Egg hatching protocol and an *in vitro* scoring system in *Parascaris univalens* larvae after exposure to anthelmintic drugs

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Abstract:

Ascaris is a genus of parasitic worms (helminths) found in the small intestine of various mammalian hosts, including *Ascaris lumbricoides* in humans, *Parascaris equorum* and *P univalens* in horses, *Ascaris suum in pigs*, *Toxocara cati* in cats and *Toxocara canis* in dogs. To date, *Parascaris* spp. are the only *Ascaris* worms that have developed resistance to anthelmintic drugs. The mechanisms of resistance in *Parascaris* spp are incompletely understood, partly due to the absence of robust *in-vitro* models. Further complicating *in-vitro* studies, *Parascaris* spp lack a free-living larval stage as their larva only hatch within the host. The aim of this study was to develop *in-vitro* methods for hatching, scoring the viability of *Parascaris* L3 larvae and exposing them to the anthelmintic drugs ivermectin, pyrantel, thiabendazole, and the herbal extract carvacrol. This study shows that mechanical *Ascaris* egg breaking using a homogenizer resulted in a hatching rate of 98%. Our viability scoring system could distinguish an ivermectin resistant larvae from an ivermectin susceptible larvae derived from different farms. This indicates that this method may have utility for the screening of larvae ivermectin resistance on the level of farm populations. Interestingly, a highly paralytic effect observed after carvacrol exposure. Carvacrol shows direct paralytic effects on *Parascaris* larvae in a dose-dependent manner, as higher concentrations were lethal to all exposed larvae. This result presents a potential future opportunity for carvacrol used in the treatment of *Ascaris* infections. To conclude our results, we have successfully developed an *in-vitro* model as well as a scoring system for the viability of *Parascaris* L3 stage larvae, which can be used for assaying the effect on larvae after drug exposure

Popular summary:

Parascaris is a subtype of *Ascaris* parasitic worm which causes infection in young horses causing mild to severe symptoms, potentially resulting in small intestinal obstruction or even death. Horses older than 1 year usually have no problems with this parasite because their immune system has developed immunity against this parasite. A worldwide overuse of anthelmintic drugs has resulted in increasing resistance to macrocyclic lactones (ML) and sporadic cases of resistance to other drug classes with economic and animal welfare consequences. The mechanisms of this resistance are unknown, due in part to the lack of a model to study *Parascaris* in the laboratory (*in vitro*). This parasite lacks the free-living stage (only the eggs). Larva only hatches inside the host which makes this parasite complicated to study *in* vitro. Inside the host, the pregnant female can lay more than 170,000 eggs and that means around 60 million eggs in a year. Because of very thick outer layers, the egg can survive in the environment for more than 10 years. This study aimed to develop a model in order to study the effects of anthelminthic drugs on *Parascaris* under laboratory conditions.

Keywords:

Parascaris, Equine, Larvae, Anthelminthic Resistance, Hatching, Parascaris univalens, Carvacrol, Thiabendazole, Pyrantel, Ivermectin

1- Introduction

1-1: Ascarid worms:

Ascaris are large parasitic worms found in the small intestinal of various mammalians including humans (*Ascaris lumbricoides)*, horses (*Parascaris. equorum* and *univalens),* pigs *Ascaris suum*, cats *Toxocara cati*, and dogs *Toxocara canis*. *A. suum* is the *Ascaris* species which shows high potential zoonotic infection between pigs and humans (CDC, 2018), but there is also some evidence that *A. lumbricoides* can infect dogs (Scott Weese, *et al*.,2010). *A. lumbricoides* belong to the soil-transmitted helminth (STH) which currently infect approximately 1.5 billion people annually (Lee, *et al.,*2012). *Ascaris* infections may induce starvation in the host as they can parasitize a substantial amount of nutrition. Growth and development in children or young animals may be stunted by *Ascaris* infections.

1-2: The equine roundworms:

The *Parascaris spp, P. equorum,* and *P. univalens* are intestinal parasites that can infect horses, zebras, and donkeys (Colin, 2000) and are the most common parasites in foals worldwide (Pusteria & Nicola, 1967). Interestingly, *Parascaris* spp are the only *Ascaris* worms that have developed resistance to anthelmintic drugs (Nielsen, 2016). The most susceptible to infection are young horses that are 2 to 6 months old (Lyons, *et al*., 1976). By the age of one-year, most horses develop natural immunity to this parasite infection (Clayton & Duncan, 1977). Symptoms of infestation with ascarids vary from asymptomatic to highly pathogenic and include nasal discharge, respiratory syndromes, impaired condition, coughing and cessation of growth, intestinal blockage, and potentially lethal complications such as intestinal rupture (Clayton & Duncan, 1978; Austin *et al*., 1990; Reinemeyer, 2009). *P. equorum* and *P. univalens* are morphologically identical, and similar in their life cycles as well as all other biological characteristics. One notable difference between the two is that *P. equorum* has two pairs of chromosomes while *P. univalens* has only one pair. Recent studies have shown that *P. univalens* is the most common species infecting horses today (Alan, *et al*., 2019).

1-3: Life cycle of Parascaris spp:

The life cycle of *Parascaris spp.* is a direct sexual life cycle (Figure 1) and starts when parasite eggs are passed out in faeces and contaminate the environment. Larvae start to develop from first larval stage (L1) to second stage (L2) and third stage (L3) infectious larvae. The host gets infected after ingesting eggs containing an infectious larva (L3), often via consumption of contaminated grass. In the intestine, L3 larvae are hatched and then start to migrate by penetrating the wall of the small intestine. They then migrate to the liver and thereafter, via blood vessels to the alveoli and finally the lungs in a 7-day process (Fagerholm, 2000). Thereafter, the larvae migrate up to the respiratory tree, get coughed up, and again swallowed by the host. Larvae eventually find their way to the small intestine where they mature to adults, reproduce and excrete copious amounts of eggs. These eggs will be passed out in host faeces to the environment (Dickson, 1987). Eggs of *Parascaris* spp are surrounded with a very thick lipid layer that protects the eggs and gives it the ability to stick to any surface (Briggs & Karen, 2004; Roberts, *et al*., 2008). This lipid layer is what makes the eggs resistant to unfavourable environmental conditions (Shanahan, 2007; Roberts, *et al*., 2008).

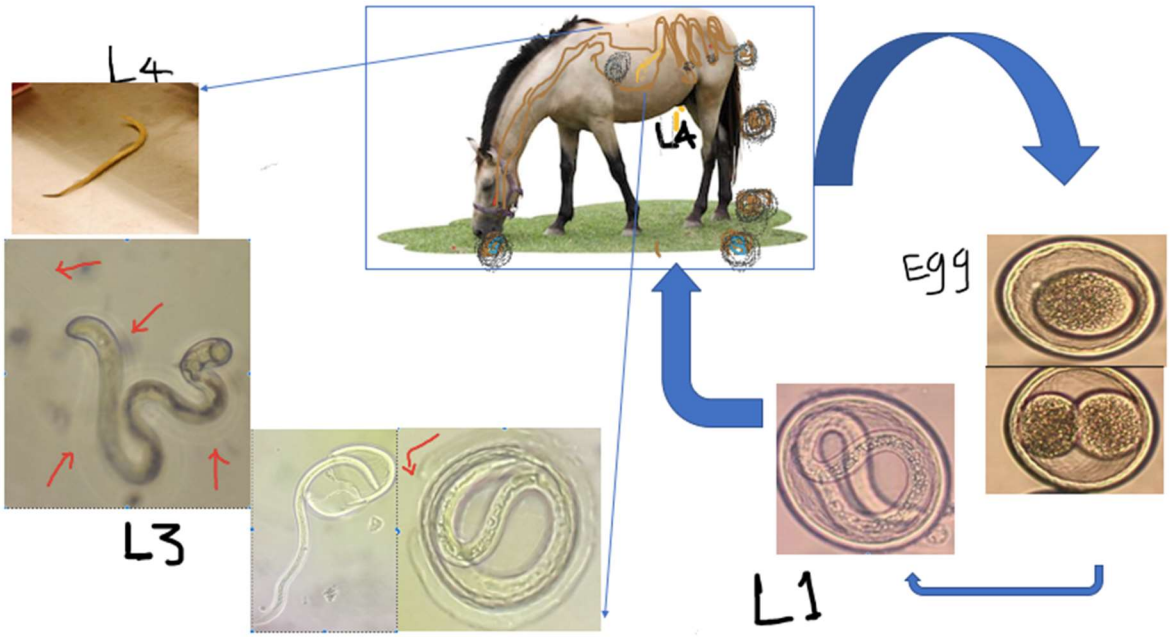


Figure 1. (Eggs of *Parascaris equorum* photo by *Dimah Alshenah, SLU, Uppsala*. Eggs with a thick multilayered shell will be expelled with feces to the environment (Briggs & Karen, 2004)). Horses can get infected by ingesting eggs with stage L3 larva (an infectious egg) by contaminated water or grass. In the digestive system, the thick outer layer will be lost then the larva can hatch and migrate through the lymphatic system to the liver (Nielsen, *et al*., 2018; Roberts, *et al*., 2008), then to the lungs. The parasite will be coughed up and swallowed again back to the small intestines where it will mature and reproduce. The adult worm is around 30 to 50 cm in size. Hundreds of thousands of eggs are shed daily from infected individuals, contaminating the environment and continuing infection transmission in new hosts. The infective eggs can stay viable in the environment for years (Martin, *et al*., 2018).

The female is morphologically larger (around 40 cm) than the male (15-28 cm). In one day, a pregnant female can lay more than 170,000 eggs, translating to around 60 million eggs over a year (Roberts, *et al*., 2008). During the migratory phase in the lungs, symptoms such as coughing and nasal discharge are often observed (Hilary, *et al*., 1977). The adult worms in the small intestine can cause intestinal blockage and potentially develop intestinal rupture (Briggs & Karen, 2004)

1-4: Anthelminthic drugs and resistance:

Three classes of broad-spectrum anthelminthic drugs have been used for the treatment of *Parascaris* infections; **1.** Macrocyclic lactones (ML), **2.** Benzimidazoles (BZ), and **3.** Tetrahydro pyrimidines (PYR). MLs act by binding to glutamate-gated chloride ion channels (GGCI) in nerve cells and muscle cells, causing increased ion permeability of the cell membrane resulting in paralysis and death (Drug Bank). PYR also induces paralysis to the parasite as it blocks neuromuscular depolarization via inhibition of the nicotinic acid receptor. (Arion & Emilio, 2017). BZ binds to b-tubulin and disrupts the formation of tubulin, resulting in starvation and death of the parasite (Lacy, 1988).

An indiscriminate control of horse nematodes with anthelmintic for over 40 years has resulted in resistance (Kaplan, 2002). In 2002, the first case of ML-resistance was reported for *P. equorum.* Since then several countries have reported ineffective treatment of nematodes with ML (Fagerholm, *et al*., 2000). In 2007, multi-resistance to ML and PYR was reported for the first time in the U.S and in 2018 in Sweden (Jabbar, *et al*., 2014; Nielsen, *et al*., 2014; Martin,

et al., 2019). Reduced efficacy of BZ in Australian farm foals was reported for the first time in 2014 (Armstrong, *et al*., 2014).

1-5: New potential substances for controlling Parascaris infections:

Due to the widespread resistance developed by *Parascaris* spp to anthelmintic drugs, there is an urgent need to investigate alternative approaches to infection control. Over the ages, there were a huge number of different oil-bearing plants that have been used and studied as therapeutics with the aim of curing intestinal parasite infections (Youssfie, 2019). Several plant-borne compounds could be the alternative not only to antibacterial, but also work well against viruses, fungi, and even parasites (Youssfie, 2019). One such example is Carvacrol, which is a phenolic monoterpene sourced from many medicinal aromatic plants such as Oregano (*Origanum vulgare*) (Health Benefit, 2020). A recent study showed that monoterpenoids have very high bioactivity toward parasites such as *Trypanosoma cruzi*, *Entamoeba histolotica*, *Giardia,* and *Leishmania* (Yousffie, 2019). The mechanism of action is believed to inhibit the GABA (Gamma-Aminobutyric acid) receptor with lethal paralytic effects (Trailovic, 2015).

1-6: In vitro models and challenges:

The mechanism of anthelminthic resistance in *Parascaris* spp. is incomplete and that is partly due to the absence of *in vitro* models. This parasite lacks the free-living stage as the larva only hatch inside the host and need a host to complete their life cycle (Clayton & Duncan, 1978) which makes this parasite complicated to study *in* vitro (ECCAP, 2019). Furthermore, adult worms are difficult to maintain under *in vitro* conditions for more than a few days (ECCAP, 2019).

2- Aim of the study

This study aimed to develop an *in-vitro* model for *Parascaris* spp. The specific objectives of this thesis were:

1. To evaluate different hatching methods for *Parascaris* larvae.
2. To develop a scoring protocol of the larvae motility under *in vitro* conditions based on a protocol developed for adult worms (Scare, *et al*., 2018).
3. To use the scoring system to investigate the larvae motility after exposure to different concentrations of IVM, PYR, TBZ, and CARV.

3- Materials and methods

Faeces were collected from naturally infected foals on a breeding farm as foals get infected naturally with *Parascaris univalens* from May to September. *Parascaris* eggs were isolated from faecal samples.

3-1: Eggs washing and isolation.

A 15 ml tube containing the eggs was centrifuged at 1500 rpm for 3 minutes, (eggs will be in the pellet). 50 ml Milton 2 solution (sodium hypochlorite 2% + sodium chloride 16,5%) was added to the egg pellet. Tubes were centrifuged again at 1500 rpm for 3 minutes. A 1 ml pipette was used to collect the eggs that were then transferred to a new 50 ml tube containing 25 ml of water. Several washing steps with water were performed, those tubes were centrifuged at 3000 rpm for 3 minutes and the supernatant was discarded.

3-2: Eggs incubation:

After washing steps, the eggs were incubated for 12 days in 25C°.

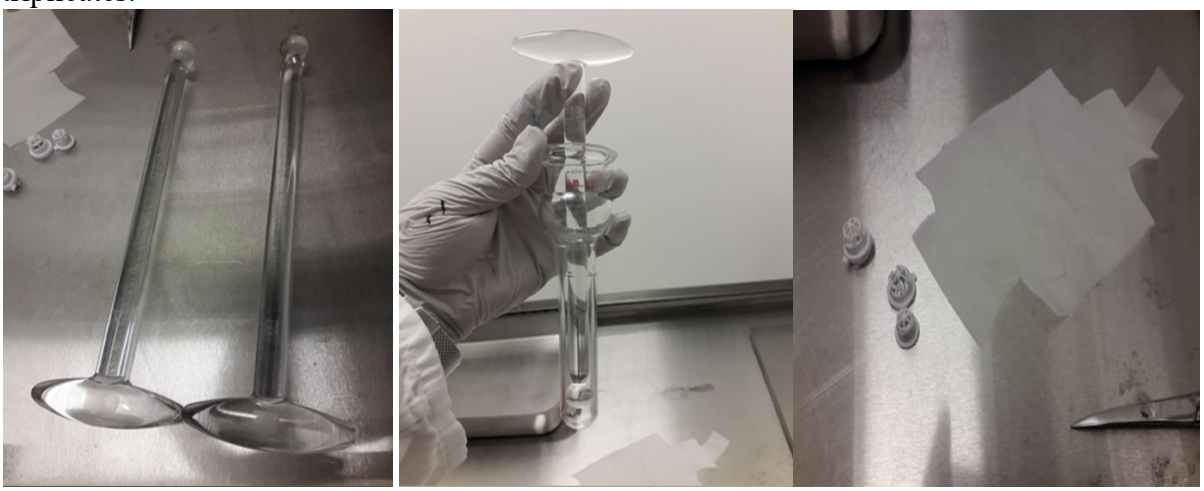
3-3: Hatching and counting of Larvae:

Eggs were counted with consideration for fertilized (larva within the egg) and nonfertilized eggs in three 10 µl drops. The average number of larvae was calculated.

3-4-1: Hatching:

3-4-1-1: Using glass Homogenizer alone & centrifugation matter:

The eggs were first counted and then hatched. Egg counting was made by taking three 10 µl drops and counting the hatched and unhatched larvae in each drop, then the number of larvae per 10 ml was calculated. Hatching was induced in a homogenizer using a glass pestle to assist larval escape from the eggs. Eggs were then hatched in 5 ml Hanks Balanced Salt Solution (HBSS) or PBS. We compared three groups containing between 200 to 100 eggs using a 5 ml Kontez glass homogenizer, two types of pestles were used for hatching (A and B) and variable numbers of crushing pestle strokes. Pestle B was larger than A, with pestle A having a mechanical clearance of 0.889–0.165 mm between the pestle and the homogenizer wall, while pestle B had 0.025–0.076 mm (Figure 2, A and B). Hatched larvae were resuspended in 1 ml media (RMPI-1640 media + 10% FBS+ 1% L-glut + 1% AMAB (Antimycotic antibiotic), transferred to 21 tissue culture plates for counting and evaluation under light microscopy. The total amount of larvae and unhatched eggs in the well were counted and compared to the results. Another improvement of the hatching protocol was an increasing centrifugation to 3000 rpm for 5 minutes from the original protocol of 1500 rpm for 3 minutes. Eggs were counted before hatching and then larvae were counted after hatching after centrifugation. The larvae were recounted in both the supernatant and the deposit. All the experiments were performed in triplicates.



(A) (B) (C)

Figure 2: *Different pestles were used in our protocols* photo by *Dimah Alshenah, SLU, Uppsala*: First figure(A) show homogenizer A size (on right) and B size (on the left).The second show the glass which was used to crush the eggs with the chosen homogenizer. Figure C shows the 20 µM sieve filter which was tested to filter the larvae after hatching.

3-4-1-2: Using HCL protocol for hatching:

Eggs were resuspended in 15 ml HBSS + 2 ml sodium bicarbonate 1M. 1-2 drops of 5 M HCL were added carefully to the egg´s tube to lower the pH to 2. The tube was then incubated at 37°C in 5% CO_2 for 30 minutes. After 30 minutes pH was normalized (pH=7) with 1M NaOH. The eggs were washed in HBSS and then crushed with a glass homogenizer in 5 ml HBSS. 2 to 3 strokes of the pestle were taken. Hatched larvae were counted and then centrifuged in 1500

rpm for 5 minutes. The larvae were resuspended in 8 ml RPMI 1640 media + 10% FBS + 1%L-glut + 1% AMAB). and transferred to a small tissue culture flask. Thereafter, were incubated at 37°C in 5% CO_2. Media was changed every two to three days.

3-4-2: *Using the 20uM sieve to purify the Larvae after hatching protocol*:

20 µM sieve (3D printed at INRA) was tested to be used directly after hatching or after they had been first incubated for 24 hours in tissue culture flask after they were hatched. Directly after hatching, the larvae were filtered through a 20-uM sieve (Figure 2, C). The sieve was placed top on the tube and hatched larvae were carefully filtered into the tube using a pipette. The tube then was filled with PBS or HBSS to the top until it was reaching the filter. This tube then was incubated at 37°C for 2 h. After that, the larvae that had succeeded to migrated through the sieve were transferred back to a tissue culture flask with 8 to 10 ml RPMI and incubated at 37°C, 5% CO_2. The media was changed every two to three days. The tissue culture flask was then examined via light microscopy to count and evaluate the motility and viability of the larvae every 2 days.

