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Anthelmintic Resistance in Nematode Parasites of Sheep in Denmark with Special Emphasis on Levamisole Resistance in *Ostertagia circumcincta*

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Bjørn, H., J. Monrad and P. Nansen: Anthelmintic resistance in nematode parasites of sheep in Denmark with special emphasis on levamisole resistance in *Ostertagia circumcincta*. Acta vet. scand. 1991, 32, 145–154. – This study was undertaken to elucidate the presence of anthelmintic resistance in nematode parasites of sheep in Denmark. Twenty two flocks of sheep were selected for Faecal Egg Count Reduction (FECR) tests, based on a prior history of either the same anthelmintic, or anthelmintic class having been used 3 times or more over the previous 5 years. Evidence of anthelmintic resistance was detected in 7 flocks. Two flocks showed FECR of 88 % and 94 % after treatment with thiabendazole, FECR of another 2 were 90 % and 94 % following treatment with fenbendazole. Three flocks showed FECR of 73 %, 89 % and 94 %, respectively following the use of levamisole. *Ostertagia circumcincta* was isolated from 1 of the latter flocks and subjected to an *in vivo* controlled slaughter assay. Following treatment with levamisole at the recommended dose rate of 7.5 mg/kg, FECR was 44.5 % and worm counts were reduced by 67.7 %. These results were further substantiated by an *in vitro* egg hatch paralysis assay and by measuring pepsinogen levels in treated and non-treated lambs. This is the first instance of anthelmintic resistance in sheep nematodes in Scandinavia.

nematodes; endoparasites; chemotherapy; small ruminants; control.

Introduction

Resistance to anthelmintics in sheep nematodes was first reported in 1957 by *Drudge et al.* (1957) in Kentucky, USA, to phenothiazine, the commonly used anthelmintic at that time. Since then, numerous reports of resistance in nematodes of sheep and other domestic animals have been documented (for reviews see *Waller* 1986, *Waller & Prichard* 1986). Anthelmintic resistance in sheep and goat nematodes is now widespread in Australia, certain countries of the African and South American continents and is of increasing importance in New Zealand. However, reports of resistance in European countries did not emerge until somewhat later, with the first record of resistance from Switzerland (*Jordi et al.* 1980). Since then, reports have been made from U.K. (*Britt* 1982, *Britt & Oakley* 1986, *Cawthorne & Whitehead* 1983, *Cawthorne & Cheong* 1984), the Netherlands (*Boersema et al.* 1982, 1984), France (*Kerboeuf & Hubert* 1985, *Kerboeuf et al.* 1989) and the GDR (*Düwel et al.* 1987, *Bauer et al.* 1988).

Prichard et al. (1980) defined anthelmintic resistance as "a significant increase in the ability of individuals within a strain to tolerate doses of a compound (toxicant) which

would prove lethal to the majority of individuals in a normal population of the same species." Recently a working party on anthelmintic resistance in Australia has provided a more precise interpretation on resistance being present in a flock when 1) the observed percentage reduction in faecal egg counts after treatment is less than 95 % and 2) the lower 95 % confidence limit of the percentage reduction is less than 90 % (*Anon.* 1989). This definition was used in the present investigation.

At present there is only 2 classes of anthelmintics available for treatment of nematodiasis in sheep in Denmark, namely the (pro)-benzimidazoles (Group I) and the neuromuscular agents, i.e. levamisole and tetramisole (Group II). All drugs in group I act by interfering with the intracellular polymerisation of tubulin subunits to microtubules (*Lacey* 1988). This means, that resistance against 1 benzimidazole automatically confers resistance against other compounds in this group. The anthelmintics in group II act as cholinergic agonists (*Coles et al.* 1975, *Harrow & Gration* 1985), causing paralysis of worms, resulting in loss of motility and subsequent removal from predilection site by gut motility.

So far, no anthelmintic resistance in sheep nematodes has been described in the Scandinavian countries. This study was designed to establish whether resistance to anthelmintics was present in sheep nematodes in Denmark. Thus, this study comprises a survey in the field, using *in vivo* faecal egg count reduction (FECR) tests and an experimental controlled *in vivo* and *in vitro* study in the laboratory to confirm resistance against levamisole in *Ostertagia circumcincta* of sheep.

