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Date: 24-11-28

Final report

Läkemedelsresistenta spolmaskar - nya metoder för kontroll och övervakning av resistenta parasiter

Drug Resistant Equine Roundworms - novel methods for monitoring and control of resistant parasites

Project number: H-20-47-556

Project period: 20210501-20241201

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Part 1: Detailed summary

Bakgrund och syfte

Den alarmerande utvecklingen av trippelresistenta Parascaris spp. utgör ett allvarligt hot mot fölens hälsa och hästnäringen i Sverige. Det är därför av största vikt att öka kunskapen om läkemedelsresistens hos parasitära maskar och identifiera molekylära markörer för resistens, för att säkerställa långsiktigt hållbara rutiner för parasitkontroll. Detta är särskilt kritiskt eftersom inga nya anthelmintika för hästar har introducerats sedan 1980-talet.

Mål att uppnå inom forskningsprojektet:

I. Utvärdera effekten av bensimidazoler (fenbendazol - Axilur® Intervet) och övervaka anthelmintikaresistens på stuterier i Sverige. Bensimidazoler utgör den sista effektiva läkemedelsklassen för behandling av Parascaris spp.-infektioner på majoriteten av svenska gårdar. (Studie I)
II. Undersöka molekylära biomarkörer associerade med resistens mot bensimidazoler, i syfte att utveckla ett screeningsverktyg för att tillämpa i hållbara kontrollprogram för övervakning av resistens. (Studie II-III)
III. Utveckla ett in vitro-system för Parascaris spp. för att: a) studera mekanismer som leder till läkemedelsresistens och b) screena resistenta parasitpopulationer. Avsaknaden av ett *in vitro*-system är för närvarande en stor begränsning för att förstå uppkomsten av resistenta maskar och därmed för att utforma effektiva övervakningsprogram. (Studie IV-V)

Metoder

Föl med en naturlig parasitinfektion har deltagit i effektstudien. Totalt medverkade 21 stuterier och 92 föl i studie I. Parasitinfektion analyserades genom träckprover och behandlades enligt rekommendation. Parasitmaterial till övriga studier (I-V), renades from från träckprover från föl med en positiv parasitinfektion.

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Resultat och slutsatser

Resultaten från Studie I, som utvärderade effekten av fenbendazol på svenska stuterier, ger avgörande bevis för den snabba spridningen av anthelmintikaresistens i Sverige. Detta är särskilt betydelsefullt för föl, som ofta behandlas utan föregående parasitologisk diagnostik, vilket understryker behovet av att övervaka läkemedelseffH20ektiviteten i denna sårbara grupp. Resultaten från studien har direkt bidragit till uppdaterade rekommendationer för parasitkontroll hos hästar, såsom framgår i Hästens mag-tarmparasiter: att förebygga och behandla.

Att identifiera molekylära markörer för tidig upptäckt av mutationer kopplade till resistens är en hög prioritet. Resultaten från Studier II och III visar dock att resistensmekanismer skiljer sig åt mellan parasitarter. Noterbart är att Parascaris spp., en ascaridmask, verkar ha en unik resistensmekanism mot bensimidazoler, sannolikt kopplad till dess unika uppsättning av β-tubulingener, till skillnad från andra tarmparasiter.

Forskning om Parascaris har historiskt hämmats av bristen på *in vitro*-tekniker, eftersom dessa maskar normalt inte kläcks utanför värddjuret. I Studie IV utvecklade vi en ny kläckningsteknik som utgör en grund för in vitro-metoder att följa resistensutveckling hos Parascaris. I samarbete med INRAE och Invensis har vi även etablerat en dos-responskurva för pyrantel på känsliga Parascaris-populationer med hjälp av migration trap assay-teknik.

Detta arbete är ett viktigt steg mot att utveckla tillförlitliga *in vitro*-metoder för att screena resistens på gårdsnivå. Genom att möjliggöra tester på larvstadiet kan sådana metoder bidra till evidensbaserat val av läkemedel, förbättrad behandlingseffektivitet och en mer hållbar parasitkontroll inom hästsektorn.

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Part 2: Main report (max. 10 pages)

Introduction

Background and objective.

The alarming development of triple resistant *Parascaris* spp. is a threat to foal health and the equine industry in Sweden. It is therefore crucial to gain more knowledge about drug resistance in parasitic worms and to find molecular markers for resistance to ensure long-term sustainable parasitic control routines since no new anthelmintic drugs have been made available for use in horses since the 1980s.

