

Mathilde Schipper Student number: 5652863 Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine Utrecht University, Utrecht

Supervisors Dr. D.C.K. van Doorn Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine Utrecht University, Utrecht

Dr. F.N.J. Kooyman Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine Utrecht University, Utrecht

Abstract

Background: A widespread resistance of cyathostomins against macrocyclic lactones has been reported. Changes in the glutamate-gated chloride (GluCl) channels of cyathostomins are suspected to contribute to this development of anthelmintic resistance. To monitor the development of anthelmintic resistance it is important to know the genetic basis of the proteins involved. This research builds further on research where cDNA variations were found in *in vitro* resistant L3 stadia. The variation lies in the 5'end of the *glc-3* gene coding for the GluCl channel protein. Cwiklinski et al (2013) mapped with the use of DNA sequencing the biggest part of the genome of *Cylicostephanus goldi*, but did not succeed to sequence the 5'end of the *glc-3* gene.

Objectives: To map the DNA-sequence of the *glc-3* gene in cyathostomins and gain insight into the variation of this gene within individual cyathostomins.

Methods: Steps were taken to optimise the current lysis and polymerase chain reaction (PCR) protocol, especially for cyathostomins which have been stored in ethanol. Moderations were made in lysis type, Proteinase K amounts, inactivation temperature and purification manner. With PCR, different variations of the *glc-3* gene in individual cyathostomins were detected. PCR, multi displacement amplification, restriction enzymes, ligation and precipitation were used to map the unknown part of the *glc-3* gene. **Results:** No satisfactory PCR protocol has been found for adult cyathostomins which have been fixated in alcohol. The results of detecting different variations of the *glc-3* gene could be mapped.

Conclusions; A different research method must be developed to map the DNA sequence of the glc-3 gene in cyathostomins, as the used method proved to be inadequate.

Introduction

Cyathostomins, also known as small strongyles, are parasites of the equine intestine and are present in the caecum, ventral colon and dorsal colon of the horse (Collobert-Laugier *et al.*, 2002). Cyathostomin infections have a pathogenic effect on horses, with adult stadia of the worm causing damage to the intestine, resulting in lethargy, weight loss and diarrhoea. More severe however, is the simultaneous migration of many L4's, causing diarrhoea, colic and mortality, especially in younger horses (Love, Murphy and Mellor, 1999)

Cyathostomin infections are controlled with good management measures and partially with drugs: benzimidazoles, pyrantel or macrocyclic lactones are registered anthelmintic for horses and are used to diminish the egg output of the affected horses by killing adult cyathostomins. Because of the widespread resistance of cyathostomins against benzimidazoles and pyrantel, macrocyclic lactones are currently the most used drugs against cyathostomins (Kaplan, 2004; Nielsen *et al.*, 2018).

However, Geurden *et al.*, (2014) found a shortened egg reappearance period after treatment with ivermectin and moxidectin in more than half the researched study sites in Belgium, Italy and The Netherlands. The study of Kooyman *et al.*, (2016) showed that a shortened egg reappearance period is an indication for a reduced susceptibility to anthelmintics rather than an indication for faster development of immature stadia. This indicates, alongside other studies, that also resistance of the cyathostomins against macrocyclic lactones as ivermectin and moxidectin has developed (Lyons *et al.*, 2008; Näreaho, Vainio and Oksanen, 2011; Molena *et al.*, 2018).

Because of the lack of other drugs that can control cyathostomin infections in horses, this resistance forms a serious problem.

Targets of the macrocyclic lactones which possibly plays a role in anthelmintic resistance are the ABC transporters, 8-tubulin molecules and glutamate gated chloride channels (GluCl channels) (Mottier and Prichard, 2008). Great interest is in the GluCl channels. These are transmembrane proteins that play a role in the impulse transfer and are only present in invertebrates. Stimulated by glutamate, the GluCl channels are opened and chloride ions flow into the cell, giving a short hyperpolarisation of the membrane. Macrocyclic lactones such as ivermeetin can bind to the GluCl receptor and activate them, giving a permanent opening of the channels and thus flow of chloride ions into the cell. This results in a permanent hyperpolarisation, causing paralysis and death of the worms (Wolstenholme and Rogers, 2005). Different genes code for GluCl channels. *Glc-3* codes for GluCla4 subunit, present in cyathostomins (Wolstenholme, 2012). Changes in the GluCl channel is suspected to be related to the development of anthelmintic resistance of cyathostomins against macrocyclic lactones. Suspectedly, there is a variation in the glc-3 gene coding for the GluCl channel (Wolstenholme, 2011). To monitor the development of anthelmintic resistance it is important to know the genetic basis of the proteins involved. If more is known about the molecular mechanisms of the anthelmintic resistance, possibly in the future resistance could be mapped and tracked more exactly.

In vitro studies within the department on pools of L3s demonstrate that within cDNA of the 5'end of the *glc-3* gene four variants existed: It showed that Variation A was present both in pools of sensitive and in pools of resistant cyathostomins to Ivermectin. Variation B, C and D were only present in pools of resistant L3s for Ivermectin (Unpublished). Variation A and Variation B are transcripts which code for a putative functional protein. Variation C and D are truncated, meaning that the genes do not code for a functional protein. These variations C and D are however promising, as no functional protein means no target and consequently no effect of the drug. Variation A consist of a low complexity part with a ligand binding domain. Variation B consists of a false translation initiation, signal peptide and a ligand binding domain. The ligand binding domains in both variations are identical and are called the conserved coding part. There are multiple explanations for the existence of the *glc-3* gene variation for the functional protein. It is possible that Variation A and B are splice variations or possibly that they are allele variations.

Cwiklinski *et al* (2013) with the use of whole genome sequencing mapped the draft genome of *Cylicostephanus goldi*. In this study only the 3' part of the *glc-3* gene was sequenced. In the database of GenBank the 5'end of the *glc-3* is still missing.

The mapping of the unknown 5'end of the DNA sequence of the *glc-3* gene can in theory be done with genome walking. This is a method where -step by step- from a primer on a known part of the genome, an unknown adjacent part of the genome is amplified and consequently sequenced. By repeating this process multiple times, each time with a new primer pair, the sequence of a gene can be discovered. An overview of the genome walking method can be seen in Figure 1. The *glc-3* gene has a conserved coding part, which is present in each variation and of which the DNA-sequence is known. This is the starting point for the genome walking, by which the unknown 5'end will be uncovered.



