# COMPARISON OF AUTOMATED AND MANUAL DNA EXTRACTION METHODS IN ISOLATING *ENTEROBIUS VERMICULARIS* DNA.

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**ABSTRACT**

**The aim** of the present study was to compare automated and manual DNA extraction method from *Enterobius vermicularis* (human pinworm) eggs.

**Materials and methods**. Scotch tape samples positive for *Enterobius vermicularis* eggs via microscopic examination, collected from 16 patients aged between 2 and 12 years, were included in the study. Using manual spin-column-based and automated procedures, the isolation of parasite DNA was carried out under identical conditions including: number of eggs, type and quantity of storage solution before the extraction process, and sample pretreatment (number of freeze-thaw cycles). The concentration and purity of the isolated DNA with both methods was measured with a spectrophotometer. Nested PCR was applied to amplify a segment of the mitochondrial gene encoding for the cytochrome c oxidase subunit 1 (*cox*1) of *E. vermicularis*, and the products were separated by agarose gel electrophoresis.

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**Results.** Higher concentrations of DNA with excellent purity (A260/A280 – 1.0-3.0 were obtained with the automated extraction method. The DNA extracts obtained with the Nucleic Acid Automated Extraction System contained fewer contaminants than those isolated by the manual procedure as detected by analyzing the A260/A230 and A260/A280 ratios. As compared to the manual extraction method, a greater sensitivity in percent yield of positive samples was demonstrated by the automated method. All samples (n=16) subjected to the automated isolation were PCR positive and products with the expected size of 379 bp were detected in 100% of the samples vs. 2 false negatives (12.5%) with the manual extraction procedure.

**Conclusion**. Data from the comparative study of the two methods for DNA extraction from *E. vermicularis* eggs showed that the automated extraction procedure provides an excellent quality and yield of isolated DNA samples as compared to the manual processing. The extracted DNA has a lower content of organic or carbohydrate contaminants, including proteins, which is a prerequisite for successful conduct of subsequent molecular genetic analyses.

**Keywords**. *Enterobius vermicularis*, DNA extraction, PCR.

**INTRODUCTION**

Enterobiasis is one of the globally prevalent intestinal helminth infections in humans, mainly affecting children. Its causative agent is *Enterobius vermicularis*, and humans are its only host. Poor hygiene habits including nail biting, finger sucking, as well as not washing hands after using the toilet contribute to the cosmopolitan spread of the parasite.

Infection occurs through the ingestion of mature eggs, from which a larva is released following the small intestine entry. There it reaches sexual maturity and migrates to the colon. The parasite lives in the lower half of the small intestine and the cecum. After fertilization, male worms die and females migrate to the rectum. At night, they actively come out through the anus to deposit their eggs in the perianal folds. Under suitable conditions, including moisture and temperature of 37$^{o}$C, the eggs mature within 5-6 hours and are already infective. The female can lay up to 15,000 eggs per day, and the lifespan of *E.*

*vermicularis* is about 3-4 weeks (1). The diagnosis of *E. vermicularis* infection is made by *pinworm* eggs demonstration after microscopic examination of a scotch tape obtained from the patient's perianal skin folds or, *less commonly*, when eggs and/or female pinworms are detected in stool samples (2,3).

Molecular tools are widely used to track pathogen transmission, to assess the epidemiology in the general population and in specific subgroups, to distinguish persistent from recurrent infections. PCR-based analyses are employed to detect and identify parasitic organisms, including pinworms. They can help to determine the pathogenetic role of the parasite in some gastrointestinal disorders. The application of PCR for pinworm detection in human stool samples has been reported (5,7,8). These publications were focused on the diagnostic application of the PCR method in patients with suspected *E. vermicularis* infection.

Mitochondrial (mt) DNA is a commonly used genetic marker of molecular and phylogenetic studies (9,10,11). The complete mtDNA of *E. vermicularis* has been sequenced and published by Kang, S et al.(12) Three distinct clusters (subtypes) A, B and C in *E. vermicularis* isolates from samples of captive chimpanzees and humans from Asian and European countries, based on phylogenetic studies using the gene encoding mitochondrial cytochrome c oxidase subunit 1 (*cox*1) have been identified (5, 7, 13). A and B genotypes were isolated from humans (14) and captive chimpanzees (*Pan troglodytes*), while genotype C has only been identified in chimpanzees (15 ).