Tasks to achieve within the research project:

1. Assess the efficacy of benzimidazoles (fenbendazole - Axilur® Intervet) and monitor the emergence of anthelmintic resistance on breeding farms in Sweden. Benzimidazoles is the last effective drug class for treatment of *Parascaris* spp. infections on the majority of Swedish farms. **(Study I)**
2. Evaluate molecular biomarkers associated with benzimidazoles drug resistance in order to develop a screening tool to be applied in sustainable control programs for monitoring of resistance. **(Study II-III)**
3. Develop an in vitro system for Parascaris spp. to a) study mechanisms leading to drug resistance, b) screen resistant parasite populations. Currently the lack of an *in vitro* system is a major constraint to understanding the emergence of resistant worms and consequently to design an effective monitoring program. **(Study IV-V)**

Material and methods

1. Study I: Assess the efficacy of benzimidazoles

1.1 Study Design

Since karyotyping was not conducted, the study refers to *Parascaris* spp. Stud farms with at least four foals under one year old, shedding ≥50 *Parascaris* spp. eggs per gram of feces (EPG), were eligible. Fecal samples were collected pre-treatment and 9-16 days post-treatment with fenbendazole (Axilur® vet. 19%, MSD Animal Health, Sweden) oral paste at 7.5 mg/kg. Foal weights were estimated with a weight tape (Boehringer Ingelheim AB, Sweden) and rounded up to avoid underdosing. All foals had written owner consent.

1.2 Fecal Analysis

Paired fecal egg counts (FECs) were conducted pre- and post-treatment using a modified McMaster technique (Coles et al., 1992) with a multiplication factor of 12.5.

1.3 Data Analysis

Egg count reductions were classified as resistant or susceptible per WAAVP guidelines and the online tool FECRT.com (Kaplan et al., 2023). Resistance was indicated if the upper 90% credible interval fell below the target 99.9% efficacy of fenbendazole. Susceptibility was indicated by a significant p-value from the Beta Negative Binomial (BNB) method. For descriptive purposes, efficacy was also calculated using a Bayesian hierarchical model on shiny-eggCounts.

1.4 Questionnaire Data

Participating farms were interviewed on factors like, numbers of foals, treatment routines, and pasture management. Using a Generalized Linear Model (GLM), associations were examined between farm resistance status, farm practices, and treatment frequency in the first year. A GLM also examined the relationship between the number of foals and treatments given, with a quasibinomial error distribution used to address overdispersion. Analyses were performed in R v4.3.2.

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2. Study II-III: Evaluate molecular biomarkers associated with benzimidazoles drug resistance

2.1 Parasite material (study II and III)

The study included 63 foals across two farms. Foals with ≥100 EPG were treated with fenbendazole at 7.5 mg/kg. Reduction in egg counts was calculated using WAAVP guidelines. Farm 1, having foals with resistant phenotype have used fenbendazole for 15 years. Farm 2, with 100% documented fenbendazole efficacy, served as a control, susceptible phenotype. Parascaris eggs from the Farm 1 (resistant) were collected post treatment and eggs from farm 2 (susceptible) were collected prior treatment. Eggs from each farm were pooled prior DNA extraction. Primers were designed to target specific SNPs (study II) and primers for complete β-tubulin genes (study III).

2.2 β-Tubulin SNPs Examination (study II)

Using RNAseq data and BLAST searches, β-tubulin genes in *P. univalens* were identified. Fort study II: Amplicons were generated, sequenced, and analyzed to detect mutations related to fenbendazole resistance, position 167, 198, 200.

2.3 Phylogenetic Analysis (study II)

Orthologous β-tubulin sequences from related nematode species were retrieved and aligned. Phylogenetic relationships were examined using maximum likelihood methods, visualized with PhyML, and plotted using R's ggtree package.

2.4 Sequencing of complete B-tubulin genes (study III)

Primers were designed to cover the entire coding regions, introns, and UTRs of seven *P. univalens* β-tubulin genes, with sequences obtained from WormBase Parasite. Missing sequence for gene Pun-tb-E was completed using in-house transcriptome data. Each primer included unique barcodes for gene and population identification, yielding amplicons of 6.5 to 11 kb. PCR specificity was confirmed with Platinum SuperFi PCR Master Mix, visualized by agarose gel.

2.5 Amplicon Generation and Sequencing of complete B-tubulin genes (study III)

Amplicons were created in triplicate PCR reactions, pooled based on concentration and length, and sequenced using PacBio Sequel at Uppsala Genome Center. HiFi reads were processed using pbaa for clustering and minimap2 for sequence orientation. Aligned coding sequences were compared between resistant and susceptible populations for key resistance-associated amino acids. Results and raw data are available in the ENA database, with analysis code on GitHub.