Figure 1: Brief schematic overview of Genome Walking. By repeating this process multiple times, the sequence of a gene can be discovered. The blue arrows show the direction of amplification. Modified from Tsaftaris, Pasentzis and Argiriou (2010)

In the past, within the division of Clinical Infectiology, Department of Infectious Diseases & Immunology, other students have worked on the mapping of the glc-3 gene. My study continues the work of these students. The aim of this research is to map the 5'end of the DNA sequence of the glc-3 gene and gain insight into the variation of this gene within individual cyathostomins.

Through our previous studies with adult cyathostomins it became known that with the current protocols it was difficult to amplify DNA from adult worms and perform downstream applications with the PCR products. To succeed in mapping the 5'end of the DNA sequence of the glc-3 gene, it is necessary to optimise the current PCR protocol for adult cyathostomins.

Materials and methods

Worm collection

Adult worms were collected from the dorsal colon, ventral colon and caecum of horses at a slaughterhouse in Nijkerk, the Netherlands at multiple occasions. Worms were separated from gut contents by agar gel method (Van Wyk, Gerber and Groeneveld, 1980). The isolated worms were stored under different conditions. In 70 % ethanol at room temperature, at -20 °C or at -80 °C. For morphological determination the worms were decapitated and the heads were exposed to Chloride-Lactophenol and determined according to Lichtenfels *et al.*, (2008)

Lysis method

Several lysis methods were tested during this study. As control for adult worms we used cyathostomin L3 pools of unknown species composition. Because a L3 without tail is about 1/10 of the length of an adult worm, 1000 L3s were considered the equivalent of 1 adult worm.

Worm Lysis Buffer (WLB) method.

One adult worms or 1000 L3s were put into 100 µl Worm Lysis Buffer (WLB) containing 0.5 µl Proteinase K (20 mg/mL, InvitrogenTM, ThermoFisher Scientific, the Netherlands) (van Doorn *et al.*, 2010). Next, the sample was frozen for 15 min at -80 °C, followed by overnight incubation at 56 °C. The next morning the sample was heated for 15 min at 95 °C to inactivate the Proteinase K.

QiaAmp mini DNA kit (Qiagen) lysis

Adult worms or L3s were lysed according the manufacturers manual for tissues. One adult worm or 1000 L3s were lysed in 100 μ l buffer ATL, 80 μ l Phosphate buffered saline and 20 μ l Proteinase K overnight at 56 °C. Next, 200 μ l AL buffer was added, vortexed and incubated for 10 min at 70 °C.

Alkaline lysis

Worms were lysed with a 100 μ l alkaline lysis solution (400 mM KOH, 100 mM DTT, 10 mM EDTA). The samples were incubated (10 min., on ice) and neutralized with 35 μ l of neutralization solution (4 ml of 1 M HCl, 6 ml of 1 M Tris·HCl buffer, pH 7.5, final pH 0.6).

Test fragmentation of template DNA

After lysis with the WLB method the possible fragmentation of the genomic DNA was tested. The DNA samples (100 μ l) were precipitated by mixing with 50 μ l 3M sodium acetate (pH 5.3) and 500 μ l 100% ethanol. The sample was frozen at -80 °C for 45 minutes, centrifuged for 15 minutes and the supernatant removed. Two hundred μ l 70% cold ethanol was added, the sample was centrifugated and the supernatant removed. The pellet was dissolved in 10 μ l Tris-EDTA (TE) buffer.

Purification Template DNA

In order to increase the purity of the DNA isolates, several purification methods were performed.

Purification with QiaAmp purification mini DNA kit (Qiagen kit)

Purification was performed according the manual of the manufacturer. In brief, four hundred μ l lysed isolate was added to 100 μ l 96% ethanol, mixed and centrifugated. The mixture was applied to the spin column and 500 μ l AW1 Buffer was added. After mixing,

centrifuging and placing the spin column in a new collection tube, $500 \ \mu$ l AW2 was added. Thereafter the spin column was mixed, centrifuged and placed in a new collection tube, after which 200 μ l Buffer AE was added. The mixture was incubated at room temperature for 1 minute and centrifugated for 1 minute. The spin column was placed in a new collection tube, another 200 μ l Buffer AE was added and was incubated at room temperature and centrifugated again for 1 minute.

The dsDNA within the fractions was quantified with the Qubit Fluorimetric Quantification (ThermoFisherScientific, the Netherlands)

Purification with GFXTM PCR DNA and Gel Band Purification Kit (GFX kit)

Purification was performed according the manual of the manufacturer. In brief, 100 μ l lysed isolate were added to 300 μ l capture buffer, mixed and brought to the column. The column was washed according the manual of the manufacturer and eluted with 10 or 50 μ l elution buffer.

The dsDNA within the fractions was quantified with the Qubit Fluorimetric Quantification (ThermoFisherScientific, the Netherlands)

Polymerase Chain Reaction

DreamTaq PCR

2.5 µl template was added to a DreamTaq PCR mix, the contents added as Adjective 1. The DreamTaq PCR was run for 3 minutes at 95 °C, 35 cycles of consecutively 30 seconds at 95 °C, 30 seconds at 55-60 °C and 60 seconds at 72 °C, finished with 7 minutes at 72 °C.

Phusion Hotstart PCR

1 µl template was added to a Phusion Hotstart PCR mix, the contents added as Adjective 1. The Phusion Hotstart PCR was run for 30 seconds at 98 °C, 35 cycles of consecutively 10 seconds at 98 °C, 20 seconds at 54-60 °C and 60 seconds at 72 °C, finished with 7 minutes at 72 °C.

Primers

The used primer pairs were; CY1-CY18, coding for rRNA of cyathostomins, F532-R823, coding for the GluCl- α (*glc-3* gene), F350-R370, coding for the Conserved Coding Part of the *glc-3* in cyathostomins, FA250- RA200, coding for the Variation A of the *glc-3*, FB140-RB30, coding for the Variation B of the *glc-3*

Genome walking

Multi Displacement Amplification (MDA)

 $5~\mu l$ isolate was replicated with the REPLI-g Mini Kit (Qiagen, the Netherlands) according to the manufacturer's instructions, the protocol added as Adjective 3, and incubated overnight at 30 °C for 16 hours.