The process of DNA extraction, which constitutes the initial stage of the PCR procedure, is of paramount significance. The quality and integrity of the isolated materials are pivotal in determining the outcome of the subsequent PCR experiment.

The objective of the present study is to compare the efficacy of automated and manual methodologies for the extraction of deoxyribonucleic acid (DNA) from Enterobius vermicularis (human sedge) eggs.

## MATERIALS AND METHODS

***Patients infected with E. vermicularis***

Sixteen patients with microscopically proven *E. vermicularis* infection were included in the study. All patients were children, 9 (56.3%) girls and 7 (43.7%) boys, aged 2 – 12 (age distribution: 4 in the group of 0-4; 10 children in the group of 5-9; 2 in the group of 10-14 years). In the cohort of patients participating in the study, a triple testing of samples was performed.

***Specimen preparation***

Samples were obtained from the patient's perianal region using transparent tape and subsequently examined under a light microscope at 100x and 400x magnification. The egg-positive samples (slides) were stored at 4°C until DNA extraction. A sample was considered positive if at least one *E. vermicularis* egg was found. For collection of pinworm eggs from the adhesive tape surface, the slide was placed in a petri dish and soaked in sterile saline solution. The eggs were then recovered from the tape surface by scraping with a disposable scalpel under a light microscope and transferred to 200 µL of sterile saline solution into a clean 1.5 mL *microcentrifuge tube*. Distilled water was used to avoid inhibiting factors in the subsequent molecular processing of the samples. Samples were subjected to three freeze-thaw cycles in order to destroy the egg shell. Five eggs recovered from each slide were aliquoted into two 1.5 mL microcentrifuge tubes for DNA isolation using the two methods (manual and automated) and stored at -20°C until use.

***Manual spin-column-based extraction of DNA***

Manual DNA extraction was conducted using the PureLink Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific, North America) according to the manufacturer's guidelines. Two hundred microliters (µl) of ATL Digestion buffer and 30 µl of proteinase K (20 mg/mL) were added to a microcentrifuge tube containing the eggs. The DNA was then eluted in 100 µl of elution buffer and stored at -20°C until needed.

***Automated extraction of DNA***

DNA extraction was performed using the Techstar Nucleic Acid Automated Extraction System (model YC702, China). Parasite DNA was isolated using the Nucleic Acid (DNA/RNA) Extraction Kit from Techstar, following the manufacturer's instructions. The kit includes two types of wash buffer, proteinase K (30 µl, 20 mg/mL), and elution buffer (100 µl). The volumes of the reagents used were identical to those in the manual extraction method. The extracted DNA was stored at 4°C for short-term use (2-3 days) and at -20°C for long-term storage.

***DNA yield and purity.***

DNA was extracted from five *E. vermicularis* eggs per sample and quantified utilizing a Biochrom microvolume spectrophotometer (BioDrop µLite+, Harvard Bioscience Co. Ltd.; Shanghai, China). Initially, the elution buffers provided with each extraction kit were employed to blank the spectrophotometer prior to the measurement process. Subsequently, the absorbance of the DNA samples was recorded. The spectrophotometer computes the concentration of DNA for each sample based on the absorbance measured at 260 nm (A260), which is then multiplied by a conversion factor of 50 µg/ml per 1 A260 unit for double-stranded DNA. To assess the purity of the DNA extracts, absorbance was measured at 280 nm (A280) and 230 nm (A230). The average ratios of A260 to A280 (A260/A280) and A260 to A230 (A260/A230) were calculated for all samples. Samples exhibiting A260/A280 ratios between 1.8 and 2.0 are regarded as free from significant contamination (6,16).