3. Study IV-V: Develop an in vitro system for *Parascaris* spp

3.1 Hatching (study IV)

Parascaris eggs do not hatch outside the host, which makes it difficult to develop *in vitro* models. Therefore, a protocol for hatching *P. univalens* eggs was developed for use of larvae *in vitro* experiments. Eggs were isolated from faces and decorticated by 2 % sodium hypochlorite in 16.5 % sodium chloride followed by additional washing steps. Decorticated eggs were incubated at 28 °C for 21 days, allowing larvae to develop inside the egg. The eggs were hatched by six slow strokes in a Kimble Kontes 15 ml glass homogeniser, using a pestle leaving 0.16 mm clearance.

3.2 The Invenesis Migration Trap Assay (MTA) (study V)

The migration trap assay (MTA) measured the effect of compounds on parasite larvae to detect resistance in an early stage. Hatch larvae are exposed to the treatment in each well of a multi-well plate. After 24 hours of exposure to pyrantel, the worms are transferred into a migration plate causing a 4-fold dilution of the drug. The migration of the worms towards a destination

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trap is measured over time and efficacy is expressed as a % reduction of migration compared to negative controls.

3.3 Dose response curves

Dose response curve for pyrantel av been established on *Parascaris* larvae with a susceptible phenotype using the MTA set up. Concentration of pyrantel 0.01, 0.1, 1.0, 10, 100 uM using the migration trap assay.

3.4 Parasite material

Farms with a naturally parasite infection were included. Paired fecal egg counts (FECs) were conducted pre- and post-treatment using a modified McMaster technique with a multiplication factor of 12.5. Infected foals were treated with pyrantel embonate paste (Banminth® Pharmaxim) at a dosage of 19 mg/kg bodyweight (6.6 mg of pyrantel base). All foals had written owner consent.

Results and discussion

1. Study I: Assess the efficacy of benzimidazoles

1.1 Fecal Egg Count Reduction Test (FECRT) Results

After screening fecal samples from 21 farms, 11 stud farms with 92 foals under 12 months old were included in the fecal egg count reduction test (FECRT). Four farms (1, 7, 10, and 11) were classified as resistant per WAAVP guidelines, with efficacy values of 83%, 45%, 84%, and 96% using a Bayesian hierarchical model. Post-treatment egg shedding was observed in 67%, 83%, 62%, and 50% of foals on these farms, respectively. The remaining seven farms showed 100% efficacy ($p < 0.001$) and were classified as susceptible, with no post-treatment egg shedding. See Table 1.

Table 1. Included stud farms, sorted by the year FECRT was performed, number of participating foals, total number of foals on the farm, age of participating foals, number of FBZ treatments the first year, mean (range) EPG pre- and post-treatment, total eggs counted, proportion of horses excreting eggs post treatment, efficacy according to Bayesian hierarchical model and classified as either susceptible (S) or resistant (R) (90-99.9) according to the clinical protocol of the WAAVP guidelines (Kaplan et al., 2023).

Farm no	Year tested	Participating foals (total foals at the farm)	Age of included foals (months)	Number of FBZ treatments first year	Mean (range) EPG pre treatment	Mean (range) EPG post treatment	Total no of eggs counted	% horses excreting eggs post treatment	Efficacy % (UCL-LCL)[4]	Classification incl UCL and LCL for R[5] and p for S[6] at two weeks post-treatment
1	2021	9 (40)	< 12[1]	6	611 (350-1350)	106 (0-350)	110[2]	67%	83% (81.5-83.9)	R 66.3% - 94.2%
2	2021	6 (10)	6-8	2-3	457 (50-1513)	0 (0-0)	219	-	100% (99.9-100)	S p< 0.001
3	2021	6 (20)	7-8	2-3	267 (125-588)	0 (0-0)	128	-	100% (99.9-100)	S p< 0.001
4	2021	5 (9)	6-9	2	553 (63-1250)	0 (0-0)	221	-	100% (99.9-100)	S p< 0.001
5	2022	4 (10)	6-9	2	204 (63-388)	0 (0-0)	65	-	100% (99.9-100)	S p< 0.001
6	2022	6 (16)	4-5	2	271 (75-975)	0 (0-0)	130	-	100% (99.9-100)	S p< 0.001
7	2022	23 (60)	4-7	2-3	1164 (100-6150)	642 (0-4038)	536[3]	83%	45% (43.7-45.9)	R -3.9% - 79.7%
8	2023	5 (10)	7-9	2	435 (125-663)	0 (0-0)	174	-	100% (99.8-100)	S p< 0.001
9	2023	5 (20)	7-10	2	295 (138-550)	0 (0-0)	118	-	100% (99.8-100)	S p < 0.001
10	2023	13 (50)	5-8	4-5	320 (138-813)	51 (0-225)	333	62%	84% (82.5-85.3)	R 72.5% - 92.8%
11	2023	10 (80)	6-8	2-3	1993 (275-4900)	75 (0-538)	1594	50%	96% (95.9-96.5)	R 89.9% - 99.6%