3-5: In Vitro method for Larvae viability and scoring:

After hatching, larvae were cultured in optimum media using a 50 ml Tissue Culture Flask from (Inter Med, Nunclon, Delta). RPMI-1640 (Roswell Park Memorial Institute) was used as a liquid culture media +10% fetal bovine serum (FBS, Gibco), 1% Penicillin and Streptomycin (Life technologies) or 1% Antibiotic Antimycotic Solution (Sigma, Germany) and 1% L-glutamine (Lonza) as a mix of 45 ml RPMI + 5 ml FBS + 0.5 ml L-Glutamine +0.5 (antimycotic antibacterial). Between 8 to 10 ml from this mix were used to culture the larvae in a tissue culture flask and incubated at 37°C in 5% CO_2. For testing the viability, scoring two independent experiments were performed. In both experiments, hatched larvae were incubated in RPMI media at 37°C in a 5% CO_2 incubator. Media was changed every 2nd to 3rd day after centrifugation in 3000 rpm for 5 minutes. In experiment 1, three small culture flasks (X1, Y1, and Z1) incubated for 10 days (X1, Y1, and Z1 experiments were performed in technical triplicates under identical conditions and nearly identical hatched *Parascaris* larvae). In experiments 2, small culture flasks X, Y, and Z were incubated for 41 days in technical triplicates. The viability of the larvae were evaluated every other day under light microscopy. 50 larvae were collected and evaluated for scoring of their motility from 0 to 6 according to the motility method (Table 1).

Table 1: Motility and Viability Scoring table Based on Scare, J.*et al*.,2018

Scoring	Explanation	Note
0	Dead (paralyzed)	Larvae are not moving and showing no resistance when using external stimulation
1	Very weak movement	Larvae moving very rarely once after more than 40 seconds
2	Poor movement	Larvae move after more than 30 seconds
3	Fairly good movement	Larvae move after more than 20 seconds
4	Good movement	Larvae move after 10 to 15 seconds
5	Very good movement	Larvae moving from the first second but in the same field
6	Very motile (excellent movement)	Continuous movement and can move from one place to another in the field of the slide.

Scoring was calculated by using this formula:

Score ={Σ(scoring at a time× number of larvae performing this score)/Total number of Larvae.

3-7: Exposure to drugs:

For each exposure experiment, a variable number of L3 larvae were incubated with anthelmintic drugs with around 5000 to 8000 eggs hatched in the presence of PBS. Eggs were counted before hatching including eggs with larvae (fertilized) and unfertilized ones. Larvae were also counted after hatching to include live, dead, or unhatched larvae. Technical triplicates and biological triplicates were performed for each experiment with corresponding controls. Freshly hatched larvae were cultured in 48 well plates from (Nunclon Delta Surface) or 24 well plates (TPP) tissue culture plates were used for each exposure experiment. Every column on the plate consisted of the same concentration for all technical replicates. Light microscopy was used for reading the plates. 200 larvae per well were cultured in RPMI-1640 media mixed (refer to "Scoring motility") for 24 h at 37 °C in an atmosphere containing 5 % CO_2 plus anthelminthic drug for 24 hours. The drugs used in our experiments were: Ivermectin (IVM), thiabendazole (TBZ), pyrantel citrate (PYR), and the natural essential oil carvacrol. All drugs were dissolved in DMSO except for carvacrol in ethanol.

A stock solution of 0.1 M Ivermectin was prepared and diluted in DMSO to reach the final concentration in the plate of 10^{-3}, 10^{-4}, 10^{-6}, $10^{-8,}$ and 10^{-10} M.
A stock of 1 M Carvacrol in EtOH was prepared and diluted in 99,5 % ethanol to reach the final concentration of 10^{-2}, 10^{-4}, 10^{-6}, $10^{-8,}$ and 10^{-10} M.
A stock of 0.1 M Thiabendazole was made and diluted in DMSO to reach final concentrations of 10^{-3}, 10^{-4}, 10^{-6}, $10^{-8,}$ and 10^{-10} M.
A stock of 0.1 M Pyrantel was prepared and diluted in DMSO to reach the final concentration of 10^{-3}, 10^{-4}, 10^{-6}, $10^{-8,}$ and 10^{-10} M.

For all drugs exposure experiments, the last two rows contained controls, first one-row containing only media, the second row containing media + 0.1 % DMSO drug cocktail except, Carvacrol as DMSO was replaced with 0.1% EtOH. The larvae were incubated for 24 hours at 37°C in 5% CO_2 during the exposure work. After 24 hours each well in the plates was read under the light microscopy. Larvae counting and motility scoring were performed according to the table above. All results were translated to a table Using Excel and curves using Prism 8 (GraphPad, San Diego, USA) as shown in the results below.

4- Results

4-1: *Eggs washing and isolation:*

In order to isolate the eggs from faeces, samples were washed to isolate the eggs using different types of filters. Thereafter, the eggs were stored in water to the next step. There more purification for cleaning and decortication was performed for the eggs and to remove the thick outer layers of the eggs, for that, a tube containing a suspension of water and *Parascaris* eggs were centrifuged on 1500 rpm for 3 minutes. 50 ml Milton 2 solution then was added to the eggs for cleaning and decortication (removal of external chitinous lipid layer and to eliminate bacteria or fungal contaminants). Tubes were centrifuged again at 1500 rpm for 3 minutes where eggs floating up in the upper part of the supernatant. Eggs were collected from the upper part of the supernatant (around 5 ml) then transferred to a new 50 ml tube containing 25 ml water. This step was repeated three to four times to make sure no eggs remained. Several washing

steps with water were performed after Milton step as Milton solution is toxic to the eggs. This tube was centrifuged at 1500 rpm for 3 minutes and the supernatant was discarded, and the deposit was kept in the last washing step with 10 ml water at 25 C° incubator.

4-2: Larval development:

Larvae were allowed to develop into stage 3 (Fig. 1) within the eggs by incubating the washed eggs for 12 days at 25 C° for the development of larvae in water.

4-1: Hatching protocol:

To improve hatching frequencies a random number of eggs were used that had been counted before and after hatching with the help of two types of crushing glass homogenizer aiming to improve the hatching method. The eggs were hatched in 5 ml (HBSS) or PBS as mention in (3-4- 1- 1). 5 ml Kontez glass homogenizer was used, and two types of pestles, A and B. 3 groups were tested to be hatched with both types, then they were examined under a microscope. The result of using pestle type A shows, that around 26 to 30 unhatched eggs remained of a total 150 to 90 eggs. Whereas 10 to 20 unhatched eggs remained when using type B. In other words, hatching with homogenizer A resulted in 70% hatched larvae while homogenizer B resulted in 90-80 % hatched larvae. After the B type homogenizer was selected, our hatching protocol was further optimized with an additional crushing stroke (six strokes total) resulting in up to 10 unhatched eggs only remains from a total of 130 to 100 eggs. Therefore, we achieved and optimal hatching rate of 90-99% and discovered that an additional crushing stroke reduced hatch yields by up to 10 eggs. Comparing with, between 30 to 36 unhatched eggs remained of 100-150 eggs after following the original 5-stroke protocol, translating to an efficiency of 60 to 70%. In subsequent experiments in this study, six crushing strokes were used. Overall to get the best result the strokes should be done carefully to avoid crushing the larvae. Another improvement of the hatching protocol was an increase of length and speed to 3000 rpm for 5 minutes of the centrifugations step as this was used when media was changed. A large proportion of larvae remained in supernatant while using 1500 rpm for 3 minutes according to the original protocol. Eggs were counted before hatching and then the larvae were counted direct after hatching and then after the centrifugation step. The larvae were then, counted in both supernatants and the pellet. It was noted that the number of the larvae were decreased in after using 1500 rpm for 3 minutes protocol. The optimized protocol using 3000 rpm for 5 minutes resulted in very high larvae yields almost the same of the original number.

4-2: Using a 20uM filter:

A 20-uM filter was used to increase the purity of the extracted larvae, on the other word, to purify the larvae from eggshells and debris after hatching protocol. In this step, our goal was to determine filter efficiency (evaluated)by calculating the number of larvae that would be lost with each use of this filter.3 groups were tested of hatched L3 larvae. The first groups stand for 10000 hatched larvae and the larvae counting after using 20uM filter were 8000 Larvae. The second group consisted of 5000 hatched larvae to became 4000 larvae after using the filter and the last group stands for 10500 larvae to reach 8000 larvae after using the filter. In another word, the loss of L3 *parascaris* larvae was around 20%. This method could work very well for a short incubation period study but not so recommended when it comes to a long incubation period study since the ability to develop contamination is high as tow of this groups develop contamination growth of nonsignificant growth of mixed bacteria in the 4th and 5^{th} day as this filter is not sterile.

4-3: Viability scoring:

For testing the viability scoring, hatched larvae were incubated in RPMI media at 37°C, 5% CO_2 incubator and evaluated for the viability every 2 days under light microscopy. Three cultures of larvae (X, Y, and Z) were examined for viability scoring for 41 days. As shown in figure 3. Every 1 to 2 days the flasks were evaluated under a light microscope. For that, 50 larvae were counted and evaluated for scoring of their motility from score 0 to 6 with the formula (Table 1) mentioned above "3-6 Scoring motility". In each checking point (checking time), 50 larvae were counted and each was evaluated a cording to their motility**,** scoring at the time× number of larvae performing this score then the sum was taken and multiply with the total number of larvae. At time 0, the scoring motility was around 4. In between the 96 h to 600 h the larvae show very high scoring in all the 3 repetitions from score 4 to 5. In day 20 viability was around 85 % then it decreased. In the day 40 (960 h) or 42 (1000 h) most larvae were dead (figure 3) and (table 3). It's also good to mention, in order to reduce or prevent any growth contamination of bacteria or fungal, microbiological contamination was checked for both media preparation and media flasks after culturing the larvae so any changes in the color of the media could be significant. For that one to two drops of the media were streaking on Horses blood agar plate and incubated for 3 days in 38 C°+CO_2 incubator. In the first group X1, Y1 and Z1, we found one contamination growth of *Candida. spp.* in one flask which takes us to stop this group and start with the second groups (the one we mention up X, Y, and Z)which contain also of three repetitions of tissue culture flasks containing media and *Parascaris* larvae.

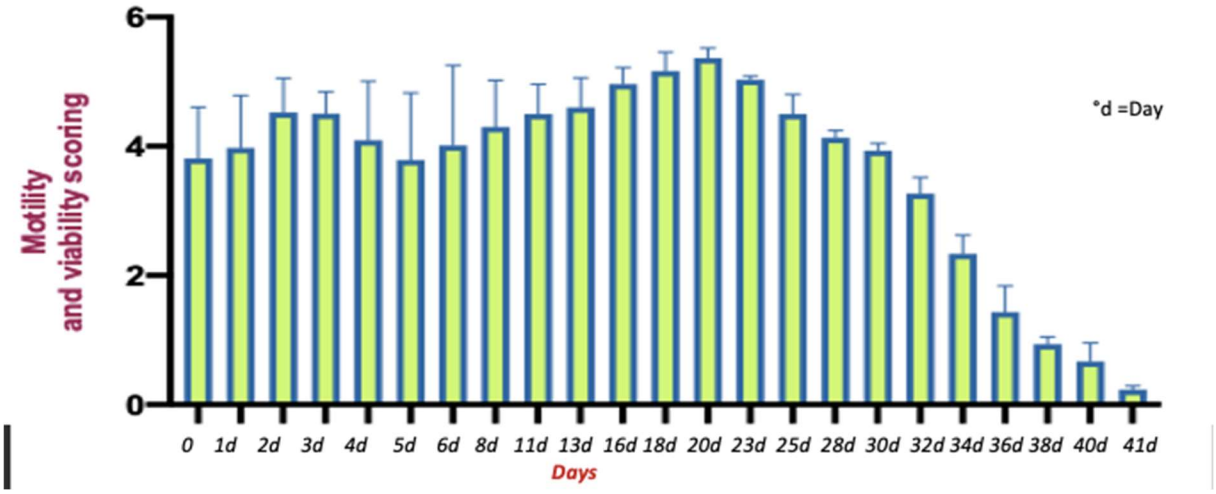


Figure3; In vitro viability of Parascaris larvae monitored for 42 days (1000 hours): The average was taken for the 3 experiment X, Y, and Z taking to account the STDEV to perform this figure with a help of prism showing the relation between the motility scoring and every checking point hour there to check the Larvae in the culture flasks. Microscopy was used to read and evaluated the results.

Table 3: In vitro survival monitored for 42 days to *Parascaris* larvae in RPMI media:

Hours	Motility score for Experiment (X)	Motility score for Experiment (W)	Motility score for Experiment (Z)	Average	STDEV
0	4.2	3.0	4.3	3.8	0.6
24	3.4	3.7	4.9	4.0	0.7
48	4.1	4.4	5.1	4.5	0.4
72	5.0	4.3	4.3	4.5	0.3
96	4.7	4.6	3.0	4.1	0.7

120	4.6	4.1	2.6	3.8	0.8
144	5.0	4.4	2.6	4.0	1.0
192	5.0	4.5	3.5	4.3	0.6
264	5.0	4.6	4.0	4.5	0.4
312	5.0	4.7	4.1	4.6	0.4
384	5.2	4.7	5.0	5.0	0.2
432	5.5	5.0	5.0	5.2	0.2
480	5.4	5.2	5.5	5.4	0.1
552	5.0	5.1	5.0	5.0	0.0
600	4.2	4.8	4.5	4.5	0.3
672	4.0	4.2	4.2	4.1	0.1
720	3.9	4.0	4.0	4.0	0.1
768	3.0	3.3	3.5	3.3	0.2
816	2.5	2.0	2.5	2.3	0.2
864	1.5	1.0	1.8	1.4	0.3
912	1.0	0.8	1.0	1.0	0.1
960	1.0	0.5	0.5	0.7	0.2
1000	0.2	0.3	0.2	0.2	0.0

Three repetitions cultures of larvae (X, Y, and Z) were examined for viability scoring for 41 days. The first column shows every checking point hour there where the Larvae in the culture flasks were read and evaluated under light microscopy. The next columns show each experiment (X), (Y), and (Z) and it is scoring motility of indifferent checking point. The 4th column is the average of the 3 repetitions experiments and the last column shows the standard deviation between these 3 repetitions.

4-3: Parascaris larvae incubated in PBS:

After hatching the larvae, PBS was used as a culture media instead of RMPI. The result showed that larvae were very motile at the beginning and stayed very active with a higher score (6) for 2 days of incubation but then larvae motility decreased till they died on the 3rd day (figure 4). This protocol could be used for studies requiring a short incubation period since there are no nutrient substances in PBS that the larvae can use it to survive longer periods.

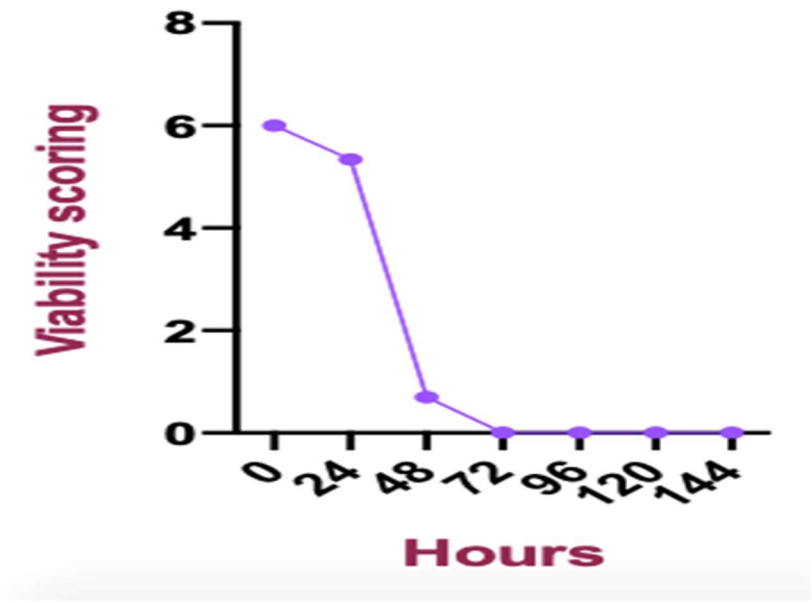


Figure 4; ***Motility and Viability scores of Parascaris larvae incubated in PBS*** After hatching the L 3 larvae they were incubated directly in PBS and incubated in a tissue culture flask in 37°c incubator. The average was taken for the 3 repetitions experiments. First 2 days the larvae motility in PBS were high as it reached almost score 6. The figure was done by using prism program.

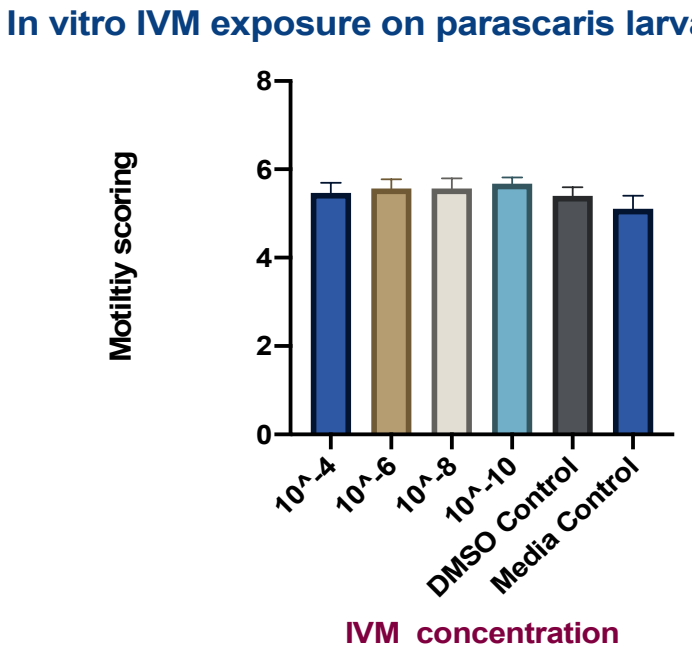
4-4: Drug exposure:

Three classes of broad-spectrum anthelminthic drugs have been used for the treatment of *Parascaris* infections; **1.** Macrocyclic lactones (ML), **2.** Benzimidazoles (BZ), and **3.** Tetrahydro pyrimidines (PYR). In our experiment, we use Ivermectin (IVM) as one type of (ML), Thiabendazole (TZP) as its one type of (BZ) and Pyrantel as a type of (PYR) all to be tested on *Parascaris* larvae. An indiscriminate control of horse nematodes with anthelmintic for over 40 years has resulted in resistance (Kaplan, 2002). In 2002 the first case of ML-resistance was reported for *P. equorum.* Since then several countries have reported ineffective treatment of nematodes with first time in the U.S and 2018 in Sweden (Jabbar, *et al*., 2014; Nielsen, *et al*.; Martin, *et al*., 2019). Reduced efficacy to the third drug class BZ in foals on farms in Australia
was reported for the first time in 2014 (Armstrong, *et al*., 2014). As *Parascaris* is the only type of *Ascaris* that has developed resistance to antihelminth drugs and this resistance is increasing there is an urgent need to look into other alternatives that what takes us to test one natural oil from an aromatic-plants call Carvacrol which has been registered as an effective agent on other parasites. However, we were the first to test carvacrol on *Parascaris* L3 stage larvae. After scoring protocol for larvae motility under *in vitro* condition has been developed based on a protocol developed for adult worms (Scare, *et al*., 2018), as the aim here was to use this scoring system to investigate the larvae motility after exposure to different concentrations of different drugs for other futures drugs exposures studies. *Parascaris* eggs were isolated from those faeces as mention in (3- 3) eggs were incubated at 25°C for 14 days as mention in (3- 4) to allow the Larvae to inter the L3 stage. In total around (24000 to 36000) eggs were used which means around (6000 to 9000) Parascaris eggs for each drug exposing experiment. That compensated around 5000 to 8000 fresh hatched larvae as mention in (3- 4- 1- 1). A total of 24 tissue culture 48 well plates were utilized (Figure 2 (A, B)). Around 200 larvae/well were cultured in RPMI-1640 media mixed (described under "Scoring motility") for 24 h at 37 °C in an atmosphere containing 5%CO_2 for 24 h. Each well contains (1 ml media + 200 larvae). After 48 well plates

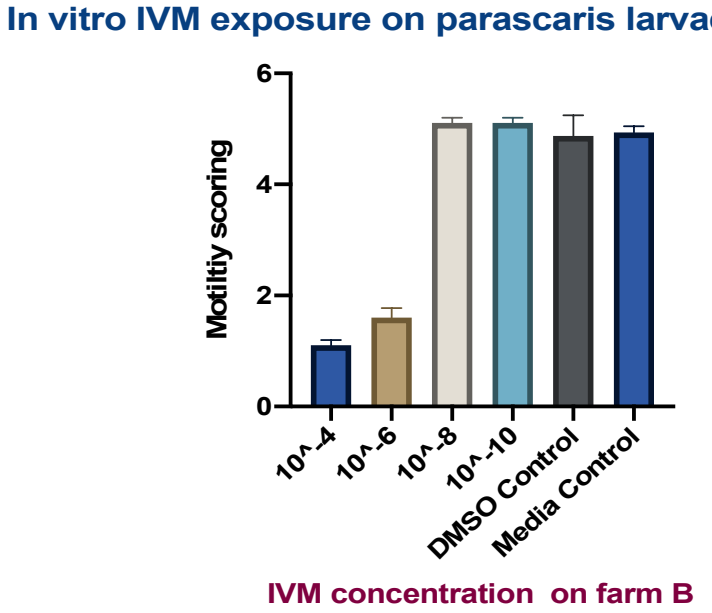
were left for 24 h different concentrations of the drugs were added after a certain dilution was performed for each. Plates were then incubated again for 24 h in the same incubator under the same circumstances. Every column on the plate consisted of the same concentration and its technical replicates (3 technical replicates). 3 biological repetitions for each drug were performed. Light microscopy was used for reading and evaluated the larvae viability in the tissue culture plates after they were incubated for 24h. All drugs were dissolved in DMSO but carvacrol with ethanol.

