Evaluation of Macrocyclic Lactone Resistance in Gastrointestinal Nematodes of First Season Grazing Cattle

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Cover: Bursa (posterior end) of male *Cooperia oncophora*
(photo: M. Areskog)

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Abstract

Parasitic gastrointestinal nematodes (GIN) are common world-wide among grazing cattle, and cause welfare problems and associated economic losses due to reduced performance of their hosts. In Sweden, the most important GIN include *Cooperia oncophora* and the more pathogenic *Ostertagia ostertagi*, which are usually present as mixed infections in first season grazing cattle (FSG). Strategic treatments with anthelmintic drugs remain the principal means of control of helminth infections in grazing livestock in conventional cattle farming. A new challenge for European livestock farmers is the increasing evidence of emerging anthelmintic resistance (AR), which today seems to be an emerging global problem among GIN in cattle. The reasons for AR development, and the mechanisms behind it have not been fully investigated. The primary aim of this thesis was therefore to investigate the effect of one of the most commonly used anthelmintic groups (macrocyclic lactones, ML) against GIN in Swedish cattle, including both dairy and beef herds. An additional aim was to seek an explanation for AR by further characterising worm isolates *in vitro* and comparing changes in gene expression before and after treatment.

Two field trials carried out in consecutive years, one in Northern Europe and one in Sweden, both showed reduced efficacy of ML in FSG, with *C. oncophora* being the predominant surviving species post anthelmintic treatment. To further investigate this, a controlled pen trial was performed using two isolates collected from the field trials. The results were ambiguous when animals were successfully dewormed according to the standard, but nevertheless several adult worms of *C. oncophora* survived the treatment. To seek explanations for the varying results, another controlled pen trial was performed in which the pharmacokinetic behaviour of the anthelmintic drug was studied in interaction with concomitant use of dexamethasone (DXM) in FSG. The results showed a significant difference in plasma levels of the anthelmintic drug in combination with DXM.

Finally, gene expression regarding P-glycoproteins (PGP), which are transmembrane efflux transporters, was investigated in the same worm isolates before and after ML treatment. Surviving male worms showed a tendency for increased gene expression of putative *Con-pgp-9*, but amplified fragment length polymorphism (AFLP) testing showed no sign of changes in gene diversity among the surviving worms. The differing results of these studies illustrate the complexity of the AR problem, which poses a great challenge for future research in this area.

Keywords: Anthelmintic resistance, ivermectin, *Cooperia oncophora, Ostertagia ostertagi*

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Svensk sammanfattning

Löpmagsmasken *Ostertagia ostertagi* och tunntarmsmasken *Cooperia oncophora* är parasitära nematoder som orsakar skador i mag-tarmkanalen framför allt hos unga nötkreatur och förekommer över hela världen. Skadorna leder till nedsatt tillväxt hos värddjuret och därigenom ekonomisk förlust för djurägaren. Vid kraftigare infektioner kan de även orsaka klinisk sjukdom och utgöra ett djurskyddsproblem. Förebyggande åtgärder tillämpas i viss utsträckning i det svenska lantbruket men avmaskning med anthelmintika utgör alltjämt hörnstenen i en effektiv parasitkontroll bland förstagångsbetande nötkreatur på konventionella gårdar. Under det senaste decenniet har resistens mot avmaskningsmedel bland nötkreaturens parasiter påvisats i en allt högre grad världen över, i synnerhet på södra halvklotet där läkemedlen traditionellt använts i hög utsträckning. Undersökningar från USA och Europa, främst Storbritannien och Belgien, har pekat på att problemet finns även på norra halvklotet och bör påverka hur vi tänker kring antiparasitär behandling av boskap. Denna avhandling baseras på fem publikationer och syftar till att utreda resistensläget bland svenska nötkreatur samt undersöka möjliga mekanismer bakom anthelmintikaresistens.

Ett tvåårigt fältförsök genomfördes genom ett internationellt samarbete, där Tyskland, Sverige och Belgien deltog under betessäsongerna år 2006 och 2007. Fem svenska gårdar valdes ut och 10-15 förstagångsbetare avmaskades efter inledande träckprovtagning och följdes sedan upp med nya prover efter 7 och 21 dagar. Träckproverna undersöktes med avseende på förekomst av maskägg och art av mask. Resultaten av våra mätningar bland svenska besättningar pekade mot förekomst av anthelmintikaresistens, då vi såg en otillfredsställande effekt av avmaskningsmedlet ivermektin. Främst överlevde maskar av arten *C. oncophora* men också *O. ostertagi*.

Försöket följdes upp med en fältstudie inkluderande svenska nötbesättningar av både mjölk- och köttras under somrarna 2009 och 2010. Initialt mättes nivåerna av parasitsmitta bland 107 respektive 64 besättningar via inskickade träckprover och ca 40 % av de anmälda betesgrupperna hade ett sådant parasittryck att de inkluderades i ett avmaskningsförsök. Mjölkrasbesättningarna och köttrasbesättningarna hade lika stor parasitbörda. I försöket fick djurägarna själva samla prover och sedan behandla djuren efter tydliga instruktioner och enligt gällande doseringsrekommendationer. Avmaskningsmedlen som förskrevs efter träckprovtagning och analys var s.k. pour-on preparat av typen makrocykliska laktoner. En till två veckor efter behandling provtogs djuren igen och nivåer av kvarvarande parasitsmitta mättes. Resultaten visade än en gång att avmaskningsmedlen gav en otillfredsställande effekt, då färre än 40 % av besättningarna uppnådde en acceptabel reduktion av maskägg i träcken. Molekylärbiologiska analyser av framkläckta larver visade vilken art som överlevt

behandlingen, och även denna gång dominerade *C. oncophora*. Löpmagsmasken, *O. ostertagi*, kunde dock påvisas efter behandling i 15 % av besättningarna.

Effekten av avmaskningsmedlet ivermektin utreddes vidare genom ett kontrollerat försök på universitetets forskningsanläggning, där kalvar infekterades med larver av både löpmagsmask och tunntarmsmask från två av de svenska gårdarna som deltog i fältförsöket 2006 och 2007. Maskägg samlades genom träckprovtagning under tre veckors tid för att senare analyseras med hjälp av laborativa och molekylärdiagnostiska metoder, innan djuren avmaskades. Denna gång visade sig avmaskningsmedlet ge acceptabel effekt enligt globala riktlinjer för detektion av anthelmintikaresistens. Fortfarande kunde dock en liten mängd överlevande maskar av båda arterna påvisas och åter igen var tunntarmsmasken, *C. oncophora*, mest motståndskraftig.

Resultaten från fältförsöken skiljde sig alltså från de kontrollerade försöken med experimentellt infekterade djur. Detta pekar mot att flera faktorer än behandling och dosering skulle kunna påverka resultatet av läkemedlet. För att undersöka eventuella sådana faktorer, utfördes ytterligare ett kontrollerat försök på forskningsanläggningen, där ivermektinets effekt studerades under interaktion med glukokortikoiden dexametason. Båda läkemedlen gavs samtidigt till en av djurgrupperna och blodprovsanalys från dessa kalvar visade att plasmanivåerna av ivermektin sänktes betydligt under påverkan av kortisonet, jämfört med kontrollgruppens. Detta påverkade även avmaskningsresultatet, där kontrollgruppen hade en signifikant högre reduktion av maskägg i träcken.

Mekanismerna bakom nematodernas utveckling av resistens mot ivermektin är ännu inte fullt kartlagda men problemet tycks vara multifaktoriellt. En gemensam nämnare mellan ivermektin och dexametason är P-glykoproteiner, s.k. PGP. Proteinet fungerar som en membranpump med syfte att transportera in eller ut ämnen ur celler i kroppen. Båda de givna läkemedlen påverkar eller påverkas av PGP. För att närmare undersöka proteinets roll i effekten av ivemektin, studerade vi vuxna maskar som vi samlat in före respektive efter avmaskning i ett av de kontrollerade försöken. Överlevande hanliga maskar av arten *C. oncophora* tenderade att uttrycka genen för en sorts PGP, *Con-pgp-9*, i högre utsträckning än innan de utsatts för ivermektin. Dock kunde vi med vårt underlag inte säkerställa resultaten statistiskt och genetiska studier med metoden AFLP kunde inte påvisa någon skillnad i genetisk mångfald före respektive efter avmaskning.

Resultaten av de olika studierna pekar åt olika håll och visar på hur komplex problematiken med resistens mot makrocykliska laktoner är. I samtliga studier överlevde ett varierande och mer eller mindre acceptabelt antal maskar behandlingen med avmaskningsmedel och *C. oncophora* var ständigt den dominanta arten. Vi kunde också se att den uteblivna behandlingseffekten i fält inte nödvändigtvis beror på genetisk resistens, då samma maskpopulation gav ett helt annat resultat i kontrollerade

försök på stall. Vi kunde inte heller säkerställa en genetisk skillnad hos de maskar som överlevt en avmaskning jämfört med maskar som undersökts innan behandlingen, men vi anade en tendens mot ökat genuttryck av *Con-pgp-9* hos överlevarna. Det finns fortfarande en uppsjö av ännu oprövade mekanismer bakom anthelmintikaresistens att utreda i framtida forskning.