***E. vermicularis*-specific PCR**

To compare the performance of different extraction methods, the PCR amplification success with *E. vermicularis*-specific primers was used. Nested PCR targeting a 379-bp segment of mitochondrial *cox*1 gene of *E. vermicularis* was applied according to Piperaki et al. (2011) (4), using two sets of primers. Primer sequences of the outer primer pair are as follows:

EVM1 5'-TTTTTGGTCATCCTGAGGTTTATATTC-3',

EVM2 5'-CCACATTATCCAAAATAGGATTAGCC-3'.

Primers for the inner amplification reaction have the following sequences:

EVIF 5'-TTGGTCATCCTGAGGTTTATATTC-3',

EVIR 5'-TCCAAAATAGGATTAGCCAACA-3'.

The first amplification reaction was performed in a 25-µl volume, using AmpliTaq Gold 360 Master Mix (AppliedBiosystems by Thermo Fisher Scientific) containing enhancer, 0.2 mM of each primer, and 3 µl DNA. The thermal cycling protocols were as follows: an initial denaturation at 95°C/ 10 minutes, followed by 45 cycles of 95°C/1 minute, 57°C/1 minute, and 72°C/10 minute; final extension step at 72°C for 10 minutes.

Three µl of the first PCR were used as a template for the inner amplification reaction (25-µl volume). The conditions of this second reaction differed from the first one in the annealing temperature (53°C), and the number of cycles (30). One positive (*E. vermicularis* DNA from adult pinworms) and one negative (PCR water instead of template DNA) control were included in all reactions to check for amplification success and DNA carry-over contamination, respectively. PCR amplifications were performed in a GeneExplorer Thermal Cycler instrument (Hangzhou Bioer Technology Co., Ltd.). PCR products were separated by agarose gel electrophoresis on 3% gel. A 50 bp marker (GeneRuler™ 50bp DNA Ladder, Thermo Scientific, Lithuania) was used to determine the sizes of the resulting products. Ten µl of the products from the second PCR reaction were mixed with 2 µl of fluorescent dye (peqGREEN DNA/RNA dye, VWR International GmbH, Germany) and loaded on the gel, visualized on a UV transilluminator, and photographed using SYNGENE gel documentation system (GelVue Model No. GVM20, Synoptics Ltd, UK).

## RESULTS

***Specimens***

Sixteen transparent Scotch tape slides that tested positive for eggs were included in the study. Each slide was examined under direct light microscopy, confirming the presence of at least one egg, which validated their positive status. The study aimed to compare the PCR results of DNA samples extracted using manual and automated methods. Therefore, the samples from the 16 patients were prepared in duplicates, ensuring an identical number of parasite eggs in each sample. All manipulations during the pretreatment phase, including an equal number of freeze-thaw cycles, were carried out under consistent conditions.

***Comparison of DNA Extraction methods***

Two methods for isolation of genomic DNA from *E. vermicularis* eggs were applied. The comparison between the procedures was performed using a PCR assay developed by Piperaki et al. (2011), which optimized for our conditions (5). *E. vermicularis* DNA was detected in all 16 samples (n=16, 100%) processed by automated extraction method and the presence of a 379-bp band was determined for all examined samples. Two specimens (12.5%) obtained by manual extraction procedure were read as false negatives by PCR. This may be due to loss of sensitivity, associated with DNA yield and purity. DNA samples isolated by