4-4-1: Ivermectin exposure (IVM);

Ivermectin is a broad-spectrum anti-parasite drug and is a substance in macrocyclic lactone group (ML). (IVM) was first known as Stromectol® effective against nematode except for tapeworms, 2012 was used and approved as a treatment for head lice and can be used from 6 months and older. Here the name changes to Sklice™, l. Ivermectin very effective against onchocerciasis, and also show effective against other worms like strongyloidiasis, ascariasis, trichiasis, and enterobiasis. This anthelmintic agent is semisynthetic and is an ivermectin a group of pentacyclic sixteen-membered lactone execrated from soil bacteria (Streptomyces ivermictilis) (drug bank). However, Include Asia and Australia, south and north America, Europe Resistant was reported against IVM in *Parascaris.* spp. (Martin, F.; Höglund, J, 2018). To prove this, two different *Parascaris* isolates (A and B) were exposed to Ivermectin (Sigma, Germany). Isolate A was collected from a farm with known resistance to ivermectin. Isolate B was collected from a farm susceptible to ivermectin. The viability of isolates A and B after exposure was compared and evaluated as shown in (figure 5) for each concertation. Scoring was calculated according to Scoring ={Σ(scoring at the time× number of larvae performing this score)⁄ Total number of Larvae, and then average and STDV was taken for every replicate for each concertation as well as to all 3 repetitions. The viability of isolate A and B after exposure was compared. The concentrations are based on two previous studies by Janssen *et al*., 2013 and Jonsson, 2019. In the highest concentration, 10^{-3} M crystal formation was noticed, and this concentration was therefore not included, and the concentration 10^{-4} M was the highest in our experiment. On-farm A, which was resistant to ivermectin, no paralysis effect was observed. An interesting finding was that in the higher concentration the larvae were more active compared to the control (Figure 2 A). Farm B, which was susceptible to ivermectin, the mobility scoring was reduced to 1-2 in the highest concentrations (10^{-4} M and 10^{-6} M) (figure 2 B).



(A)



(B)

Figure5 (A and B); *In Vitro IVM exposure on Parascaris Larvae from Farm A and B*: Two different isolates (A and B) were exposed to Ivermectin. Isolate A were collected from a farm with known resistance to ivermectin. Isolate B was collected from a farm susceptible to ivermectin. The viability of isolate A was done by taking the average of all 3 repetitions and has shown high viability scoring both in the higher concentration of (IVM) and in the lower one. That translated as, no good effect of ((IVM) on farm A. The viability scoring of isolates from farm B was done also by taking the average of all the 3 repetitions from farm B to give, low viability scoring in the presence of high concentration (10^{-4} and $10^{-6}$of (IVM), that translated as, good effect of (IVM)on farm b. Both figures were done with the help of a prism program.

4-4-2: Thiabendazole exposure (TBZ);

TBZ is a 3rd generation heterocyclic antihelminth compound that can be used as antifungal and antiparasitic therapy, as well as a food preservative. Benzimidazole was introduced in early 1962 as an active agent in treatment against several nematodes (Smith & Reynard, 1992). Interestingly, TBZ is vermicidal against *Ascaris lumbricoid*. Here we will be the first to test it on *Parascaris* larvae regardless that *Parascaris* has shown resistance for other anthelmintics unlike the *A. lumbricoides.* The mechanism of action of TBZ has been shown to suppress the egg and the larvae production. It could be involved in inhibiting the development of eggs and larvae before they are passing in the faeces. Our results show that in the highest concentration (10^{-3} M) larvae moved weakly in all 3 of the biological replicates and reached a motility scoring of 1 as shown in figure 6. That is considered a remarkable effect of TBZ on Parascaris L3 larvae, whereas all other lower concentrations did not show a noticeable effect on the larvae since the motility scoring was high (3 to 5). All the 3 replicates observed had closely related results and varied approximately with one score as the STDV gave acceptable results between them.

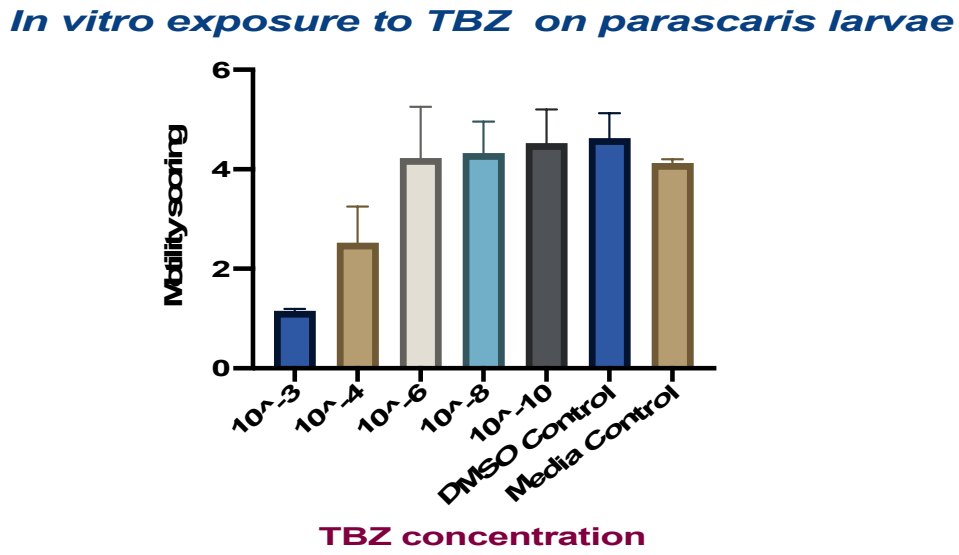


Figure 6*: In vitro exposure to thiabendazole on Parascaris Larvae*:
The figure shows the results of 24 h *in vitro* exposure of different concentrations of TBZ on Parascaris larvae by taking the average of the 3 repetitions. The figure shows that the effect of the first concentration was noticeable were Parascaris larvae move so weakly. 3 biological replicates and 3 technical repetitions were done, and the average considered for all the three repetitions to perform this figure. Each plate experiment was evaluated on different days and read under light microscopy Motility scoring was counted separately for each in Excel(not attached) and then average (for the 3 replicates)and SDTEV was also calculated for all 3 experiments together. Prism 8 was used for drawing this graph

4-4-3: Carvacrol exposure:

Carvacrol, is an example of a phenolic monoterpene which is excreted from many medicinal aromatic plants, specially *Oregano* as it is known (*Origanum vulgare*) (healthbenifet,2020). A recent study shows that monoterpenoids have very effective bioactivity that is antagonistic toward *Trypanosoma cruz*i, *Entamoeba histolotica*, *Giardia,* and *Leishmania*. (Youssfie, 2019) This could be due to their accessibility from abundant natural sources as well as the low toxicity because they are generally recognized as safe (GRAS). Discovering new drugs against parasites is a very consuming project both in time and money as it takes years to produce a new drug,
The need for safe, effective drugs is dominant in order to replace current ones that have become ineffective due to resistance. The mechanism of action for carvacrol can be explained by the effect of carvacrol on GABA (Gamma-Aminobutyric acid)which known as the master inhibitory neurotransmitter in the

central nervous system which is responsible for reducing the neural excitability of the nervous system. Carvacrol binds with GABA and Tyramine receptors causing inhibition of the contraction of the L3 larvae (Sasa, 2015). However, we were first to test the effect of CARV on *in vitro* atmosphere on *Parascaris* larvae and to explore if CARV can be a replacement for the other anthelmintics drugs against *Parascaris.*

Exposure of carvacrol in our results showed a dose-dependent effect on the larvae. The highest concentrations of carvacrol (10^{-2} and 10^{-4}) showed a very high paralytic effect with 100% dead larvae (scoring 0) (Figure 7). A very rapid effect of carvacrol was observed already after 1 hour as 100 % of larvae dead in the highest concentrations. On the 10^{-6}, a remarkable effect was noticed reaching score 1. There was less effect from CARV in the lower concentration as the score was around 5 which was similar to the control (unexposed).

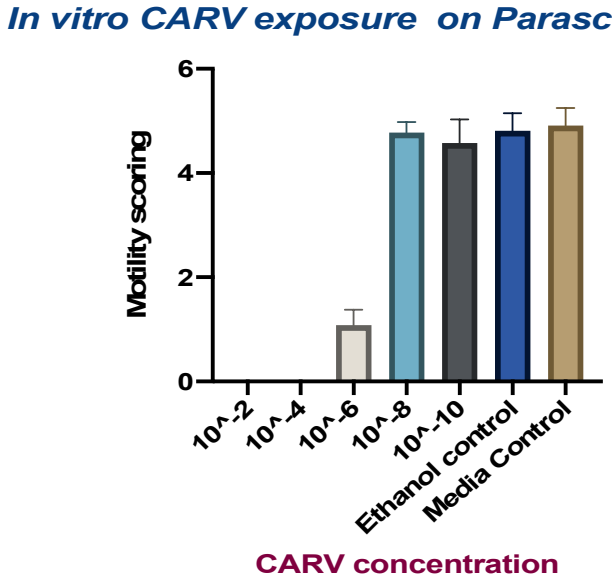


Figure 7; In vitro Carvacrol exposure on parascaris larvae: Show the result of the average of 3 repetitions experiments using different concentrations of Carvacrol on *Parascaris* larvae after 24 h of incubation with drug + RPMI media. The first highest concentration in all the 3 repetitions gives motility scoring 0 for the parascaris larvae which shows the effect of CARV on the larvae at this concentration. Good paralysis effect on the larvae as motility scoring was around 1.

4-4-4: Pyrantel citrate exposure on Parascaris larvae (PYR);

Pyrantel is one of the safest and most effective medicines in the health system according to the WHO's list of essential medicines. This drug was first described in 1965 by international Pfizer researches who explored this drug as an anthelmintic looking for drug specificity and duration. Pyrantel is a broad-spectrum anthelmintic, a pyrimidine-originate compound that works against*, Ascaris lumricodies* (roundworms), *Thricho strongyliasis, Trichinella*, (hookworms) and *Enterobius enterobiosis* (pinworms) infections (AHFS, 2020).This drug is known to affect the nerves causing paralysis to the parasite as it depolarizes neuromuscular -blocking material which activates the nicotinic receptor which will lead to sudden contraction and then paralysis of the worms. The helminth will lose power on the intestinal wall and be thrown out of the body in the faeces (Arion & Emilio, 2017). Based on this information we tested Pyrantel in *Parascaris* larvae on *in-vitro* atmosphere to evaluate the Pyrantel effect against *Parascaris* larvae. After they were exposure to Pyrantel as described in the method the results show that in the highest concentration (10^{-3}M) larvae showed reduced motility with a score 1 (figure 8). An interesting observation was that the larvae were coiled on themselves like a snail or spiral shape

figure (9) and (10) and they were more swollen compared to the larvae size in the control (Figure 10). Surprisingly, in 10^{-4} concentration (figure 11) about 100% of the larvae were paralyzed or dead. Also, in this concentration, the larvae body show some abnormality (figure 11) and was swollen in comparison with the larvae in the control (Figures 9 and 10). In 10^{-6} concentration the larvae scoring was around 1 showing that this concentration is also effective as all larvae moved so slowly. In the 10^{-8} concentration, the motility has been not affected by the drug as it gives a score 4 which was close to the larvae control´s scoring on the same plate.

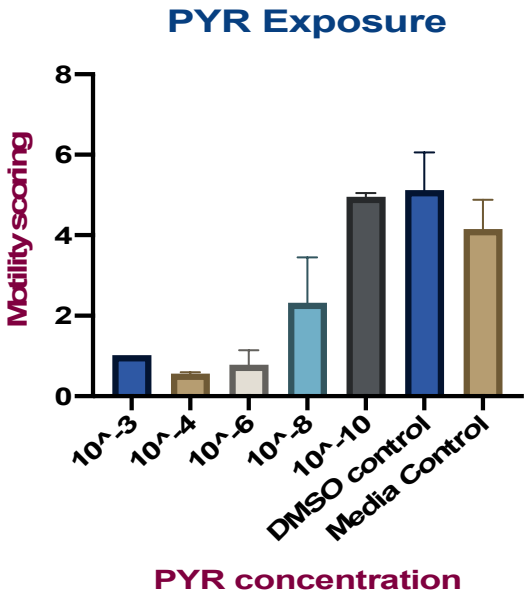


Figure 8*; In vitro PYR exposure on parascaris larvae*: The figure shows, the result of the average of the 3 repetitions of PYR exposure on Parascaris L3 stage larvae, with different PYR concentrations and its effect on ***Parascaris* larvae** motility after 24 h of incubation with the drug + RPMI media. 3 biologicals replicates were done. Each plate experiment was evaluated on different days and read under a light microscope. Motility scoring was counted separately for each in Excel (not attached) and then average and SDTEV was done for each plate separately and then all 3 experiments together. SDTEV in all was under 1 which makes our result noteworthy.

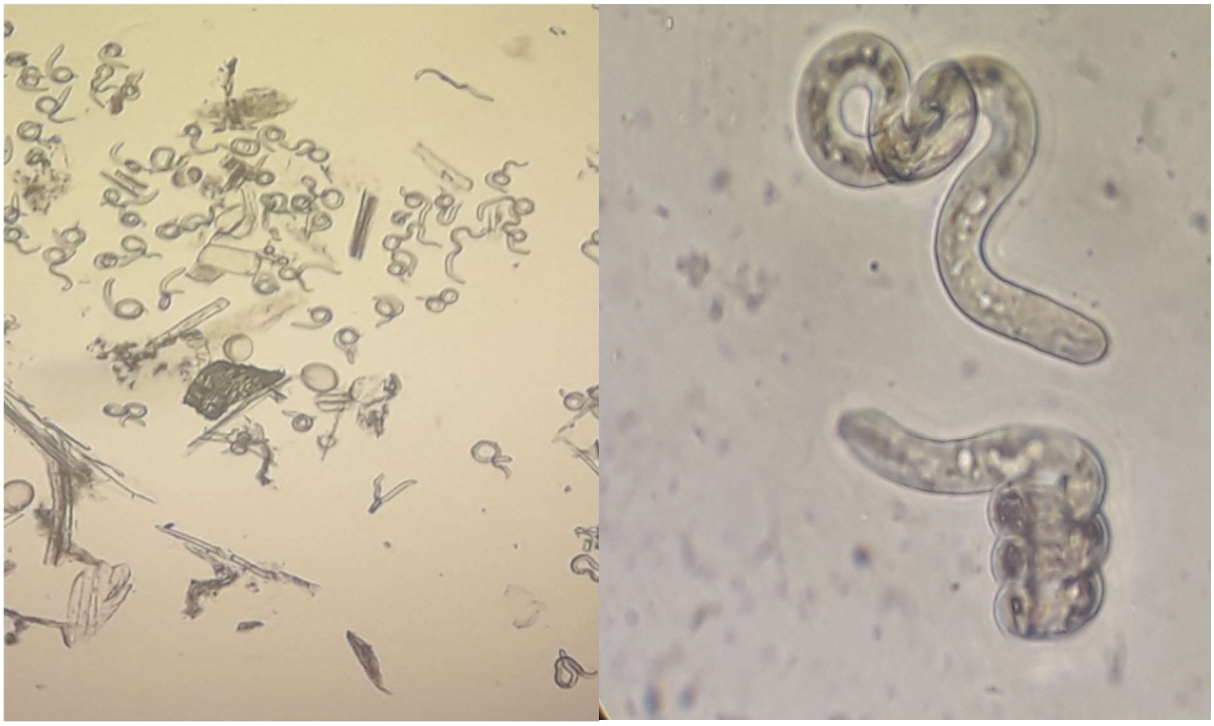
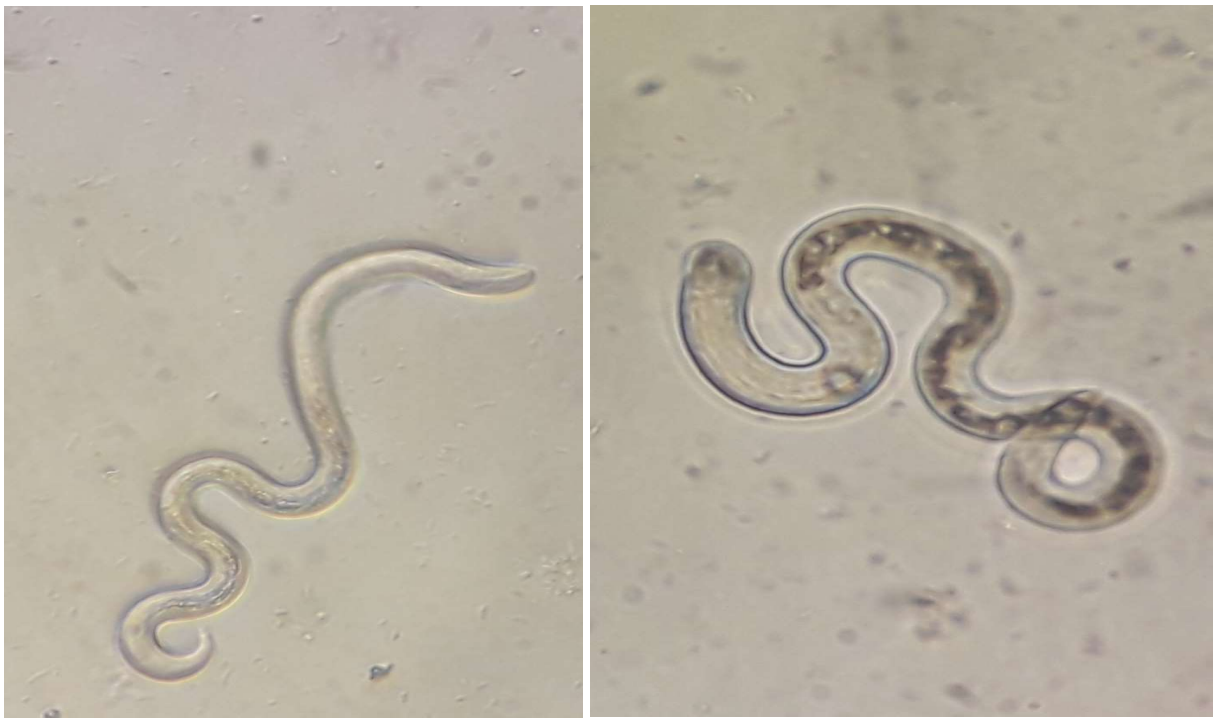


Figure 9*: The effect of PYR on Parascaris larva on the* 10^{-3} ***concentration***. by *Dimah Alshenah, SLU, Uppsala* Larvae were incubated in RPMI media for 24 h in 37°C +5% CO_2 incubator in the presence of PYR 10^{-3} M. The results were evaluated under light microscopy in the highest (left) and lowest (right) magnification. Larva moved slowly and majority of them take snail shape and all are swollen compared with normal parascaris larva in the media control wells of the same plate



(A) **(B)**

Figure 10; **Different body size between parascaris larva in the control (A) with the one under PYR exposure in the** 10^{-3} **concentration(B)**. by *Dimah Alshenah, SLU, Uppsala* Figure

A show parascaris larva size in the media control. Figure B show Parascaris larva body size after exposure to PYR for 24h in the 10^{-3}concentration.