Materials and methods

Survey

One hundred and five sheep farmers in Denmark, registered by the Danish Sheep Breeding Committee, were included in a questionnary survey on worm control practices and on anthelmintic use (the results of this survey will be published elsewhere). Twenty two farms from this survey were selected for faecal egg count reduction (FECR) tests. The farms had between 10 and 450 ewes with an average flock size of 21 ewes. The prerequisite for a farm to be included in the survey was that the same anthelmintic, or drugs from the same class of anthelmintic, had been used for the last 5 years and that 3 or more anthelmintic treatments were performed each year. The lambs had to be left untreated with any anthelmintic at least 6 weeks before the performance of the test. Furthermore, a minimum of 15 lambs should be available for a FECR-test on the farm.

Thus, at least 15 lambs from each sheep farm were selected for a FECR trial, using the drug applied during the last 5 years. Individual lambs were identified by eartag numbers, weighed, faecally sampled (at least 10 g) and treated according to the recommended dose rate of the drug. The same lambs were re-sampled 10–14 days later. All samples were identified by eartag numbers and stored in a cooling box during transportation to the laboratory and here they were kept at 4°C until examination. The nematode egg concentration in faeces was examined by a modified McMaster technique, as described by *Henriksen* (1981), and the number of eggs per gram (epg) was recorded. From lambs showing positive egg counts composite faecal samples, collected before and after treatment were mixed with vermiculite in equal proportions (w/w) and water was added until the mixture appeared moist. Cultures were incubated for 10 days at room temperature and third stage larvae were harvested by the Baerman technique. At least 100 larvae of each culture were identified

according to *Douvres* (1957) and the proportion of the species isolated was calculated.

Experimental studies

Isolation of parasite strains. This was made from 1 of the flocks (flock no 20) showing a low response to treatment with levamisole. Approximately 1 kg of faeces was obtained from 2 lambs with faecal egg counts between 200–500 epg. Faecal cultures for production of L_3 larvae were prepared and harvested as described above. The larvae were stored at 15°C in 20 ml of distilled water in 50 ml flat tissue culture flasks. Faecal cultures yielded infective third stage (L_3) larvae, which were found to be either *Trichostrongylus* spp. or *Ostertagia* spp.

Two lambs, reared under parasite free conditions, were each inoculated intra-ruminally with 10,000 of these larvae. On day 33 post inoculation (p.i.) the lambs were killed and the gastrointestinal tract was removed for worm recovery. The abomasum, the small intestine and the large intestine were examined separately. No worms were recovered in the small or the large intestine, but numerous *O. circumcincta* were isolated from the mucosal surface and the contents of the abomasum. Approximately 200 female worms were sampled randomly from each lamb. The worms were placed on a wire mesh sieve (aperture 212 µm), washed with distilled water and then transferred to a sterile mortar and ground with a pestle, to release eggs from the uteri. This homogenate was mixed with 50 grams of lambs faeces, free of helminth eggs and a faecal culture was then established to produce L_3 larvae. This strain was designated KYSE. An anthelmintic susceptible strain of *O. circumcincta,* designated MIAS, was obtained from Moredun Institute of Animal Science, Edinburgh, United Kingdom. Both strains were used to each infect 2 worm free lambs to build up stocks of L_3 for the *in vivo* controlled study.

In vivo tests for resistance

Twenty two wether lambs of mixed breed reared under parasite free conditions were used for the *in vivo* controlled study (*Powers et al.* 1982) to detect levamisole resistance. According to live weight the lambs were divided into 2 similar groups. In 1 group each lamb was inoculated intra-ruminally with 10,000 L_3 larvae from the KYSE strain. For determination of epg, faecal samples were collected per rectum from all lambs at the day of inoculation (day 0) and hereafter on day 13 and subsequently every third day for 9 occasions.

Based upon the average epg of individual lambs on days 26 and 27 p.i. (mean epg of the two days), each of the two groups of lambs (MIAS and KYSE) were divided into 2 comparable subgroups. At day 27 p.i. 1 subgroup of each category (KYSE-T and MIAS-T) was treated orally with levamisole at the recommended dose rate (7.5 mg/kg bodyweight) and 2 other subgroups were left as untreated controls (KYSE-C and MIAS-C). FECR was calculated at day 33 p.i. (day 6 after treatment) in groups MIAS-T and KYSE-T, respectively. On day 33 p.i. all lambs were killed and the gastrointestinal tract was removed. The abomasum and the small intestine were separated and the large intestine was discarded. The abomasum and the small intestine of each lamb were examined separately. The abomasum was cut open and the contents transferred into a plastic bucket. Using warm tapwater, the mucosa of the abomasum was rinsed thoroughly, and all the washings were collected in the bucket, making a total volume of 5 liters. After a thorough suspension of the abomasal contents, a 500 ml subsample was taken and washed carefully through a wire mesh sieve

(aperture 212 μm), using tapwater. The retentate on the sieve was then transferred to a 250 ml plastic container, and 10 ml of a strong iodine solution was added to preserve the sample. The small intestine was processed likewise.