Digestion with Restriction Enzymes

5 µl MDA product isolate was added to 1 µl restriction enzyme, 2.5 µl 10X Fast Digest buffer and 16.5 µl Nuclease Free water. The used restriction enzymes were EcoRI, XBal, BamH1 and XHol. The mix is incubated at 37°C for 10 minutes (XbaI, BamH1 and XhoI) or 20 minutes (EcoRI) and subsequently the enzyme is inactivated with a heating block of 80°C for 5 minutes (EcoRI, BamH1 and XHoI) or 65°C for 20 minutes (XBaI).

Ligation

Twenty μl of the isolate was mixed with 50 μl 10X T4DNA ligase buffer, 5 μl T4 ligase and

 $425~\mu l$ Nuclease Free Water. After incubation at room temperature for two hours, the ligase was inactivated in a 70 °C heating block for 5 minutes.

Precipitation

The ligation products (500 μ l) were precipitated by mixing with 50 μ l 3M sodium acetate (pH5.3) and 500 μ l 100% ethanol. The sample was frozen at -80 °C for 45 minutes, centrifugated for 15 minutes and the supernatant removed. 200 μ l 70% cold ethanol was added, the sample was centrifugated and the supernatant removed. The sediment was dissolved in 20 μ l TE buffer.

PCR with Genome Walking Primers

The precipitation product was amplified with PCR. The primer pair used is F523-R370, primers of the known conserved coding part of the *glc-3* gene and adjacent to an unknown part of the gene, with the direction of the primers outwards.

Gel electrophoresis analysis

PCR and MDA products were analysed with gel electrophoresis, in an 1.0-1.5% agarose gel containing Midori Green (Nippon Genetics Europe). Before application to the gel Phusion Hotstart PCR products and MDA products were mixed 3:1 with DNA Gel Loading Dye 6X (Thermo ScientificTM, The Netherlands). The gel was processed by a Molecular Imager[®] Gel DocTM XR System (BioRad Laboratories B.V., Veenendaal, Nederland).

Results

Morphological determination of cyathostomin species

For morphological determination of cyathostomins it was necessary to conserve the head of worms in lactophenol. Worm heads following this procedure without fixation in ethanol, lost their structure after exposure to chloride-lactophenol. Consequently, the species of the worm could not be determined.

Fixating worms in 70% ethanol helped to retain the original structure of the worm during the procedure. Four hours fixation turned out to be insufficient, but overnight fixation of the worms in ethanol gave acceptable results. For this reason, the heads of the cyathostomins were removed and fixated in ethanol for at least 24 hours to fixate them, while the remaining body of worm was stored in -20°C.

Fixation methods and Proteinase K amounts

Individual adult non-differentiated worms, fixed in ethanol or in -20°C, were lysed with the WLB method in duplicate. In order to estimate the effect of Proteinase K, the amount Proteinase K was either standard or 10 times higher. The success of the lysis was estimated by performing DreamTaq PCR on the lysate, with CY1-CY18 primers. L3s were included as positive control. The results of the amplification are shown in Figure 2.

All the L3 samples (positive control) show a product on the gel, regardless of fixation or amount of Proteinase K. Only 1 L3/ethanol sample shows a more diminished product. Non-differentiated adult worms, taken from -20°C, show also a clear product, but all adult worm samples which have been stored in ethanol show no product on the gel. Regular or a tenfold more Proteinase K makes in this no difference. For adult worms a freezer fixation seems thus benifitial over a fixation in alcohol. Because the largest collection of *Cylicostephanus goldi* in the department is fixated in alcohol, for this research we focus on finding a working PCR protocol for cyathostomins fixated in alcohol.



Figure 2: Non-differentiated adult or L3 worms, fixated in either ethanol or -20°C and 1 or ten times the standard Proteinase K amount were lysed with WLB and are amplified with a DreamTaq PCR, the PCR products are shown on gel

Gaining insight in the fragmentation after lysis

Because the lysis of ethanol fixed adult worms resulted in a template not suitable for amplification, we checked the fragmentation of the DNA after lysis. A too big fragmentation makes DNA unsuitable as template for PCR.

Samples consisting of 10 cyathostomins, either frozen or from ethanol were lysed with WLB lysis method and precipitated. The pellet was run on gel (Figure 3). No difference in size or fragmentation could be seen.

As cyathostomins fixated in ethanol and fixated frozen are on similar height on the gel, there is no indication that the difference in the PCR outcome in Figure 2 of cyathostomins fixated in ethanol and fixated frozen is the result of a difference in fragmentation. The used method is a very rough method to measure the length of the fragments after lysis. It gives mainly information if a difference in fragment length is shown on gel, when no difference is shown, no definite conclusions can be drawn.



Figure 3: Gel electrophoresis of WLB method lysed non-differentiated cyathostomins, either fixated frozen or in ethanol, to study the fragmentation of the DNA after lysis.

Alkaline lysed cyathostomins

The experiment of Figure 3 gave no indication of a difference in the lengths the fragments, but did not give information about the integrity of the gDNA after lysis. It was hypothesized that the combination of a WLB lysis and fixation in ethanol damaged the DNA of the sample too much to use successfully as a template for PCR. Investigated was whether a milder lysis improves the PCR of adult cyathostomins fixated in ethanol. Adult worm samples, taken from -20 °C and taken from ethanol, were lysed with an alkaline lysis. The templates were amplified with a DreamTaq PCR with CY1-CY18 primers and run on gel (Figure 4). No sample shows a product on gel. An alkaline lysis does not improve the PCR of adult cyathostomins fixated frozen can

be amplified after Proteinase K/WLB lysis (Figure 2), but not after alkaline lysis. This change in lysis method was not an improvement in the PCR protocol for adult worms fixated in ethanol.