Figure 11*:* <u>The effect of PYR on *Parascaris* larva on the 10^{-4} concentration.</u> by *Dimah Alshenah, SLU, Uppsala* Larva almost not moving only very slow movement for the head or the tails as all are paralyzed and show some abnormality in the body and swollen comparing with the larvae in the media control wells or DMSO control wells.

5-Discussion:

It is a well-established fact that parasite infection are a significant cause of morbidity and mortality worldwide in both humans and animals. Hugo, *et al*. 2001 has assumed that over 28,000 nematode species have been described of which 16,000 are parasitic. Severe economic damage and loss of welfare of animals as well as the physical suffering for both humans and animals have been a result of parasitic worms' infestation. Around 50 roundworm species can infect humans, with many classified as neglected tropical diseases e.g. *Ascaris* causing ascariasis (Payne & Fitchett, 2010). Anthony *et al*., 2007 suggests that more than 3 billion people are infected by parasitic nematodes worldwide. Furthermore, increasing anthelminthic drug resistance has been reported virtually everywhere. Indeed, increasing the dose during treatment may not necessarily yield better therapeutic results in most cases. This was seen in our larvae exposure experiment to ivermectin, there increasing the dose of ivermectin did not cause death or any paralysis effect on the larvae from the farm with an ivermectin resistant *Parascaris* spp. population.

The complete picture of the resistance mechanisms in *Parascaris* spp. is poorly understood, partly due to the absence of *in-vitro* models. Because the larva only hatch inside the host, it makes this parasite complicated to study *in vitro* (ECCAP, 2019). This study evaluated different hatching protocols. It has been shown that *A. lumbricoides* and *A. suum* have a chitinous layer 2-4µm thick (Lysek, *et al*., 1985: Rogers, 1956). This thickness of the eggshell is a problem for

normal hatching protocols. The results from this study show that the mechanical breaking of the eggs with a homogenizer resulted in a hatching rate of 98%. This protocol was based on a previous protocol used on another Ascaris called *Toxocara canis* (Ponce-Macotela, *et al*, 2011) but we modified it by adding 6 strokes by a glass homogenizer. Taking into consideration especially in the bottom where the risk for smashing the larvae is higher if they were hatched by hard strokes. On the other hand, we found that soft slow strokes give better results and more chances to minimize the loss of hatched L3. Using the filter for hatch L3 larvae direct after hatching can be used to purify the L3 from the debris and eggshells. The filter method is not so recommended when it comes to a long incubation period as the loss of L3 larvae can be up to 20%. Furthermore, the hatched larvae were incubated in RPMI at 37°C to study the survivors. The *in vitro* survival study gave a very good picture and gave evidence that *Parascaris* larvae can be used to study for a long period *in vitro* as previous studies did not examine more than three weeks (Burk, *et al*., 2014: Jonsson, *et al*., 2017). In our study, we found that *Parascaris* larvae can survive *in vitro* in RMPI media for more or less than one month. 85% of the larvae were alive on day 20, 58% of them were alive on day 32. On day 38 around 20% of the larvae were still surviving and 5 to 10 % larvae on day 40. Further studies should be performed to confirm this find. A modification of centrifugation was needed when changing the RPMI media. Centrifugating the larvae for 3 minutes at 1500 rpm was not enough because many larvae were found in the supernatant. Therefore, the centrifugation was modified to 5 minutes at 3000 rpm. This gave an improved result with a reduced loss of larvae, as only 0-2 % of larvae remained in the supernatant. Our model gives a wider ability for further studies to explore novel drugs for the treatment of *Parascaris* infection.

In 2002 the first case of ML-resistance was reported for *P. equorum.* Since then several countries have reported ineffective treatment with ML (Fagerholm, *et al*., 2000). Shortly after, in 2007, multi-resistance to ML and PYR was reported for the first time in the U.S and 2018 in Sweden (Jabbar, *et al*., 2014: Nielsen, *et al*., 2014: Martin, *et al*., 2018). In 2014, the first report about reduced efficacy to the third drug class BZ in foals on farms in Australia was highlighted (Armstrong, *et al*., 2014). However, it is important to note that our experiment supports this finding as our results showed a substantial difference between the two A and B isolates. Isolate A was from a farm with known resistance to IVM and isolate B from a farm with known susceptibility to IVM. It was a marked difference between A and B in our in vitro assay. No paralytic effect was observed in isolate A in the highest IVM concentration (10^{-4}) but in isolate B, the mobility scoring was reduced to 1-2 scores. This suggests that the *in-vitro* method for drug exposure developed in this thesis could be a good method for evaluating the resistance status on farms. This *in-vitro* method could be complemented to FECRT (The fecal egg count reduction test), which is the gold standard for evaluation of anthelmintic resistance in the field.

Thiabendazole is known to be effective as an antifungal and anthelminthic and is also used as a food additive (E233) (Setzinger, *et al*., 1965). Iqual- Adel 2004, has shown in his article that TBZ has strong effects in controlling roundworms, hookworms, and others helminth spp. which infect both animal and human. TBZ is the original of benzimidazoles (BZ) that are still effective, but it has been reported that 2 two of 4 farms have developed resistance against BZ in Australia (Armstrong, *et al*., 2014). We showed in our experiment that the larvae that were exposed to a high-concentration dose of TBZ show a good effect of the drug on the larvae as they were paralyzed or had very slow movement. A similar observation was also observed at the second-highest concentration. That leads us to consider TBZ to be still effective on *Parascaris* larvae.

This is the first study evaluating the effect of *Parascaris* larvae exposure of PYR *in-vitro.* The exposure showed a dose-dependent effect with reduced scoring in the higher concentrations. Interesting findings were observed in the higher concentrations where larvae were swollen and exhibited a supercoiling behavior. More studies are needed to understand the toxic effects of PYR.

The history of thymol *(Thymus vulgaris*), one of the organic aromatic oils, was used commonly for worm infection specifically *Ascaris* and hookworms (kaplen *et al*., 2014). Due to the emerging problem with resistance, compounds from plants can be explored in the future. Carvacrol is a phenolic monoterpene extracted from many medicinal aromatic plants specially Oregano (*Origanum vulgare*) (Health Benefit, 2020). A recent study shows that monoterpenoids has very effective bioactivity toward parasites such as *Trypanosoma cruzi*, *Entamoeba histolotica*, *Giardia,* and *Leishmania* (Yousffie, 2019). The mechanism of action is believed to be on GABA receptors (Gamma-Aminobutyric acid) which is known as the master inhibitory neurotransmitter in the central nervous system of parasites which reduces the neural excitability of the nervous system causing paralysis and death (Trailovic, 2015). Carvacrol was tested *in vitro* on L3 larvae of *Anisakis simplex* by Hierro *et al*., (2004). Abdelrahman *et al*.,2013, have also published that carvacrol has a nematocidal effect against *Caenorhabditis elegans.* Carvacrol was also tested in *A. suum* muscular tissue from the worm and showed highly significant inhibition for the acetylcholine contractions (Trailović *et al*., 2016). The results from this study show that *in-vitro* exposure of carvacrol is promising. Carvacrol show a direct paralytic effect on *Parascaris* larvae in the higher concentration wells($10^{-2}$and 10^{-4}), as all larvae were dead or paralyzed . Indeed, we were the first to test the effect of Carvacrol on *Parascaris* on L3 larvae and our result opens an important opportunity for future research.

6-Conclusion:

To conclude our results, we have successfully developed and validated an *in-vitro* model with scoring system for the viability of *Parascaris* L3 stage larvae. The results from this show that mechanical breaking of the eggs with a homogenizer was an effective method as it resulted in the hatching of 98% of the embryonated eggs. Our viability scoring system could distinguish larvae from an Ivermectin resistant farm from larvae from an Ivermectin susceptible farm. This indicates that this method could be used for screening of Ivermectin resistant larvae. An interesting finding was the high paralytic effect observed after carvacrol exposure. Carvacrol shows direct paralysis effect on *Parascaris* larvae in the higher concentration as all larvae were dead or paralyzed. This result may indicate a potential role for carvacrol for the treatment of *Ascaris* infections (ascariasis). Taken together, the *in-vitro* model developed in this project can be used for assaying the effect on larvae after drug exposure.

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important person who was always beside me in all my way when I was alone or with a company when I was happy or sad the words is not enough to thank you My Lord and my Guide.

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Appendix:
Table 4;The Exposure of Ivermectin to 2 different isolates A and B

IVM A concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-4}	5.6	5.2	5.6	5.4	0.2
10^{-6}	5.4	5.8	5.5	5.6	0.2
10^{-8}	5.3	5.7	5.7	5.6	0.2
10^{-10}	5.8	5.7	5.5	5.7	0.2
DMSO-Control	5.4	5.6	5.2	5.4	0.2
Media-Control	5.4	5.1	4.8	5	0.3

IVM B concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-4}	1.01	1.2	1.14	1.12	0.1
10^{-6}	1.51	1.45	1.81	1.6	0.2
10^{-8}	5.14	5.21	5.03	5.13	0.1
10^{-10}	5.1	5.02	5	5.04	0.04
DMSO-Control	4.63	5.3	4.74	4.9	0.3
Media-Control	5	5	4.8	4.9	0.1

Table 5 ; ***In vitro exposure to thiabendazole:***

TBZ concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-3}	1.2	1.1	1.12	1.14	0.04
10^{-4}	1.7	3.24	2.61	2.52	0.63
10^{-6}	2.9	4.6	4.9	4.13	0.9
10^{-8}	3.7	4.2	4.9	4.3	0.5
10^{-10}	5	3.7	4.81	4.5	0.6
DMSO-Control	4.9	4.81	4	4.6	0.4
Media-Control	4.14	4.21	3.9	4.1	0.13

Table 6; ; In vitro Carvacrol exposure :

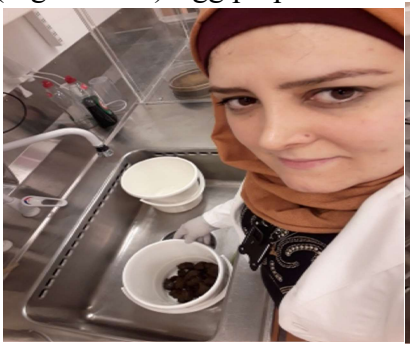
CAR concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-2}	0	0	0	0	0
10^{-4}	0	0	0	0	0
10^{-6}	1.42	0.84	1	1.1	0.24
10^{-8}	4.6	4.7	4.9	4.7	0.13
10^{-10}	4.3	5.1	4.35	4.6	0.4
Ethanol-Control	5.02	5	4.41	4.8	0.3
Media-Control	5.12	5.12	4.52	5	0.3

Table 7*; In vitro PYR exposure on Parascaris larvae*

PYR concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-3}	0.9	0.9	0.9	0.9	0

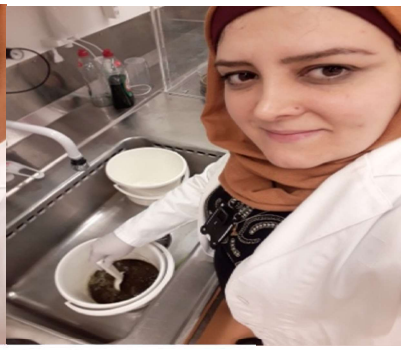
10^{-4}	0.5	0.5	0.6	0.5	0.05
10^{-6}	1.2	0.5	0.6	0.75	0.31
10^{-8}	3.24	1	2.7	2.3	0.95
10^{-10}	4.8	4.9	4.9	4.7	0.1
DMSO-Control	5.9	4.1	5.2	5.1	0.74
Media-Control	3.7	3.7	5	4.1	0.61

(Figure 1-10) Egg preparation and isolation from faeces



Figur (1) Bag of horse stool

Figure (2)Stool in the pail



Figur 3 adding water

Figure (4) Mixing

Figure (5)Using 100 mm filter

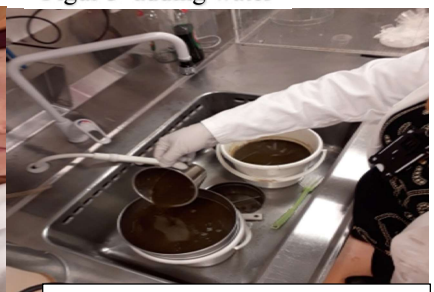


Figure (7) Pouring the mix in the 150 uM filter

Figur (8) Funnel 80 uM

(A) 48 well plate containing larvae +RPMI media(1ml). (B): 24 well plate containing larvae+ RPMI media(1ml)

Table show an example of how we calculated in different concentration of PYR the scoring motility.

PYR 1 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A2(10'-4)	170	160	10	0	0	0	0	330	
score at the time*true score	0	160	20	0	0	0	0	180	0,529411765
B2(10'4)	150	150	10	0	0	0	0	310	
score at the time*true score	0	150	20	0	0	0	0	170	0,548387097
C2(10'-4)	170	140	7	0	0	0	0	317	
score at the time*true score	0	140	14	0	0	0	0	154	0,485804416
Average									0,521201093
PYR2 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A2(10'-4)	120	70	10	0	0	0	0	200	
score at the time*true score	0	70	20	0	0	0	0	90	0,45
B2(10'4)	110	75	5	0	0	0	0	190	
score at the time*true score	0	75	10	0	0	0	0	85	0,440414508
C2(10'-4)	100	80	10	0	0	0	0	190	
score at the time*true score	0	80	20	0	0	0	0	100	0,518134715
Average									0,469516408
PYR 3 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A2(10'-4)	220	100	10	0	0	0	0	330	
score at the time*true score	0	100	20	0	0	0	0	120	0,545454545
B2(10'4)	190	150	10	0	0	0	0	350	
score at the time*true score	0	150	20	0	0	0	0	170	0,735930736
C2(10'-4)	195	90	10	0	0	0	0	295	
score at the time*true score	0	90	20	0	0	0	0	110	0,441767068
Average									0,640692641
total average	0,527226								

PYR 1 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A7 (media)CONTROL	8	61	0	0	0	20	62	151	
score at the time*true score	0	61	0	0	0	100	372	533	3,52980132
B7 (media)CONTROL	10	50	0	0	0	25	55	140	
score at the time*true score	0	50	0	0	0	125	330	505	3,60714286

C7 (media)CONTROL	5	40	0	0	0	20	50	115	
score at the time*true score	0	40	0	0	0	100	300	440	3,82608696
Average									3,65434371
PYR 2 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A7 (media)CONTROL	5	78	0	0	0	30	90	203	
score at the time*true score	0	78	0	0	0	150	540	768	3,78325123
B7 (media)CONTROL	10	80	0	0	0	25	75	190	
score at the time*true score	0	80	0	0	0	125	450	655	3,44736842
C7 (media)CONTROL	8	80	0	0	0	20	100	208	
score at the time*true score	0	80	0	0	0	100	600	780	3,75
Average									3,66020655
PYR 3 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A7 (media)CONTROL	8	40	0	0	0	20	200	268	
score at the time*true score	0	40	0	0	0	100	1200	1340	5,21400778
B7 (media)CONTROL	10	50	0	0	0	20	190	270	
score at the time*true score	0	50	0	0	0	100	1140	1290	4,77777778
C7 (media)CONTROL	7	60	0	0	0	15	210	292	
score at the time*true score	0	60	0	0	0	75	1260	1395	4,77739726
Average									4,99589278
total average	4,1								

PYR 1 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	Sum	total score=(sum/total nr of larvae)
A5(10'-10)	10	60	0	0	0	0	100	170	
score at the time*true score	0	60	0	0	0	0	600	1150	6,7647059
B5(10'-10)	8	60	0	0	0	0	99	167	
score at the time*true score	0	60	0	0	0	0	594	654	3,9161677
C5(10'-10)	10	70	0	0	0	0	85	165	
score at the time*true score	0	70	0	0	0	0	510	580	3,5151515
Average									4,7320084
PYR 2 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	Sum	total score=(sum/total nr of larvae)
A5(10'-10)	7	51	0	0	0	0	150	208	
score at the time*true score	0	51	0	0	0	0	900	1141	5,4855769
B5(10'-10)	5	70	0	0	0	0	140	215	
score at the time*true score	0	70	0	0	0	0	840	910	4,2325581
C5(10'-10)	5	55	0	0	0	0	135	195	
score at the time*true score	0	55	0	0	0	0	810	965	4,9487179

Average									4,888951
PYR 3 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	sum	total score=(sum/total nr of larvae)
A5(10'-10)	10	100	0	0	0	0	150	260	
score at the time*true score	0	100	0	0	0	0	900	1000	3,8461538
B5(10'-10)	5	110	0	0	0	0	120	235	
score at the time*true score	0	125	0	0	160	200	720	1375	5,8510638
C5(10'-10)	7	100	0	0	0	0	110	217	
score at the time*true score	0	100	0	0	0	0	660	760	3,5023041
Average									4,8486088
total average	4,711								

PYR 1 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	sum	total score=(sum/total nr of larvae)
A4(10'-8)	6	30	0	0	50	40	10	136	
score at the time*true score	0	30	0	0	200	200	60	490	3,60294118
B4(10'-8)	5	40	0	0	60	50	10	165	
score at the time*true score	0	40	0	0	240	250	60	590	2,96482412
C4(10'-8)	3	50	0	0	55	45	20	173	
score at the time*true score	0	50	0	0	220	225	120	615	3,15384615
average									3,24053715
PYR 2 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	sum	total score=(sum/total nr of larvae)
A4(10'-8)	15	110	40	0	0	0	0	165	
score at the time*true score	0	110	80	0	0	0	0	190	1,15151515
B4(10'-8)	10	115	40	0	0	0	0	165	
score at the time*true score	0	115	80	0	0	0	0	195	1,18181818
C4(10'-8)	13	120	30	0	0	0	0	163	
score at the time*true score	0	120	60	0	0	0	0	100	0,61349693
average									0,98227676
PYR 3 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	sum	total score=(sum/total nr of larvae)
A2(10'-4)	10	100	0	0	20	40	5	175	
score at the time*true score	0	100	0	0	80	200	30	410	2,34285714
B4(10'-8)	7	125	0	0	40	40	5	217	
score at the time*true score	0	125	0	0	160	200	30	685	2,96536797
C4(10'-8)	8	130	0	0	40	30	0	208	
score at the time*true score	0	130	0	0	160	150	0	440	2,11538462
average									2,65411255
total average	2,2474								

Egg hatching protocol and an *in vitro* scoring system in *Parascaris univalens* larvae after exposure to anthelmintic drugs

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Master's degree project in infection Biology, 45 credits. 2020
Department: Biomedical Sciences and Veterinary Public Health, SLU
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Co-Supervisor: Frida Martin, PhD

Abstract:

Ascaris is a genus of parasitic worms (helminths) found in the small intestine of various mammalian hosts, including *Ascaris lumbricoides* in humans, *Parascaris equorum* and *P univalens* in horses, *Ascaris suum in pigs*, *Toxocara cati* in cats and *Toxocara canis* in dogs. To date, *Parascaris* spp. are the only *Ascaris* worms that have developed resistance to anthelmintic drugs. The mechanisms of resistance in *Parascaris* spp are incompletely understood, partly due to the absence of robust *in-vitro* models. Further complicating *in-vitro* studies, *Parascaris* spp lack a free-living larval stage as their larva only hatch within the host. The aim of this study was to develop *in-vitro* methods for hatching, scoring the viability of *Parascaris* L3 larvae and exposing them to the anthelmintic drugs ivermectin, pyrantel, thiabendazole, and the herbal extract carvacrol. This study shows that mechanical *Ascaris* egg breaking using a homogenizer resulted in a hatching rate of 98%. Our viability scoring system could distinguish an ivermectin resistant larvae from an ivermectin susceptible larvae derived from different farms. This indicates that this method may have utility for the screening of larvae ivermectin resistance on the level of farm populations. Interestingly, a highly paralytic effect observed after carvacrol exposure. Carvacrol shows direct paralytic effects on *Parascaris* larvae in a dose-dependent manner, as higher concentrations were lethal to all exposed larvae. This result presents a potential future opportunity for carvacrol used in the treatment of *Ascaris* infections. To conclude our results, we have successfully developed an *in-vitro* model as well as a scoring system for the viability of *Parascaris* L3 stage larvae, which can be used for assaying the effect on larvae after drug exposure

Popular summary:

Parascaris is a subtype of *Ascaris* parasitic worm which causes infection in young horses causing mild to severe symptoms, potentially resulting in small intestinal obstruction or even death. Horses older than 1 year usually have no problems with this parasite because their immune system has developed immunity against this parasite. A worldwide overuse of anthelmintic drugs has resulted in increasing resistance to macrocyclic lactones (ML) and sporadic cases of resistance to other drug classes with economic and animal welfare consequences. The mechanisms of this resistance are unknown, due in part to the lack of a model to study *Parascaris* in the laboratory (*in vitro*). This parasite lacks the free-living stage (only the eggs). Larva only hatches inside the host which makes this parasite complicated to study *in* vitro. Inside the host, the pregnant female can lay more than 170,000 eggs and that means around 60 million eggs in a year. Because of very thick outer layers, the egg can survive in the environment for more than 10 years. This study aimed to develop a model in order to study the effects of anthelminthic drugs on *Parascaris* under laboratory conditions.