Plötsligt såg jag hur hon
fick något akademiskt
i blicken.

Isabelle söder

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text. Papers I-V are reproduced with the kind permission of the publishers.

I Demeler, J., van Zeveren, A.M.J., Kleinschmidt, N., Vercruysse, J., Höglund, J., Koopmann, R., Cabaret, J., Claerebout, E., Areskog, M. & von Samson-Himmelstjerna G. (2009). Monitoring the efficacy of ivermectin and albendazole against gastro intestinal nematodes of cattle in Northern Europe. *Veterinary Parasitology* 160, 109-115.

II Areskog, M., Ljungström, B. & Höglund, J. (2013). Limited efficacy of pour-on anthelmintic treatment of cattle under Swedish field conditions. *International Journal of Parasitology: Drugs and Drug Resistance.* 3, 129-134.

III Areskog, M., Sollenberg, S., Engström, A., von Samson-Himmelstjerna, G. & Höglund, J. (2014). A controlled study on gastrointestinal nematodes from two Swedish cattle farms showing field evidence of ivermectin resistance. *Parasites & Vectors* 7, 13. doi: 10.1186/1756-3305-7-13.

IV Areskog, M., von Samson-Himmelstjerna, G., Alvinerie, M., Sutra, J.F. & Höglund, J. (2012). Dexamethasone treatment interferes with the pharmacokinetics of ivermectin in young cattle. *Veterinary Parasitology* 190, 482-8.

V Areskog, M., Engström, A., Tallkvist, J., von Samson-Himmelstjerna, G. & Höglund, J. (2013). PGP expression in *Cooperia oncophora* before and after ivermectin selection. *Parasitology Research*. 112, 3005-12.

Abbreviations

AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
AR	Anthelmintic resistance
ATP	Adenosine triphosphate
BCS	Body condition scoring
BZ	Benzimidazole
CET	Controlled efficacy test
CI	Confidence interval
DNA	Deoxyribonucleic acid
DOR	Doramectin
DXM	Dexamethasone
EPG	Eggs per gram faeces
FEC	Faecal egg count
FECRT	Faecal egg count reduction test
FSG	First-season grazing
GABA	Gamma-aminobutyric acid
GI	Gastrointestinal
GIN	Gastrointestinal nematodes
GLM	General linear model
GluCl	Glutamate-gated chloride (channel)
IVM	Ivermectin
KRAV	Kontrollförening för ekologisk produktion (Swedish certification body for organic production)
L3	Infective third stage trichostrongylid nematode larva/-e
LEV	Levamisole
LMIT	Larval migration inhibition test
ML	Macrocyclic lactones
PCR	Polymerase chain reaction

PGE	Parasitic gastroenteritis
PGP	Permeability glycoprotein (P-glycoprotein)
RNA	Ribonucleic acid
RT PCR	Reverse transcription polymerase chain reaction
SLB	Svensk Låglandsboskap (Swedish Holstein)
SNP	Single nucleotide polymorphism
SRB	Swedish Red and White breed
TST	Targeted selective treatment

1 Introduction

The parasitic gastrointestinal (GI) nematodes *Cooperia oncophora* and *Ostertagia ostertagi* can cause severe gastroenteritis in first season grazing (FSG) cattle and economic losses even if only subclinical levels of infection are reached. Repeated natural low-level infections eventually result in protective immunity, but to avoid escalating levels of pasture contamination and severe infections in naïve calves, anthelmintic drugs are frequently used in the cattle farming industry. World-wide emergence of anthelmintic resistance (AR) in parasitic nematodes of veterinary importance is a critical issue for both small ruminant and cattle farming, leading to impaired animal welfare and reduced profitability for farmers. The development of new antiparasitic drugs is often an expensive, uneconomical and slow process, and few new drug classes have entered the market since the breakthrough of the "wonderdrug" ivermectin (IVM) in the 1980s. Thus, restraint in the use of anthelmintics and early detection of developing resistance are increasingly important, and new methods for effective recognition of AR are under constant development. The overall aim of this thesis was to investigate AR against macrocyclic lactones (ML) in *C. oncophora* and *O. ostertagi* in Swedish FSG cattle, and to come one step closer to identifying the mechanisms behind AR to the ML derivative IVM.

This work presented in the thesis includes results from field trials where farmers contributed material from their farms, controlled infection trials with collected nematode populations, and laboratory *in vitro* trials investigating molecular changes in the genes expressed in the worms before and after anthelmintic treatment. Background information to these studies is provided below.

2 Background

2.1 Swedish Cattle Production

Swedish cattle farmers currently face major challenges due to a stiffening competition on the market and declining profitability, which is draining the sector and annually decreasing the number of cattle. The total number of cattle in Sweden in December 2012 was 1 443 584, an 11% decrease since the year 2000. The greatest decrease, 19%, occurred within the dairy sector, where the number of cows declined to 345 500, with on average 70 cows per herd, by December 2012 (SJV 2013). The predominant dairy breeds are Swedish Red and White (SRB) and Swedish Holstein (formerly SLB). Beef breeds are purebred or crossbreeds of Charolais, Hereford, Simmental, Highland Cattle, Limousin, Aberdeen Angus and Blond d'Aquitaine (SJV 2012). The grazing season usually runs from May to October, depending on geographical and geological conditions, and animals are generally housed during the winter season. All cattle older than 6 months, bulls excluded, must have outdoor access during the grazing season for at least 2-4 months, depending on region (Nilsson 1973; DFS 2007).

Cattle production, both dairy and beef, accounts for a significant proportion of the agricultural net value in Sweden and represents almost 20% of the livestock value. Cattle farming plays a significant role in the development of rural areas in general by creating an attractive open landscape, and is absolutely necessary to preserve biodiversity and cultural values in pasture. However, the profitability of cattle farming nowadays is poor due to low milk, meat and crop prices, inadequate compensation for landscape conservation efforts and high production costs at farm level. In cattle farming, two-thirds of the production value has disappeared since 1975 and the Swedish market now imports more than 50% of the meat consumed in the country (Anonymous 2012). The decline in prices and the depressed profitability place increasing

demands on Swedish farmers, who require good preventative animal health to achieve cost-effective production.

Another challenge to Swedish farmers is the growing market and demand for organic products, leading to more farmers converting their production systems to certified organic farming. The Swedish organic certification brand KRAV® is growing, and the number of organically certified cattle (both dairy and beef) increased from 150 000 animals in 2009 to nearly 250 000 in 2011 (KRAV® 2012).

2.2 Gastrointestinal Nematodes: *Cooperia oncophora and Ostertagia ostertagi*

Gastrointestinal nematodes in livestock are common world-wide, and in Sweden they were first studied by Hoflund and Koffman (1948). During the 1970s and 1980s, further investigations on the epidemiology of GIN in Swedish cattle were performed by Nilsson and Sorelius (1973), Olsson and Holtenius (1980) and Törnquist and Tolling (1983, 1987). Later assessments of animal productivity have repeatedly shown that losses in live weight gain can be considerable during the first grazing season in Sweden (Dimander *et al.* 2000, 2003; Larsson *et al.* 2007). It has also recently been demonstrated that GIN are widespread and that there is a negative interaction between exposure to GIN and individual daily milk yield in Swedish dairy herds, even when the overall exposure is relatively low (Charlier *et al.* 2009; Höglund *et al.* 2010; Blanco-Penedo *et al.* 2012).

In temperate regions of the world such as Sweden, the most important GIN include the small intestinal worm *C. oncophora* and the more pathogenic abomasal worm *O. ostertagi*, which are usually present as mixed infections in pasture-based cattle (Höglund 2010). Both species belong to the superfamily Trichostrongyloidea and are 5-9 mm long, hair-like worms. Males can be recognised by the presence of a disproportionately large bursa at the posterior end and paired spicules close to the bursa (Figure 1). The tail end of the females tapers to a point. The females of *C. oncophora* also have a square-ended anterior, containing refractile bodies.

Figure 1. Male *C. oncophora*: mouthparts at pointed anterior end and characteristic bursa and spiculae at posterior end.

Both *O. ostertagi* and *C. oncophora* have direct life cycles, where eggs are passed in faeces of the host to the pasture. Trichostrongylid eggs can be identified by microscopic examination due to the almost parallel walls of the egg. In the pasture environment, the egg develops into three different larval stages, L1, L2 and the infective L3 (Figure 2). The time of development, however, is dependent on temperature and weather conditions (Frankena 1987). The optimal temperature, based on the rate of development and percentage of eggs developing into infective larvae, is 25 ˚C. No development beyond the gastrula stage occurs below 6 ˚C. Above 32 ˚C, development is faster than at lower temperatures, but mortality rate of the preinfective stages is very high (Ciordia & Bizell 1963). L3 larvae are ingested by the host.