1.2 Questionnaire Results

Detailed responses, including anthelmintic routines and pasture management, are summarized in Table 2. Farms with >40 foals were significantly more likely to have resistant *Parascaris* populations (GLM, $t = 70.39$, $p < 0.001$). Fenbendazole was the primary drug used, with two farms rotating between fenbendazole and pyrantel. Treatment frequency ranged from 2 to 6 treatments during the foals' first year. Larger farms conducted more frequent treatments (GLM, $t = 2.76$, $p < 0.05$). Four farms evaluated treatment efficacy, while others did not. Resistance signs, such as *Parascaris*-induced impaction, were reported on two resistant farms, with reduced efficacy confirmed by FECRT on another. Most farms reused pastures annually and did not remove feces. Infection pressure was managed through co-grazing, rotational grazing, or pasture reseeding (6 farms). New arrival management varied;

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however, none applied quarantine protocols with post-treatment efficacy testing to prevent resistant parasites. Six farms expressed concerns about resistance, emphasizing reduced treatment frequency, improved pasture management, and better education on parasite control, including appropriate timing of fecal testing and treatment recommendations.

Table 2 Questionnaire data collected from participating farms.

Question	Response alternative	Response
Q3. Anthelmintic routine for *Parascaris* sp.	i) routinely at week 8-10 and 16-18 ii) routinely at week 8-10, 16-18 and after weaning if detected at faecal diagnostics iii) routinely at week 8-10 and 16-18 and after weaning iv) treatment only if detected at faecal diagnostics v) own treatment plan described in free text.	3 farms 1 farm 2 farms 1 farm 4 farms
Q4. Estimated number of treatments with FBZ during the foals first year.	i) 2 ii) 2-3 iii) 4-5 iv) 6	5 farms 4 farms 1 farm 1 farm
Q5[1]. Anthelmintic substances used to treat *Parascaris* sp.	i) FBZ ii) PYR + FBZ	9 farms 2 farms
Q6. Have seen signs of reduced efficacy of FBZ.	i) Yes ii) No	3 farms 8 farms
Q7. Regular control of FBZ efficacy. If yes, how often?	i) Yes, free text ii) No	4 farms 7 farms
Q8. Use of same pastures for foals every year.	i) Yes ii) No	10 farms 1 farm
Q9 Faecal removal from pastures.	i) Yes ii) No	0 farm 11 farms
Q10[1,2] Other pasture management routines.	i) harrowing ii) bi-annual rotation or co-grazing with ruminants iii) ploughing/reseeding, time frame in free text iv) other - free text	0 farm 3 farms 6 farms 1 farm
Q11 Management of new arrivals (foals).	i) kept in separate fields ii) faecal sampling iii) treatment with anthelmintic drug iv) drug treatment and control of efficacy v) no special routines vi) other	5 farms 0 farm 3 farms 0 farm 1 farm 2 farms
Q12[2] Are you worried about anthelmintic resistance in parasites? If yes, have this affected your routines?	i) Yes, free text ii) No	6 farms 4 farms
Q13[2] Is more information needed about anthelmintic resistance?	i) Yes ii) No	10 farms 0 farm

1.3 Discussion

This study confirms the presence of fenbendazole resistance in *Parascaris spp.* on Swedish breeding farms, with four of the 11 farms classified as resistant according to WAAVP guidelines. Efficacies ranged from 45% to 96%, with over half the foals on resistant farms shedding eggs post-treatment. Seven farms remained susceptible, achieving 100% efficacy. Larger farms (>40 foals) were significantly associated with resistance development, aligning with findings from other studies linking higher stocking densities to increased resistance risk. Similar cases of fenbendazole resistance in *Parascaris* spp. have been reported globally, alongside resistance in related ascarids, suggesting an emerging trend. On farm 7, resistance