Keywords:

Parascaris, Equine, Larvae, Anthelminthic Resistance, Hatching, Parascaris univalens, Carvacrol, Thiabendazole, Pyrantel, Ivermectin

1- Introduction

1-1: Ascarid worms:

Ascaris are large parasitic worms found in the small intestinal of various mammalians including humans (*Ascaris lumbricoides)*, horses (*Parascaris. equorum* and *univalens),* pigs *Ascaris suum*, cats *Toxocara cati*, and dogs *Toxocara canis*. *A. suum* is the *Ascaris* species which shows high potential zoonotic infection between pigs and humans (CDC, 2018), but there is also some evidence that *A. lumbricoides* can infect dogs (Scott Weese, *et al*.,2010). *A. lumbricoides* belong to the soil-transmitted helminth (STH) which currently infect approximately 1.5 billion people annually (Lee, *et al.,*2012). *Ascaris* infections may induce starvation in the host as they can parasitize a substantial amount of nutrition. Growth and development in children or young animals may be stunted by *Ascaris* infections.

1-2: The equine roundworms:

The *Parascaris spp, P. equorum,* and *P. univalens* are intestinal parasites that can infect horses, zebras, and donkeys (Colin, 2000) and are the most common parasites in foals worldwide (Pusteria & Nicola, 1967). Interestingly, *Parascaris* spp are the only *Ascaris* worms that have developed resistance to anthelmintic drugs (Nielsen, 2016). The most susceptible to infection are young horses that are 2 to 6 months old (Lyons, *et al*., 1976). By the age of one-year, most horses develop natural immunity to this parasite infection (Clayton & Duncan, 1977). Symptoms of infestation with ascarids vary from asymptomatic to highly pathogenic and include nasal discharge, respiratory syndromes, impaired condition, coughing and cessation of growth, intestinal blockage, and potentially lethal complications such as intestinal rupture (Clayton & Duncan, 1978; Austin *et al*., 1990; Reinemeyer, 2009). *P. equorum* and *P. univalens* are morphologically identical, and similar in their life cycles as well as all other biological characteristics. One notable difference between the two is that *P. equorum* has two pairs of chromosomes while *P. univalens* has only one pair. Recent studies have shown that *P. univalens* is the most common species infecting horses today (Alan, *et al*., 2019).

1-3: Life cycle of Parascaris spp:

The life cycle of *Parascaris spp.* is a direct sexual life cycle (Figure 1) and starts when parasite eggs are passed out in faeces and contaminate the environment. Larvae start to develop from first larval stage (L1) to second stage (L2) and third stage (L3) infectious larvae. The host gets infected after ingesting eggs containing an infectious larva (L3), often via consumption of contaminated grass. In the intestine, L3 larvae are hatched and then start to migrate by penetrating the wall of the small intestine. They then migrate to the liver and thereafter, via blood vessels to the alveoli and finally the lungs in a 7-day process (Fagerholm, 2000). Thereafter, the larvae migrate up to the respiratory tree, get coughed up, and again swallowed by the host. Larvae eventually find their way to the small intestine where they mature to adults, reproduce and excrete copious amounts of eggs. These eggs will be passed out in host faeces to the environment (Dickson, 1987). Eggs of *Parascaris* spp are surrounded with a very thick lipid layer that protects the eggs and gives it the ability to stick to any surface (Briggs & Karen, 2004; Roberts, *et al*., 2008). This lipid layer is what makes the eggs resistant to unfavourable environmental conditions (Shanahan, 2007; Roberts, *et al*., 2008).

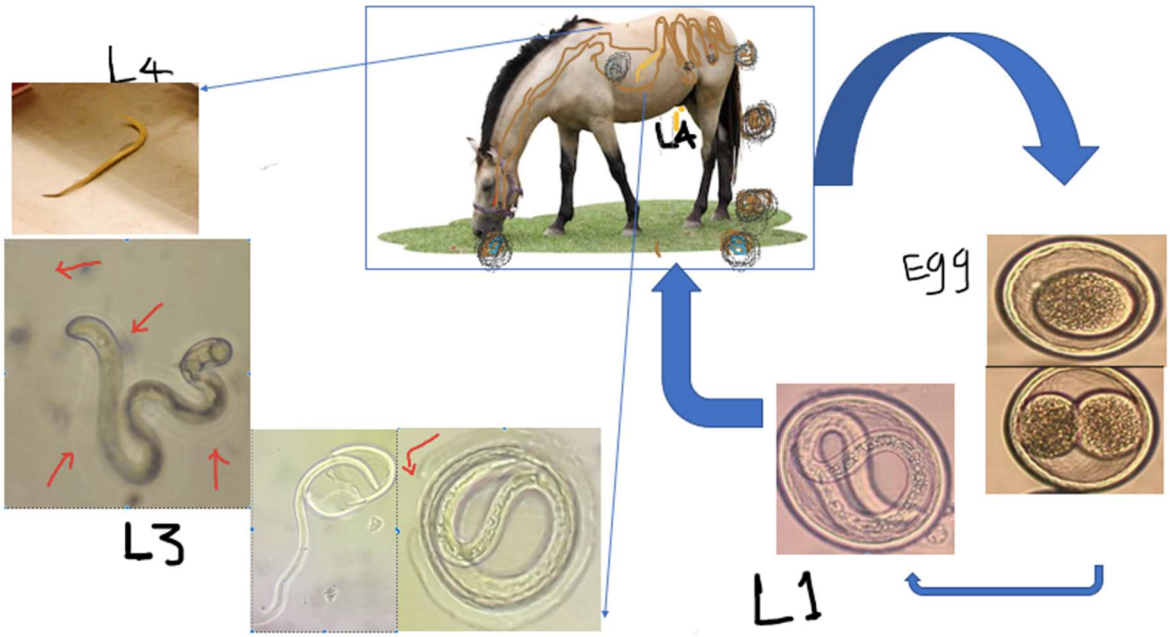


Figure 1. (Eggs of *Parascaris equorum* photo by *Dimah Alshenah, SLU, Uppsala*. Eggs with a thick multilayered shell will be expelled with feces to the environment (Briggs & Karen, 2004)). Horses can get infected by ingesting eggs with stage L3 larva (an infectious egg) by contaminated water or grass. In the digestive system, the thick outer layer will be lost then the larva can hatch and migrate through the lymphatic system to the liver (Nielsen, *et al*., 2018; Roberts, *et al*., 2008), then to the lungs. The parasite will be coughed up and swallowed again back to the small intestines where it will mature and reproduce. The adult worm is around 30 to 50 cm in size. Hundreds of thousands of eggs are shed daily from infected individuals, contaminating the environment and continuing infection transmission in new hosts. The infective eggs can stay viable in the environment for years (Martin, *et al*., 2018).

The female is morphologically larger (around 40 cm) than the male (15-28 cm). In one day, a pregnant female can lay more than 170,000 eggs, translating to around 60 million eggs over a year (Roberts, *et al*., 2008). During the migratory phase in the lungs, symptoms such as coughing and nasal discharge are often observed (Hilary, *et al*., 1977). The adult worms in the small intestine can cause intestinal blockage and potentially develop intestinal rupture (Briggs & Karen, 2004)

1-4: Anthelminthic drugs and resistance:

Three classes of broad-spectrum anthelminthic drugs have been used for the treatment of *Parascaris* infections; **1.** Macrocyclic lactones (ML), **2.** Benzimidazoles (BZ), and **3.** Tetrahydro pyrimidines (PYR). MLs act by binding to glutamate-gated chloride ion channels (GGCI) in nerve cells and muscle cells, causing increased ion permeability of the cell membrane resulting in paralysis and death (Drug Bank). PYR also induces paralysis to the parasite as it blocks neuromuscular depolarization via inhibition of the nicotinic acid receptor. (Arion & Emilio, 2017). BZ binds to b-tubulin and disrupts the formation of tubulin, resulting in starvation and death of the parasite (Lacy, 1988).

An indiscriminate control of horse nematodes with anthelmintic for over 40 years has resulted in resistance (Kaplan, 2002). In 2002, the first case of ML-resistance was reported for *P. equorum.* Since then several countries have reported ineffective treatment of nematodes with ML (Fagerholm, *et al*., 2000). In 2007, multi-resistance to ML and PYR was reported for the first time in the U.S and in 2018 in Sweden (Jabbar, *et al*., 2014; Nielsen, *et al*., 2014; Martin,

et al., 2019). Reduced efficacy of BZ in Australian farm foals was reported for the first time in 2014 (Armstrong, *et al*., 2014).

1-5: New potential substances for controlling Parascaris infections:

Due to the widespread resistance developed by *Parascaris* spp to anthelmintic drugs, there is an urgent need to investigate alternative approaches to infection control. Over the ages, there were a huge number of different oil-bearing plants that have been used and studied as therapeutics with the aim of curing intestinal parasite infections (Youssfie, 2019). Several plant-borne compounds could be the alternative not only to antibacterial, but also work well against viruses, fungi, and even parasites (Youssfie, 2019). One such example is Carvacrol, which is a phenolic monoterpene sourced from many medicinal aromatic plants such as Oregano (*Origanum vulgare*) (Health Benefit, 2020). A recent study showed that monoterpenoids have very high bioactivity toward parasites such as *Trypanosoma cruzi*, *Entamoeba histolotica*, *Giardia,* and *Leishmania* (Yousffie, 2019). The mechanism of action is believed to inhibit the GABA (Gamma-Aminobutyric acid) receptor with lethal paralytic effects (Trailovic, 2015).

1-6: In vitro models and challenges:

The mechanism of anthelminthic resistance in *Parascaris* spp. is incomplete and that is partly due to the absence of *in vitro* models. This parasite lacks the free-living stage as the larva only hatch inside the host and need a host to complete their life cycle (Clayton & Duncan, 1978) which makes this parasite complicated to study *in* vitro (ECCAP, 2019). Furthermore, adult worms are difficult to maintain under *in vitro* conditions for more than a few days (ECCAP, 2019).

2- Aim of the study

This study aimed to develop an *in-vitro* model for *Parascaris* spp. The specific objectives of this thesis were:

1. To evaluate different hatching methods for *Parascaris* larvae.
2. To develop a scoring protocol of the larvae motility under *in vitro* conditions based on a protocol developed for adult worms (Scare, *et al*., 2018).
3. To use the scoring system to investigate the larvae motility after exposure to different concentrations of IVM, PYR, TBZ, and CARV.

3- Materials and methods

Faeces were collected from naturally infected foals on a breeding farm as foals get infected naturally with *Parascaris univalens* from May to September. *Parascaris* eggs were isolated from faecal samples.

3-1: Eggs washing and isolation.

A 15 ml tube containing the eggs was centrifuged at 1500 rpm for 3 minutes, (eggs will be in the pellet). 50 ml Milton 2 solution (sodium hypochlorite 2% + sodium chloride 16,5%) was added to the egg pellet. Tubes were centrifuged again at 1500 rpm for 3 minutes. A 1 ml pipette was used to collect the eggs that were then transferred to a new 50 ml tube containing 25 ml of water. Several washing steps with water were performed, those tubes were centrifuged at 3000 rpm for 3 minutes and the supernatant was discarded.

3-2: Eggs incubation:

After washing steps, the eggs were incubated for 12 days in 25C°.

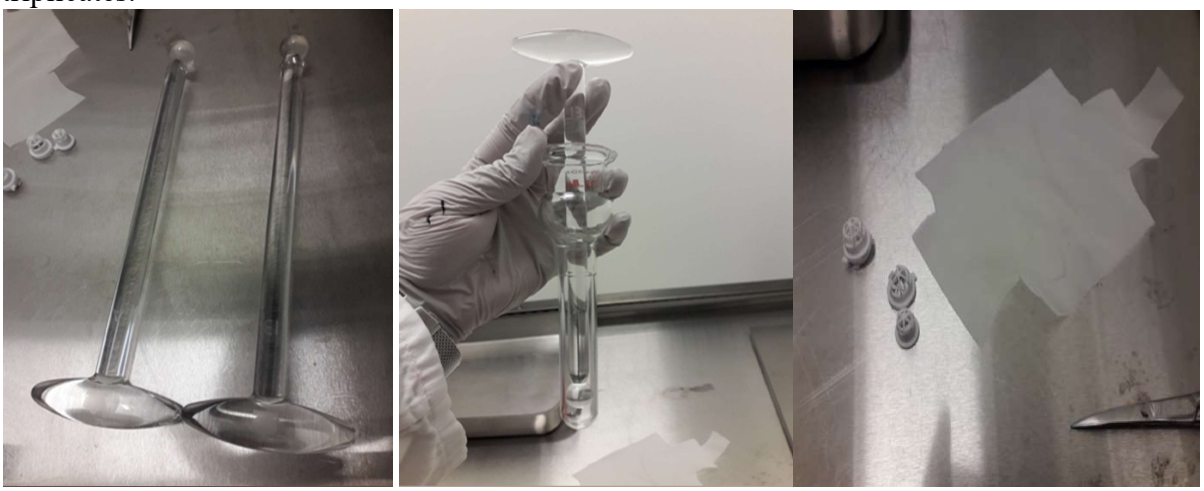
3-3: Hatching and counting of Larvae:

Eggs were counted with consideration for fertilized (larva within the egg) and nonfertilized eggs in three 10 µl drops. The average number of larvae was calculated.

3-4-1: Hatching:

3-4-1-1: Using glass Homogenizer alone & centrifugation matter:

The eggs were first counted and then hatched. Egg counting was made by taking three 10 µl drops and counting the hatched and unhatched larvae in each drop, then the number of larvae per 10 ml was calculated. Hatching was induced in a homogenizer using a glass pestle to assist larval escape from the eggs. Eggs were then hatched in 5 ml Hanks Balanced Salt Solution (HBSS) or PBS. We compared three groups containing between 200 to 100 eggs using a 5 ml Kontez glass homogenizer, two types of pestles were used for hatching (A and B) and variable numbers of crushing pestle strokes. Pestle B was larger than A, with pestle A having a mechanical clearance of 0.889–0.165 mm between the pestle and the homogenizer wall, while pestle B had 0.025–0.076 mm (Figure 2, A and B). Hatched larvae were resuspended in 1 ml media (RMPI-1640 media + 10% FBS+ 1% L-glut + 1% AMAB (Antimycotic antibiotic), transferred to 21 tissue culture plates for counting and evaluation under light microscopy. The total amount of larvae and unhatched eggs in the well were counted and compared to the results. Another improvement of the hatching protocol was an increasing centrifugation to 3000 rpm for 5 minutes from the original protocol of 1500 rpm for 3 minutes. Eggs were counted before hatching and then larvae were counted after hatching after centrifugation. The larvae were recounted in both the supernatant and the deposit. All the experiments were performed in triplicates.



(A) (B) (C)

Figure 2: *Different pestles were used in our protocols* photo by *Dimah Alshenah, SLU, Uppsala*: First figure(A) show homogenizer A size (on right) and B size (on the left).The second show the glass which was used to crush the eggs with the chosen homogenizer. Figure C shows the 20 µM sieve filter which was tested to filter the larvae after hatching.

3-4-1-2: Using HCL protocol for hatching:

Eggs were resuspended in 15 ml HBSS + 2 ml sodium bicarbonate 1M. 1-2 drops of 5 M HCL were added carefully to the egg´s tube to lower the pH to 2. The tube was then incubated at 37°C in 5% CO_2 for 30 minutes. After 30 minutes pH was normalized (pH=7) with 1M NaOH. The eggs were washed in HBSS and then crushed with a glass homogenizer in 5 ml HBSS. 2 to 3 strokes of the pestle were taken. Hatched larvae were counted and then centrifuged in 1500

rpm for 5 minutes. The larvae were resuspended in 8 ml RPMI 1640 media + 10% FBS + 1%L-glut + 1% AMAB). and transferred to a small tissue culture flask. Thereafter, were incubated at 37°C in 5% CO_2. Media was changed every two to three days.

3-4-2: *Using the 20uM sieve to purify the Larvae after hatching protocol*:

20 µM sieve (3D printed at INRA) was tested to be used directly after hatching or after they had been first incubated for 24 hours in tissue culture flask after they were hatched. Directly after hatching, the larvae were filtered through a 20-uM sieve (Figure 2, C). The sieve was placed top on the tube and hatched larvae were carefully filtered into the tube using a pipette. The tube then was filled with PBS or HBSS to the top until it was reaching the filter. This tube then was incubated at 37°C for 2 h. After that, the larvae that had succeeded to migrated through the sieve were transferred back to a tissue culture flask with 8 to 10 ml RPMI and incubated at 37°C, 5% CO_2. The media was changed every two to three days. The tissue culture flask was then examined via light microscopy to count and evaluate the motility and viability of the larvae every 2 days.