Figure 4: Non-differentiated adult worms, fixated in either ethanol or -20°C, were alkaline lysed. The template was amplified with a DreamTaq PCR and the PCR products are shown on gel

Inactivation temperatures and the effect of purification

Contamination can influence the suitability of the template for amplification. For this experiment we tried to remove all elements of the worm excluding its DNA from the sample, in order to remove possible inhibitors of the PCR. Non-differentiated adult worms fixated in ethanol were WLB method lysed, purified with the QiaAmp purification mini DNA kit and amplified with DreamTaq PCR with CY1-CY18 primers. Non-differentiated adult worms, taken from -20 °C, which were not purified after lysis, are included as a positive control. The results of the amplification are shown in Figure 5 (Lane 1-4).

Figure 4 shows only a product at the positive control (Lane 4). Purification of the lysed sample by QiaAmp kit, in order to remove possible inhibitors, has had no effect. To gain more insight in the effect of purification on the cyathostomin sample dsDNA concentrations were measured with the Qubit Fluorometric Quantification. Before purification the sample contained 73 ng dsDNA per worm. After purification the sample contained 12,5 ng dsDNA per worm, thus containing still 17% dsDNA of the original sample.

The same figure shows the effect of a lower Proteinase K inactivation temperature of 70°C instead of 95°C. Non-differentiated adult worms fixated in ethanol or fixated frozen were WLB method lysed, with different inactivation temperatures and amplified with DreamTaq PCR. The results of the amplification are shown in Figure 5 (Lane 5-7). Using a milder inactivation temperature (Lane 5 and 6), did not improve the amplification as it did not result in a product on gel.

As no successful method to amplify the DNA of ethanol stored cyathostomins was found, we chose to continue with cyathostomins stored frozen.



Figure 5: Non-differentiated adult worms, fixated in ethanol, are WLB method lysed, purified and amplified with DreamTaq PCR and put on gel (Lane 1 and 2). Non-differentiated adult worms, fixated in ethanol or fixated frozen, are WLB method lysed but inactivated with different temperatures (70 °C or 95°C), amplified with DreamTaq PCR and put on gel (Lane 5-7).

Purification with Qiagen and GFX kit

Genome walking is done with products of MDA. MDA requires very pure DNA samples. Different purification kits are available to purify DNA samples.

The effect of GFX kit purification of lysed adult worms fixated frozen was measured. Simultaneously, the effect of a 10 times higher amount of Proteinase K than the standard amount was investigated. Hereto, lysed individual adult worms were divided in 2 equal parts and 1 part was purified with the GFX kit before amplification. The template was amplified with a DreamTaq PCR with CY1-CY18 primers and the PCR product was put on gel, see Figure 6. This was all done in duplicate.

If comparing the purified samples with the non-purified samples of the same adult, all with a 1x Proteinase K concentration, it can be noted that in one of the two cases the GFX purification has had a negative effect on the amplification. Sample 1 shows a diminished product on gel, while sample 2 shows a clear product. The 10x Proteinase K samples show only a faint product on gel compared to the 1x Proteinase K samples. This experiment shows that a higher Proteinase K amount during lysis reduced the success of the amplification with PCR.



Figure 6: Non-differentiated adult worms were WLB method lysed with standard Proteinase K amounts or the tenfold and half of the samples were purified with the GFXTM PCR DNA and Gel Band Purification Kit. The templates were subsequently amplified with DreamTaq PCR and put on gel.

To further evaluate the effect of the purification and evaluate potential loss of dsDNA as a result of the purification, dsDNA concentrations of non-differentiated adult worms, fixated frozen and fixated in ethanol and lysed with the WLB method, were measured before and after GFX purification with the Qubit Fluorometric Quantification, as can be seen in Table 1.

Table 1: dsDNA concentrations in WLB lysed worms before and after GFX purification measured with Qubit. Each sample consisted out of 10 undifferentiated adult worms fixed in ethanol or at -20C°.

	Lysed worms before purification			Lysed worms after purification			
<u>Fixation</u>	<u>volume</u> <u>(ul)</u>	<u>conc. dsDNA</u> (ng/ul)	<u>total</u> dsDNA (ng)	<u>volume</u> <u>(ul)</u>	<u>conc.</u> <u>dsDNA</u> (ng/ul)	<u>total</u> dsDNA (ng)	<u>recovery</u> <u>dsDNA</u> <u>(%)</u>
Ethanol fixed	100	0,935	93,5	10	too low	/	/
Ethanol fixed	100	0,935	93 <i>,</i> 5	50	0,014	0,70	1%
-20 C°	100	0,871	87,1	10	too low	/	/
-20 C°	100	0,871	87,1	50	0,0135	0,68	1%

A dsDNA loss of more than 98% is not a satisfactory result for a purification method, so another purification method must be investigated.

As purification with the GFX kit did not give acceptable results, purification with the Qiagen kit was tested.

Non-differentiated adult worms, fixated in -20°C, were lysed with the WLB method, were purified with the QiaAmp purification mini DNA kit, amplified with DreamTaq PCR and put on gel, as can be seen in Figure 7. The PCR was run with primers CY1-CY18, coding for the rRNA of cyathostomins as well as with the primers R832 and F532, coding for amplification of a part of the *glc-3* gene. This was done to determine whether after purification the *glc-3* gene was present in the sample. DsDNA concentrations before and after purification were measured (Table 2).

Figure 7 shows that all samples have been amplified. No negative effect of the purification is seen. The samples that have been amplified with R832-F532 primers show that, even with the possible reduction of gDNA because of the purification, the *glc-3* gene was present in the sample as this gene is successfully replicated.

More information on the possible reduction of the amount of gDNA was established by measurement with Qubit Fluorometric Quantification, as can be seen in Table 2. The table shows a dsDNA amount per cyathostomin after purification that is almost four times as high than in the sample before purification. A spontaneous increase in DNA amount is impossible, so the purified sample was remeasured multiple times, consequently giving the same outcome as seen in Table 2. This demonstrates that this manner of determining dsDNA amounts is not an accurate method for this type of sample. As Figure 7 shows that the *glc-3* gene is present in the sample after purification and is amplified, we chose to carry on with this Qiagen purification kit, even though we had no conclusive measurement outcomes of DNA amounts in the samples.



