Iranian J Parasitol: Vol.2, No.2, 2007, pp. 34-39

Original Article

Isolation of DNA from A Single Helminth Using New Developed Kit in Iran and Its PCR Analysis

**P Shayan [1, 2], H Borji[1], A Eslami[1], S Zakeri[3]*

[1]*Dept. of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Iran*
[2]*Research Unit "Molecular Biological System Transfer (MBST)", Tehran, Iran*
[3]*Dept. of Biotechnology, Pasteur Institute of Iran, Tehran*

(Received 12 Sep 2006; accepted 10 Apr 2007)

Abstract

Background: Nematodes are among the most common and important parasites of man and animal. DNA of a single worm can be used for several purposes, such as identification to the species, subspecies, strain and antihelmenthic resistance. DNA extraction from a single small worm using traditional methods such as phenol extraction technique faces serious problems.

Methods: DNA from 20 single *Haemonchus contortus* was isolated using DNA isolation kit newly designed in Iran by the Research Unit of Molecular Biological System Transfer (MBST) based on the specific binding of DNA to the carrier. The genomic DNA was amplified using specific primers derived from β-tubulin isotype 1 in PCR. The specificity of the PCR products was determined using semi-nested PCR technique. Specific PCR-product from β-tubulin gene could be amplified with 1 ng, 100 pg and 10 pg DNA.

Results: The used DNA extraction method was safe, with high quality and quantity, fast, easy to handle and not costly for genetic analysis of even a single small worm.

Conclusion: The Iran produced DNA extraction Kit is grounded on a selective binding of nucleic acids to a silica-based membrane and is recommended for the isolation of DNA from even small amount of biological materials.

Keywords: *Genomic DNA, PCR, Semi-nested PCR, Iran*

Introduction

The utilization of molecular biology techniques brought new views to different biomedical areas. The molecular helminthology laboratory specializes in the use of molecular biology tools for investigations on the field of host-parasite interactions (1-4). The other aspects of the use of molecular biology techniques in the helminthology are the characterization and determination of species, subspecies and strain or generated mutations during the treatment of animals with the anthelmintics (3, 5-10).

For any biological sample, the efficiency of the DNA extraction methods is determined through DNA quality and recovery rate. The extraction of DNA with high quality and quantity is a key step in the genetic analysis. Numerous direct DNA extraction methods have been used in the preparation of DNA from various organisms. The isolation of the genomic DNA from small amounts of biological materials, such as blood smears (11) or small single worms, is not always applicable using the traditional DNA isolation methods based on the phenol/chloroform/ isoamyl alcohol, which is the most used technique for DNA extraction in developing countries. In contrast, the most commonly used DNA extraction method in industrial countries based on the selective binding of the nucleic acids on the silica based carriers (2, 11-13). The advantage of the latter mentioned method compared to

***Corresponding author:** Tel: +98 21 66924469, E-mail: pshayan@ut.ac.ir

the traditional method can be summarized in the better recovery, purity, safety and speed. The only disadvantage of this method is that is costly. Since all DNA extraction kits based on the selective binding of DNA to the carrier are produced in industrial countries, so are expensive to obtain in Iran, it is of great importance to develop a specific comparable DNA isolation method in Iran. The objective of this study was to present a simple, safe, easy to handle and not costly method for the isolation of DNA from small amount of any biological specimens including a single worm.

Materials and Methods

Parasite

Twenty adult male and female *Haemonchus contortus* were collected directly from the abomasums of sheep. They were stored in 70% ethanol solution until used. The worms were removed from ethanol either direct used or dried and washed twice in PBS (phosphate saline buffer) and stored for 1-2 d without any buffer in 1.5 ml tubes at -20 °C. All tissues had been obtained with consent given according to institutional guidelines.

DNA extraction

DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. This kit was newly developed by the first author of the paper in the Investigating Unit-Molecular Biological System Transfer (MBST) in Iran and based on a selective binding of nucleic acids to a silica-based membrane. Briefly, the single worm was lysed in 180 µl lysis buffer and 20 µl proteinase K (10 mg/ml) for 20 min-2 h at 55 °C. After adding of 360 µl binding buffer and incubation for 10 min at 70 °C, 270 µl ethanol (96%-100%) was added to the solution. Then, it was vortexed and the complete volume was transferred to the MBST-column. The MBST-column was first centrifuged at 8000×g and then washed twice with 500 µl washing-buffer at 8000×g. The columns were then centrifuged with 12000×g to remove the rest ethanol from the carrier. After that, the DNA was eluted from the carrier with 100 µl elution buffer. Finally, the DNA was visualized on 0.8% agarose gel in 0.5 time TBE buffer using ethidium bromide and UV-transilluminator. The quantity of the DNA in the solution was calculated from the absorbance of 260 nm (A260) and the purity was calculated by the ratio of A260/A280.