3-5: In Vitro method for Larvae viability and scoring:

After hatching, larvae were cultured in optimum media using a 50 ml Tissue Culture Flask from (Inter Med, Nunclon, Delta). RPMI-1640 (Roswell Park Memorial Institute) was used as a liquid culture media +10% fetal bovine serum (FBS, Gibco), 1% Penicillin and Streptomycin (Life technologies) or 1% Antibiotic Antimycotic Solution (Sigma, Germany) and 1% L-glutamine (Lonza) as a mix of 45 ml RPMI + 5 ml FBS + 0.5 ml L-Glutamine +0.5 (antimycotic antibacterial). Between 8 to 10 ml from this mix were used to culture the larvae in a tissue culture flask and incubated at 37°C in 5% CO_2. For testing the viability, scoring two independent experiments were performed. In both experiments, hatched larvae were incubated in RPMI media at 37°C in a 5% CO_2 incubator. Media was changed every 2nd to 3rd day after centrifugation in 3000 rpm for 5 minutes. In experiment 1, three small culture flasks (X1, Y1, and Z1) incubated for 10 days (X1, Y1, and Z1 experiments were performed in technical triplicates under identical conditions and nearly identical hatched *Parascaris* larvae). In experiments 2, small culture flasks X, Y, and Z were incubated for 41 days in technical triplicates. The viability of the larvae were evaluated every other day under light microscopy. 50 larvae were collected and evaluated for scoring of their motility from 0 to 6 according to the motility method (Table 1).

Table 1: Motility and Viability Scoring table Based on Scare, J.*et al*.,2018

Scoring	Explanation	Note
0	Dead (paralyzed)	Larvae are not moving and showing no resistance when using external stimulation
1	Very weak movement	Larvae moving very rarely once after more than 40 seconds
2	Poor movement	Larvae move after more than 30 seconds
3	Fairly good movement	Larvae move after more than 20 seconds
4	Good movement	Larvae move after 10 to 15 seconds
5	Very good movement	Larvae moving from the first second but in the same field
6	Very motile (excellent movement)	Continuous movement and can move from one place to another in the field of the slide.

Scoring was calculated by using this formula:

Score ={Σ(scoring at a time× number of larvae performing this score)/Total number of Larvae.

3-7: Exposure to drugs:

For each exposure experiment, a variable number of L3 larvae were incubated with anthelmintic drugs with around 5000 to 8000 eggs hatched in the presence of PBS. Eggs were counted before hatching including eggs with larvae (fertilized) and unfertilized ones. Larvae were also counted after hatching to include live, dead, or unhatched larvae. Technical triplicates and biological triplicates were performed for each experiment with corresponding controls. Freshly hatched larvae were cultured in 48 well plates from (Nunclon Delta Surface) or 24 well plates (TPP) tissue culture plates were used for each exposure experiment. Every column on the plate consisted of the same concentration for all technical replicates. Light microscopy was used for reading the plates. 200 larvae per well were cultured in RPMI-1640 media mixed (refer to "Scoring motility") for 24 h at 37 °C in an atmosphere containing 5 % CO_2 plus anthelminthic drug for 24 hours. The drugs used in our experiments were: Ivermectin (IVM), thiabendazole (TBZ), pyrantel citrate (PYR), and the natural essential oil carvacrol. All drugs were dissolved in DMSO except for carvacrol in ethanol.

A stock solution of 0.1 M Ivermectin was prepared and diluted in DMSO to reach the final concentration in the plate of 10^{-3}, 10^{-4}, 10^{-6}, $10^{-8,}$ and 10^{-10} M.
A stock of 1 M Carvacrol in EtOH was prepared and diluted in 99,5 % ethanol to reach the final concentration of 10^{-2}, 10^{-4}, 10^{-6}, $10^{-8,}$ and 10^{-10} M.
A stock of 0.1 M Thiabendazole was made and diluted in DMSO to reach final concentrations of 10^{-3}, 10^{-4}, 10^{-6}, $10^{-8,}$ and 10^{-10} M.
A stock of 0.1 M Pyrantel was prepared and diluted in DMSO to reach the final concentration of 10^{-3}, 10^{-4}, 10^{-6}, $10^{-8,}$ and 10^{-10} M.

For all drugs exposure experiments, the last two rows contained controls, first one-row containing only media, the second row containing media + 0.1 % DMSO drug cocktail except, Carvacrol as DMSO was replaced with 0.1% EtOH. The larvae were incubated for 24 hours at 37°C in 5% CO_2 during the exposure work. After 24 hours each well in the plates was read under the light microscopy. Larvae counting and motility scoring were performed according to the table above. All results were translated to a table Using Excel and curves using Prism 8 (GraphPad, San Diego, USA) as shown in the results below.

4- Results

4-1: *Eggs washing and isolation:*

In order to isolate the eggs from faeces, samples were washed to isolate the eggs using different types of filters. Thereafter, the eggs were stored in water to the next step. There more purification for cleaning and decortication was performed for the eggs and to remove the thick outer layers of the eggs, for that, a tube containing a suspension of water and *Parascaris* eggs were centrifuged on 1500 rpm for 3 minutes. 50 ml Milton 2 solution then was added to the eggs for cleaning and decortication (removal of external chitinous lipid layer and to eliminate bacteria or fungal contaminants). Tubes were centrifuged again at 1500 rpm for 3 minutes where eggs floating up in the upper part of the supernatant. Eggs were collected from the upper part of the supernatant (around 5 ml) then transferred to a new 50 ml tube containing 25 ml water. This step was repeated three to four times to make sure no eggs remained. Several washing

steps with water were performed after Milton step as Milton solution is toxic to the eggs. This tube was centrifuged at 1500 rpm for 3 minutes and the supernatant was discarded, and the deposit was kept in the last washing step with 10 ml water at 25 C° incubator.

4-2: Larval development:

Larvae were allowed to develop into stage 3 (Fig. 1) within the eggs by incubating the washed eggs for 12 days at 25 C° for the development of larvae in water.

4-1: Hatching protocol:

To improve hatching frequencies a random number of eggs were used that had been counted before and after hatching with the help of two types of crushing glass homogenizer aiming to improve the hatching method. The eggs were hatched in 5 ml (HBSS) or PBS as mention in (3-4- 1- 1). 5 ml Kontez glass homogenizer was used, and two types of pestles, A and B. 3 groups were tested to be hatched with both types, then they were examined under a microscope. The result of using pestle type A shows, that around 26 to 30 unhatched eggs remained of a total 150 to 90 eggs. Whereas 10 to 20 unhatched eggs remained when using type B. In other words, hatching with homogenizer A resulted in 70% hatched larvae while homogenizer B resulted in 90-80 % hatched larvae. After the B type homogenizer was selected, our hatching protocol was further optimized with an additional crushing stroke (six strokes total) resulting in up to 10 unhatched eggs only remains from a total of 130 to 100 eggs. Therefore, we achieved and optimal hatching rate of 90-99% and discovered that an additional crushing stroke reduced hatch yields by up to 10 eggs. Comparing with, between 30 to 36 unhatched eggs remained of 100-150 eggs after following the original 5-stroke protocol, translating to an efficiency of 60 to 70%. In subsequent experiments in this study, six crushing strokes were used. Overall to get the best result the strokes should be done carefully to avoid crushing the larvae. Another improvement of the hatching protocol was an increase of length and speed to 3000 rpm for 5 minutes of the centrifugations step as this was used when media was changed. A large proportion of larvae remained in supernatant while using 1500 rpm for 3 minutes according to the original protocol. Eggs were counted before hatching and then the larvae were counted direct after hatching and then after the centrifugation step. The larvae were then, counted in both supernatants and the pellet. It was noted that the number of the larvae were decreased in after using 1500 rpm for 3 minutes protocol. The optimized protocol using 3000 rpm for 5 minutes resulted in very high larvae yields almost the same of the original number.

4-2: Using a 20uM filter:

A 20-uM filter was used to increase the purity of the extracted larvae, on the other word, to purify the larvae from eggshells and debris after hatching protocol. In this step, our goal was to determine filter efficiency (evaluated)by calculating the number of larvae that would be lost with each use of this filter.3 groups were tested of hatched L3 larvae. The first groups stand for 10000 hatched larvae and the larvae counting after using 20uM filter were 8000 Larvae. The second group consisted of 5000 hatched larvae to became 4000 larvae after using the filter and the last group stands for 10500 larvae to reach 8000 larvae after using the filter. In another word, the loss of L3 *parascaris* larvae was around 20%. This method could work very well for a short incubation period study but not so recommended when it comes to a long incubation period study since the ability to develop contamination is high as tow of this groups develop contamination growth of nonsignificant growth of mixed bacteria in the 4th and 5^{th} day as this filter is not sterile.

4-3: Viability scoring:

For testing the viability scoring, hatched larvae were incubated in RPMI media at 37°C, 5% CO_2 incubator and evaluated for the viability every 2 days under light microscopy. Three cultures of larvae (X, Y, and Z) were examined for viability scoring for 41 days. As shown in figure 3. Every 1 to 2 days the flasks were evaluated under a light microscope. For that, 50 larvae were counted and evaluated for scoring of their motility from score 0 to 6 with the formula (Table 1) mentioned above "3-6 Scoring motility". In each checking point (checking time), 50 larvae were counted and each was evaluated a cording to their motility**,** scoring at the time× number of larvae performing this score then the sum was taken and multiply with the total number of larvae. At time 0, the scoring motility was around 4. In between the 96 h to 600 h the larvae show very high scoring in all the 3 repetitions from score 4 to 5. In day 20 viability was around 85 % then it decreased. In the day 40 (960 h) or 42 (1000 h) most larvae were dead (figure 3) and (table 3). It's also good to mention, in order to reduce or prevent any growth contamination of bacteria or fungal, microbiological contamination was checked for both media preparation and media flasks after culturing the larvae so any changes in the color of the media could be significant. For that one to two drops of the media were streaking on Horses blood agar plate and incubated for 3 days in 38 C°+CO_2 incubator. In the first group X1, Y1 and Z1, we found one contamination growth of *Candida. spp.* in one flask which takes us to stop this group and start with the second groups (the one we mention up X, Y, and Z)which contain also of three repetitions of tissue culture flasks containing media and *Parascaris* larvae.

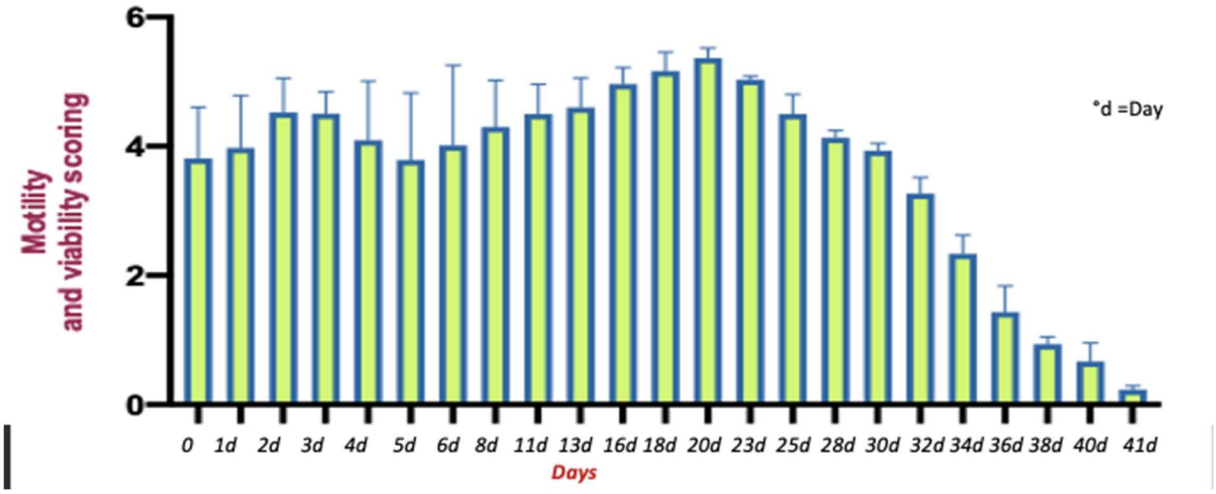


Figure3; In vitro viability of Parascaris larvae monitored for 42 days (1000 hours): The average was taken for the 3 experiment X, Y, and Z taking to account the STDEV to perform this figure with a help of prism showing the relation between the motility scoring and every checking point hour there to check the Larvae in the culture flasks. Microscopy was used to read and evaluated the results.

Table 3: In vitro survival monitored for 42 days to *Parascaris* larvae in RPMI media:

Hours	Motility score for Experiment (X)	Motility score for Experiment (W)	Motility score for Experiment (Z)	Average	STDEV
0	4.2	3.0	4.3	3.8	0.6
24	3.4	3.7	4.9	4.0	0.7
48	4.1	4.4	5.1	4.5	0.4
72	5.0	4.3	4.3	4.5	0.3
96	4.7	4.6	3.0	4.1	0.7

120	4.6	4.1	2.6	3.8	0.8
144	5.0	4.4	2.6	4.0	1.0
192	5.0	4.5	3.5	4.3	0.6
264	5.0	4.6	4.0	4.5	0.4
312	5.0	4.7	4.1	4.6	0.4
384	5.2	4.7	5.0	5.0	0.2
432	5.5	5.0	5.0	5.2	0.2
480	5.4	5.2	5.5	5.4	0.1
552	5.0	5.1	5.0	5.0	0.0
600	4.2	4.8	4.5	4.5	0.3
672	4.0	4.2	4.2	4.1	0.1
720	3.9	4.0	4.0	4.0	0.1
768	3.0	3.3	3.5	3.3	0.2
816	2.5	2.0	2.5	2.3	0.2
864	1.5	1.0	1.8	1.4	0.3
912	1.0	0.8	1.0	1.0	0.1
960	1.0	0.5	0.5	0.7	0.2
1000	0.2	0.3	0.2	0.2	0.0

Three repetitions cultures of larvae (X, Y, and Z) were examined for viability scoring for 41 days. The first column shows every checking point hour there where the Larvae in the culture flasks were read and evaluated under light microscopy. The next columns show each experiment (X), (Y), and (Z) and it is scoring motility of indifferent checking point. The 4th column is the average of the 3 repetitions experiments and the last column shows the standard deviation between these 3 repetitions.

4-3: Parascaris larvae incubated in PBS:

After hatching the larvae, PBS was used as a culture media instead of RMPI. The result showed that larvae were very motile at the beginning and stayed very active with a higher score (6) for 2 days of incubation but then larvae motility decreased till they died on the 3rd day (figure 4). This protocol could be used for studies requiring a short incubation period since there are no nutrient substances in PBS that the larvae can use it to survive longer periods.

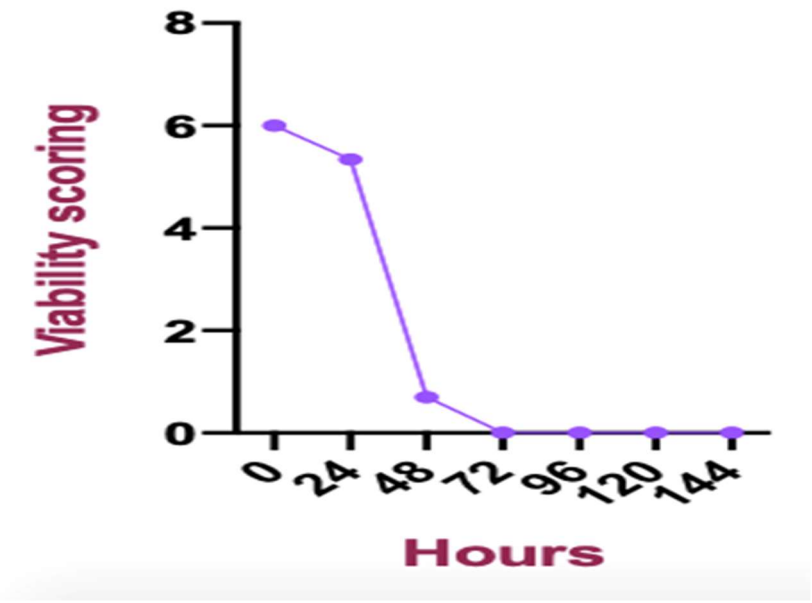


Figure 4; ***Motility and Viability scores of Parascaris larvae incubated in PBS*** After hatching the L 3 larvae they were incubated directly in PBS and incubated in a tissue culture flask in 37°c incubator. The average was taken for the 3 repetitions experiments. First 2 days the larvae motility in PBS were high as it reached almost score 6. The figure was done by using prism program.

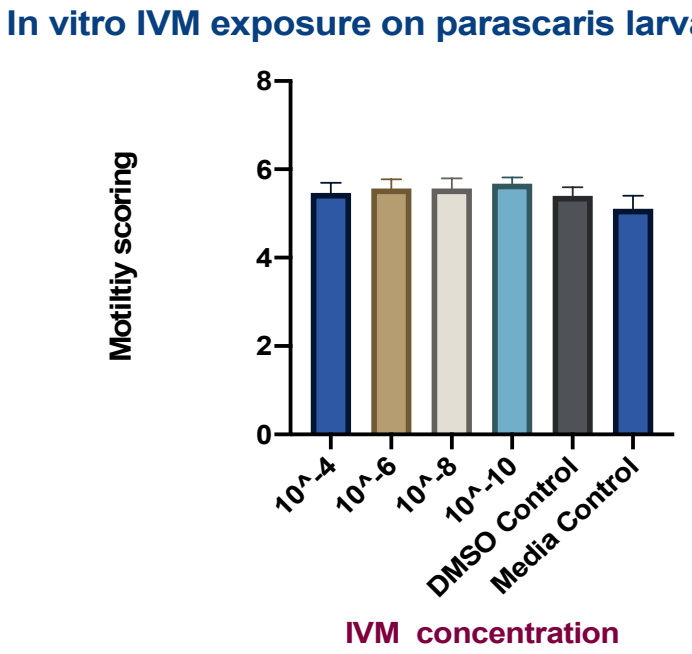
4-4: Drug exposure:

Three classes of broad-spectrum anthelminthic drugs have been used for the treatment of *Parascaris* infections; **1.** Macrocyclic lactones (ML), **2.** Benzimidazoles (BZ), and **3.** Tetrahydro pyrimidines (PYR). In our experiment, we use Ivermectin (IVM) as one type of (ML), Thiabendazole (TZP) as its one type of (BZ) and Pyrantel as a type of (PYR) all to be tested on *Parascaris* larvae. An indiscriminate control of horse nematodes with anthelmintic for over 40 years has resulted in resistance (Kaplan, 2002). In 2002 the first case of ML-resistance was reported for *P. equorum.* Since then several countries have reported ineffective treatment of nematodes with first time in the U.S and 2018 in Sweden (Jabbar, *et al*., 2014; Nielsen, *et al*.; Martin, *et al*., 2019). Reduced efficacy to the third drug class BZ in foals on farms in Australia
was reported for the first time in 2014 (Armstrong, *et al*., 2014). As *Parascaris* is the only type of *Ascaris* that has developed resistance to antihelminth drugs and this resistance is increasing there is an urgent need to look into other alternatives that what takes us to test one natural oil from an aromatic-plants call Carvacrol which has been registered as an effective agent on other parasites. However, we were the first to test carvacrol on *Parascaris* L3 stage larvae. After scoring protocol for larvae motility under *in vitro* condition has been developed based on a protocol developed for adult worms (Scare, *et al*., 2018), as the aim here was to use this scoring system to investigate the larvae motility after exposure to different concentrations of different drugs for other futures drugs exposures studies. *Parascaris* eggs were isolated from those faeces as mention in (3- 3) eggs were incubated at 25°C for 14 days as mention in (3- 4) to allow the Larvae to inter the L3 stage. In total around (24000 to 36000) eggs were used which means around (6000 to 9000) Parascaris eggs for each drug exposing experiment. That compensated around 5000 to 8000 fresh hatched larvae as mention in (3- 4- 1- 1). A total of 24 tissue culture 48 well plates were utilized (Figure 2 (A, B)). Around 200 larvae/well were cultured in RPMI-1640 media mixed (described under "Scoring motility") for 24 h at 37 °C in an atmosphere containing 5%CO_2 for 24 h. Each well contains (1 ml media + 200 larvae). After 48 well plates

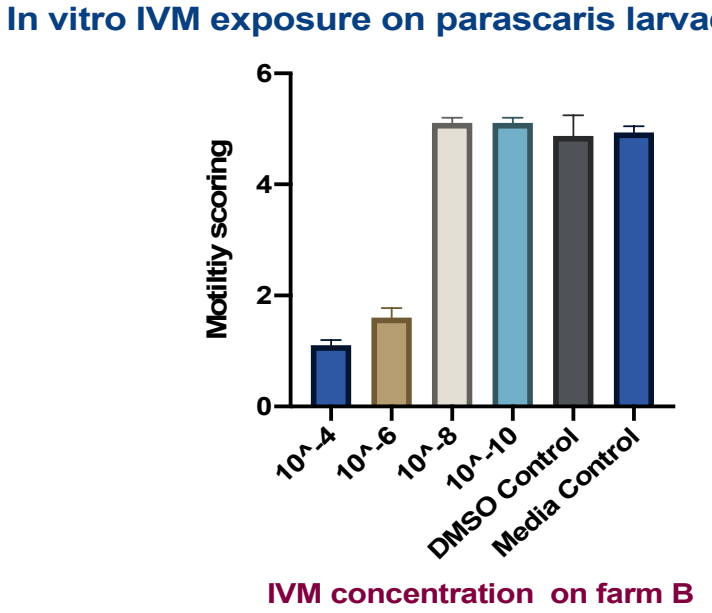
were left for 24 h different concentrations of the drugs were added after a certain dilution was performed for each. Plates were then incubated again for 24 h in the same incubator under the same circumstances. Every column on the plate consisted of the same concentration and its technical replicates (3 technical replicates). 3 biological repetitions for each drug were performed. Light microscopy was used for reading and evaluated the larvae viability in the tissue culture plates after they were incubated for 24h. All drugs were dissolved in DMSO but carvacrol with ethanol.