Ostertagia ostertagi burrows into the abomasal glands, where it moults twice, and then returns to the lumen, where it develops into the sexually mature stage. The larvae damage the parietal cells in the abomasal glands and cause subclinical or clinical parasitic gastroenteritis (PGE). The outcome is watery diarrhoea, anorexia, rough coat, weight loss and sensitivity to secondary infections (Anderson *et al.* 1965). The prepatent period from infection to sexual reproduction and the start of the egg shedding is approximately three weeks.

Unlike *O. ostertagi*, *C. oncophora* burrows into the wall of the small intestine and thus causes less harm to the enzymatic digestive function when it reproduces. However, in heavy infections it too causes reduced weight gain and diarrhoea, due to damage to the intestinal mucosa. Both species can undergo arrested L4 development, where the parasite goes dormant in a hyperbiotic stage for several months in the mucosa of the GI tract, to help the species survive cold winter months (Frankena 1987). Both species also overwinter on pasture if the weather conditions are favourable, *i.e*. with dry summers and subsequent poor disintegration of faecal pats (Dimander *et al.* 2000), and thus are able to infect new hosts in the next spring.

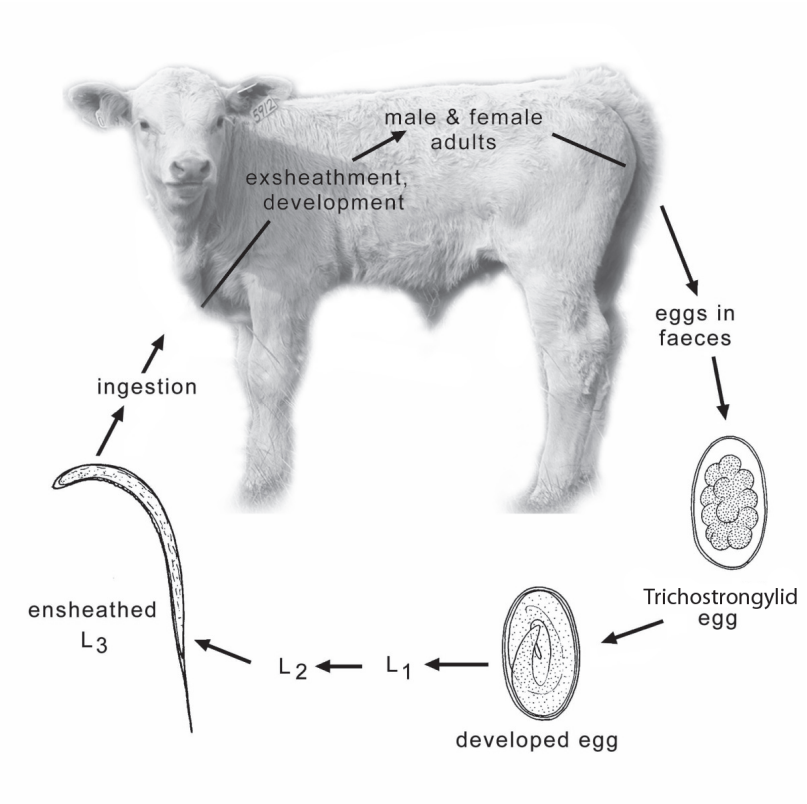

Figure 2. Life cycle of *Ostertagia ostertagi* and *Cooperia oncophora.*

First season grazing calves have not yet acquired immunity to the parasite and are the most susceptible hosts. Their inability to fight the initial parasite infection causes them to shed large amounts of nematode eggs onto the pasture, which becomes contaminated. Therefore, use of the same turn-out pastures for FSG calves year after year is not recommended (Törnquist & Tolling, 1987).

2.3 Gastrointestinal Nematode Prevention and Control

2.3.1 Anthelmintics

Attempts have been made to control GIN infections through various means, but the principal method of control, and the cornerstone in Swedish cattle farming, has been the repeated strategic use of anthelmintics or dewormers to prevent the disease in calves which have not yet developed natural immunity to the parasites (Dimander 2003). Anthelmintics are drugs containing substances active against nematode infections. The principal aim of anthelmintic treatment is to prevent clinical or sub-clinical disease, and hence eliminate some of the costs associated with PGE, whilst allowing the development of natural immunity (Urquhart *et al.* 1996; Molento 2009). Anthelmintics are available in various forms and can be given orally or through parenteral administrations (*e.g*. injectables and pour-on preparations).

Several classes of anthelmintics have been used for decades to treat worm infections. The two most common chemical families of broad-spectrum anthelmintics used in cattle are the benzimidazoles (BZ), and the ML, each group with a different mode of action (Taylor, Coop & Wall 2007). Another broad spectrum anthelmintic, levamisole (LEV), was released onto the market in 1968. That drug belongs to the imidazothiazole /tetrahydropyrimidine class of anthelmintics, which also includes pyrantel and the previously used morantel (McKellar & Jackson 2004), but LEV is now only available as a licensed product in Sweden. A new class of drugs, the aminoacetonitrile derivatives (AAD), was recently discovered by Kaminsky *et al.* (2008), but has so far only been launched onto the market for small ruminants in New Zealand and the UK.

Benzimidazoles

The BZ were introduced in the 1960s and are effective against a broad range of parasites. The mode of action of this drug class is disruption of the tubulinmicrotubule within the parasite, resulting in cell lysis and inhibiting motility and feeding (von Samson-Himmelstjerna *et al.* 2007).

Macrocyclic lactones

The broad spectrum anthelmintics released in the 1960s changed the management strategies for nematode control in livestock and companion animal veterinary practice. The introduction of ivermectin (IVM; Figure 3) in 1981 elevated worm control to new levels. Ivermectin is an ML that was first isolated from *Streptomyces avermitilis* in 1974. The unique endectoparasitic properties of IVM made it popular as a broad spectrum anthelmintic. The long

persistence and wide safety margins in the host animal were other advantages (McKellar & Jackson 2004; Alvinerie *et al.* 2008; Omura 2008).

Macrocyclic lactones act by binding to glutamate-gated chloride channels (GluCl) and GABA receptors, blocking neurotransmission by interfering with neuromuscular synapses (Blackhall *et al.* 1998a; Martin *et al.* 1998; Blackhall, Prichard & Beech 2003; Rana & Misra-Bhattacharya 2013). The GluCl are common in pharyngeal and somatic muscles of nematodes, insects and ticks, and the drug paralyses the nematode pharynx. However, ML lack activity against cestodes and trematodes (Taylor, Coop & Wall 2007). Ivermectin products have become available for several application routes, such as slow release devices (boluses), injectable formulations and topically applied (pour-on) formulae (Geary 2005). When fully effective, IVM has an efficacy of almost 100% against GIN. As a result, it has become the largest selling anti-parasitic drug in livestock and has essentially revolutionised the animal health industry (McKellar & Jackson 2004; Geary 2005; Van Zeveren 2009).

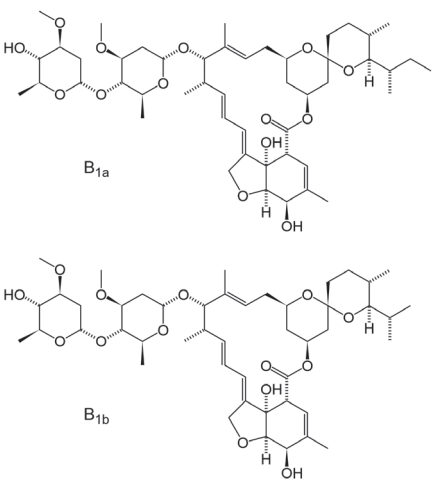

Figure 3. Skeletal formula of 22, 23-dihydroavermectin B_{1a}, and 22, 23-dihydroavermectin B_{1b}

The ML family has several members that derive from either *S. avermitilis* (avermectins) or *S. cyanogriseus* (milbemycins) (Takahashi *et al.* 2002; Taylor, Coop & Wall 2007) The two ML subclasses, the avermectins (*e.g.* IVM) and milbemycins (*e.g.* moxidectin), share the same central ML ring structure, only differing at the side chains, and both are lipophilic molecules (Lespine *et al.* 2007; Ardelli *et al.* 2009; Lifschitz *et al.* 2010).

According to Paper II, 76% of Swedish farmers who dewormed their cattle used topical avermectin formulations such as IVM, DOR and eprinomectin. An intraruminal intermittent release device with BZ (*e.g.* oxfendazol) was used by 16%, while only 3% used injectable compounds such as IVM. In 2010, 73% of Swedish dairy farmers dewormed their cattle. The corresponding figure for beef producers was 58%, but 31% of those only used anthelmintics at housing. Swedish farmers usually treat their cattle once to twice per year, mainly at turnout and 6-8 weeks after.

2.3.2 Alternative control strategies

A consequence of organic certifications is that the use of anthelmintics is restricted. Preventive suppressive treatments are not allowed. Deworming upon diagnosis of the presence of parasites and/or disease is accepted, although the withdrawal period after use of pharmaceuticals, including anthelmintics, is doubled. Nevertheless, avermectins, which today represent a large proportion of available pour-on anthelmintics on the Swedish market, should be avoided in organic farming and reserved for when other drugs are not expected to give the desired effect (KRAV® 2013). This places greater demands on farmers to be more attentive of parasitic infections than in conventional livestock farming, which may lead to parasite problems being disregarded.