Polymerase chain reaction (PCR)

For DNA amplification, different amounts of DNA solution (50 ng, 10 ng, 1 ng, 100 pg and 10 pg) were used. The PCR was performed on 100 µl total volume including one time PCR buffer, 2.5 U Taq Polymerase (Cinagen, Iran), 2 µl of each primer (20 mM, Cinagen, Iran), 200 µM of each dATP, dTTP, dCTP and dGTP (Fermenta) and 1.5 mM $MgCl_2$ in automated Thermocycler (Premius 96, MWG, Germany) with the following program: 5 min incubation at 95 °C to denature double strand DNA, 35 cycles of 45 s at 94 °C (denaturing step), 45 s, at 58 °C (annealing step) and 45 s at 72 °C (extension step). Finally, the amplification was completed with the additional extension step for 10 min. The PCR products were analyzed on 1.8% agarose gel in 0.5 times TBE buffer and visualized using ethidium bromide and UV-transilluminator.

To control the specificity of the PCR products from the beta-tubulin gene, semi-nested PCR technique was used, in which the additional primer (β-tubulin-Forward 2) was designed within the DNA sequence between the first two forward and reverse primers. The primers are listed in the Table 1.

Semi-nested PCR was performed with the PCR product isolated from agarose gel using the MBST-Kit according to the manufacturer's instructions. Briefly, the DNA bands were cut from the gel using UV-transilluminator and dissolved in the binding buffer at 60 °C. The dissolved agarose was transferred into the MBST-column. After washing, the bound DNA was eluted using 50 µl TE-buffer. One to five µl of the eluted DNA was amplified with the semi-nested forward and reverse primers.

PCR product extraction from the agarose gel

Ten µl of PCR product was run on a 1.5% agarose in TBE buffer. After visualization of the posi-

tive band using ethidium bromide under UV, the PCR product was extracted from the gel using DNA extraction kit from agarose gel (MBST, Iran) according to the manufacturer's. The PCR product was cut from the gel using UV-transil-luminator and dissolved in 340 µl binding buffer for 5 min by 60 °C. After addition of 255 µl ethanol (96%) to the sample, the mixture was applied to the spin column and centrifuged for 1 min at 8000 g. The column was washed twice with washing buffer and the adsorbed DNA was eluted from the column using 50 µl elution buffers (11).

Table 1: The primers derived from *Haemonchus contortus* β-tubulin gene with accession No. X80046, version X80046.1 GI: 897752.

Primer	Nucleotide sequence
β-tubulin-Forward 1	5` gttctc cgttgttcca tcaccc 3`
β-tubulin-Forward 2	5` ccctt tccgtccatc aactggtaga-gaacaccgatgaaacat 3`
β-tubulin-Reverse	5` cgtgacaccagacattgtgacag 3`

Results

Our results showed that with this procedure the effect of proteinase K was much better and the amount of isolated DNA from worm increases compared to the direct DNA isolation after washing with PBS buffer. The amount of the extracted DNA was low, when the worms were used for the DNA extraction promptly after removing from 70% ethanol solution. Analysis of extracted DNA on agarose gel was visualized on 0.8% agarose gel using ethidium bromide using UV-transilluminator (Fig. 1 A).

The amount of the DNA in the probes was measured using spectrophotometer from the absorbance of 260 nm. The amount of the DNA in the solutions varied between non measurable (to low, when the DNA was extracted from the worms directly after removing from 70% ethanol solution) and 12 µg/ml. The average of the DNA in the probes was calculated as 5 µg/ml. Although in some few samples, it was not possible to measure the quantity of the DNA in solution, but the DNA could be amplified with the primers derived from *H. contortus* β-tubulin gene to obtain a PCR product with 402 bp in length (Fig.1 B lane 1). The amplification of *H. contortus* β-tubulin gene was successfully performed with 50 ng, 10 ng, 1 ng, 100 pg and 10 pg of the isolated DNA solution respectively (Fig. 1 C).