4-4-1: Ivermectin exposure (IVM);

Ivermectin is a broad-spectrum anti-parasite drug and is a substance in macrocyclic lactone group (ML). (IVM) was first known as Stromectol® effective against nematode except for tapeworms, 2012 was used and approved as a treatment for head lice and can be used from 6 months and older. Here the name changes to Sklice™, l. Ivermectin very effective against onchocerciasis, and also show effective against other worms like strongyloidiasis, ascariasis, trichiasis, and enterobiasis. This anthelmintic agent is semisynthetic and is an ivermectin a group of pentacyclic sixteen-membered lactone execrated from soil bacteria (Streptomyces ivermictilis) (drug bank). However, Include Asia and Australia, south and north America, Europe Resistant was reported against IVM in *Parascaris.* spp. (Martin, F.; Höglund, J, 2018). To prove this, two different *Parascaris* isolates (A and B) were exposed to Ivermectin (Sigma, Germany). Isolate A was collected from a farm with known resistance to ivermectin. Isolate B was collected from a farm susceptible to ivermectin. The viability of isolates A and B after exposure was compared and evaluated as shown in (figure 5) for each concertation. Scoring was calculated according to Scoring ={Σ(scoring at the time× number of larvae performing this score)⁄ Total number of Larvae, and then average and STDV was taken for every replicate for each concertation as well as to all 3 repetitions. The viability of isolate A and B after exposure was compared. The concentrations are based on two previous studies by Janssen *et al*., 2013 and Jonsson, 2019. In the highest concentration, 10^{-3} M crystal formation was noticed, and this concentration was therefore not included, and the concentration 10^{-4} M was the highest in our experiment. On-farm A, which was resistant to ivermectin, no paralysis effect was observed. An interesting finding was that in the higher concentration the larvae were more active compared to the control (Figure 2 A). Farm B, which was susceptible to ivermectin, the mobility scoring was reduced to 1-2 in the highest concentrations (10^{-4} M and 10^{-6} M) (figure 2 B).



(A)



(B)

Figure5 (A and B); *In Vitro IVM exposure on Parascaris Larvae from Farm A and B*: Two different isolates (A and B) were exposed to Ivermectin. Isolate A were collected from a farm with known resistance to ivermectin. Isolate B was collected from a farm susceptible to ivermectin. The viability of isolate A was done by taking the average of all 3 repetitions and has shown high viability scoring both in the higher concentration of (IVM) and in the lower one. That translated as, no good effect of ((IVM) on farm A. The viability scoring of isolates from farm B was done also by taking the average of all the 3 repetitions from farm B to give, low viability scoring in the presence of high concentration (10^{-4} and $10^{-6}$of (IVM), that translated as, good effect of (IVM)on farm b. Both figures were done with the help of a prism program.

4-4-2: Thiabendazole exposure (TBZ);

TBZ is a 3rd generation heterocyclic antihelminth compound that can be used as antifungal and antiparasitic therapy, as well as a food preservative. Benzimidazole was introduced in early 1962 as an active agent in treatment against several nematodes (Smith & Reynard, 1992). Interestingly, TBZ is vermicidal against *Ascaris lumbricoid*. Here we will be the first to test it on *Parascaris* larvae regardless that *Parascaris* has shown resistance for other anthelmintics unlike the *A. lumbricoides.* The mechanism of action of TBZ has been shown to suppress the egg and the larvae production. It could be involved in inhibiting the development of eggs and larvae before they are passing in the faeces. Our results show that in the highest concentration (10^{-3} M) larvae moved weakly in all 3 of the biological replicates and reached a motility scoring of 1 as shown in figure 6. That is considered a remarkable effect of TBZ on Parascaris L3 larvae, whereas all other lower concentrations did not show a noticeable effect on the larvae since the motility scoring was high (3 to 5). All the 3 replicates observed had closely related results and varied approximately with one score as the STDV gave acceptable results between them.

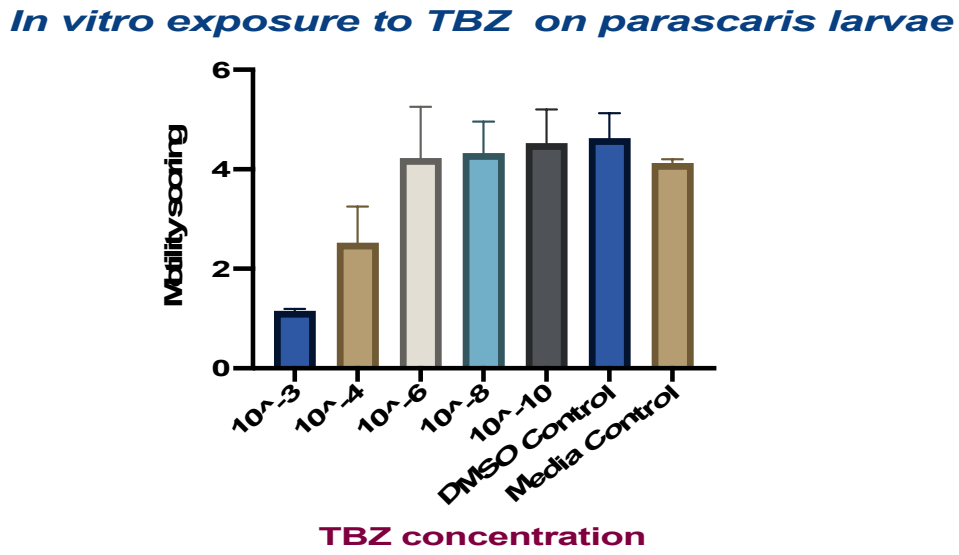


Figure 6*: In vitro exposure to thiabendazole on Parascaris Larvae*:
The figure shows the results of 24 h *in vitro* exposure of different concentrations of TBZ on Parascaris larvae by taking the average of the 3 repetitions. The figure shows that the effect of the first concentration was noticeable were Parascaris larvae move so weakly. 3 biological replicates and 3 technical repetitions were done, and the average considered for all the three repetitions to perform this figure. Each plate experiment was evaluated on different days and read under light microscopy Motility scoring was counted separately for each in Excel(not attached) and then average (for the 3 replicates)and SDTEV was also calculated for all 3 experiments together. Prism 8 was used for drawing this graph

4-4-3: Carvacrol exposure:

Carvacrol, is an example of a phenolic monoterpene which is excreted from many medicinal aromatic plants, specially *Oregano* as it is known (*Origanum vulgare*) (healthbenifet,2020). A recent study shows that monoterpenoids have very effective bioactivity that is antagonistic toward *Trypanosoma cruz*i, *Entamoeba histolotica*, *Giardia,* and *Leishmania*. (Youssfie, 2019) This could be due to their accessibility from abundant natural sources as well as the low toxicity because they are generally recognized as safe (GRAS). Discovering new drugs against parasites is a very consuming project both in time and money as it takes years to produce a new drug,
The need for safe, effective drugs is dominant in order to replace current ones that have become ineffective due to resistance. The mechanism of action for carvacrol can be explained by the effect of carvacrol on GABA (Gamma-Aminobutyric acid)which known as the master inhibitory neurotransmitter in the

central nervous system which is responsible for reducing the neural excitability of the nervous system. Carvacrol binds with GABA and Tyramine receptors causing inhibition of the contraction of the L3 larvae (Sasa, 2015). However, we were first to test the effect of CARV on *in vitro* atmosphere on *Parascaris* larvae and to explore if CARV can be a replacement for the other anthelmintics drugs against *Parascaris.*

Exposure of carvacrol in our results showed a dose-dependent effect on the larvae. The highest concentrations of carvacrol (10^{-2} and 10^{-4}) showed a very high paralytic effect with 100% dead larvae (scoring 0) (Figure 7). A very rapid effect of carvacrol was observed already after 1 hour as 100 % of larvae dead in the highest concentrations. On the 10^{-6}, a remarkable effect was noticed reaching score 1. There was less effect from CARV in the lower concentration as the score was around 5 which was similar to the control (unexposed).

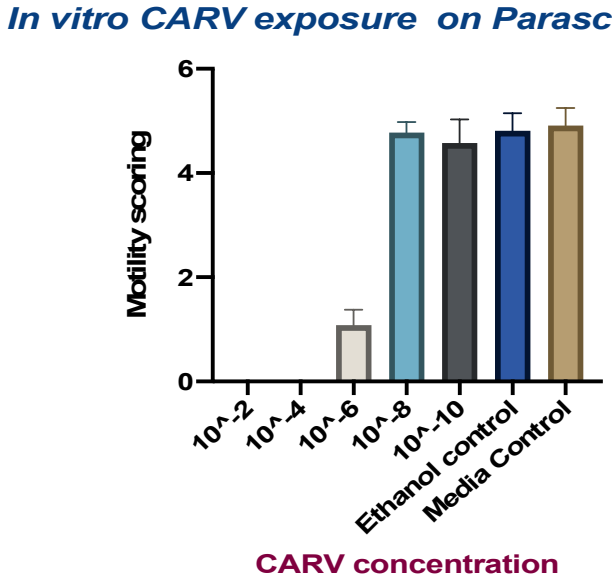


Figure 7; In vitro Carvacrol exposure on parascaris larvae: Show the result of the average of 3 repetitions experiments using different concentrations of Carvacrol on *Parascaris* larvae after 24 h of incubation with drug + RPMI media. The first highest concentration in all the 3 repetitions gives motility scoring 0 for the parascaris larvae which shows the effect of CARV on the larvae at this concentration. Good paralysis effect on the larvae as motility scoring was around 1.

4-4-4: Pyrantel citrate exposure on Parascaris larvae (PYR);

Pyrantel is one of the safest and most effective medicines in the health system according to the WHO's list of essential medicines. This drug was first described in 1965 by international Pfizer researches who explored this drug as an anthelmintic looking for drug specificity and duration. Pyrantel is a broad-spectrum anthelmintic, a pyrimidine-originate compound that works against*, Ascaris lumricodies* (roundworms), *Thricho strongyliasis, Trichinella*, (hookworms) and *Enterobius enterobiosis* (pinworms) infections (AHFS, 2020).This drug is known to affect the nerves causing paralysis to the parasite as it depolarizes neuromuscular -blocking material which activates the nicotinic receptor which will lead to sudden contraction and then paralysis of the worms. The helminth will lose power on the intestinal wall and be thrown out of the body in the faeces (Arion & Emilio, 2017). Based on this information we tested Pyrantel in *Parascaris* larvae on *in-vitro* atmosphere to evaluate the Pyrantel effect against *Parascaris* larvae. After they were exposure to Pyrantel as described in the method the results show that in the highest concentration (10^{-3}M) larvae showed reduced motility with a score 1 (figure 8). An interesting observation was that the larvae were coiled on themselves like a snail or spiral shape

figure (9) and (10) and they were more swollen compared to the larvae size in the control (Figure 10). Surprisingly, in 10^{-4} concentration (figure 11) about 100% of the larvae were paralyzed or dead. Also, in this concentration, the larvae body show some abnormality (figure 11) and was swollen in comparison with the larvae in the control (Figures 9 and 10). In 10^{-6} concentration the larvae scoring was around 1 showing that this concentration is also effective as all larvae moved so slowly. In the 10^{-8} concentration, the motility has been not affected by the drug as it gives a score 4 which was close to the larvae control´s scoring on the same plate.

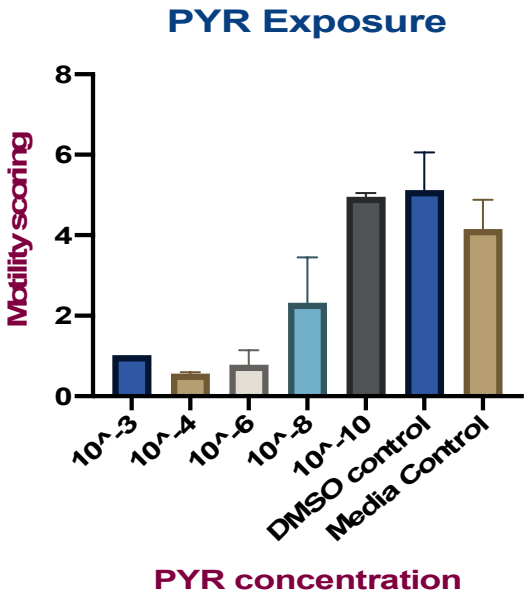


Figure 8*; In vitro PYR exposure on parascaris larvae*: The figure shows, the result of the average of the 3 repetitions of PYR exposure on Parascaris L3 stage larvae, with different PYR concentrations and its effect on ***Parascaris* larvae** motility after 24 h of incubation with the drug + RPMI media. 3 biologicals replicates were done. Each plate experiment was evaluated on different days and read under a light microscope. Motility scoring was counted separately for each in Excel (not attached) and then average and SDTEV was done for each plate separately and then all 3 experiments together. SDTEV in all was under 1 which makes our result noteworthy.

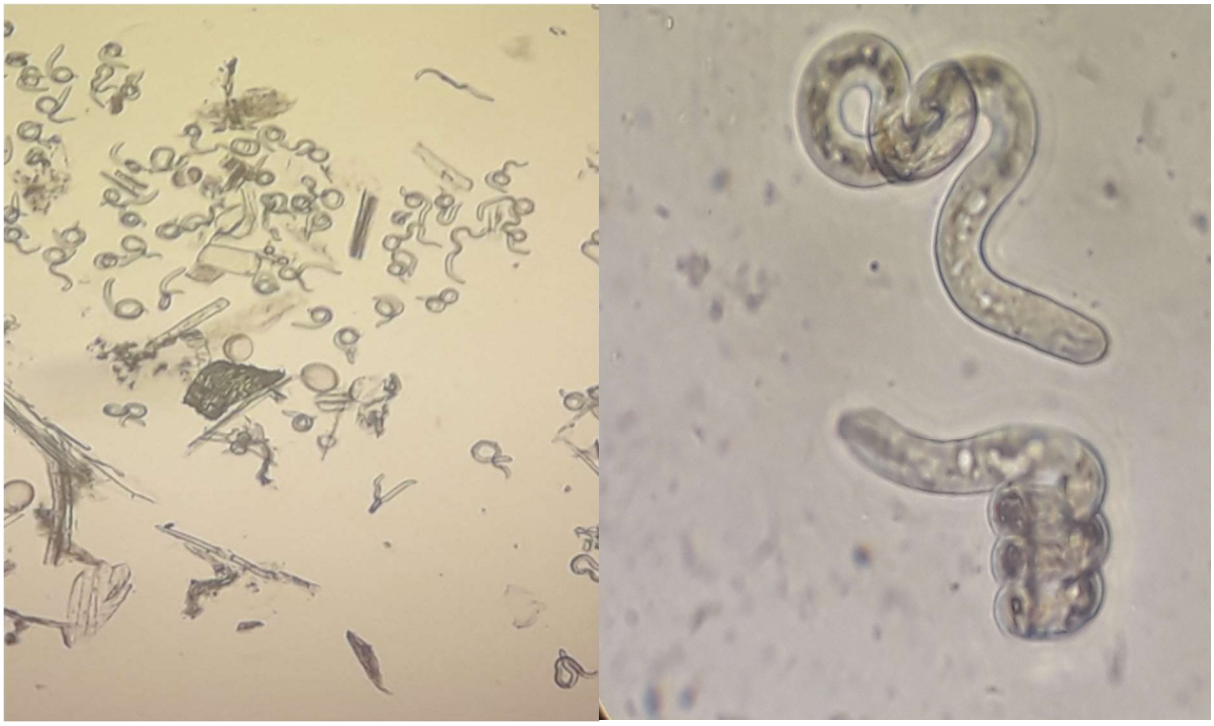
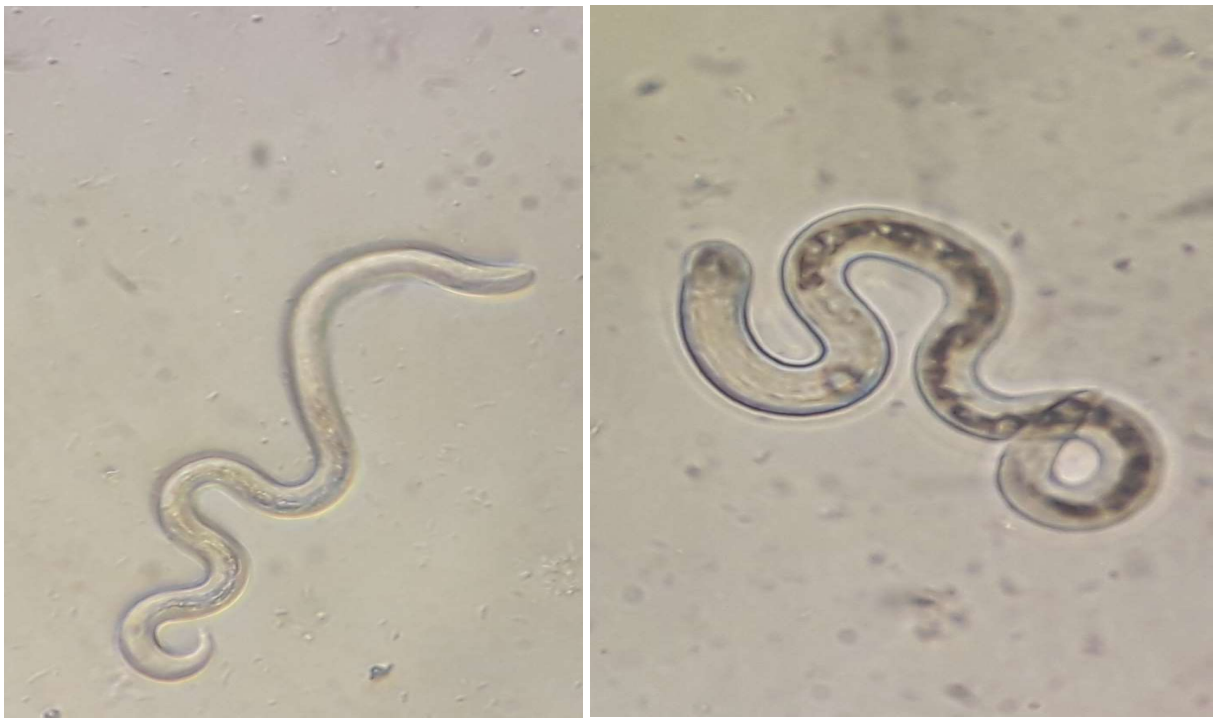


Figure 9*: The effect of PYR on Parascaris larva on the* 10^{-3} ***concentration***. by *Dimah Alshenah, SLU, Uppsala* Larvae were incubated in RPMI media for 24 h in 37°C +5% CO_2 incubator in the presence of PYR 10^{-3} M. The results were evaluated under light microscopy in the highest (left) and lowest (right) magnification. Larva moved slowly and majority of them take snail shape and all are swollen compared with normal parascaris larva in the media control wells of the same plate



(A) **(B)**

Figure 10; **Different body size between parascaris larva in the control (A) with the one under PYR exposure in the** 10^{-3} **concentration(B)**. by *Dimah Alshenah, SLU, Uppsala* Figure

A show parascaris larva size in the media control. Figure B show Parascaris larva body size after exposure to PYR for 24h in the 10^{-3}concentration.