The growing popularity of organically produced food and concerns about drug residues entering the food chain, together with a suspected increase in AR, has generated interest in control strategies that do not rely on anthelmintics only. However, none of these alternative control strategies on its own offers an effective alternative to the use of anthelmintics (taking into account the work required).

Pasture management can reduce the number of anthelmintic treatments required, but cannot replace them entirely, and in many cases it is not a realistic option for Swedish farmers (Höglund 2011). Effective vaccines and non-chemical means of control, such as plant extracts or reliable biological control, are still some distance in the future (Dimander, Höglund & Waller 2003; Wolstenholme *et al.* 2004).

When animals are treated with anthelmintics, a certain proportion of the parasite population will not be exposed to the drug during treatment. These parasites are said to be in refugia, and include the stages of the parasite present outside the host (eggs to L3). Use of a refugia-based control strategy, such as targeted selected treatment (TST), allows part of the herd to remain untreated, for example animals which have already built up resilience to parasites while only treating those showing poor weight gain, high faecal egg counts (FEC) or clinical symptoms of PGE. This increases the numbers of unselected parasites in refugia. Thus TST is generally considered to optimise treatment and slow AR development in ruminant livestock (Kenyon & Jackson 2012). So far, the principles of weight-based TST in cattle have been evaluated under Swedish conditions both by applying a theoretical approach using archived data (Höglund *et al.* 2009b), and by conducting an on-farm grazing experiment (Höglund *et al.* 2013a). In the latter, calves were selected for treatment when individual calf performance was low, but the results showed that even when using this labour-intensive method, the average weight gain in animals

subjected to TST was always significantly lower than in animals dewormed regularly (Höglund *et al.* 2013a).

2.4 Anthelmintic Resistance Globally and in Sweden

The use of modern broad spectrum anthelmintics, since their introduction in the 1960s, has been a convenient and often efficient method to control parasite infections in grazing livestock. However, recent reports have shown that the sometimes extensive use of anthelmintics has led to the world-wide spread of AR in the cattle industry (Gasbarre *et al.* 2009; Sutherland & Leathwick 2011).

Anthelmintic resistance is defined as the ability of parasites to survive a dose of a drug that would normally be lethal. It is a heritable trait and a non-reversible condition (Wolstenholme *et al.* 2004). Resistance to available veterinary anthelmintic drugs was reported soon after their introduction. Resistance among sheep nematodes to BZ (thiabendazole) was reported already in 1964, only four years after the drug's entry onto the market. Resistance to IVM was first reported in 1988 among both laboratory-selected and field strains of *Haemonchus contortus* in South African sheep (Kaplan 2004). In general, *H. contortus* in sheep has a very high propensity to develop resistance to anthelmintics and is the parasite species which has developed resistance most rapidly and in which resistance is most widespread (a.a.).

Resistance to IVM appears to be more common in nematode parasites of small ruminants than those of cattle, probably due to deviant pharmacokinetics and cattle being able to generate a stronger immune response to the parasites. Therefore they also require fewer anthelmintic treatments (Geary 2005). However, *Cooperia* spp. in cattle are considered to be the dose-limiting parasites for ML and reports of AR are common (Sutherland & Leathwick 2011). Furthermore, these parasites are often also resistant to BZ. The rather low pathogenicity of *Cooperia* spp. and thus their relative lack of importance, could have caused resistance to develop without a notable increase in parasite-induced pathology (a.a.).

Anthelmintic resistance is a global problem but seems to be more prevalent in the Southern Hemisphere, for instance in New Zealand, where over 90% of cattle farms surveyed in 2005 contained resistant parasites (Meija *et al.* 2003; Kaplan 2004; Waghorn *et al.* 2006; Soutello, Seno & Amarante 2007; Suarez & Christel 2007). In Europe, AR among cattle nematodes to ML, the market-dominating anthelmintic family, has been reported both in the UK (Coles, Watson & Anziani 2001; Sargison, Wilson & Scott 2009; Orpin 2010; Sargison *et al.* 2010; Stafford, Morgan & Coles 2010; McArthur *et al.* 2011;

Geurden *et al.* 2013) and in Belgium (El-Abdellati *et al.* 2010). Widespread resistance was also reported in our multinational European survey, including German, Belgian and Swedish farms (Paper I) and in Germany and France in another recent study by Geurden *et al.* (2013).

In Sweden, the resistance situation has so far only been investigated systematically in the sheep farming and horse breeding industries (Nilsson *et al.* 1993; Lind et al 2007; Höglund *et al.* 2009a). Over 10 years ago, resistance to BZ derivatives was detected among small blood worms, *Cyathostominae* spp., in Swedish horses. Follow-up studies have shown that the situation has deteriorated further, with resistance to pyrantel (Lind *et al.* 2007). A few years ago, impaired efficacy of IVM against the horse roundworm, *Parascaris equorum,* was also reported (Lindgren *et al.* 2008; Lind & Christensson 2009).

To date, AR among cattle parasites has been very sparingly mapped in Sweden. The aim of this thesis was therefore to further investigate the presence of AR among Swedish cattle, and to examine some of the mechanisms involved.

2.5 Mechanisms of Anthelmintic Resistance

2.5.1 General mechanisms

The mechanisms and genetics of AR are complex. Different species of parasites seem to present different patterns of resistance, depending on whether the genetics are sex-linked, controlled by one gene, polygenic, dominant or autosomal recessive. It is also likely that the various modes of action of the anthelmintic drug classes mean they each have their own mechanism of resistance, which may also differ between different species of parasites.

Several factors seem to contribute to the development of AR, including dosing interval, pasture management and genetic exchange in the worm population (Prichard 2001). Resistance can be seen as either a heritable decline in a drug's effectiveness or a reduction in the length of time that a drug is able to exert its effects on the parasites within the host (James, Hudson & Davey 2009).

Development of AR can be divided into specific and nonspecific mechanisms (Wolstenholme *et al.* 2004). Specific mechanisms include mutations that cause a change in the target molecule of the drug, leading to lowered binding affinity of the anthelmintic drug. Nonspecific mechanisms, *i.e.* mechanisms not related to the precise mode of action of the drug, lead to a lower concentration of the drug at its target site. One example of such mechanisms is altered expression levels of cell membrane transport proteins

(non-target proteins) used by the parasite to handle drugs and toxins in general, *e.g*. P-glycoproteins (PGP) (a.a.).

There can also be a change in the metabolism of the drug, preventing it from being metabolised into its active form, or causing it to be removed from its target sites. Alternatively, an alteration in the distribution of the drug within the parasite can prevent it from reaching its target site, or a change in gene expression in the drug's target can overcome its therapeutic action (Wolstenholme *et al.* 2004; Dicker 2010).

Differences in expression levels of genes may be constitutive, where a gene is always expressed differentially between anthelmintic-susceptible and resistant strains of the parasite. They may also be inducible, where a change in gene expression is observed only in parasites exposed to an anthelmintic compound (Dicker 2010). Changes in gene expression pattern may be caused by either upregulation or downregulation of the gene, or an increased and/or decreased gene copy number. Changes in genes or in gene expression levels in response to drugs enable the organism to survive treatment and might reflect evolution in a toxic environment in which drug resistance leads to "survival of the fittest" (a.a.). Worms carrying the resistant allele/s become evolutionarily superior and can give rise to the next generation of resistant GIN. Once AR is present in a population, reversal or loss of resistance has never been observed (Wolstenholme *et al.* 2004).

A parasite resistant to one anthelmintic compound will probably also be resistant to all other anthelmintics in that chemical class, through so-called side resistance (Kaplan *et al.* 2007). In addition, it is possible to have multiple resistances, where parasites develop resistance to several anthelmintic classes (Wolstenholme *et al.* 2004).

2.5.2 Benzimidazole resistance

Regarding BZ-resistant populations of GIN, genetic studies have shown that a few specific changes in the β-tubulin coding sequence lead to single amino acid exchanges and result in reduced drug susceptibility (von Samson-Himmelstjerna *et al.* 2007; Dicker 2010). The major genetic determinant of BZ resistance in *Teladorsagia circumcincta*, *Trichostrongylus colubriformis*, *H. contortus* and *C. oncophora* has been shown to be a point mutation, leading to a substitution of tyrosine (Tyr) for phenylalanine (Phe) at codon 200 (Phe200Tyr or F200Y) in the isotype 1 -tubulin gene (Kwa, Veenstra & Roos 1994; von Samson-Himmelstjerna *et al.* 2007).

However, there is also convincing evidence of drug efflux-mediated, PGP-associated mechanisms of BZ resistance in nematodes (von Samson-

Himmelstjerna, Krücken & Demeler 2013). In addition, upregulation of enzymes belonging to the broad family of cytochrome P450 oxidases, affecting drug metabolism, has been found to contribute to AR in trematodes and trichostrongyles. Thus, BZ resistance can probably be achieved by at least two different mechanisms and it is still unknown whether several routes of resistance frequently occur simultaneously in the field and what effect this may have on levels of resistance (a.a.).