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worsened over time, with efficacy dropping from 100% in 2010 to 45% in 2022. Frequent treatments for foals shedding eggs accelerated selection for resistance, illustrating how resistance continues to increase once established, consistent with other nematodes. The study adhered to WAAVP guidelines but included some foals with lower fecal egg counts to meet farm-level inclusion criteria. The absence of untreated control groups, due to ethical concerns, may have influenced results. Recent Swedish guidelines recommend routine treatments at specific ages, but adherence among farms was inconsistent, with treatment frequencies varying widely. Larger farms tended to administer more treatments, but no statistical link was found between treatment frequency and resistance. However, frequent treatments likely increase selection pressure for resistance. The findings highlight the urgent need for improved management practices, including selective treatment strategies, enhanced pasture management, and regular efficacy testing. Fenbendazole remains a critical drug for *Parascaris* spp., but its declining efficacy underscores the need to explore alternative control methods. Multi-drug-resistant *Parascaris* spp. populations pose significant risks, emphasizing the importance of proactive interventions to mitigate resistance development. Results from study I is published.

2. Study II-III: Evaluate molecular biomarkers associated with benzimidazoles drug resistance

2.1 Parasite material for fenbendazole drug efficacy (study II and III)

On farm 1, resistant farm, showed post-treatment of egg shedding of 38% (group A), 74% (group B), and 36% (group C) of foals. Additionally, 6% (group A) and 23% (group B) shed more eggs post-treatment than pre-treatment. On farm 2, susceptible farm, (group D) showed 100% efficacy (Figure 1).

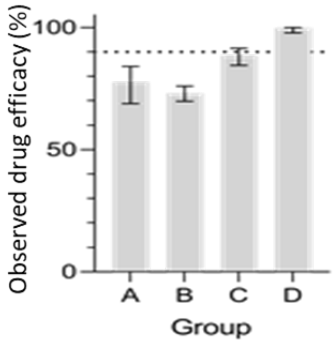

Figure 1.
Efficacy below the dotted line 90% indicate treatment failure due to resistance. All foal groups (A-C) from one stud farm shows evolving resistance. Almost 100% efficacy was noted for the control group (D)

2.3 Phylogenetic Analysis (study II)

Seven β-tubulin genes were identified in *P. univalens*, with two corresponding to previously known isotypes (Pun-bt-B and Pun-bt-G) and five as novel isotypes. Phylogenetic analysis grouped these genes into four major clusters alongside β-tubulin genes from other nematodes in clades III and V. Two genes, Pun-bt-F and Pun-bt-E, clustered with homologs in *Haemonchus contortus* and *Caenorhabditis elegans*. The remaining three genes (Pun-bt-A, Pun-bt-C, and Pun-bt-D) clustered with unnamed clade III β-tubulin genes, including ascarids.

2.3 β-Tubulin Gene Examination (study II)

PacBio sequencing provided 1,741–20,611 consensus reads per amplicon. Amplicon sequencing of the seven β-tubulin genes from farms (resistant) and farm 2 (susceptible) detected no resistance-associated SNPs at positions 167, 198, or 200.

2.4 Amplicon Generation and Sequencing of complete B-tubulin genes (study III)

Amplicon sequencing of the complete β-tubulin genes revealed a single mutation in the isolate from the resistant Farm (1-R) in Pun-bt-B within cluster 1. This mutation was at position 167 with amino acid S (serine) instead of F (Phenylalanine). No other mutations were identified, either at previously known BZ-resistance-associated sites such as E198, F200, or at the in silico

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docking sites Q134, N165, V236, L253, N256 or A315. As confirmed by pervious sequencing, Isotype Pun-bt-D contain Y (Tyrosine) at position 200 in all clusters on both Farm 1-R and Farm 2-S.

3.2 Expression levels of B-tubulin genes in the anterior end and intestine of adult *Parascaris univalens*

Tissue-specific RNA-seq analysis of the anterior end and intestine of adult *P. univalens* revealed distinct β-tubulin isotype expression patterns (Figure 2). Pun-bt-A and Pun-bt-B were the most abundant in both tissues, while Pun-bt-G had the lowest expression. In the anterior end, Pun-bt-C, D, E, and F showed relatively uniform expression, with Pun-bt-F modestly higher but still markedly lower than Pun-bt-A and B. In contrast, the intestine exhibited a more varied profile: Pun-bt-C ranked third in abundance, followed by Pun-bt-D, while Pun-bt-E and F were minimally expressed. Pun-bt-C also showed the highest variability between tissue types, whereas other isotypes displayed consistent expression across individual worms.

