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Kısa Bildiri ***Short Communication***

Comparison of Two Methods for the Isolation of Genomic DNA from Cyathostomin Adult Parasites

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Abstract

Cyathostomins are the most common and important group of large intestine nematodes, infecting horses worldwide. The current control strategy is associated with the development of anthelmintic resistance, which has been reported worldwide. Therefore, experiments with this family of parasites have become progressively important to provide their monitoring and control strategies. The aim of the present study was to propose a faster and more economic assay for isolation of genomic DNA from the adult stage of Cyathostomin parasites than reported. Adult parasites were collected from a single horse from a farm in São José dos Pinhais, PR, Brazil, and were identified. Genomic DNA was isolated from ten individual female adult parasites using a standardized procedure developed. Then, extraction from ten individual female was carried out by another DNA extraction method. DNA concentration from both methods were measured and compared. We obtained a good DNA quality with this standardized procedure. As a result of this analysis, we propose a modified phenol-chloroform method, which will contribute to assays that require DNA extraction from adult worms for genomic DNA sequences of cyathostomin, or species-specific identification.

Introduction

Cyathostomins (Strongylida), also known as small strongyles, are the most common and important group of large intestine nematodes (Love et al., 1999) infecting horses worldwide being present in more than 90% of the infected animals (Molento et al., 2012). Infection with adults can cause intermittent diarrhea, weight loss, lethargy, loss of appetite and peripheral edema (Matthews and Morris, 1995) but the most pathogenic life stage is the migrating larvae, especially as a result of a mass emergence of the fourth stage (L4) from the intestinal wall. This potentially fatal condition is known as larval cyathostomiasis and can result in severe colitis, blood loss, enteropathy, acute diarrhea, and subcutaneous edema (Lyons et al., 1999).

The control of parasite infections relies on the regular anthelmintic treatment with short intervals. This control strategy imposes a high selection pressure to the parasite population and is associated to the lack of efficacy (Canever et al., 2013; Peregrine et al., 2014). This is emerging as one of the most serious problems to cyathostome control, creating a challenge to equine health programs (Peregrine et al., 2014). In Brazil, multidrug resistant cyathostomes are prevalent (Molento et al., 2008) and the lack of alternative treatments will make accurate diagnosis of anthelmintic resistance increasingly important (Kaplan and Vidyashankar, 2012). Therefore, experiments with this family of parasites have become progressively important to provide better control strategies and to understand their epidemiology. The aim of this study was to determine a faster, effective and more economic assay for the isolation of genomic DNA from adult stage Cyathostomin parasites.

Materials and Methods

Adult parasites were collected from a single horse from a farm in São José dos Pinhais, PR, Brazil, and were identified under a compound microscopy at x100 and x400 by morphological criteria (Tolliver, 2000), (Table 1). All worms were immediately washed twice with phosphate buffered saline (PBS) and stored in Trizol (Invitrogen, Carlsbad, CA, USA) at -20◦ C until use. Genomic DNA was isolated from ten individual female adult parasites using a standardized procedure

Table 1. Yield of genomic DNA material extracted from both DNA extraction protocols from Cyathostomin adult parasites identified according to the species*. DNA extraction methods are represented as: (A) modified phenol-chloroform DNA extraction method; (B) DNA extraction method from Truett et al. (2000).

DNA extraction methods		
	Species	**Genomic material concentration (ng/mL)**
(A)	Cylicodontophorus bicoronatus	2.4×10^4
	Triodontophorus serratus	1.1×10^4
	Triodontophorus tenuicollis	9.8×10^3
	Cylicostephanus calicatus	4.3×10^4
	Cylicocyclus brevicapsulatus	1.0×10^4
	Cylicostephanus calicatus	3.8×10^4
	Cylicocyclus leptostomus	2.6×10^4
	Cylicostephanus goldi	1.9×10^4
	Cylicocyclus brevicapsulatus	7.3×10^3
	Cylicostephanus longibursatus	2.7×10^4
(B)	Cylicocyclus brevicapsulatus	5.9×10^2
	Cylicocyclus leptostomus	4.5×10^2
	Cylicocyclus nassatus	6.3×10^2
	Cylicocyclus elongates	5.1×10^2
	Coronocyclus coronatus	6.4×10^2
	Coronocyclus labiatus	5.2×10^2
	Cylicostephanus minutes	6.4×10^2
	Cylicostephanus goldi	5.6×10^2
	Cylicostephanus longibursatus	6.0×10^2
	Petrovinema poculatus	5.1×10^2

*The species were identified by a morphological key (Tolliver, 2000).

developed, derived from phenol-chloroform method, as follows. Adult parasites were placed in a microcentrifuge tube where 500 µL of extraction buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 0.5 M NaCl, 1% SDS, 1 mM DTT, and 1 mg/mL Proteinase K were added. The tube was placed in an incubator at 56° C for 2 h, mixing at every 30 minutes. The sample was treated with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and homogenized. After centrifugation at 1300 x g for 5 min, the surface, which contained the DNA material was transferred to another microtube. We then added 1 mL of absolute ethanol at 8°C and the solution was mixed for 30 min. After another centrifugation at 1300 x g for 10 min at 5°C, the supernatant was discarded and 1 mL of 70% ethanol at 8°C was added to the sample. After the third centrifugation at 1300 x g for 2 min at 5°C, the super-natant was discarded and the microcentrifuge tube was inverted on a paper for 3 h to allow the DNA pellet to dry. The pellet was resuspended in 50 µL of ultrapure water.

Then, another DNA extraction from ten individual female was carried out by the DNA extraction method described by Truett et al. (2000), that use an alkaline lysis reagent, with a brief incubation in hot sodium hydroxide and pH adjustment with a Tris solution (HotSHOT), and neutralization with a suitable buffer. For more details about the HotSHOT method see Truett et al. (2000). The DNA concentration from both methods was measured by the Qubit dsDNA assay kit and the data were subjected to F test performed at a 1% significance level. The quality and integrity of the isolated DNA was confirmed with samples of the two DNA extraction methods in a 0.8% agarose gel electrophoresis.

Results

Our data suggests that in terms of ease and reproducibility, the tested DNA extraction methods were satisfactory, but the yield of genomic DNA material extracted by the modified protocol was significantly better than the method described by Truett et al. (2000) (Table 1).

Discussion

Given that quite a few protocols for the isolation of genomic DNA from Cyathostomin adult parasites have been reported (Tandon et al., 2005). It is natural to add new modifications into these protocols and to adapt to other nematodes, although most of them include freezing with nitrogen (Chagas et al., 2011; Fontenele et al., 2013). The DNA isolation procedure that eliminates the requirement of the liquid nitrogen step was accurate and less expense, and can be used mainly in areas where obtaining liquid nitrogen is a limitation. All the other

protocols are laborious and time-consuming, making them limited in their application. In this report, we described a very simple, fast, reproducible and effective DNA extraction procedure, significantly reducing the number of hours in the incubator, and the number of centrifugations, when compared to the other techniques for the isolation of genomic DNA from adult nematodes with phenol-chloroform (Chagas et al., 2011).

We obtained a good DNA quality with this modified phenol-chloroform procedure developed with the objective to reduce the number of steps and manipulation time, using smaller quantities of reagents. This is especially convenient if several samples are going to be prepared in parallel. As a result of this analysis, we propose a simplified method, which will contribute to assays that require DNA extraction from adult worms for genomic DNA sequences (cyathostomins or other nematode), or species-specific identification. Also, it could represent a basic step toward the development of a rapid and simple molecular test for the early detection of drug resistant parasites.

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