2.5.3 Macrocyclic lactone resistance

Resistance genetics in several parasites of sheep have been investigated to some extent, while very little information is available about inheritance in important GIN of cattle. However, IVM resistance in *Cooperia* spp. in cattle appears to involve polygenic inheritance, probably selected through repeated under-dosing with, for example, topically applied anthelmintics (Sutherland & Scott 2010).

Genes implicated in IVM resistance are the likely molecular targets of IVM and include glutamate- and GABA-gated Cl channels (Njue *et al.* 2004; Gilleard 2006; Dicker 2010). It has been shown both for the model organism *Caenorhabitidis elegans* (Cully *et al.* 1994; Dent *et al.* 2000; McCavera, Walsh & Wolstenholme 2007; Lynagh & Lynch 2010) and for *C. oncophora* (Njue *et al.* 2004) that IVM resistance is associated with mutations and/or altered expression levels (El-Abdellati *et al.* 2011) in GluCl, resulting in receptor conformation changes and lowered sensitivity to IVM. Changes in allele frequencies of Glu- and GABA- Cl subunits have been observed in *H. contortus*, but no single allele has been associated with AR in different IVM-resistant populations (Blackhall *et al.* 1998a; Blackhall, Prichard & Beech 2003).

One study has associated an amino acid point mutation, from leucine to phenylalanine at codon 256 (Leu256Phe) in the GluClα3 subunit of *C. oncophora* with IVM resistance, but this has not yet been found in other isolates of the worm or in other GIN species (Njue *et al.* 2004; Van Zeveren 2009; El-Abdellati *et al.* 2011). The majority of previous studies dealing with AR have investigated target site mutations (Wolstenholme *et al.* 2004). However, the mechanisms behind IVM resistance are complex, and there are also many studies that, irrespective of GluCl channel, suggest the involvement of PGP (Pouliot *et al.* 1997; Blackhall *et al.* 1998b; Xu *et al.* 1998; Demeler *et al.* 2013). Changes or upregulation in helminth PGP gene expression has been suggested to enhance the parasite's ability to survive exposure to IVM. The

potential effect of this may be increased membrane transport, and thus faster drug elimination

P-glycoproteins – a cause of resistance?

P-glycoproteins are part of a larger superfamily of transmembrane efflux transporters that have been implicated as a primary cause of AR both in *C. elegans* and in *H. contortus* (Blackhall *et al.* 1998b; Kerboeuf *et al.* 2003; James & Davey 2009). The PGP, for which IVM is a well-known substrate (Lespine *et al.* 2008; Kerboeuf & Guegnard 2011), are members of the ATP binding cassette (ABC) superfamily of genes coding for molecules involved in active transport of endogenous and exogenous hydrophobic molecules (Jones & George 2005). Altogether, 14 isoforms of PGP, plus one pseudogene, have been annotated in the *C. elegans* genome database (WormBase, version: WS234). The complete PGP repertoire is not yet known for any of the GIN of ruminants (Demeler *et al.* 2013).

Dupuy *et al.* (2010) have shown that PGP are involved in the pharmacokinetics of IVM. Kerboeuf and Guegnard (2011) recently found evidence that ML activate transport activity in nematode PGP, and suggest that several substituents in the ML structure are involved in modulating the stimulatory effect. Janssen *et al.* (2013) found that absence of, or blocked, PGP resulted in higher IVM susceptibility of *C. elegans*, which suggests that PGP are important for IVM detoxification in this model organism.

Recently, PGP expression in 10 variants of PGP in IVM-resistant isolates of *T. circumcincta* from sheep was investigated (Dicker, Nisbet & Skuce 2011). One of these, *Tci-Pgp-9*, showed increased expression at the mRNA level and nucleotide sequences also showed high levels of polymorphism, which could play an important role in helminth survival of IVM exposure. Pachnicke (2009) performed similar studies in a BZ-selected *O. ostertagi* population and found evidence of increased PGP-related signals in eggshells from BZ-selected *O. ostertagi*, but no signs of increased PGP gene expression in adult worms. On the other hand, De Graef *et al.* (2013) showed 3- to 5-fold increased transcript levels of *Con-pgp-11* in resistant *C. oncophora* in both adult worms and L3, after IVM selection, suggesting that resistant worms have acquired the ability to upregulate the number of efflux transporters upon exposure to IVM. In Paper V, we found a tendency for upregulation of C*on-pgp-9* in *C. oncophora* 10 days after exposure to IVM. Furthermore, Demeler *et al.* (2013) showed that the PGP inhibitor verapamil could reverse ML resistance in an *in vitro* test, while a similar finding was made in *C. elegans* in a study by Ardelli and Prichard (2013).

Whether altered PGP expression is the key to ML resistance is still unclear an in fact AR is likely to be multifactorial. Thus, the molecular basis of resistance to IVM, as well as to other anthelmintic drug classes in trichostrongyle parasites, still remains to be elucidated (Geary 2005; Prichard *et al.* 2007; De Graef *et al.* 2013).

2.6 Detection of Anthelmintic Resistance - A Brief Overview of Methodologies Used on Cattle Nematodes

With the development and spread of AR in nematodes of livestock, the need for methods to detect resistance has evolved simultaneously. Different *in vivo* and *in vitro* tests are available. However, currently available methods for diagnosis of helminth infections in livestock and detection of AR are labour-intensive and time-consuming, and often only detect single species in pooled or single samples. The development of molecular tests, mainly with the focus on polymerase chain reaction (PCR) technology is therefore also progressing.

2.6.1 The controlled efficacy test

The controlled efficacy test (CET) is the "gold standard" for detecting AR. The efficacy of an anthelmintic is determined by comparing parasite populations in groups of treated and/or non-treated animals. Worm burdens of animals artificially infected with susceptible or suspected resistant isolates of nematodes are compared after treatment, when necropsy is carried out and the parasites are recovered, identified and counted (FAO 2004; Coles *et al.* 2006). Resistance is confirmed when the reduction in worm count is less than 90%, or more than 1000 worms that survived treatment are found at necropsy (Coles *et al.* 1992; Taylor, Hunt & Goodyear 2002). An untreated group is often included as a control. The downside is that CET is expensive and labour-intensive. There are also ethical concerns about the use of experimental animals.

2.6.2 Faecal egg count reduction test

Detection of AR is usually based on the faecal egg count reduction test (FECRT). This was originally designed for sheep, but has since been adapted for use in cattle, swine and horses (FAO 2004). Treatment by modern anthelmintics should normally result in a reduction in faecal egg count (FEC) of more than 95%, and the FECRT provides an estimation of anthelmintic efficacy. The FEC of animals is compared before treatment and 10-17 days after treatment, depending on the anthelmintic being tested (Gill *et al.* 1998). For monitoring of normal fluctuations, the treated group can be compared with

non-treated controls. This test is particularly suitable for field surveys. For cattle, the ideal is to use animals with a minimum individual count of 100 eggs per gram faeces (EPG). If initial egg counts are below 150 EPG, egg counting may require the use of a method more sensitive than the modified McMaster technique, *e.g* FECPAK (Coles *et al.* 2006). Macrocyclic lactone-resistance in ruminant parasitic nematodes is declared when the reduction after ML treatment is ≤ 95%, and when the lower 95% confidence interval is ≤ 90%. If only one of these two criteria is met, resistance against anthelmintics is suspected (Coles *et al.*1992).

In addition to FECRT and CET, a range of *in vitro* bioassays have been developed and recently validated for detection of AR in cattle nematodes. Examples of these are the egg hatch test (EHT), larval development test (LDT), and larval migration inhibition test (LMIT) (Demeler *et al.* 2010; Demeler, Küttler & von Samson-Himmelstjerna 2010). *In vitro* assays are easier, faster and cheaper to perform than *in vivo* tests and do not require the use of animals, which eliminates ethical concerns and removes any inter-host variation (Dobson, Le Jambre & Gill 1996). A characteristic common to both *in vivo* and *in vitro* assays, however, is that they might not detect resistance if less than 25% of the tested population is resistant (Dicker 2010).

2.6.3 Egg hatch test

The EHT was originally developed to differentiate between resistant and susceptible strains of GIN of sheep to BZ and LEV, since ML are not ovicidal (Coles 2005). It is based on determination of the proportion of eggs that fail to hatch in solutions of increasing drug concentration in relation to the control wells. This enables the percentage hatch rate to be observed and allows the discriminating dose, or effective dose where 50% inhibition is observed, to be calculated from a dose response curve. It is important that eggs are fresh from the host to prevent embryonation from starting, since sensitivity to BZ decreases with age of the eggs. The test has only been shown to work on nematode species in which the eggs hatch rapidly (Coles *et al.*1992, 2006). It is comparatively more rapid and economical to conduct than the FECRT.

2.6.4 Larval development test

The LDT is the only *in vitro* test that allows the detection of AR against all anthelmintics, irrespective of their mode of action (FAO 2004). In this test, nematode eggs isolated from faecal samples are applied to the wells of micro-

titre plates and the larvae hatch and develop to the L3 stage in the presence of increasing concentrations of the active substance, with *Escherichia coli* as a food source. The concentration of anthelmintic required to block development is related to *in vivo* efficacy. A commercial LDT called “Drenchrite” has been developed (Coles 2005).