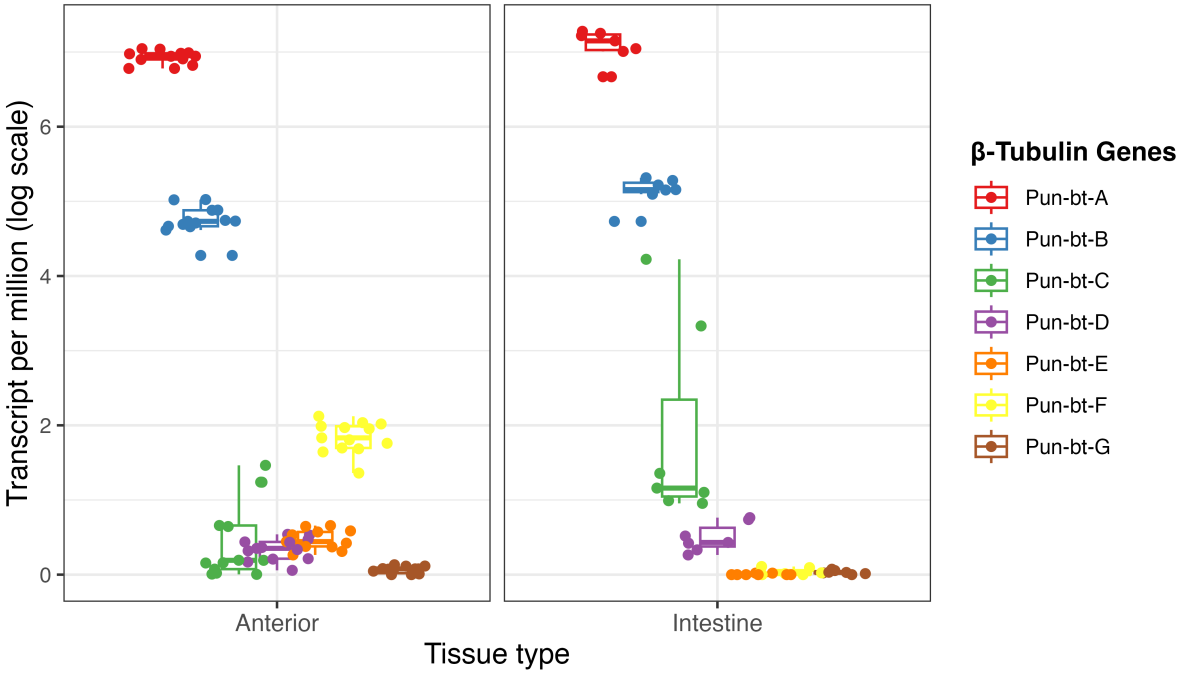

Figure 2 Tissue-specific RNA-seq analysis of the anterior end and intestine of adult P. univalens revealed distinct β-tubulin isotype expression patterns

Discussion

The first amplicon sequencing only covering the known SNPs revealed no resistance-associated SNPs at positions 167, 198, or 200 in any β-tubulin genes, aligning with previous studies on *Ascaris lumbricoides*. However, the extended sequencing covering the entire β-tubulin genes revealed one cluster with SNP at the position 167 in isotype Pun-bt-B from the resistant Farm (1-R). This SNP might explain part of the resistant phenotype of fenbendazole. Moreover, this study identified seven β-tubulin genes in *P. univalens*, including two previously described isotypes (Pun-bt-B and Pun-bt-G) and five novel genes. Phylogenetic analysis showed that Pun-bt-B and Pun-bt-G, Pun-bt-A, and Pun-bt-C cluster with ascarid β-tubulins and are potential targets for BZ interaction. However, Pun-bt-F, Pun-bt-E, and Pun-bt-D, based on their expression and sequence, are unlikely to be involved in BZ binding or resistance. We will need to sequence several resistant farms in Sweden as well to include farms from other countries. We also aim to analyze regulatory elements in the intron of β-tubulin genes to elucidate any marker for resistance to fenbendazole. To summarize, further research is needed to identify novel resistance mutations and develop molecular markers for early detection. Regular efficacy monitoring and enhanced biosecurity on stud farms are essential to mitigate resistance spread

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and maintain the efficacy of available anthelmintics. Results from study II is published and results from study III is soon submitted.

3. Study IV-V: Develop an in vitro system for Parascaris spp

3.1 Hatching (study IV)

The hatching ratio of this method was 92% and resulted in larvae that are viable for several weeks in tissue culture media at 37 °C, 5 % CO2. This technique was used to study drug response in larvae after drug exposure.