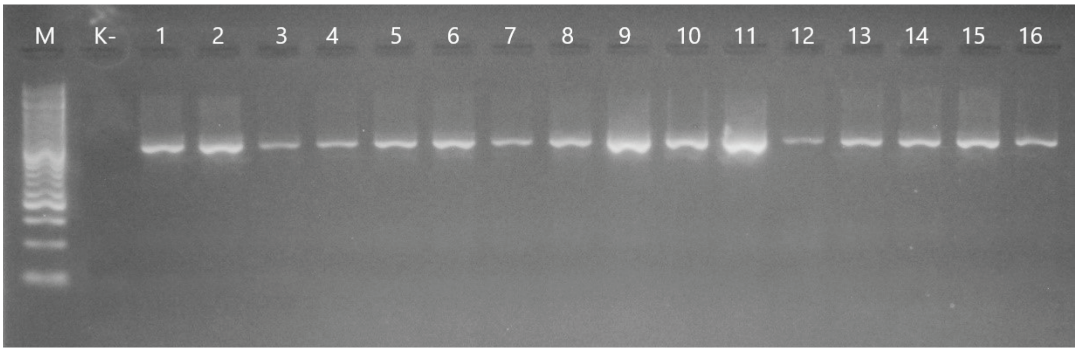


**Fig. 1.** Amplified PCR products analyzed by 3% agarose gel electrophoresis - automatic isolation. Starts: 1-DNA marker, bands across 50bp, 2-negative control, 1-16 *E. vermicularis* positive samples. Presence of band ~379 bp

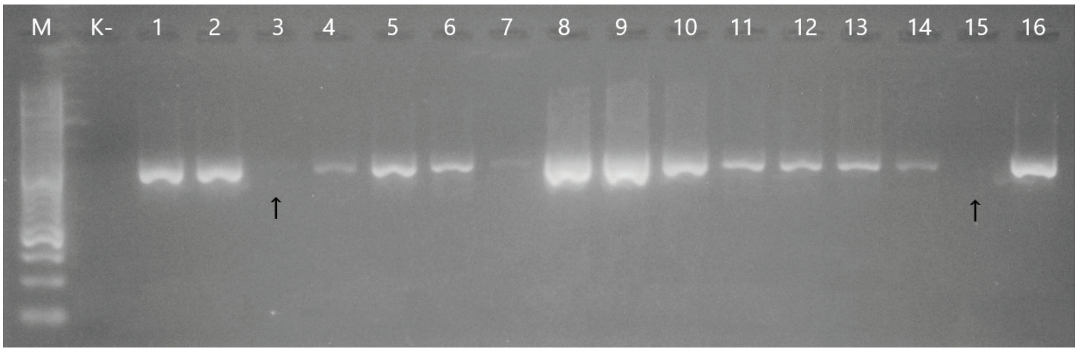


**Fig. 2.** Amplified PCR products analyzed by 3% agarose gel electrophoresis – manual extraction. Starts: 1-DNA marker, bands across 50bp, 2-negative control, 1-16- samples – 3 and 15 without presence of band ~379 bp

the automated procedure showed highest concentrations: 6 mg/ml and excellent purity (A260/A280 – 1.0 – 3.0). They contained less contaminants, compared to the manual extraction (fig.1 and fig.2). The manual extraction procedure using the PureLink Genomic DNA Mini Kit yielded DNA with ratios ranging from (A260/A280 – 0.6 – 2.0). The DNA concentration values were in the range of 1 – 5 ɱg/ml.

## DISCUSSION

This study was conducted to compare the performance of DNA extraction methods (automated and manual procedures) with regard to DNA yield and purity by using *E. vermicularis*-specific PCR. The sensitivity of PCR assays can be influenced by different factors, such as DNA yield, DNA purity from PCR inhibitors, and DNA damage. The selection of the appropriate DNA extraction method is essential for the detection of the target DNA region of the studied pathogen. The adequate quality and purity of the isolated DNA are essential for its long-term storage and to guarantee good-quality results in subsequent analyses. We tested the effects of two different procedures used for isolation of genomic DNA from pinworm eggs on the sensitivity of the detection of a segment of mitochondrial *cox*1 gene of *E. vermicularis*. Using *E. vermicularis*-specific primers, the automated extraction method resulted in higher rate of amplifiable *E. vermicularis* DNA, which is expressed as 100% PCR amplification success.

A good correlation between the manual and automated extraction methods has been reported (17,18,19). Good average values of DNA concentration and purity were obtained by Utaminingsih (20) using the column-based method for DNA isolation and the extracted DNAs were successfully used as templates in their real-time PCR analysis.