2.6.5 Larval migration inhibition test

In the LMIT, which is suitable for the detection of LEV and ML resistance, L3 are incubated in serial dilutions of anthelmintic and then placed, in solution, above a nylon mesh and incubated for a specific time (Figure 4). Mesh size depends on the species tested. Resistant larvae, which are not paralysed by the drug solution, will be able to migrate through the mesh by a twisting rotating movement. The number of migrated and non-migrated larvae is counted, the percentage migration can be calculated and a dose response curve can be calculated (Demeler *et al.* 2010).

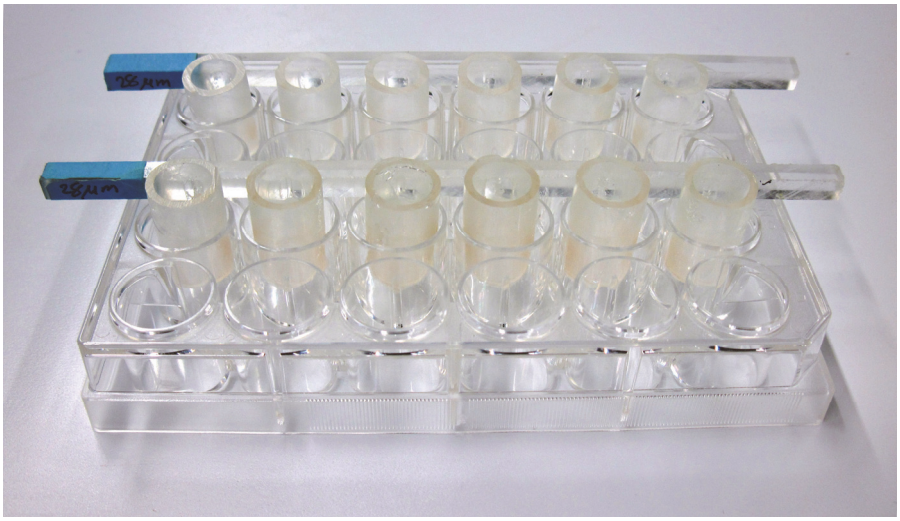

Figure 4. A 24-well incubation plate with sieves (mesh size 28 µm) for larval migration inhibition testing.

2.6.6 Molecular markers

DNA-based tests are suitable for detection of resistance, since there is most likely a genetic basis, either through qualitative changes (mutations) or quantitative changes (alterations in expression of genes). Development of PCR-based tests has allowed the detection of AR at lower levels than in earlier

in vitro methods. However, to date PCR tests have only been established for detection of BZ resistance in GIN. Single nucleotide polymorphism (SNP), partly associated with resistance, has only been identified for this drug class as yet. The most common SNP, which confers BZ resistance in small ruminant trichostrongyles, strongyles in horses and *C. oncophora* in cattle, is a mutation at codon 200 of the isotype 1 β-tubulin gene, where phenylalanine is changed to tyrosine (Kwa, Veenstra & Roos 1994; von Samson-Himmelstjerna *et al.* 2007). Resistance mechanisms to LEV and the other imidazothiazoles /tetrahydropyrimidines, including PYR, have been less well researched (Kopp *et al.* 2008).

Some studies have also sought to develop molecular-based tests to identify ML resistance, where the genes involved are most likely the molecular targets of ML and include glutamate- and GABA-gated Cl-channels (Njue *et al.* 2004; Gilleard 2006). Allele frequency changes in Glu- and GABA-Cl subunits in *H. contortus* have been observed, but no single allele has been identified as a cause of resistance in IVM-resistant populations of *H. contortus* (Blackhall *et al.* 1998a; Blackhall, Prichard & Beech 2003).

One of the modes of action of IVM is starvation of nematodes, caused by inhibition of the pharyngeal pump (Dent *et al.* 2000). Altered *avr-14B* gene transcription patterns, encoding GluCl α-type subunits expressed in the extrapharyngeal nervous system, have been found in resistant *C. oncophora* and *O. ostertagi* (El-Abdellati *et al.* 2011). McCavera, Walsh & Wolstenholme (2007) also found a polymorphism in *avr-14* in *C. oncophora*, making the subunit less sensitive to IVM. One study found a mutation at codon 256 in the GluClα3 subunit of *C. oncophora* as a possible cause of IVM resistance, but this has not been found in *H. contortus*, *T. circumcincta*, *O. ostertagi* or other *C. oncophora* isolates (Njue *et al.* 2004; Van Zeveren 2009).

Most of the AR mechanisms identified to date have tested candidate genes to identify qualitative changes conferring resistance. The success as regards ML has been limited, as classical genetic studies to identify resistance genes are difficult when the mode of action of the drug is partly unclear. Thus, it is difficult to propose candidate resistance genes for further investigations (Dicker 2010). Alternative mechanisms of resistance such as changes in gene expression level have previously been rather unexplored, but lately several studies have been performed to investigate nematode-specific PGP gene expression (Dicker, Nisbet & Skuce 2011; Paper V; De Graef *et al.* 2013; Demeler *et al.* 2013).

3 The Present Investigation

3.1 Objectives and Hypothesis of the Thesis

The overall aims of this thesis were to investigate the presence of ML resistance among *C. oncophora* and *O. ostertagi* in FSG cattle in Sweden, and to investigate possible mechanisms responsible for reduced anthelmintic efficacy of treatments with IVM.

The specific objectives of Papers I-V were as follows:

I. To investigate the efficacy of IVM and albendazole against GIN of cattle in Belgium, Germany and Sweden.

II. To investigate the effect of avermectin pour-on anthelmintics under field conditions among Swedish cattle, including dairy and beef herds; and to introduce and test a novel TST concept, where deworming decisions were based on the information from FEC in fresh faecal samples collected directly from the pasture.

III. To investigate suspected AR in *O. ostertagi* and *C. oncophora* isolates after IVM treatment, by performing a CET, but also by using a range of available *in vivo* and *in vitro* methods for further characterisation of the AR and species composition status.

IV. To investigate possible negative interactions between immunosuppression by dexamethasone injections and the efficacy of IVM treatment in young cattle.

V. To investigate genetic selection and PGP expression in three different isolates of adult *C. oncophora* before treatment and after IVM injection.

3.2 Experiments and Design

A brief description of materials and methods used in this thesis is presented below. More detailed information can be found in Papers I-V.

3.2.1 Animals

First season grazing cattle of the Swedish SRB and Holstein (SLB) breeds were used in all infection trials. The calves used in Papers III-V originated from Kungsängen Research Centre in Uppsala, while calves used as 'incubators' for propagating L3 for the trials were bred and housed at Götala Research Centre near Skara. The experimental calves were between 3 and 7 months old, weaned and parasite-naïve before inoculation with L3 and kept indoors from birth until the end of the trials.

The field trials (Papers I-II) were conducted on conventional and organic farms with FSG of Holstein Frisians, Blue cross breed (Belgium), SRB and purebred or crossbred beef cattle, *e.g.* Charolais, Hereford and Simmental.

3.2.2 Worm isolates

In the field trials (Papers I-II), all animals were naturally infected with GIN present in pasture during their first grazing season. In the infection trials (Paper III-V), calves received different isolates of fresh trichostrongyle L3 of *C. oncophora* and *O. ostertagi,* mixed in 50:50 proportions.

"TiHo"

This *O. ostertagi* and *C. oncophora* mix originated from a Weybridge isolate and was obtained from BAYER Animal Health AG in 2002. Since then, it had been maintained for several years at Tierärzliche Hochschule in Hannover, Germany. It had no history of previous exposure to ML. In all passages it remained susceptible to therapeutic doses of ML, resulting in a 100% reduction in egg count in infected calves up to 28 days after treatment.

"Gråmunkehöga" and "Kolsta"

These two isolates, both containing a mixture of *O. ostertagi* and *C. oncophora*, originated from two dairy farms in Uppland, Sweden, where previous field trials indicated resistance to injectable IVM (Paper I). For these

experiments, faeces from FSG calves on the two farms were collected rectally, pooled by farm and mixed with vermiculite. Following incubation for 14 days under moist conditions at 25 ˚C, L3 were harvested with the inverted cover glass technique and stored until use in flasks in the refrigerator at 8 ˚C.

3.2.3 Larval cultures and infection

All isolates were passaged once and propagated in three different parasite-naïve calves at Götala Research Centre. Faeces was harvested through individual rectal samples during several weeks and incubated using the same method as mentioned above, in order to get sufficient numbers of larvae to infect all calves in the pen trials. On the first day of the infection trials, each calf received approximately 40,000 L3 (50% *O. ostertagi* and 50% *C. oncophora*) orally in a small volume of water. Faecal samples were then analysed and checked for trichostrongyle eggs after three weeks to detect patent infections.