One micro liter of PCR product was directly amplified with reverse primer and second forward primer designed within the first forward primer and reverse primer region, to demonstrate the specificity of the PCR product. In all cases we obtained an expected PCR product with 222 bp in length (Fig. 1 B lane 2). Furthermore, the first PCR product (402 bp) was isolated from the 1.5% agarose gel using MBST-gel extraction kit. One to five micro liters from 50 µl of extracted PCR-product solution was amplified using the second forward primer and reverse primer. In all cases a PCR product with 202 bp could be demonstrated (Fig. 1 B lane 3).

Discussion

H. contortus a blood sucking parasites, is one of the most important gastrointestinal nematode of ruminant of Iran. It is an abomasal worm of ruminants of Iran and can cause high economical losses (14, 15). The discovery of the molecular mechanisms involved in the parasite/host interaction is one of the most interesting issues for investigating in helminthology (1-3). In many cases, we need the genomic DNA of a parasite. This genomic DNA must be pure and suitable for further analysis. DNA extraction from single helminths such as *Telodorsagia* sp. or *Haemonchus* sp. using methods based on the phenol/chloroform is problematic. We faced many difficulties in the DNA isolation of helminthes with phenol/chloroform or Tripure kit (Roche, Germany). The isolation of the DNA from the small quantity of biological materials by phenol based extraction requires high experimental experiences. It is easy possible to loose the small DNA pellet after ethanol precipitation. Ethanol pellets may be too small to see and may also be loosely at-

tached to the side of the tube. Therefore, it can be easily aspirated with the supernatant. Apart from this critical point, it is known, that the optimal ethanol precipitation of the low quantity of the DNA needs usually carrier molecules. Jiang et al. (12) have isolated the DNA from different number of oocystes of *Cryptosporidium* using 6 methods (traditional phenol-chloroform method, QIAamp DNA minikit after oocyst isolation by IMS, QIAamp DNA minikit, QIAamp DNA stool minikit, UltraClean soil DNA isolation kit and FastDNA SPIN kit for soil), from which the last 5 based on the selective binding of the DNA on the carriers. Due to the poor performance of the phenol-chloroform DNA extraction method, only five other methods were evaluated for the detection efficiency of *Cryptosporidium* spp. Shayan *et al*. (11) have analyzed the DNA from blood smear of infected sheep with primers specific for *Theileria* spp. and *Babesia* spp. They could isolate the sufficient DNA form blood smear only using the kit based on the selective DNA binding to the carrier and not with the traditional phenol-chloroform method.

Therefore, in the present investigation we used MBST-kit for the extraction of DNA from twenty single *H. contortus*. This kit is based on the selective binding of the DNA on the carrier localized in the special spin columns. The worm after lysing and proteinase digestion was transferred into the column. By centrifugation, the DNA bound to the carrier material in the column. All contaminants (proteins, carbohydrates, fatty acids and salts) were easily washed from the carrier by centrifugation. In the last step, the DNA was eluted from the column in the sterile tube also simply by centrifugation using elution buffer. The extraction of DNA using this kit required neither phenol nor ethanol precipitation, and the eluted DNA was ready to use.

The concentration of the DNA in solution was measured from the absorbance of 260 nm. Although A260 values between 0.1 and 1.0 are very reproducible, A260 readings <0.1 and > 1.0 lead to considerably lower reproducibility. When working with small amounts of DNA, quantification by agarose gel electrophoresis may be more reliable. The amount of the extracted DNA was low, when the worms were used for the DNA extraction promptly after removing from 70% ethanol solution. The important critical point for the DNA extraction is to ensure that the worm is well disrupted, homogenized, the cells are completely lysed and the DNA is free in the solution. Otherwise, the debris or non homogenized and insoluble materials can easily disrupt the DNA binding carrier in the column and the DNA isolation can not be performed efficiently. Due to the insufficient lysis of the worms, the extraction of the DNA from worms directly after removing from 70% ethanol solution was not satisfactory and our results confirmed the importance of the abovementioned critical point by the DNA extraction. The concentration of the DNA in the solutions varied between not demonstrable (very low amount, in the case of DNA extraction promptly after removing of the worm from 70% ethanol solution) and 12 µg/ml. The average amount of the DNA in the solution was 5 µg/ml. The purity of the DNA in the solution was calculated by measurement of optic density at A260 and A280. The A260: 280 ratio was between 1.7 and 2.0. The results indicated that the nucleic acids were pure without protein contamination in the solution. Lower values indicate protein contamination. Although the DNA concentration in the solution was in some few cases very low, the DNA could be amplified with the primers derived from β-tubulin-gene. The amplification resulted in the expected PCR product of 402 bp. The specificity of the PCR products was controlled with additional forward primer localized within the first forward primer and reverse primer. Our results showed that the specific PCR could be obtained with 50 ng, 10 ng, 1 ng, 100 pg and 10 pg genomic DNA respectively (Fig. 1 C). In several experiments, that we carried out, better amplification was achieved with 100 pg and 10 pg genomic DNA, respectively.