Migration Trap Assay (study V)

The MTA developed by invenesis and INRAE was used to establish a dose response curve for pyrantel on a susceptible farm from France. The dose response curve revealed an EC50 of 1.1 uM Pyrantel (Figure 3)

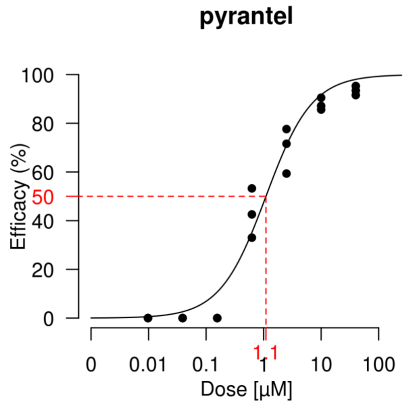

Figure 3.
Shows the EC of pyrantel indicated by the red line. EC50 corresponds to the dose which kills 50% of the larval population

FECRT identified two farms resistant to pyrantel and one farm susceptible to pyrantel. Larvae with either a resistant phenotype or susceptible phenotype were isolated and hatched as described above. After establishment of EC50 on a susceptible farm (A) the MTA was further tested on two resistant farms (B and C). However, the EC50 value was not different between the susceptible and resistant farms (Figure 4).

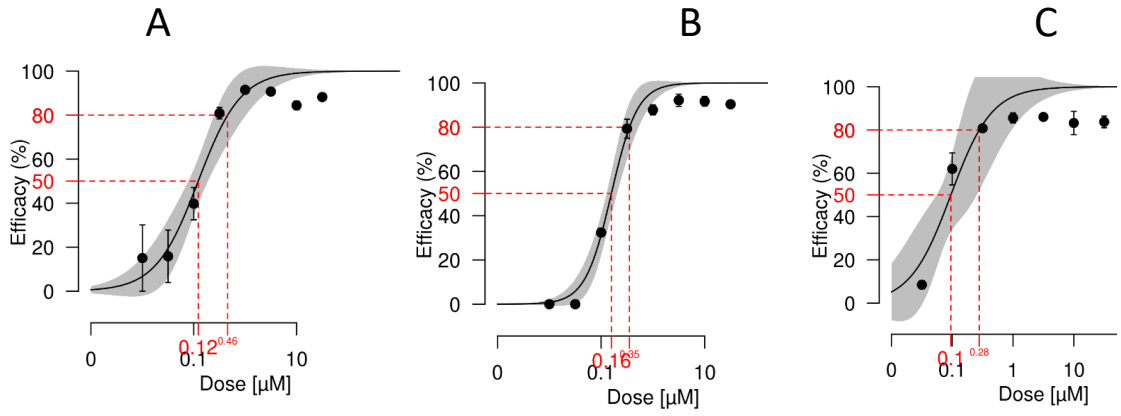

Figure 4. Shows the EC50 value in a susceptible farm A and in two resistant farms B and C.

Discussion

Parascaris spp. typically do not hatch outside the host, presenting a significant challenge for conducting *in vitro* studies using larval populations. In Study IV, we successfully developed a high-throughput hatching method to overcome this limitation. Using this method, we

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established an *in vitro* assay to evaluate drug efficacy in study V. However, the migration-trap-assay (MTA) was unable to reliably differentiate between larvae from susceptible and resistant *Parascaris* populations. Interestingly, this assay demonstrated a 10-fold difference in EC50 when applied to larvae from a resistant population of a related intestinal parasite in sheep. These findings highlight the need for further validation and optimization of the MTA for use with *Parascaris* larvae to ensure its reliability and applicability in tracking anthelmintic resistance. The hatching assay from study IV is published.

Conclusions

1. Study I: Assess the efficacy of benzimidazoles

This study confirms the presence of fenbendazole resistance in *Parascaris* spp. on Swedish breeding farms, with four of the 11 farms classified as resistant according to WAAVP guidelines. Efficacies ranged from 45% to 96%, with over half the foals on resistant farms shedding eggs post-treatment. The findings highlight the urgent need for improved management practices, including selective treatment strategies, enhanced pasture management, and regular efficacy testing. Fenbendazole remains a critical drug for *Parascaris* spp., but its declining efficacy underscores the need to explore alternative control methods. Multi-drug-resistant *Parascaris* spp. populations pose significant risks, emphasizing the importance of proactive interventions to mitigate resistance development.