3.2.4 Necropsy and worm sampling

The abomasum and small intestine were collected from euthanised animals in the infection trials (Papers III-V). The process was in general accordance with that described by Larsson *et al.* (2007), with 20 mL subsamples collected from 4 L each of abomasal and small intestine contents. In Paper III, adult worms were also recovered one-by-one in Eppendorf tubes and directly frozen at -80 ˚C to enable RNA isolation for PCR tests (Paper V). However, RNA has a short half-life and it is impossible to know how much it was degraded during the hours between euthanasia of the calves and freezing of the worms. Nevertheless, all samples were subjected to the same handling upon collection.

3.2.5 Field study

In a two-year field study (Paper II), farmers received step-by-step instructions on when and how to collect, handle and post the faecal samples. If FSG calves were infected with ≥100 EPG they were included in the trial, and the farmers received prescriptions of anthelmintics and clear instructions on how to apply the drugs and send in follow-up samples. Some farmers never sent in the second samples after deworming, or sent in samples too late, and a few farmers waited a month before treating, which made the results unreliable. Others dewormed their animals in time, but did not send in new samples until several weeks after treatment, which also made the material unsuitable for use. In total, 18 farms were excluded from the study.

4 Publications and Manuscripts – Results and Discussion

Anthelmintics are the cornerstone of modern parasite nematode control and, in some areas of the world, the levels of resistance to ML exhibited by GIN of cattle is rising. In the long run, this may threaten economically viable farming. However, IVM is still the mainstay of many treatment strategies for reducing the burden of PGE in livestock world-wide and it is also used in mass drug treatment programmes for some human parasites, *e.g*. to treat river blindness (onchocerciasis) (Omura 2008; Dicker 2010). The development of IVM resistance, coupled with climate change and rising temperatures potentially causing an increase in disease incidence, is a worrying development (a.a.).

In Europe, ML resistance seems to be increasing and multi-drug-resistant parasite isolates have been reported, although mainly for small ruminants in the UK (Sargison *et al.* 2007). However, in Sweden no routine monitoring is carried out to investigate the level of AR in nematodes of veterinary importance and thus its incidence is still largely unknown. This thesis was a step towards exploring AR to the major drug classes used against GIN of Swedish cattle, especially IVM.

The general findings made in Papers I-V of this thesis are briefly described below. More detailed information can be found in the individual papers, which are contained in the appendix.

4.1 Anthelmintic resistance in gastrointestinal nematodes of cattle is, by definition, present in Belgium, Germany and Sweden (I)

A FECRT using IVM and BZ was conducted to investigate the prevalence of AR in GIN on cattle farms in Germany, Belgium and Sweden in 2006 and 2007.

Based on sufficient numbers of eggs prior to the study, between 3 and 10 farms per country were selected and 10-15 animals were randomly selected per farm and subcutaneously treated with IVM. Faecal samples were collected individually from every animal on day 0 (treatment), day 7 (Belgium & Sweden) or 14 (Germany), and day 21 (Germany, Belgium and Sweden). A FEC was performed on each sampling occasion to estimate the EPG and the reduction in eggs after treatment.

The FECRT using IVM in 2006 revealed a mean reduction in egg count of between 69-100% (95% confidence interval (CI) 19-102) on day 7/14 and 35-96% (95% CI 0-102) on day 21.

Farms showing indications of an AR problem were re-visited in 2007 and, except for one case, all results obtained in 2006 were confirmed in 2007.

Larvae obtained from faecal cultures were identified morphologically and *C. oncophora* was the predominant species detected after treatment, but *O. ostertagi* was also identified post-treatment in samples from three farms in Germany and three farms in Sweden.

In 2007, an additional FECRT using BZ was conducted in Germany and Sweden, using oral albendazole (10%) at a dose of 8 mg/kg. For BZ, an efficacy of 100% was obtained on all farms tested in both Sweden and Germany. This was the first multinational anthelmintic efficacy investigation in cattle in Europe. The results suggested that testing of anthelmintic efficacy should be performed more intensively due to possible insufficient efficacy of the current drugs. The need for a semi-quantitative, PCR-based test for species identification that can be used on non-invasive samples was also identified.

4.2 Topical ivermectin treatments have limited efficacy under Swedish field conditions (II)

In Paper II, the effect of topical ML against GIN in Swedish FSG cattle was investigated during the grazing seasons of 2009 and 2010. Herds across the country were recruited through the farming press and both dairy and beef cattle farms were encouraged to participate.

A questionnaire revealed that 64% of participating farmers (n=59) had dewormed their animals in previous years and of these, 76% had used topical formulations with ML. Four to six weeks after turnout, 107 (2009) and 64 (2010) farmers sent in individual faecal samples from 6-10 FSG. The FEC were determined by the FECPAK® method in 2009 and the McMaster method in 2010, when larvae were also cultured.

An average FEC of ≥100 EPG was seen in 39% of the herds in 2009 and 42% in 2010, with arithmetic means of 258 ±110 and 252 ±350 EPG, respectively. Interestingly, FSG in dairy and beef herds had similar mean FEC values.

In herds with mean FEC ≥100 EPG, farmers dewormed all FSG in the tested grazing group with IVM or doramectin (DOR) pour-on. In 2009 33 (31%) and in 2010 26 (40%) of the herds were retested 7-16 days post treatment. The mean reduction was 89% (95% CI 83-93) in 2009, and 88% (CI 81-93) in 2010, and it was ≥95% in only 12 (36%) and 10 (38%) herds respectively. Beef herds had mean reductions similar to those in dairy herds. No significant difference (P=0.66) in reduction was seen between the groups treated with three different pour-on formulations, nor was there any correlation between the previous year's usage of anthelmintics and their efficacy.

Larvae from post-treatment cultures in 2010 were analysed with a species-specific ITS2 qPCR. The results showed that in Paper II too, *C. oncophora* was the predominant species after deworming. Four (15%) groups also harboured surviving *O. ostertagi* post-treatment.

4.3 "Resistant" field isolates become susceptible in pen trials (III)

The effect of IVM was investigated in three mixed isolates of GIN from cattle to further characterise the AR status by a range of *in vivo* and in *vitro* methods. One ("TiHo") was an IVM-susceptible laboratory isolate, whereas the remaining two isolates ("Gråmunkehöga" and "Kolsta") originated from the Swedish cattle farms where results in Paper I indicated AR against IVM.

Three groups, each of 11 calves, were inoculated with L3 and FEC were monitored from 0 to 45 days post infection (d.p.i.). L3 were cultured continuously for use in a LMIT and identification was performed using a species-specific PCR (Höglund *et al.*, 2013b). At 31 d.p.i., one calf from each group was necropsied and adult worms were recovered pre-treatment. At 35 d.p.i., calves from all groups were injected with IVM at the recommended dose (0.2 mg/kg body weight). At 45 d.p.i., another two animals from each group were sacrificed and established GIN were collected and counted.

A few animals in all three groups were still excreting eggs (50-150 EPG) 10 days post IVM injection. However, there was no significant difference in the FEC reductions in the groups "TiHo" (95%; 95% CI 81-99), "Gråmunkehöga" (98%; 92-100) and "Kolsta" (99%; 97-100) between 35 and 44 d.p.i. Furthermore, LMIT showed no significant difference between the three groups.

Approximately 100 adult *O. ostertagi* were found in the abomasum of one calf ("Gråmunkehöga" group), whereas low to moderate numbers (400-12 200) of *C. oncophora* remained in the small intestine of the calves in all three groups at 45 d.p.i. A PCR test on L3 harvested from faecal samples up to 10 days post treatment showed a ratio of 100% *C. oncophora* in the calves inoculated with "TiHo" and "Gråmunkehöga" isolates, whereas those inoculated with "Kolsta" isolate also had 8% *O. ostertagi*.

Overall, Paper III showed that the animals were successfully treated according to the FECRT standard (≥95% reduction). However, once again, several adult worms of the dose-limiting species *C. oncophora* demonstrably survived the IVM treatment.

4.4 Dexamethasone affects the efficacy of ivermectin in first-season grazing cattle (IV)

Paper IV studied possible interactions between immunosuppression as a result of dexamethasone (DXM) treatment and the efficacy of IVM treatment in young cattle.

Two groups, each of seven calves, were experimentally inoculated with a mixture containing equal numbers of L3 larvae of *C. oncophora* and *O. ostertagi*, and with no history of being resistant to any anthelmintics ("TiHo" isolate). Blood parameters and FEC were monitored from day 0 until 35 d.p.i. The calves in one group received intramuscular injections of short-term and long-term acting DXM at 22 and 24 d.p.i., respectively. The other group remained as a control. Three days post patency (24 d.p.i.), both groups were injected subcutaneously with IVM.

A significant difference ($p<0.001$) in FEC patterns was observed between the groups. Although both groups still excreted eggs (100-200 EPG) 11 days post anthelmintic treatment, the control group had a significantly higher reduction between 23 and 35 d.p.i. ($p=0.025$).

After 35 days, four animals per group were euthanised and worms in the GI tract were counted. No *O. ostertagi* were found in the abomasum, but again, low to high numbers (800-6200) of *C. oncophora* remained in the small intestine in both groups.