DNA from single adult *H. contortus* can be extracted probably with phenol based techniques. Recently, Tiwari *et al*. had used phenol extrac-

tion technique for the isolation of DNA from single adult worm and developed a new RFLP-PCR method for the detection of BZ resistance in the β-tubulin gene isotype 1 gene of *H. contortus* (16). They used 2 µl from 30 µl DNA solution and had not given any information about the concentration of the DNA in the solution. Comparing with our DNA extraction results, it seems that the DNA yield with phenol method is much less than with the kit used in the present study. For the genetic analysis of larva they used its DNA directly after proteinase K digestion, probably due to the small amount of DNA, without extraction. Therefore, it could be concluded that phenol extraction is not recommended for the isolation of DNA from low amount of biological materials. Coles *et al*. have digested first the single larva of Strongyles species (*Teladorsagia circumcincta, Teladorsagia colubriformis, H. contortus*) with proteinase K and the digested material was directly used for PCR analysis (2). These authors recommended using fresh larva to obtain reliable results. They used also nucleospin 1 tissue kit for the isolation of the DNA from single strongyles larva (*Cylicocyclus nassatus, C. insigne, C. elongatus, C. radiatus, Cyathostomum pateratum, C. catinatum and C. coronatum*) or Schwab *et al*. (13) extracted the DNA from single microfilariae of *Wuchereria bancrofti* directly in the PCR tube using the Qiagen DNeasy tissue kit .

Our literature search revealed that at present the isolation of DNA from small amount of biological materials is not any more recommended by the traditional method, but according to our results using the kit presented in this paper is safe, with high quality and quantity, fast, easy to handle and not costly for genetic analysis of even a single small worm.

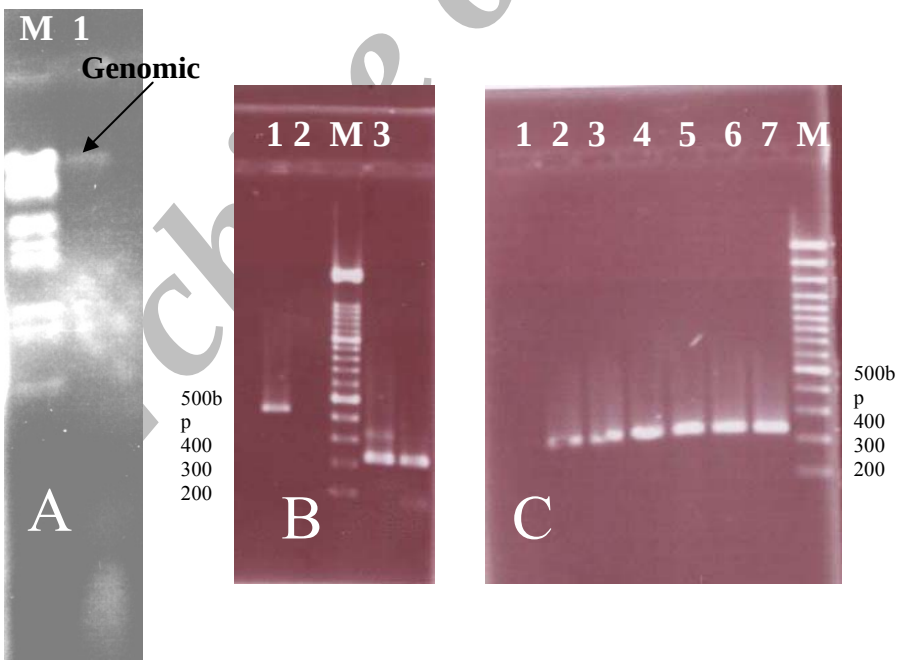

Fig. 1: A: DNA was extracted from single *Haemonchus contortus* worm and analyzed on 0.8 % agarose gel (lane 1), M was Hind III DNA-marker. B: DNA was amplified with β-tubulin-Forward 1 and reverse primer (lane 1). One micro liter of PCR product was amplified with β-tubulin-Forward 2 and reverse primer (lane 3) or the PCR product was first isolated from the agarose gel and then amplified with β-tubulin-Forward 2 and reverse primer (lane 4). Lane 2 was negative control and M was 100 bp DNA-marker. C. Genomic DNA was amplified with β-tubulin-Forward 2 and reverse primer in different dilutions: negative control (lane 1), positive control (lane 2), 5 µl (lane 3), 1µl (lane 4), 1:10 (lane 5), 1:100 (lane 6) and 1:1000 (lane 7). M was 100 bp DNA-marker.