Before the worms were finally recovered the total volume of the plastic container was again placed on a sieve (aperture 212 μm) and washed, using tapwater, until the washings were almost colorless. Subsamples were examined in a petri dish on a light-board, adding 5–6 drops of a 3 % sodium thiosulphate solution and 10–15 ml of water. The worms were recovered and transferred to a glass tube with 2 % phosphate buffered formaldehyde solution for a later counting, identification and sex differentiation.

In vitro test for resistance

For the *in vitro* egg hatch assay approximately 100 grams of faeces were collected directly from the rectum of lambs infected with either the KYSE or the MIAS strain. The faecal samples were soaked in tap water (approximately 10°C) for 30 min and the entire volume was then mixed to a homogenous slurry. Egg recovery was performed according to the method described by *Whitlock* (1959). The *in vitro* egg hatch paralysis assay was undertaken as described by *Dobson et al.* (1986).

Measurement of pepsinogen concentration in serum

For the determination of the serum concentration of pepsinogen, blood samples were taken from the jugular vein in plain vacutainer tubes, at the day of inoculation (day 0) and again at days 7, 14, 21, 28 and 33 p.i. Blood samples were allowed to rest overnight for clotting and then two 5 ml subsamples of serum were obtained from each sample and then stored at –18°C until examination. The concentration of pepsinogen in serum was determined by a colorimetric method as described by *Ross et al.* (1967).

Calculations and statistical analysis

In the field survey only lambs excreting more than 200 epg were incorporated in the estimation of FECR. The daily average excretion of nematode eggs in faeces and worm numbers in groups of lambs were calculated as geometric means (GM) according to the formula: $GM = \exp[1/n \sum \ln(x+1)] - 1$, where x is epg or worm number of individual lambs in a group. The faecal egg count reduction (FECR) was computed according to the formula:

$FECR = \{1 - [\exp(1/n (\sum ECR))]\} \times 100\ \%$

where the egg count ratio $ECR = \ln(EPG_2 + 1)/(EPG_1 + 1)]$, EPG2 and EPG1 are epg after and before treatment with the anthelmintic of choice. The lower (LC) and the upper confidence limit (UC) of FECR were calculated as follows:

$$LC = [1 - \exp(\frac{\sum ECR}{n}) + ci)] \times 100\ \%$$

$$UC = [1 - \exp(\frac{\sum ECR}{n}) - ci)] \times 100\ \%,$$

where $ci = t_{.05,\,df} \times SD(ECR) \times n^{1/2}$, $t_{.05,\,df}$ is the t-value at 5 % level at df = n–1 degrees of freedom, SD(ECR) is the standard deviation of ECR and n is the number of observations in the group. Worm count reduction (WCR) was calculated according to the formula:

$WCR = [(WN_u - WN_t)tWN_u] \times 100\ \%$

where WN is the group geometric mean of worm numbers and subscript u and t denote the corresponding untreated and treated groups, respectively. The transformed figures of epg and worm numbers of each group of lambs were compared by using Student's t-test.

In the *in vitro* egg hatch assay the percentage of unhatched eggs was transformed to probits for graphical presentation of log dose-probit regression lines and for probit analysis (*Finney* 1971). The concentration of

levamisole, where 50 % of the eggs were killed (LC_{50}) was estimated for eggs from the KYSE and the MIAS strain and the ratio between LC_{50} of the KYSE and the MIAS strain, i.e. the resistance ratio (RR) = $LC_{50, KYSE}/LC_{50, MIAS}$ was calculated.

The pepsinogen levels before treatment in lambs infected with the MIAS and the KYSE isolate were compared by Student's t-test.

Results

The field study consisted of FECR tests in 22 flocks of sheep. In Table 1 are presented

Table 1. Results of a faecal egg count reduction (FECR) test on the efficacy of some anthelmintics tested in 22 flocks of sheep.