Figure 7: Non-differentiated adult worms, lysed with the WLB method, were purified with QiaAmp purification mini DNA kit. Subsequently the templates were amplified with DreamTaq PCR with primers for ribosomal DNA (CY1 & CY18) and for the glc-3 gene (F832 & F532) and put on gel

	Lysed worms before purification		Lysed worms after purification				
<u>Fixation</u>	<u>volume</u> <u>(ul)</u>	<u>conc.</u> <u>dsDNA</u> (ng/ul)	<u>total dsDNA</u> <u>(ng)</u>	<u>volume</u> (ul)	<u>conc. dsDNA</u> (ng/ul)	<u>total dsDNA</u> <u>(ng)</u>	recovery dsDNA (%)
Fixated frozen	100	0,184	93,5	400	0,6315	252,6	270%

Table 2: dsDNA concentrations in WLB lysed worms before and after Qiagen purification measured with Qubit. The sample consisted out of 10 undifferentiated adult worms fixed in ethanol.

Glc-3 variation detection on individual cyathostomins

To select worms for genome walking, the *glc-3* variation type of individual adult worms must be determined. For this, differentiated adult worms, fixated frozen, were lysed with Qiagen method, purified with the QiaAmp purification mini DNA kit and amplified with either Phusion Hotstart PCR (Figure 8) or with DreamTaq PCR (Figure 9). To determine the *glc-3* variant type in the cDNA of the individual cyathostomins, the PCR was run with three primer pairs: Primers F350 and R370, for amplification of the Conserved Coding Part of the gene, which should be present in all variations. FA250 and RA200 for gene variation A and FB140 and RB30 for gene variation B.

The Phusion Hotstart PCR as can be seen in Figure 8, is, apart from some primer-dimer, without any product showing. No variation A or B is shown and neither the Conserved Coding Part.

The gel electrophoresis of the DreamTaq PCR (Figure 9) shows one Conserved Coding Part product (Lane 8). Variation A product is only shown by the positive control and variation B shows three vague products (Lane 1, 5 and 8).

The Conserved Coding Part should be present in all the gene variations of *glc-3*. On the gel however, only a product shows in lane 8 for the primer pair F350-R370 (Figure 9). Therefore, it is the question whether the PCR has worked correctly. Also, variation A is shown in none of the samples on gel and variation B only in 3 out of 8 samples.

With a small yield like this, it is not possible to say whether the variations A or B are not present in the cyathostomin samples or if the PCR has not worked correctly. No final conclusions concerning the presence of the *glc-3* variations in the individual cyathostomins can be drawn.



Figure 8: Differentiated adult worms, fixated frozen, were Qiagen lysed, purified with the QiaAmp purification mini DNA kit, amplified with Phusion Hotstart PCR, with the primers F350 & R370, for amplification of the Conserved Coding Part of the gene, FA250 & RA200 for glc-3 variation A and FB140 & RB30 for glc-3 variation B.



Figure 9: Differentiated adult worms, fixated frozen, were Qiagen lysed, purified with the QiaAmp purification mini DNA kit, amplified with DreamTaq PCR, with the primers F350 & R370, for amplification of the Conserved Coding Part of the gene, FA250 & RA200 for glc-3 variation A and FB140 & RB30 for glc-3 variation B.

Mapping the DNA-sequence of the glc-3

We did not succeed to conclusively determine which variations of *glc-3* were present in the cyathostomins (Figure 8 & 9). For this reason, randomly chosen were one *C. longibursatus*, one *C. nassatus*, both fixated frozen and one *C. goldi*, fixated in ethanol. These differentiated adult worms were Qiagen lysed and purified with the QiaAmp purification mini DNA kit. On this template an MDA was carried out. The effect of the MDA was tested with DreamTaq PCR by using a dilution series of the MDA product as template, as can be seen in Figure 10. The dilution factor is given as compared to the original lysis sample. The MDA product is already a 10 times dilution of the original lysis sample.

Only the *C. nassatus* sample shows product on gel. As the lysis sample of *C. nassatus* shows a product on gel till 100 times diluted and the MDA product sample of *C. nassatus* shows a faint product on gel at a 10,000 times dilution, this shows that with the MDA amplification of the genome was achieved.

As earlier experiments indicate that the PCR of cyathostomins, fixated in ethanol, are not possible with the current methods, it is of little surprise that also the *C. goldi* MDA shows no product on gel. Why sample 3, a *C. longibursatus*, fixated frozen, was not amplified is unclear.

Diluted lysisproduct				
C. goldi	C. longibursatus			
EtOH	-20 °C			
10X 100X 1000X 10.000X 100.000X	10X 100X 1000X 10.000X 100.000X			
	Diluted lysisprodu <u>C. goldi</u> EtOH X000 X01 1 1 1 1 1 1 1 1 1 1 1 1 1			

Diluted MDA product				
C. nassatus	C. goldi	C. longibursatus		
-20 °C	EtOH	-20 °C		
10X 100X 1000X 10.000X 100.000X 1.000.000X	10X 100X 1000X 10.000X 100.000X 1.000.000X	10X 100X 1000X 10.000X 100.000X 1.000.000X		

Figure 10: DreamTaq PCR performed on dilution series of MDA products and corresponding lysis products of differentiated adult worms

Because of this PCR outcome, the template of *C. nassatus* was chosen for the mapping of the DNA-sequence of the *glc-3*.

The MDA template was cut with restriction enzymes, ligated and precipitated. De product of these steps was amplified with DreamTaq PCR with primers of a known part of the genome (F532-R823) and put on gel to check the quality of the product, see Figure 11. No products, apart from the positive control, were seen on gel.



Figure 11: MDA product of C. nassatus was cut with restriction enzymes, ligated and precipitated. This was used as template for a DreamTaq PCR with primers F532-R823 coding for the glc-3 gene and put on gel

Despite the lack of products on gel, we tried to amplify an unknown part of the genome of the *glc-3* gene. The *C. nassatus* MDA product, which was cut with the four different restriction enzymes, ligated and precipitated, was amplified with a DreamTaq PCR of a reverse version of the primers of the known part of the gene (F532-R370). The resulting products were put on gel (Figure 12). There is no clear product on gel. There is however a faint reaction seen close by the application sites of all samples, including the lane of the negative sample. Because of the location this material is not considered an amplification product of the PCR. The positive control (Lane P1 and P2) show no product on gel. There has been no amplification of gDNA. This was expected, as these samples were not ligated. The primer pair was directed outwards, meaning that only if the template was made circular, the primer pair through PCR could form double-stranded DNA. The samples of P1 and P2 were thus technically no positive control.