Contamination of the samples is a factor responsible not only for the low quality of the isolated product, but for an insufficient yield of DNA (that is needed for the subsequent steps in genotyping) as well. The literature review showed that the automated instruments have been used for the DNA isolation for nearly two decades (21, 22, 23, 24). DNA extraction methods that utilize an automated procedure for DNA extraction were reported as effective in

**Table. 1** Purity and concentracion of automatic and manual extraction

| Sample / yields and purity | Automatic extraction | | Manual extraction | |
|---|---|---|---|---|
| | A260/A280 | concentration | A260/A280 | concentration |
| **1** | 2 | 6 μg/ml | 0,6 | 4 μg/ml |
| **2** | 3 | 6 μg/ml | 0,6 | 3 μg/ml |
| **3** | 2 | 6 μg/ml | 0,67 | 2 μg/ml |
| **4** | 1,25 | 5 μg/ml | 0,67 | 4 μg/ml |
| **5** | 1,8 | 5 μg/ml | 0,67 | 4 μg/ml |
| **6** | 1 | 4 μg/ml | 0,7 | 3 μg/ml |
| **7** | 2 | 5 μg/ml | 2 | 3 μg/ml |
| **8** | 2 | 5 μg/ml | 2 | 5 μg/ml |
| **9** | 2,5 | 5 μg/ml | 1,5 | 5 μg/ml |
| **10** | 1,5 | 4 μg/ml | 0,9 | 3 μg/ml |
| **11** | 3 | 6 μg/ml | 0,63 | 5 μg/ml |
| **12** | 1,5 | 4 μg/ml | 0,83 | 3 μg/ml |
| **13** | 1,8 | 5 μg/ml | 0,63 | 4 μg/ml |
| **14** | 2 | 5 μg/ml | 0,83 | 3 μg/ml |
| **15** | 2,23 | 5 μg/ml | 0,6 | 2 μg/ml |
| **16** | 2,2 | 5 μg/ml | 1 | 4 μg/ml |

removing PCR inhibitors (25). Compared to the manual extraction method, a greater sensitivity in terms of positive samples yield was demonstrated by the automated method (26, 27). The results of the present study support the findings of these authors. The automated DNA extraction method that we applied yielded DNA with higher purity than the manual extraction. The automated extraction system reduced contamination by using a unidirectional autosampler, UV lamps for subsequent sterilization, leading to DNA yields in required amounts for performing further analyses. Based on the observations on the sensitivity of PCR analyses in the current study we considered that the automated isolation of *E. vermicularis* DNA from the parasite eggs has some advantages over the manual method. It is faster as it provides the opportunity to process a larger number of samples in a shorter time. The products obtained contained sufficiently low amounts of impurities to be classified as pure products based on the A260/280 ratio and to be used for subsequent molecular approaches, including PCR assays and sequencing.

Considering the fact that all 16 samples were subjected to the same pretreatment conditions and procedures before applying the manual and automated DNA extraction methods from parasite eggs, the following reasons may contribute to the negative PCR results obtained for 2 of the samples: a) insufficient purification of organic contaminants such as ethanol; b) egg shell proteins that have failed to break down further; c) contamination of the columns used for isolation. We believe that the automated washing and overall processing lead to the more efficient contaminant elimination.

Genetic methods enhance diagnostic accuracy by facilitating the identification of diverse genotypes. This, in turn, enables the determination of the geographical distribution of the parasite using molecular epidemiological approaches.

## CONCLUSION

The results from our comparative study of two DNA extraction methods from pinworm eggs of *E. vermicularis* showed that the automated extraction procedures provided excellent quality and yield of isolated DNA samples as compared to manual extraction. The DNA extracts had a lower content of organic carbohydrate and protein contaminants, which is a prerequisite for the successful conduct of subsequent molecular genetic analyses. The automated method was more time-efficient when processing multiple samples, and demonstrated a higher sensitivity. Therefore, the automated DNA extraction method provides a favorable option for high-throughput clinical laboratories.

The Techstar Nucleic Acid Automated Extraction System used in our study is capable of automatically processing 1-32 samples simultaneously. It provides robust and reliable results in obtaining genomic DNA from samples containing pinworm eggs of *E. vermicularis* for downstream applications such as PCR and sequencing. The extracted DNA was free of PCR inhibitors as determined by the positive PCR results.


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