Flock number	Drug	No of sheep	Average epg		FECR (%)	Confidence limits (LC-UC)%	Larval identification*			
			EPG_1	EPG_2			T/O (%)	H (%)	Ot (%)	T/O (%)
1	TBZ	27	517	65	88	82–98	93	7		100
2	TBZ	11	223	9	99	95–100	88		12	100
3	TBZ	19	252	37	94	87–97	63	33	3	100
4	TBZ	10	720	0	100	99–100	86		14	
5	FBZ	18	631	0	100	99–100	100			
6	FBZ	4	275	38	90	22–99	96	4		100
7	FBZ	22	267	7	99	98–100	84	8	8	100
8	FBZ	5	280	0	100	97–100	98		2	
9	FBZ	6	233	0	100	98–100	94		6	
10	FBZ	21	600	64	94	89–97	96		4	100
11	ABZ	29	264	0	100	99–100	100			
12	ABZ	12	442	33	97	89–99	100			100
13	ABZ	13	573	0	100	89–100	66	10	24	
14	ABZ	6	492	0	100	99–100	96		4	
15	ABZ	16	222	0	100	99–100	75	10	15	
16	FB	12	404	25	98	94–99	84	16		
17	FB	17	206	21	97	93–97	62	38		100
18	FB	9	261	0	100	98–100	45	55		
19	FB	8	281	0	100	99–100	25	75		
20	LEV	24	288	85	73	55–84	100			100
21	LEV	13	419	69	89	68–96	92		8	100
22	LEV	11	514	41	94	87–97	42	42	16	100

TBZ = thiabendazole 44 mg/kg FB = febantel 5.0 mg/kg
FBZ = fenbendazole 5.0 mg/kg LEV = levamisole 7.5 mg/kg
ABZ = albendazole 3.8 mg/kg

EPG_1 = geometric mean eggs per gram of faeces before treatment
EPG_2 = geometric mean eggs per gram of faeces after treatment
FECR = faecal egg count reduction

LC = lower (95 %) confidence limit of FECR
UC = upper (95 %) confidence limit of FECR

T/O = Trichostrongylus/Ostertagia
H. = Haemonchus spp.
Ot = Other worm species
* = only Trichostrongylus/Ostertagia after treatment

the results of the FECR tests. Benzimidazole anthelmintics were tested on 19 farms and levamisole on 3 farms. Indications of anthelmintic resistance were found on 7 farms (flock no 1, 3, 6, 10, 20, 21 and 22). On 5 of these farms (flock no 1, 3, 6 and 10), where benzimidazoles had been used, FECR varied between 88–94 % and on 3 other farms where levamisole had been used (flock no 20, 21 and 22), FECR was estimated to vary from 73–94 %. Results of the larval differentiation showed, that from samples taken before treatment, larvae of *Ostertagia* spp. *Trichostrongylus* spp., *Haemonchus* spp., *Strongyloides* spp., *Cooperia* spp., *Nematodirus* spp. and *Chabertia* spp. were present, but following treatment only *Ostertagia* spp. or *Trichostrongylus* spp. were found.

The faecal egg excretion in all groups of lambs in the *in vivo* controlled study is depicted in Figure 1. The egg output in the MIAS-T group of lambs was reduced to zero, whereas the reduction in the egg output of the KYSE group of lambs was calculated to be only 44.5 %. It is noted, that the egg excretion in all groups show a uniform pattern and that lambs infected with the

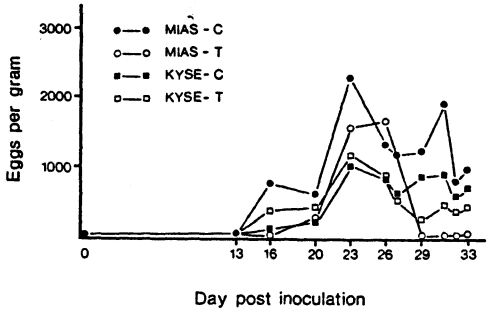

Figure 1. Geometric mean egg counts of lambs experimentally infected with either a susceptible (MIAS) or a levamisole resistant strain (KYSE) of *Ostertagia circumcincta,* untreated or treated (MIAS-C, KYSE-C or MIAS-T, KYSE-T) with levamisole at the recommended dose rate (7.5 mg/kg).

Table 2. Number of recovered worms in lambs experimentally infected with a levamisole susceptible (MIAS) or a levamisole resistant strain (KYSE) of *Ostertagia circumcincta,* and average worm burdens and worm count reduction (WCR) in groups of lambs untreated or treated (MIAS-C, KYSE-C or MIAS-T, KYSE-T) with levamisole at the recommended dose rate (7.5 mg/kg).