Acknowledgements

We thank Dr Gholamian for purchasing some samples. This work was supported by University of Tehran, Central Excellence and Grants No. 76/10195 and No. 76/9508 financial supports.

References

1. Beech RN, Prichard RK, Scott ME. Genetic variability of the beta-tubulin genes in benzimidazole-susceptible and resistant strains of *Haemonchus contortus*. Genetics. 1994; 138(1): 103-10.
2. Coles GC, Jackson F, Pomroy WE, Prich-RK, von Samson-Himmelstjerna G, Silvestre A, Taylor MA, Vercruysse J. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol. 2006; 31; 136(3-4): 167-85.
3. Humbert JF, Cabaret J, Elard L, Leignel V, Silvestre A. Molecular approaches to studying benzimidazole resistance in trichostrongylid nematode parasites of small ruminants. Vet Parasitol. 2001; 101(3-4): 405-14.
4. Jacobs DE, Schnieder T. Application of molecular biology to the diagnosis of parasitic disease. Vet Parasitol. 2006; 136(2): 67-8.
5. Kaplan RM. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 2004; 20(10): 477-81.
6. Kwa MS, Veenstra JG, Roos MH. Benzimidazole resistance in *Haemonchus con--tortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1.Mol Biochem Parasitol. 1994; 63(2): 299-303.
7. Kwa MS, Veenstra JG, Roos MH. Molecular characterisation of beta-tubulin genes present in benzimidazole-resistant populations of *Haemonchus contortus*. Mol Biochem Parasitol. 1993; 60(1): 133-43.
8. Kwa MS, Kooyman FN, Boersema JH, Roos MH. Effect of selection for benzimidazole resistance in *Haemonchus contortus* on beta-tubulin isotype 1 and isotype 2 genes. Biochem Biophys Res Commun. 1993; 191(2): 413-19.
9. Lacey E, Prichard RK. Interactions of ben--zimidazoles (BZ) with tubulin from BZ-sensitive and BZ-resistant isolates of *Haemonchus contortus*. Mol Biochem Parasitol. 1986; 19(2): 171-81.
10. Le Jambre LF, Lenane IJ, Wardrop AJ. A hybridisation technique to identify anthelmintic resistance genes in *Haemonchus*. Int J Parasitol. 1999; 29(12): 1979-85.
11. Shayan P, Rahbari S. Simultaneous differentiation between *Theileria* spp. and *Babesia* spp. on stained blood smear using PCR. Parasitol Res. 2005; 97: 281-86.
12. Jiang KJ, Alderisio KA, Singh A. L, Xiao. Development of procedures for direct extraction of *Cryptosporidium* DNA from water concentrates and for Relief of PCR inhibitors. Appl Environ Microbiol. 2005; 71(3): 1135-41.
13. Shwab AE, Boakye DA, Kyelem D, Prichard RK. Detection of Benzimidazole resistance associated mutations in the filarial nematode *Wuchereria bancrofti* and evidence for selection by albendazole and ivermectin combination treatment. Am J Trop Med Hyg. 2005; 73(2): 234-38.
14. Alvarez-Sanchez MA, Perez-Garcia J, Cruz-Rojo MA, Rojo-Vazquez FA. Real time PCR for the diagnosis of benzimidazole resistance in trichostrongylids of sheep. Vet Parasitol. 2005; 129(3-4):291-98.
15. Otsen M, Hoekstra R, Plas ME, Buntjer JB, Lenstra JA, Roos MH. Amplified fragment length polymorphism analysis of genetic diversity of *Haemonchus con-tortus* during selection for drug resistance. Int J Parasitol. 2001; 31(10): 1138-43.
16. Tiwari J, Kumar S, Kolte AP, Swarnkar CP, Singh D, Pathak KM. Detection of benzimidazole resistance in *Haemonchus contortus* using RFLP-PCR technique. Vet Parasitol. 2006; 15: 301-7.