Figure 11*:* <u>The effect of PYR on *Parascaris* larva on the 10^{-4} concentration.</u> by *Dimah Alshenah, SLU, Uppsala* Larva almost not moving only very slow movement for the head or the tails as all are paralyzed and show some abnormality in the body and swollen comparing with the larvae in the media control wells or DMSO control wells.

5-Discussion:

It is a well-established fact that parasite infection are a significant cause of morbidity and mortality worldwide in both humans and animals. Hugo, *et al*. 2001 has assumed that over 28,000 nematode species have been described of which 16,000 are parasitic. Severe economic damage and loss of welfare of animals as well as the physical suffering for both humans and animals have been a result of parasitic worms' infestation. Around 50 roundworm species can infect humans, with many classified as neglected tropical diseases e.g. *Ascaris* causing ascariasis (Payne & Fitchett, 2010). Anthony *et al*., 2007 suggests that more than 3 billion people are infected by parasitic nematodes worldwide. Furthermore, increasing anthelminthic drug resistance has been reported virtually everywhere. Indeed, increasing the dose during treatment may not necessarily yield better therapeutic results in most cases. This was seen in our larvae exposure experiment to ivermectin, there increasing the dose of ivermectin did not cause death or any paralysis effect on the larvae from the farm with an ivermectin resistant *Parascaris* spp. population.

The complete picture of the resistance mechanisms in *Parascaris* spp. is poorly understood, partly due to the absence of *in-vitro* models. Because the larva only hatch inside the host, it makes this parasite complicated to study *in vitro* (ECCAP, 2019). This study evaluated different hatching protocols. It has been shown that *A. lumbricoides* and *A. suum* have a chitinous layer 2-4µm thick (Lysek, *et al*., 1985: Rogers, 1956). This thickness of the eggshell is a problem for

normal hatching protocols. The results from this study show that the mechanical breaking of the eggs with a homogenizer resulted in a hatching rate of 98%. This protocol was based on a previous protocol used on another Ascaris called *Toxocara canis* (Ponce-Macotela, *et al*, 2011) but we modified it by adding 6 strokes by a glass homogenizer. Taking into consideration especially in the bottom where the risk for smashing the larvae is higher if they were hatched by hard strokes. On the other hand, we found that soft slow strokes give better results and more chances to minimize the loss of hatched L3. Using the filter for hatch L3 larvae direct after hatching can be used to purify the L3 from the debris and eggshells. The filter method is not so recommended when it comes to a long incubation period as the loss of L3 larvae can be up to 20%. Furthermore, the hatched larvae were incubated in RPMI at 37°C to study the survivors. The *in vitro* survival study gave a very good picture and gave evidence that *Parascaris* larvae can be used to study for a long period *in vitro* as previous studies did not examine more than three weeks (Burk, *et al*., 2014: Jonsson, *et al*., 2017). In our study, we found that *Parascaris* larvae can survive *in vitro* in RMPI media for more or less than one month. 85% of the larvae were alive on day 20, 58% of them were alive on day 32. On day 38 around 20% of the larvae were still surviving and 5 to 10 % larvae on day 40. Further studies should be performed to confirm this find. A modification of centrifugation was needed when changing the RPMI media. Centrifugating the larvae for 3 minutes at 1500 rpm was not enough because many larvae were found in the supernatant. Therefore, the centrifugation was modified to 5 minutes at 3000 rpm. This gave an improved result with a reduced loss of larvae, as only 0-2 % of larvae remained in the supernatant. Our model gives a wider ability for further studies to explore novel drugs for the treatment of *Parascaris* infection.

In 2002 the first case of ML-resistance was reported for *P. equorum.* Since then several countries have reported ineffective treatment with ML (Fagerholm, *et al*., 2000). Shortly after, in 2007, multi-resistance to ML and PYR was reported for the first time in the U.S and 2018 in Sweden (Jabbar, *et al*., 2014: Nielsen, *et al*., 2014: Martin, *et al*., 2018). In 2014, the first report about reduced efficacy to the third drug class BZ in foals on farms in Australia was highlighted (Armstrong, *et al*., 2014). However, it is important to note that our experiment supports this finding as our results showed a substantial difference between the two A and B isolates. Isolate A was from a farm with known resistance to IVM and isolate B from a farm with known susceptibility to IVM. It was a marked difference between A and B in our in vitro assay. No paralytic effect was observed in isolate A in the highest IVM concentration (10^{-4}) but in isolate B, the mobility scoring was reduced to 1-2 scores. This suggests that the *in-vitro* method for drug exposure developed in this thesis could be a good method for evaluating the resistance status on farms. This *in-vitro* method could be complemented to FECRT (The fecal egg count reduction test), which is the gold standard for evaluation of anthelmintic resistance in the field.

Thiabendazole is known to be effective as an antifungal and anthelminthic and is also used as a food additive (E233) (Setzinger, *et al*., 1965). Iqual- Adel 2004, has shown in his article that TBZ has strong effects in controlling roundworms, hookworms, and others helminth spp. which infect both animal and human. TBZ is the original of benzimidazoles (BZ) that are still effective, but it has been reported that 2 two of 4 farms have developed resistance against BZ in Australia (Armstrong, *et al*., 2014). We showed in our experiment that the larvae that were exposed to a high-concentration dose of TBZ show a good effect of the drug on the larvae as they were paralyzed or had very slow movement. A similar observation was also observed at the second-highest concentration. That leads us to consider TBZ to be still effective on *Parascaris* larvae.

This is the first study evaluating the effect of *Parascaris* larvae exposure of PYR *in-vitro.* The exposure showed a dose-dependent effect with reduced scoring in the higher concentrations. Interesting findings were observed in the higher concentrations where larvae were swollen and exhibited a supercoiling behavior. More studies are needed to understand the toxic effects of PYR.

The history of thymol *(Thymus vulgaris*), one of the organic aromatic oils, was used commonly for worm infection specifically *Ascaris* and hookworms (kaplen *et al*., 2014). Due to the emerging problem with resistance, compounds from plants can be explored in the future. Carvacrol is a phenolic monoterpene extracted from many medicinal aromatic plants specially Oregano (*Origanum vulgare*) (Health Benefit, 2020). A recent study shows that monoterpenoids has very effective bioactivity toward parasites such as *Trypanosoma cruzi*, *Entamoeba histolotica*, *Giardia,* and *Leishmania* (Yousffie, 2019). The mechanism of action is believed to be on GABA receptors (Gamma-Aminobutyric acid) which is known as the master inhibitory neurotransmitter in the central nervous system of parasites which reduces the neural excitability of the nervous system causing paralysis and death (Trailovic, 2015). Carvacrol was tested *in vitro* on L3 larvae of *Anisakis simplex* by Hierro *et al*., (2004). Abdelrahman *et al*.,2013, have also published that carvacrol has a nematocidal effect against *Caenorhabditis elegans.* Carvacrol was also tested in *A. suum* muscular tissue from the worm and showed highly significant inhibition for the acetylcholine contractions (Trailović *et al*., 2016). The results from this study show that *in-vitro* exposure of carvacrol is promising. Carvacrol show a direct paralytic effect on *Parascaris* larvae in the higher concentration wells($10^{-2}$and 10^{-4}), as all larvae were dead or paralyzed . Indeed, we were the first to test the effect of Carvacrol on *Parascaris* on L3 larvae and our result opens an important opportunity for future research.

6-Conclusion:

To conclude our results, we have successfully developed and validated an *in-vitro* model with scoring system for the viability of *Parascaris* L3 stage larvae. The results from this show that mechanical breaking of the eggs with a homogenizer was an effective method as it resulted in the hatching of 98% of the embryonated eggs. Our viability scoring system could distinguish larvae from an Ivermectin resistant farm from larvae from an Ivermectin susceptible farm. This indicates that this method could be used for screening of Ivermectin resistant larvae. An interesting finding was the high paralytic effect observed after carvacrol exposure. Carvacrol shows direct paralysis effect on *Parascaris* larvae in the higher concentration as all larvae were dead or paralyzed. This result may indicate a potential role for carvacrol for the treatment of *Ascaris* infections (ascariasis). Taken together, the *in-vitro* model developed in this project can be used for assaying the effect on larvae after drug exposure.

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important person who was always beside me in all my way when I was alone or with a company when I was happy or sad the words is not enough to thank you My Lord and my Guide.

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Appendix:
Table 4;The Exposure of Ivermectin to 2 different isolates A and B

IVM A concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-4}	5.6	5.2	5.6	5.4	0.2
10^{-6}	5.4	5.8	5.5	5.6	0.2
10^{-8}	5.3	5.7	5.7	5.6	0.2
10^{-10}	5.8	5.7	5.5	5.7	0.2
DMSO-Control	5.4	5.6	5.2	5.4	0.2
Media-Control	5.4	5.1	4.8	5	0.3

IVM B concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-4}	1.01	1.2	1.14	1.12	0.1
10^{-6}	1.51	1.45	1.81	1.6	0.2
10^{-8}	5.14	5.21	5.03	5.13	0.1
10^{-10}	5.1	5.02	5	5.04	0.04
DMSO-Control	4.63	5.3	4.74	4.9	0.3
Media-Control	5	5	4.8	4.9	0.1

Table 5 ; ***In vitro exposure to thiabendazole:***

TBZ concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-3}	1.2	1.1	1.12	1.14	0.04
10^{-4}	1.7	3.24	2.61	2.52	0.63
10^{-6}	2.9	4.6	4.9	4.13	0.9
10^{-8}	3.7	4.2	4.9	4.3	0.5
10^{-10}	5	3.7	4.81	4.5	0.6
DMSO-Control	4.9	4.81	4	4.6	0.4
Media-Control	4.14	4.21	3.9	4.1	0.13

Table 6; ; In vitro Carvacrol exposure :

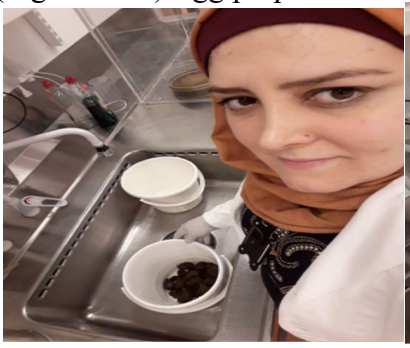
CAR concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-2}	0	0	0	0	0
10^{-4}	0	0	0	0	0
10^{-6}	1.42	0.84	1	1.1	0.24
10^{-8}	4.6	4.7	4.9	4.7	0.13
10^{-10}	4.3	5.1	4.35	4.6	0.4
Ethanol-Control	5.02	5	4.41	4.8	0.3
Media-Control	5.12	5.12	4.52	5	0.3

Table 7*; In vitro PYR exposure on Parascaris larvae*

PYR concentration	Experiment A	Experiment B	Experiment C	Average	STDEV
10^{-3}	0.9	0.9	0.9	0.9	0

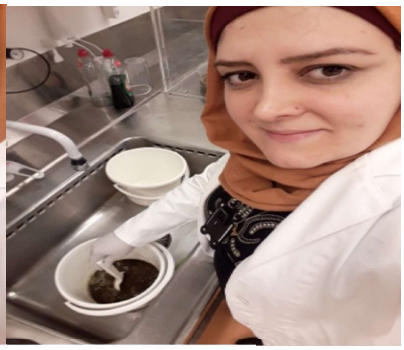
10^{-4}	0.5	0.5	0.6	0.5	0.05
10^{-6}	1.2	0.5	0.6	0.75	0.31
10^{-8}	3.24	1	2.7	2.3	0.95
10^{-10}	4.8	4.9	4.9	4.7	0.1
DMSO-Control	5.9	4.1	5.2	5.1	0.74
Media-Control	3.7	3.7	5	4.1	0.61

(Figure 1-10) Egg preparation and isolation from faeces



Figur (1) Bag of horse stool

Figure (2)Stool in the pail



Figur 3 adding water

Figure (4) Mixing

Figure (5)Using 100 mm filter

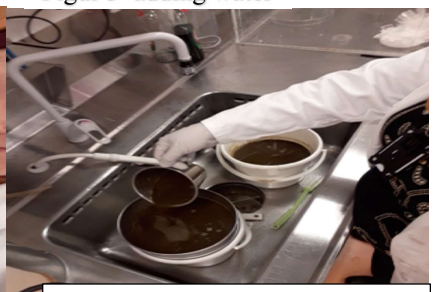


Figure (7) Pouring the mix in the 150 uM filter

Figur (8) Funnel 80 uM

(A) 48 well plate containing larvae +RPMI media(1ml). (B): 24 well plate containing larvae+ RPMI media(1ml)

Table show an example of how we calculated in different concentration of PYR the scoring motility.

PYR 1 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A2(10'-4)	170	160	10	0	0	0	0	330	
score at the time*true score	0	160	20	0	0	0	0	180	0,529411765
B2(10'4)	150	150	10	0	0	0	0	310	
score at the time*true score	0	150	20	0	0	0	0	170	0,548387097
C2(10'-4)	170	140	7	0	0	0	0	317	
score at the time*true score	0	140	14	0	0	0	0	154	0,485804416
Average									0,521201093
PYR2 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A2(10'-4)	120	70	10	0	0	0	0	200	
score at the time*true score	0	70	20	0	0	0	0	90	0,45
B2(10'4)	110	75	5	0	0	0	0	190	
score at the time*true score	0	75	10	0	0	0	0	85	0,440414508
C2(10'-4)	100	80	10	0	0	0	0	190	
score at the time*true score	0	80	20	0	0	0	0	100	0,518134715
Average									0,469516408
PYR 3 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A2(10'-4)	220	100	10	0	0	0	0	330	
score at the time*true score	0	100	20	0	0	0	0	120	0,545454545
B2(10'4)	190	150	10	0	0	0	0	350	
score at the time*true score	0	150	20	0	0	0	0	170	0,735930736
C2(10'-4)	195	90	10	0	0	0	0	295	
score at the time*true score	0	90	20	0	0	0	0	110	0,441767068
Average									0,640692641
total average	0,527226								

PYR 1 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A7 (media)CONTROL	8	61	0	0	0	20	62	151	
score at the time*true score	0	61	0	0	0	100	372	533	3,52980132
B7 (media)CONTROL	10	50	0	0	0	25	55	140	
score at the time*true score	0	50	0	0	0	125	330	505	3,60714286

C7 (media)CONTROL	5	40	0	0	0	20	50	115	
score at the time*true score	0	40	0	0	0	100	300	440	3,82608696
Average									3,65434371
PYR 2 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A7 (media)CONTROL	5	78	0	0	0	30	90	203	
score at the time*true score	0	78	0	0	0	150	540	768	3,78325123
B7 (media)CONTROL	10	80	0	0	0	25	75	190	
score at the time*true score	0	80	0	0	0	125	450	655	3,44736842
C7 (media)CONTROL	8	80	0	0	0	20	100	208	
score at the time*true score	0	80	0	0	0	100	600	780	3,75
Average									3,66020655
PYR 3 concentration	0(dead/not move)	1	2	3	4	5	6(very motile)	sum	total score=(sum/total nr of larvae)
A7 (media)CONTROL	8	40	0	0	0	20	200	268	
score at the time*true score	0	40	0	0	0	100	1200	1340	5,21400778
B7 (media)CONTROL	10	50	0	0	0	20	190	270	
score at the time*true score	0	50	0	0	0	100	1140	1290	4,77777778
C7 (media)CONTROL	7	60	0	0	0	15	210	292	
score at the time*true score	0	60	0	0	0	75	1260	1395	4,77739726
Average									4,99589278
total average	4,1								

PYR 1 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	Sum	total score=(sum/total nr of larvae)
A5(10'-10)	10	60	0	0	0	0	100	170	
score at the time*true score	0	60	0	0	0	0	600	1150	6,7647059
B5(10'-10)	8	60	0	0	0	0	99	167	
score at the time*true score	0	60	0	0	0	0	594	654	3,9161677
C5(10'-10)	10	70	0	0	0	0	85	165	
score at the time*true score	0	70	0	0	0	0	510	580	3,5151515
Average									4,7320084
PYR 2 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	Sum	total score=(sum/total nr of larvae)
A5(10'-10)	7	51	0	0	0	0	150	208	
score at the time*true score	0	51	0	0	0	0	900	1141	5,4855769
B5(10'-10)	5	70	0	0	0	0	140	215	
score at the time*true score	0	70	0	0	0	0	840	910	4,2325581
C5(10'-10)	5	55	0	0	0	0	135	195	
score at the time*true score	0	55	0	0	0	0	810	965	4,9487179

Average									4,888951
PYR 3 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	sum	total score=(sum/total nr of larvae)
A5(10'-10)	10	100	0	0	0	0	150	260	
score at the time*true score	0	100	0	0	0	0	900	1000	3,8461538
B5(10'-10)	5	110	0	0	0	0	120	235	
score at the time*true score	0	125	0	0	160	200	720	1375	5,8510638
C5(10'-10)	7	100	0	0	0	0	110	217	
score at the time*true score	0	100	0	0	0	0	660	760	3,5023041
Average									4,8486088
total average	4,711								

PYR 1 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	sum	total score=(sum/total nr of larvae)
A4(10'-8)	6	30	0	0	50	40	10	136	
score at the time*true score	0	30	0	0	200	200	60	490	3,60294118
B4(10'-8)	5	40	0	0	60	50	10	165	
score at the time*true score	0	40	0	0	240	250	60	590	2,96482412
C4(10'-8)	3	50	0	0	55	45	20	173	
score at the time*true score	0	50	0	0	220	225	120	615	3,15384615
average									3,24053715
PYR 2 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	sum	total score=(sum/total nr of larvae)
A4(10'-8)	15	110	40	0	0	0	0	165	
score at the time*true score	0	110	80	0	0	0	0	190	1,15151515
B4(10'-8)	10	115	40	0	0	0	0	165	
score at the time*true score	0	115	80	0	0	0	0	195	1,18181818
C4(10'-8)	13	120	30	0	0	0	0	163	
score at the time*true score	0	120	60	0	0	0	0	100	0,61349693
average									0,98227676
PYR 3 concentration	0(dead/not move)	1	2	3	4	5	6(very motil)	sum	total score=(sum/total nr of larvae)
A2(10'-4)	10	100	0	0	20	40	5	175	
score at the time*true score	0	100	0	0	80	200	30	410	2,34285714
B4(10'-8)	7	125	0	0	40	40	5	217	
score at the time*true score	0	125	0	0	160	200	30	685	2,96536797
C4(10'-8)	8	130	0	0	40	30	0	208	
score at the time*true score	0	130	0	0	160	150	0	440	2,11538462
average									2,65411255
total average	2,2474								