		Total number of *Ostertagia circumcincta*		
	Sheep no	Males	Females	Sum
MIAS-C	72	1880	1950	3830
	7002	2540	3270	5810
	7023	2870	2690	5560
	7027	2330	3530	5860
	7097	1160	1500	2660
GM		2059	2464	4540
MIAS-T	7003	0	0	0
	7005	0	0	0
	7012	0	0	0
	7015	0	0	0
	7146	120	190	310
	7162	0	0	0
GM		2	2	3
WCR (%)		99.9	99.9	99.9
KYSE-C	7006	2480	2490	4970
	7019	3350	3890	7240
	7043	3560	3610	7170
	7076	4190	4430	8620
	7115	1710	1860	3570
GM		2919	3104	6025
KYSE-T	7065	720	870	1590
	7092	940	1070	2010
	7112	510	540	1050
	7114	720	820	1540
	7120	1420	1530	2950
	7139	1580	1960	3540
GM		907	1036	1943
WCR (%)		68.9	66.6	67.7

GM = geometric mean worm numbers
WCR = worm count reduction

MIAS strain before treatment shed significantly ($p < 0.05$) more eggs than did lambs infected with the KYSE-strain.

The results of the worm counts are presented in Table 2. In the suspected levamisole resistant KYSE-strain, worm numbers were reduced by 67.7 % ($p < 0.005$) and in the MIAS-strain worm numbers were reduced by 99.9 % after treatment with levamisole at the recommended dose rate. In general, higher numbers of female than male worms were recovered in groups MIAS-C, KYSE-C and KYSE-T, but this difference was not significant ($p < 0.05$). No immature worms were detected in any experimental animal.

The results of the *in vitro* egg hatch assay are depicted in Figure 2. The LC_{50} of the KYSE and the MIAS population of eggs was estimated to be 0.04 and 6.05 µg LEV/ml, respectively and the resistance ratio was estimated to be 151.25.

The average concentration of pepsinogen in serum in the 4 groups is reflected in Figure 3. The pepsinogen values increased during the pre-patent period to a level below 1.0 tyrosine unit/L. After treatment of groups MIAS-T and KYSE-T with levamisole the level of pepsinogen in the MIAS-T group dropped significantly ($p < 0.01$) to a low level whereas the level of pepsinogen in the KYSE-T group stayed at the same level as

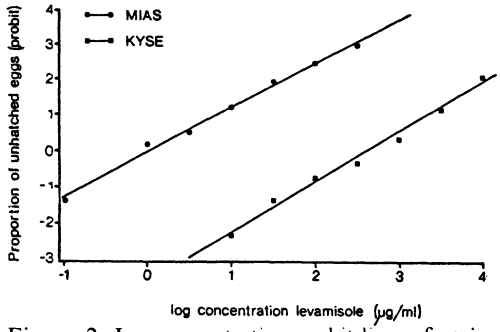

Figure 2. Log concentration probit lines of an *in vitro* egg hatch paralysis assay on eggs from a levamisole susceptible (MIAS) or levamisole resistant strain (KYSE) of *Ostertagia circumcincta.*

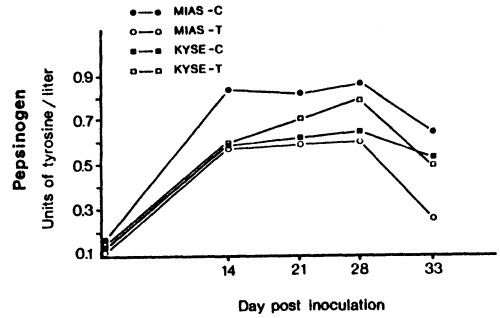

Figure 3. Aritmetic mean serum pepsinogen concentrations in groups of lambs experimentally infected with either a levamisole susceptible (MIAS) or levamisole resistant strain (KYSE) or *Ostertagia circumcincta,* untreated or treated (MIAS-C, KYSE-C or MIAS-T, KYSE-T) with levamisole at the recommended dose rate (7.5 mg/kg).

the KYSE-C group ($p > 0.05$).

The rate of establishment of the MIAS and the KYSE strain of *O. circumcincta* was estimated to 63.1 % and 47.4 %, respectively but the difference was not significant ($p > 0.05$).