Figure 12: MDA product template of C. nassatus was cut with restriction enzymes, ligated and precipitated. This was used as template for a DreamTaq PCR with genome walking primers and put on gel

Discussion

The aim of this research was to obtain the DNA-sequence of the glc-3 gene in cyathostomins and gain insight into the variation within this gene. To map this DNA-sequence it was necessary to optimise the current PCR and down stream protocols for adult cyathostomins.

As the only whole genome sequencing on cyathostomins of equids was carried out on a C. goldi, this research focused on completing this sequencing for the glc-3 on specifically this species. Since it was hard to find fresh C. goldi in the slaughterhouse material, the study depended on a batch of earlier collected C. goldi, which was fixated in ethanol. In particularly these types of material, adult cyathostomins fixated in ethanol, led to problems with the PCR. L3s which have been stored fixated in alcohol could be amplified through PCR, adult cyathostomins fixated frozen could also be amplified through PCR, but a combination of these two factors, adult and fixated in alcohol, caused problems. Within this Department these problems had also been reported in various previous experiments with adult cyathostomins fixated in ethanol.

Recent studies working with PCR's on cyathostomin material mainly investigate cyathostomins fixated frozen (Cwiklinski et al., 2012, 2013). Other studies successfully PCR amplified adult cyathostomins which have been stored in ethanol (Traversa et al., 2008; Lake et al., 2009; Peachey et al., 2017). These studies used different primers than we did, as they were investigating other genes and used different PCR master mixes, as ReadyM Mix REDTaq, Qiagen Taq PCR Master Mix Kit or BIO-X-Act polymerase, and corresponding PCR cycles. The first two master mixes were reasonably similar as the ones used within this study as also a Taq polymerase was used, whereas in the latter a different type of polymerase is used. These studies did not report problems with the PCR of adult cyathostomins in ethanol. Ethanol (70%-100%) is generally known as an excellent fixator for preserving both high-molecular weight DNA. It is shown that DNA is largely collapsed in an ethanol solution, but reverses substantially to its original form when rehydrated. This fixator can however reduce the DNA quality and quantity slightly, but the DNA remain generally sufficient to perform PCR (Gillespie et al., 2002; Srinivasan, Sedmak and Jewell, 2002). Since these studies were done on human tissue, it is not totally comparable to this experiment on cyathostomin tissue.

The seemingly small change of adult cyathostomin tissue compared to L3 cyathostomin tissue, affected how the fixation method influenced the tissue and consequently whether the PCR could be performed. Possibly the ethanol cannot perfuse the adult cyathostomins as good as the smaller L3 cyathostomins, as adult cyathostomins have a mass approximal a 1000 times higher than L3 cyathostomins. This could have resulted in a not complete fixation of the worm and thus degeneration of the DNA in the sample. The results of the test with fragmentation of the DNA after lysis did not indicate this, but the method was too rough to be able to visualize the effect of the lysis on DNA. Another possible explanation is that during the development of L3 to adult the worms developed certain inhibitors that interfered with the PCR, possibly through interaction with DNA or interference with DNA polymerase. It is interesting that even after purification following the lysis, where theoretically only the DNA remains, still no PCR on adult cyathostomin tissue fixated in ethanol was possible. Perhaps these inhibitors are bonded directly to the double stranded DNA, which makes them insusceptible for the purification process.

The results show that adult cyathostomins, fixated frozen, can be amplified with primers CY1-CY18, coding for ribosomal RNA of cyathostomins. When these worms are amplified in the same manner, but with the primers F350-R370, the PCR gives inconclusive results. These primers are coding for the conserved coding part of the glc-3 gene, a part of the genome which is present in each variation of the *glc-3* gene. Each individual cyathostomin sample should give a product on gel after DNA amplification with this primerset, because the template is always present. The results show however, that the amplification of this part of the genome did not succeed. As a result, the outcome of the experiment to establish the presence of variation A and B in individual cyathostomins could not be validated. If the sample showed no product for variation A of the gene, it was unclear whether this variation was indeed not present in the sample or whether the PCR was not successful. The inconsistency of the effectivity of the PCR is hard to explain. Possibly also a variation in the conserved coding part of the glc-3 gene is present in the cyathostomin population, causing the primers not to fit on the template of certain cyathostomins anymore. A second explanation could be that not the correct annealing temperatures were used during the PCR. The GC ratio and the master mix determine the optimal annealing temperatures. The optimal temperatures can be calculated, but as the forward and reverse primers contain different ratios of nucleobases, the optimal temperature for the forward and reverse primer can be different. The next step should include an experiment with different annealing temperatures in combination with the primers for the conserved coding part, variation A and variation B, in order to establish the optimal annealing temperature.

The start of the mapping of the unknown 5'end of the glc-3 gene was unsuccessful. No product showed on gel after PCR of the ligated MDA product, so isolation of product on gel for sequencing was not possible. Since we were unable to sequence the 5' end of the glc-3 gene, the research question cannot be answered. Unfortunately, this research has not succeeded in mapping the unknown DNA-sequence of the glc-3 gene in cyathostomins.

Somewhere in the process DNA gets lost, which has resulted in no product shown on gel. Further research should optimize the genome walking method used in this research. The restriction enzymes used were six-cutters, possibly causing a to big fragmentation of the DNA. An experiment with eight-cutter restriction enzymes could give insight if this forms a problem. Secondly, if a restriction enzyme cuts shortly after the primer application site, only a very short fragment can be amplified with PCR. We used four different restriction enzymes, so it is unlikely that all enzymes cut shortly into the unknown 5'end, but it can certainly be the case in one of the restriction enzymes. Furthermore, the concentration of DNA compared to the amount of added ligase could be out of balance. Generally, 1-10 μ g/ml concentration of DNA is recommended during ligation. Here 1.5 μ ml DNA was used, which is on the lower limit of the range. The positive side of this is that there is a low chance of self-ligation, but as a downside the amount of ligated DNA available for the next step in the process of mapping the unknown part of the *glc-3* gene is also lower.