Overall, these findings indicate that there is an interaction between the efficacy of IVM and DXM treatment. As significantly lower plasma levels of IVM were observed in the DXM group, we concluded that the impaired efficacy of IVM was due to altered pharmacokinetics rather than immunosuppression.

4.5 *Con-pgp-9* in male *Cooperia oncophora* tends to be upregulated after ivermectin treatment (V)

The detection of resistance is problematic. Current diagnostic tests, using *in vivo* and *in vitro* methods such as FECRT, EHT and LMIT are often expensive, time-consuming and insensitive (Coles *et al.* 1992; McKellar & Jackson 2004). Furthermore, the modes of action of the anthelmintics and the mechanisms of resistance employed by the target parasites are still not fully understood (Kaplan 2004; Prichard *et al.* 2007). The candidate genes believed to be the targets of the anthelmintics have been tested in studies investigating the mechanisms of AR (Demeler *et al.* 2013). This approach has not yet been successful and, in the case of ML, SNP linked to resistance are unfortunately still lacking for cattle nematodes (a.a.).

An alternative mechanism of AR that has lately begun to be investigated, relates to changes in the expression of certain genes. Examples are mechanisms allowing nematodes to survive exposure to IVM by dealing with drug transport or metabolism. These changes, if identified, could be developed into a test for diagnosis of IVM resistance in GIN. Genetic tests are more sensitive, quick and easy to perform than the tests currently available (von Samson-Himmelstjerna 2006).

In 2011, we investigated genetic selection and PGP expression in the three different isolates of *C. oncophora* (“TiHo”, “Gråmunkehöga” and “Kolsta”) before treatment and after IVM injection.

Adult parasites were recovered from the nine calves experimentally infected in Paper III, with the isolates represented by one IVM-susceptible laboratory isolate (“TiHo”), and the two field isolates “Gråmunkehöga” and “Kolsta”, which showed signs of phenotypic ML resilience according to the FECRT in Paper I, and demonstrably survived the anthelmintic treatment in the following pen trial (Paper III).

Five males and five female worms per isolate were examined both pre- and post-IVM treatment, giving a total of 60 worms. A gene sequence from *C. oncophora* (*Con-pgp*) was identified, showing 83% similarity to *Pgp-9* of *C. elegans*. In *C. elegans*, *Pgp-9* has been shown to be expressed in the intestine

and the first and second bulbs of the pharynx (www.wormbase.org). Primers specific to putative *Con-pgp-9* mRNA were designed, generating a 153 bp PCR product. Total RNA was prepared from all worms and *Con-pgp-9* expression was measured by Real time RT-PCR.

The results showed that mean PGP concentrations were 4- to 5-fold higher in female worms than in males, possibly due to the fact that nematode eggs (in pregnant female worms) also express PGP. This led us to reconsider using fecund or pregnant females in this type of test, when levels of PGP expression could be substantially different within groups.

No significant differences in gene expression between experimental groups pre- and post IVM selection were detected. However, PGP gene expression tended to be increased by IVM treatment in male worms (p=0.091), with 70% higher mean expression in treated than in untreated male worms.

Amplified fragment length polymorphism (AFLP) analysis did not demonstrate any bottleneck effect within the different isolates post treatment. The total mean gene diversity (Hj) values were 0.158 and 0.153 before and after treatment, respectively. Inbreeding coefficient in sub-populations, compared with the total population F_{ST}, was 0.0112, suggesting no genetic differentiation between or within the investigated isolates in relation to treatment.

In conclusion, comparison of *Con-pgp-9* expression showed no significant difference before and after treatment, but some tendency towards increasing expression in male worms. This was in accordance with findings by Dicker (2010) that the expression of *Teci-Pgp-9* (NBD2) tended to increase in the survivors of IVM treatment in the sheep nematode *T. circumcincta* although the differences were not statistically significant.

The change in gene expression observed in this project could be a step forward in determining how nematode parasites, such as *C. oncophora* and *O. ostertagi*, are affected by IVM exposure, and how resistant parasites are able to survive IVM treatment. Further work investigating these changes in PGP expression could help to establish the basis of an IVM resistance marker. Diagnostic tests identifying such molecular markers for ML resistance would then make it possible to monitor development of AR and to evaluate management practices aimed at delaying its spread. Once a molecular test is available, the resistance allele frequencies at which farmers will be recommended to stop using a drug will remain to be determined. The question is whether it makes sense for a farmer to stop using a drug when a molecular test indicates a low level of resistance but the anthelmintic treatment results in a 95% reduction in egg counts (see *e.g.* Kaplan & Vidyashankar 2012).

5 Summary and Concluding Remarks

Anthelmintic resistance is today a significant problem globally, especially in the small ruminants farming industry. In cattle farming the problem appears less dramatic, possibly due to the more resilient immune system of the host and less "sudden death" incidents, together with a relatively low treatment frequency. However, the animal welfare aspect and the economic importance of sustainable prevention of parasites in cattle farming should not be underestimated.

Today, Swedish consumers are exerting pressure on farmers to offer top quality meat at low prices, from animals that have been kept under excellent conditions and committed management. If available anthelmintic drugs become ineffective, there is a major risk that either the animals will suffer, or that farmers' efforts and access to larger areas of land suitable for pasture, and thus the costs, will have to increase dramatically to keep the parasite burden in animals under control. New mode of action anthelmintic drug classes may not be available in the near future. Therefore, it is important that Swedish farmers can get clear-cut advice and support from veterinarians and animal health organisations to apply scientifically based strategies for achieving a balance between good parasite control and sustainability of their control strategies. In this way, AR may be delayed and the effectiveness of anthelmintic drugs may be prolonged. This requires sensitive detection tools. With a sensitive detection technique, AR can be diagnosed at an early stage and the spread of resistance alleles in the parasite population can be prevented.

In response to the initial question of whether AR against ML is present among Swedish cattle nematodes, this thesis could not simply provide a yes or no answer, as the problem proved more complex and multifactorial than expected.

However, Papers I-V found a considerable lack of efficacy of IVM under field conditions. Whether this depends fully on genetic resistance or partly also on confounding factors such as altered pharmacokinetics remains to be elucidated.

Surprisingly the results demonstrated that if the same population of nematodes was tested under controlled conditions in pen trials, a much better control effect, totally acceptable according to the current standards, was achieved. This is an important observation and to date only a few similar studies have investigated AR, describing both field trials and CET, in GIN of small ruminants (see *e.g.* Domke *et al.* 2012; Sarre *et al.* 2012). On the other hand, significant numbers of adult *C. oncophora* and small numbers of *O. ostertagi* survived ML treatments in all critical tests. From what we know today about inheritance and mechanisms in genetics, this is a possible warning of incipient development of AR, something we need to consider in cattle management in the future. By starting now and using anthelmintics in a responsible and reflective way, preferably combined with preventive pasture management and hygiene, we can keep cattle healthy and the farming industry sustainable.

As a result of the investigations described in this thesis on AR against ML among GI nematodes in Swedish FSG cattle, the following major conclusions on parasite control can be drawn:

- Lack of anthelmintic efficacy in GIN of cattle seems to be present under field conditions in Northern Europe, including Sweden (Paper I)
- Topical ivermectin (pour-on) treatments as used today by farmers have limited efficacy under Swedish field conditions (Paper II)
- Putative 'resistant' field isolates became susceptible (according to definition) under controlled conditions in pen trials (Paper III)
- *Cooperia oncophora* is the main survivor after anthelmintic treatment with ivermectin, but some *O. ostertagi* are also able to overcome the drug's lethal properties
- Dexamethasone affects the efficacy of ivermectin in first-season grazing cattle, which may indicate that other drugs or pharmacokinetic confounders have an impact on the treatment success rate of the anthelmintic drug (Paper IV)
- *Con-pgp-9* in male *C. oncophora* tends to be upregulated after ivermectin treatment, which suggests that the cell membrane protein might be involved in this parasite's ability to overcome the toxic compounds of the anthelmintic drug (Paper V).

6 Future Research

Our present European cooperation and the world-wide interest in solving the problem and challenge of AR will hopefully continue with an interdisciplinary approach, where parasitologists work together with molecular scientists, epidemiologists, pharmacologists and animal scientists to seek answers. According to the studies performed in this thesis, the following areas of research could contribute to the general picture:

- Monitoring of FSG for FEC, together with body condition scoring (BCS) or monitoring of calves' fat reserves, and determination of pharmacokinetic parameters after anthelmintic treatment under field conditions at turnout
- Confirmation of the trend for a change in *Con-Pgp-9*, when comparing non-IVM exposed and IVM exposed male worms, in other isolates of *C. oncophora*
- Continued molecular PGP/transporter studies. Identification of changes in expression associated with IVM resistance could form the basis of a molecular marker for resistance. Lack of such a marker is currently impeding the development of targeted control strategies aimed at minimising the spread of IVM resistance in GIN cattle nematodes
- Ultimately, a sensitive and specific molecular diagnostic test for IVM resistance which can identify the IVM resistance status of each species needs to be developed. Identification of the mechanisms of IVM resistance, and the genetic changes which control these, would be a useful first step towards this.

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