Discussion

The results of the FECR tests in 22 selected sheep farms in Denmark showed, that anthelmintic resistance was indicated in 7 flocks. These flocks were selected on the basis of a previously high frequency of anthelmintic treatment and the use of the same anthelmintic for at least 5 years. These 2 factors are known to pose a risk to the development of anthelmintic resistance (*Donald* 1983). Other factors, like drenching practices and management of the sheep may have contributed to the development of resistance in these flocks. For example, in the 22 flocks selected for study, high stocking rates and intensive grazing systems, often with rotational grazing, were practiced. In the 8 flocks where anthelmintic resistance was indicated, at least 2 of the anthelmintic treatments were given from late May to the

beginning of August. Theoretically this increases the risk of selection for anthelmintic resistance because the free-living proportion (refugia) of the entire nematode population is at its lowest level relative to the proportion of worms within the host at that time (*Martin 1986, Bjørn* 1988). A factor which may further have influenced the rate of development of resistance against levamisole in flock no 20, tested by the *in vivo* controlled study, was a combination of anthelmintic treatment with move to a "clean" pasture, a procedure that had been practiced for more than 5 years in this flock. In this situation eggs deposited on the clean pasture will primarily be the progeny of more resistant worms, surviving the treatment. The drench and move practice has earlier been implicated in the development of resistance in sheep nematodes in United Kingdom (*Cawthorne & Whitehead* 1983).

Isolation and examination of the KYSE-population of the above flocks in a *in vivo* controlled study confirmed the initial observations made in the field. The results from the FECR-test, the *in vivo* study and the *in vitro* egg hatch assay were consistent and established a significant high level of resistance against levamisole in *O. circumcincta.* These observations were further supported by the effect of levamisole treatment on serum pepsinogen levels in the experimental groups. No significant difference in susceptibility of male and female worms to levamisole could be detected in the treated groups, which is in contrast to observations by others (*Dash* 1986, *Bjørn et al.* 1989) who found that male worms were significantly more susceptible to anthelmintics than were females in both anthelmintic resistant and susceptible isolates.

Our studies have confirmed the presence of levamisole resistance in *Ostertagia circumcincta* in Danish sheep. In addition comparable levels of resistance to the benzimidazoles are evident. The sheep farms in this study were not selected randomly and the results of this survey therefore do not give a true impression on how widespread anthelmintic resistance may be at sheep farms in Denmark. To get unbiased information on this subject a large scale and comprehensive survey as outlined in this paper is needed.

At this stage anthelmintic resistance does not seem to be a problem in the control of gastrointestinal nematodes of sheep in Denmark. This may be explained by the in general low frequency of treatments and the general adherence by farmers of using the correct dose of anthelmintic (*Bjørn* unpublished observations). Further, the level of nematodiasis in sheep in this study seems low and the species found generally do not cause clinical disease, unless present in considerable numbers. This precludes the observation of apparent control failure and detection of anthelmintic resistance in its early stages. Nevertheless, it would be advisable to continuously monitor the susceptibility of prevalent parasites of sheep by e.g. FECR tests, in order to detect any changes with time.

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Sammendrag

Ormemiddelresistens hos fårets parasitiske nematoder i Danmark med særlig vægt på levamisol resistens hos Ostertagia circumcincta.

Formålet med denne undersøgelse var at belyse forekomst af ormemiddelresistens hos fårets indvoldsorm i Danmark. Toogtyve fårebesætninger udvalgtes til en feltundersøgelse over ormemiddelresistens under anvendelse af en fækal æg reduktions test (eng. Faecal Egg Count Reduction (FECR) Test). Kriterierne for udvælgelse af besætningerne var, at samme ormemiddel eller ormemidler hørende til samme virkningsgruppe havde været anvendt til mindst 3 årlige behandlinger over en 5-årig periode. Der fandtes indikation på ormemiddelresistens i 7 besætninger. Efter behandling med thiabendazol fandtes FECR i to besætninger til henholdsvis 88 % og 94 %, i andre 2 besætninger var FECR 90 % og 94 % efter behandling med fenbendazol og i 3 besætninger fandtes FECR til 73 %, 89 % og 94 % ved behandling med levamisol. Fra 1 af de sidstnævnte besætninger isoleredes en population af *Ostertagia circumcincta,* som underkastedes en *in vivo* kontrolleret undersøgelse over resistens mod levamisol. Ved behandling af denne population med levamisol (7.5 mg/kg) fandtes FECR til 44.5 % og en reduktion i ormebyrden på 67.7 %, mens både ægudskillelse og ormeantallet reduceredes med 100 % i en tilsvarende anthelmintisk følsom population. Resultaterne understøttedes af en *in vitro* egg hatch paralysis test samt af målinger af serumpepsinogen. Dette er det første tilfælde af ormemiddelresistens hos fårets indvoldsorm i Skandinavien.

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