Conclusion

No satisfactory PCR protocol for adult cyathostomins which have been fixated in alcohol was established. The results of detecting different variations of the *glc-3* in individual cyathostomins were inconclusive. No previously unknown part of the *glc-3* gene could be mapped. A different research method must be developed to map the DNA sequence of the *glc-3* gene in cyathostomins, as the used method proved to be inadequate.

Adjective 1

<u>DreamTaq Master Mix</u>

	With 2.5 µl template
DreamTaq Master Mix	μΙ
Forward primer 10 uM Reversed primer 10 uM	1 1
10x DreamTaq Green Buffer	2.5
dNTP 10 mM	0.5
U/ul)	0.25
H2O	17.25
Total	22,5
	(+2.5 µl

PCR Cycle DreamTaq PCR			
3 minute 95 °C			
30 sec 95 °C			
30 sec 50-60 °C	35 x		
60 sec 72 °C			
7 min 72 °C			

Phusion Hotstart Master Mix

Phusion Hotstart Master Mix	With 1 μl template μl
Forward primer 10 uM Reversed primer 10 uM	1 1
5x HF Buffer	5
dNTP 10 mM Phusion Hotstart II (2 U/ul)	0,5 0.25
H2O	16.25

PCR Cycle Phusion Hotstart PCR <u>30 sec 98 °C</u> 10 sec 98 °C 20 sec 60 °C 35 x <u>60 sec 72 °C</u> 7 min 72 °C

Total

24 (+1 µl template)

template)

Primer	Sequence	Function	Melting Temperature (T _m)
CY1	GGT CAA GGT GTT GTA	Coding for ribosomal	60°C
	TCC AGT AGA G	RNA of cyathostomins	
CY18	CTT AGA CAT GCA TGG	Coding for ribosomal	60°C
	CTT AAT C	RNA of cyathostomins	
R823-nas	CAA AAC GAY ACC CAT	Coding for the GluCl-α	55°C
	GAT ACT ATG AC	(<i>glc-3</i> gene) in specifically	
		C. nassatus (Reversed)	
R823-cat	CAG AAC GAC ACC CAY	Coding for the GluCl-a	55°C
	GAY ACT ATG AC	(<i>glc-3</i> gene) in	
		cyathostomins except for	
		C. nassatus (Reversed)	
7500			
F532	CAG TAC TAT CCT ATG	Coding for the GluCl-a	55°C
	GAT GTG CAA AC	(glc-3 gene) in	
		cyathostomins (Forward)	
F350	CAT ATG ATC GAY AAG	Coding for the Conserved	DreamTaq: 50°C
	CUR AAU G	Coding Part of the glc-3	Hotstart: 60°C
		in cyathostomins	
P 270		(Forward)	Dream Tex: 50°C
n570	ATC AAA C	Coding Port of the <i>clar2</i>	Hotatant: 60°C
	ATG AAA G	in everthestoming	Hotstart. oo C
		(Reversed)	
FA250	TGC KGT TTT ATT AGG	Coding for the Variation	DreamTaq: 54°C
	WTT TCA GC	A of the <i>glc-3</i> in	Hotstart: 60°C
		cyathostomins (Forward)	
RA200	CGD GAA TGC TGT CTT	Coding for the Variation	DreamTaq: 54°C
	GCW CC	A of the <i>glc-3</i> in	Hotstart: 60°C
		cyathostomins (Reversed)	
FB140	ACT GCT CCT CCR YAT TCC	Coding for the Variation	DreamTaq: 50°C
	C	B of the <i>glc-3</i> in	Hotstart: 60°C
		cyathostomins (Forward)	
RB30	GTT YAG CAG TAC RGC	Coding for the Variation	DreamTaq: 50°C
	CCC	B of the <i>glc-3</i> in	Hotstart: 60°C
		cvathostomins (Reversed)	

Adjective 2

Adjective 3

Protocol for multi displacement amplification with REPLI-g Mini kit Protocol adapted from REPLI-g® Mini/Midi Handbook, Page 11-13 (<u>https://www.qiagen.com/nl/resources/resourcedetail?id=843654e0-2ccb-474b-b4b8-</u> <u>8744453ed5cb&lang=en</u>)

Protocol for amplification of 7 samples

- 1. Prepare sufficient Buffer D1 (9 μ l DLB buffer with 32 μ l Nuclease-free water) and Buffer N1 (12 μ l Stop Solution with 68 μ l Nuclease-free water).
- 2. Place 5 μ l template DNA into a microcentrifuge.
- 3. Add 5 µl Buffer D1 to the DNA. Mix by vortexing and centrifuge briefly.
- 4. Incubate the samples at room temperature for 3 min.
- 5. Add
- 6. $10 \ \mu l$ Buffer N1 to the samples. Mix by vortexing and centrifuge briefly.
- 7. Thaw REPLI-g Mini DNA Polymerase on ice. Thaw all other components at room temperature, vortex, then centrifuge briefly.
- Prepare a master mix on ice by adding 1 μl REPLI-g Mini DNA Polymerase to 29 μl REPLI-g Mini Reaction Buffer. Mix and centrifuge briefly.
- 9. Add 30 μ l of the master mix to 20 μ l of denatured DNA (step 5).
- 10. Incubate at 30° C for 10-16 h.
- 11. Inactivate REPLI-g Mini DNA Polymerase by heating the sample for 3 min at 65°C.

References

Collobert-Laugier, C. *et al.* (2002) 'Prevalence, abundance and site distribution of equine small strongyles in Normandy, France', *Veterinary Parasitology*. Elsevier, 110(1–2), pp. 77–83. doi: 10.1016/S0304-4017(02)00328-X.

Cwiklinski, K. *et al.* (2012) 'New insights into sequence variation in the IGS region of 21 cyathostomin species and the implication for molecular identification', *Parasitology*, 139(8), pp. 1063–1073. doi: 10.1017/S0031182012000467.