2. Study II-III: Evaluate molecular biomarkers associated with benzimidazoles drug resistance

The extended sequencing covering the entire β-tubulin genes revealed one cluster with SNP at the position 167 in isotype Pun-bt-B from the resistant Farm (1-R). This mutation needs further validation on other farms and countries before being stated as a “true” marker. In addition, this study provides the first comprehensive characterization of the β-tubulin gene family in *P. univalens*. Further research is needed to identify novel resistance mutations and develop molecular markers for early detection. Regular efficacy monitoring and enhanced biosecurity on stud farms are essential to mitigate resistance spread and maintain the efficacy of available anthelmintics.

3. Study IV-V: Develop an *in vitro* system for *Parascaris* spp

In Study IV, we successfully developed a high-throughput hatching method to overcome this limitation. Using this method, we established an in vitro assay to evaluate drug efficacy in study V. However, the migration-trap-assay (MTA) was unable to reliably differentiate between larvae from susceptible and resistant *Parascaris* populations. In this set-up were larvae exposed for 24 hours and the paralytic effected caused by the drug could be reversed. This will be repeated with 4-hour exposure time.

Relevance for the practical horse sector incl. recommendations

The findings from Study I, which evaluated the efficacy of fenbendazole on Swedish stud farms, provide critical evidence of the rapid spread of anthelmintic resistance in Sweden. This is particularly significant for foals, which are frequently treated without prior parasitological diagnostics, emphasizing the need to monitor drug efficacy in this vulnerable group. The outcomes of this study have directly informed updated recommendations for parasite control in horses, as outlined in *Hästens mag-tarmparasiter: att förebygga och behandla*.

Identifying molecular markers for early detection of resistance-related mutations is a key priority. However, findings from Studies II and III demonstrate that resistance mechanisms

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differ between parasite species. Notably, *Parascaris* spp., an ascarid worm, appears to possess a distinct mechanism of resistance to benzimidazole drugs, likely linked to its unique repertoire of β-tubulin genes, unlike other intestinal parasites.

Historically, *Parascaris* research has been hindered by the lack of *in vitro* techniques, as these worms do not typically hatch outside the host. In Study IV, we developed a novel hatching technique, providing a foundation for in vitro methods to track drug resistance in *Parascaris*. In collaboration with INRAE and Invensis, we have established a dose-response curve for pyrantel in susceptible *Parascaris* populations with migration trap assay technique. This work is a significant step towards developing reliable in vitro techniques to screen for resistance at the farm level. By enabling larval-stage testing, such methods could guide evidence-based drug selection, improving treatment efficacy and contributing to sustainable parasite management in the horse sector.

References

This project is continuation of the H1147027- Benzimidazolresistens hos hästens spolmask. Other references included.

Published work

Study I Martin F, Halvarsson P, Alm YH, Tydén E. Occurrence of fenbendazole resistance in Parascaris spp. on breeding farms in Sweden. Vet Parasitol. 2024 Oct;331:110272. doi: 10.1016/j.vetpar.2024.110272. Epub 2024 Jul 22. PMID: 39106597.

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Part 3: Result dissemination

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Scientific publications, *submitted*	Tydén E, Martin F, Dube F, 2024, Identification of mutation in Beta tubulin isotype B in Parascaris univalens from a farm with resistance to fenbendazole Int J Parasitol Drugs Drug Resist
Scientific publications, *manuscript*	Tyden E, Migration assay for monitoring drug resistant isolates of Parascaris univalens in vitro
Conference publications/ presentations	Eva Tydén and Frida Martin. Ascarid Research and Training Initiative (ARTI) Exploring the β-tubulin gene family in a benzimidazole-resistant Parascaris univalens population
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	E. Tyden November 2021 Nordic Equine Veterinary Conference. Expert Panelist for anthelmintic treatment and guidelines.
Other publications, *media etc.*	”Spolmask alltmer resistent mot avmaskningsmedel” 2021-10-22 https://www.tidningenridsport.se/spolmask-alltmer-resistent-mot-avmaskningsmedel/
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	”Allvarligt läge – spolmaskarna allt mer resistenta” 2021-11-09 https://www.atl.nu/spolmaskarna-allt-mer-resistenta-mot-avmaskningspreparat
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	TV4 hästmorgon tema parasiter. Maj 2024
	Tema föl: betessläpp och avmaskning. Travhästen 2024.
Oral communication, *to horse sector, students etc.*	Hippocampus seminar SVA. Researchers and clinical veterinarians May 2021 ”Läkemedelsresistens hos hästens spolmask, Parascaris univalens, i Sverige”
	Seminar for members of Stuteriveterinärföreningen November 2021. “Multiresistenta spolmaskar - vad gör vi nu?”
	Knivsta ortens ryttarförening, årsmöte KORK 2023. Förebygga parasitsjukdom hos häst och problem med resistens. Hästägare
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