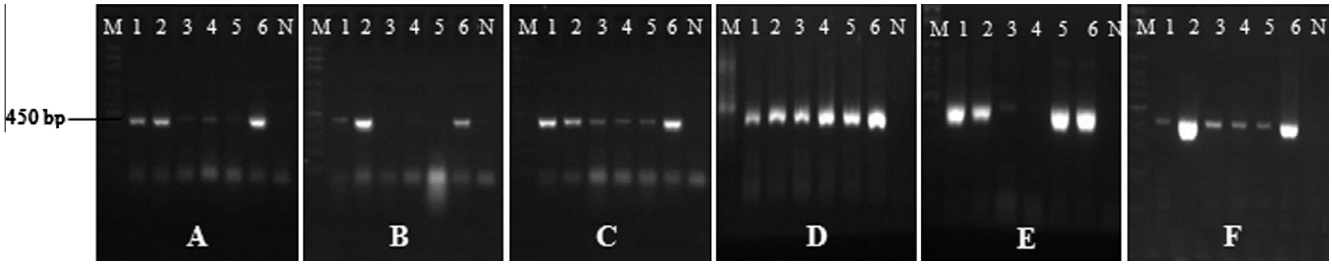

Fig. 2. PCR products of cox1 gene amplified to extract mitochondrial DNA of *Toxocara canis*, *Toxocara cati* and *Toxascaris leonina* by six different methods including: (A). Crushing, (B). Grinding, (C). Boiling, (D). Beating, (E). Freezing-thawing, and (F). Commercial kit. 1, 5 *T. canis*, 3, 4 *T. cati,* 2, 6 *T. leonina*, M: 100 bp DNA marker N: negative control.

variability for the ribosomal DNA (Fig. 1, Panel. D, Lane. 5). Although this method requires liquid nitrogen and the application of liquid nitrogen is laborious, expensive to purchase and must be kept under suitable conditions, it appears appropriate for the extraction of mitochondrial DNA of high quantity and quality .

The freeze–thawing method showed variable results in different studies. Griffiths et al. (2006) reported that the freeze-boil method was the least reliable technique for the extraction of DNA from A*spergillus fumigatus*. Sharbatkhori et al. (2009) reported that freeze–thaw method extracted sufficient DNA and showed sharp bands in electrophoresis of PCR-product of ribosomal DNA from *Echinococcus granulosus* protoscoleces. Babaei et al. (2011) recommend rupturing the cyst wall of *Giardia intestinalis* with freeze–thawing cycles in addition to agitating the samples with glass beads before using the QIAamp Stool DNA Mini Kit (Qiagen), when using stool for *Giardia* cyst DNA extraction. For DNA extraction from *Toxocara* and *Toxascaris* nematodes, the freeze–thawing method yielded high intensity bands in electrophoresis of PCR products of ribosomal and mitochondrial DNA from *T. canis*. This method also requires liquid nitrogen and application of liquid nitrogen is problematic, especially when there are a large number of samples to be examined. Other disadvantages of this method include difficulties during handlings and safety hazards.

In the boiling method, samples incubated in boiling water for only 10 min, making this method the fastest. This technique is inexpensive and needs only minimal equipment that is available in most laboratories. However, faint PCR bands and smears in the PCR amplicons of ribosomal DNA and pale PCR bands of amplified mitochondrial DNA indicate that boiling is not an appropriate method for DNA extraction from *Toxocara* and *Toxascaris* nematodes.

The crushing method followed by the mechanical grinding method was more time-consuming than the other methods. This method showed a high degree of DNA smear in gel electrophoresis of PCR amplicons of ribosomal DNA and pale bands in most of the samples in the PCR amplicons of mitochondrial DNA. The crushing method is the most inexpensive method and does not need any equipment other than two glass slides; its disadvantage is that it is more time-consuming because each sample has to be crushed separately.

The mechanical grinding method homogenizes the sample completely, but needs a mechanical grinder that is not available in all molecular laboratories and it takes more time, since each sample should be grinded separately. Thus, the crushing and grinding methods do not appear to be the most convenient methods when a large number of samples need to be analyzed (Van Burik et al., 1998; Sharbatkhori et al., 2009).

In conclusion, this is the first comparative approach for studying *Toxocara* and *Toxascaris* DNA extraction methods. Use of the commercial kit was the most expensive method, whereas the crushing and boiling methods were the cheapest. The boiling method was also inexpensive and quick but showed only smears and faint PCR bands of ribosomal and mitochondrial DNA, respectively. The crushing and grinding methods were more time consuming; therefore these methods do not appear to be convenient when many samples need to be analyzed. The Freeze–thawing method requires liquid nitrogen which is problematic, since this may often not be available. Although the beating method also needs liquid nitrogen, this method showed very distinct and bright PCR bands for mitochondrial DNA and produced a relatively low degree of smear in the ITS PCR. Hence, the beating method is the most cost-effective for mitochondrial DNA extraction from adult *Toxocara* and *Toxoascaris* while the commercial kit is recommended for ribosomal DNA extraction. These two techniques produced the highest yield of ribosomal and mitochondrial DNA suitable for molecular genetics analyses such as PCR.

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