Cwiklinski, K. *et al.* (2013) 'Transcriptome analysis of a parasitic clade V nematode: Comparative analysis of potential molecular anthelmintic targets in Cylicostephanus goldi', *International Journal for Parasitology*, 43(11), pp. 917–927. doi: 10.1016/j.ijpara.2013.06.010.

van Doorn, D. C. K. *et al.* (2010) 'In vitro selection and differentiation of ivermectin resistant cyathostomin larvae', *Veterinary Parasitology*, 174(3–4), pp. 292–299. doi: 10.1016/j.vetpar.2010.08.021.

Geurden, T. *et al.* (2014) 'Decreased strongyle egg re-appearance period after treatment with ivermectin and moxidectin in horses in Belgium, Italy and The Netherlands', *Veterinary Parasitology*, 204(3), pp. 291–296. doi: https://doi.org/10.1016/j.vetpar.2014.04.013.

Gillespie, J. W. *et al.* (2002) 'Evaluation of Non-Formalin Tissue Fixation for Molecular Profiling Studies', *The American Journal of Pathology*. Elsevier, 160(2), pp. 449–457. doi: 10.1016/S0002-9440(10)64864-X.

Kaplan, R. M. (2004) 'Drug resistance in nematodes of veterinary importance: a status report', *Trends in Parasitology*, 20(10). doi: 10.1016/j.pt.2004.08.001.

Kooyman, F. N. J. *et al.* (2016) 'Semi-quantitative differentiation of cyathostomin larval cultures by reverse line blot', *Veterinary Parasitology*. Elsevier, 216, pp. 59–65. doi: 10.1016/J.VETPAR.2015.12.009.

Lake, S. L. *et al.* (2009) 'Determination of genomic DNA sequences for beta-tubulin isotype 1 from multiple species of cyathostomin and detection of resistance alleles in third-stage larvae from horses with naturally acquired infections', *Parasites & Vectors.* BioMed Central, 2(Suppl 2), p. S6. doi: 10.1186/1756-3305-2-S2-S6.

Lichtenfels, J. R., Kharchenko, V. A., Dvojnos, G. M. (2008) 'Illustrated identification keys to strongylid parasites (strongylidae: Nematoda) of horses, zebras and asses (Equidae)', *Veterinary Parasitology*. Elsevier, 156(1–2), pp. 4–161. doi: 10.1016/J.VETPAR.2008.04.026.

Love, S., Murphy, D. and Mellor, D. (1999) 'Pathogenicity of cyathostome infection', *Veterinary Parasitology*. Elsevier, 85(2–3), pp. 113–122. doi: 10.1016/S0304-4017(99)00092-8.

Lyons, E. T. *et al.* (2008) 'Field studies indicating reduced activity of ivermectin on small strongyles in horses on a farm in Central Kentucky', *Parasitology Research*, 103(1), pp. 209–215. doi: 10.1007/s00436-008-0959-7.

Molena, R. A. *et al.* (2018) 'Cyathostomine egg reappearance period following ivermectin treatment in a cohort of UK Thoroughbreds.', *Parasites & vectors*. BioMed Central, 11(1), p. 61. doi: 10.1186/s13071-018-2638-6.

Mottier, M. de L. and Prichard, R. K. (2008) 'Genetic analysis of a relationship between macrocyclic lactone and benzimidazole anthelmintic selection on Haemonchus contortus', *Pharmacogenetics and Genomics*, 18(2), pp. 129–140. doi: 10.1097/FPC.0b013e3282f4711d.

Näreaho, A., Vainio, K. and Oksanen, A. (2011) 'Impaired efficacy of ivermectin against Parascaris equorum, and both ivermectin and pyrantel against strongyle infections in trotter foals in Finland', *Veterinary Parasitology*. Elsevier, 182(2–4), pp. 372–377. doi: 10.1016/J.VETPAR.2011.05.045.

Nielsen, M. K. *et al.* (2018) 'Anthelmintic efficacy against equine strongyles in the United States', *Veterinary Parasitology*. Elsevier, 259, pp. 53–60. doi: 10.1016/J.VETPAR.2018.07.003.

Peachey, L. E. *et al.* (2017) 'P-glycoproteins play a role in ivermectin resistance in cyathostomins', *International Journal for Parasitology: Drugs and Drug Resistance*. Elsevier, 7(3), pp. 388–398. doi: 10.1016/J.IJPDDR.2017.10.006.

Srinivasan, M., Sedmak, D. and Jewell, S. (2002) 'Effect of Fixatives and Tissue Processing on the Content and Integrity of Nucleic Acids', *The American Journal of Pathology*. Elsevier, 161(6), pp. 1961–1971. doi: 10.1016/S0002-9440(10)64472-0.

Traversa, D. *et al.* (2008) 'Haplotypic variability within the mitochondrial gene encoding for the cytochrome c oxidase 1 (cox1) of Cylicocyclus nassatus (Nematoda, Strongylida): Evidence for an affiliation between parasitic populations and domestic and wild equid hosts', *Veterinary Parasitology*. Elsevier, 156(3–4), pp. 241–247. doi: 10.1016/J.VETPAR.2008.05.031.

Tsaftaris, A., Pasentzis, K. and Argiriou, A. (2010) 'Rolling circle amplification of genomic templates for inverse PCR (RCA–GIP): a method for 5'- and 3'-genome walking without anchoring', *Biotechnology Letters*. Springer Netherlands, 32(1), pp. 157–161. doi: 10.1007/s10529-009-0128-9.

Wolstenholme, A. J. (2011) 'Ion channels and receptor as targets for the control of parasitic nematodes', *International Journal for Parasitology: Drugs and Drug Resistance*, 1(1), pp. 2–13. doi: 10.1016/j.ijpddr.2011.09.003.

Wolstenholme, A. J. (2012) 'Glutamate-gated Chloride Channels', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 287(48), pp. 40232–40238. doi: 10.1074/JBC.R112.406280.

Wolstenholme, A. J. and Rogers, A. T. (2005) 'Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics', *Parasitology*, 131(S1), pp. 85–95. doi: 10.1017/S0031182005008218.

Van Wyk, J. A., Gerber, H. M. and Groeneveld, H. T. (1980) 'A technique for the recovery of nematodes from ruminants by migration from gastro-intestinal ingesta gelled in Agar: Large-scale application', *Onderstepoort J. vet. Res*, 47, pp. 147–158.