

AUS DEM DEPARTMENT FÜR PATHOBIOLOGIE
DER VETERINÄRMEDIZINISCHEN UNIVERSITÄT WIEN
(DEPARTMENTSPECHER: UNIV.PROF.DR.RER.NAT. ARMIN SAALMÜLLER)

INSTITUT FÜR PARASITOLOGIE
(LEITERIN: UNIV.PROF.DR.MED.VET. ANJA JOACHIM)

**THE THREAT OF REDUCED EFFICACY OF ANTHELMINTICS AGAINST
GASTROINTESTINAL NEMATODES IN SHEEP FROM AN AREA CONSIDERED
ANTHELMINTIC RESISTANCE-FREE**

DIPLOMARBEIT

VETERINÄRMEDIZINISCHE UNIVERSITÄT WIEN

VORGELEGT VON
JAN KIEßLER

WIEN, NOVEMBER 2020

Betreuerinnen: In Wien: Dr.med.vet. Barbara Hinney

In Neapel: Prof. Laura Rinaldi

Begutachter: Priv. Doz. Dr. Johannes Lorenz Khol

INHALTSVERZEICHNIS

1 Einführung.....	1
2 Angaben zum Eigenanteil.....	3
2.1 Versuchsteil.....	3
2.2 Manuskript.....	3
3 Mitteilung zur Annahme der Arbeit.....	3
4 Publikation.....	4
4.1 Abstract.....	4
4.2 Background.....	5
4.3 Methods.....	7
4.3.1 Study area.....	7
4.3.2 Study farms and animals.....	7
4.3.3 Laboratory procedures.....	8
4.3.4 FECRT.....	8
4.3.5 Coprocultures.....	8
4.3.6 Follow-up study.....	9
4.3.7 Egg hatch test.....	9
4.3.8 Statistical analysis.....	10
4.4 Results.....	11
4.4.1 History of parasite management on farms.....	11
4.4.2 FECRT.....	11
4.4.3 Coprocultures.....	12
4.4.4 Egg hatch test.....	12
4.5 Discussion.....	13
4.6 Conclusions.....	16
4.7 Abbreviations.....	16
4.8 Acknowledgements and final declaration.....	17
4.9 References.....	19
4.10 List of figures.....	25
4.11 List of tables.....	30

5 Diskussion.....	34
6 Fazit.....	39
7 Zusammenfassung.....	40
8 Summary.....	41
9 Literaturverzeichnis (für Einführung, Diskussion, Fazit).....	42
Anhang 1: Nachweis zur Annahme des Manuskripts.....	48
Danksagung.....	49

1 Einführung

Infektionen mit Magen-Darm-Strongyliden (MDS) beeinträchtigen die Herdengesundheit, sowie das Wohlbefinden von Schafen und sind mit wirtschaftlichen Verlusten assoziiert, hauptsächlich als Folge subklinischer Infektionen, die sich vordergründig durch Gewichtsverluste und verringerte Milchproduktion manifestieren (Charlier et al. 2020, Glover et al. 2017, Mavrot et al. 2015, Mavrot 2016). MDS werden als endemisch betrachtet und es bestehen keine gesetzlichen Auflagen, die die Behandlung und Prävention von MDS-Infektionen vorschreiben bzw. den Viehhandel reglementieren. Aus diesem Grund liegt die Verantwortung des Entwurmungsmanagements größtenteils in den Händen der LandwirtInnen und TierärztInnen (Charlier et al. 2020).

Die effektive und nachhaltige Bekämpfung von MDS-Infektionen stellt TierärztInnen und LandwirtInnen vor eine große Herausforderung, da sie hauptsächlich von wirksamen Entwurmungsmitteln abhängig ist. Die unsachgemäße Verabreichung von Anthelmintika (z.B. durch Applikationsfehler, Unterdosierung, übermäßigen Einsatz) hat die Entwicklung von Resistenzen beschleunigt, deren Vorkommen nun weltweit in zahlreichen MDS-Spezies gemeldet wird (Furgasa et al. 2018, Kalkal 2020, Rose et al. 2015, Salgado und Santos 2016). Insbesondere in MDS von Schafen sind multiple Resistenzen gegen verschiedene Anthelmintika-Klassen beschrieben, z.B. gegen Benzimidazole und makrozyklische Laktone (Charlier et al. 2018, Glover et al. 2017, Kaplan und Vidyashankar 2012, Rose et al. 2015, Sangster et al. 2018). Aufgrund der damit verbundenen hohen wirtschaftlichen Einbußen, besteht allgemein ein großes Interesse daran, die Methodik der Früherkennung von Anthelmintika-Resistenzen (AR) auf Basis aktiver Überwachung auszubauen und pragmatisch anwendbar zu gestalten, um frühzeitig auf die Entstehung von AR reagieren zu können (Charlier et al. 2018, Charlier et al. 2020, Coles et al. 2006).

In Italien, hauptsächlich in nördlichen und zentralen Regionen, gibt es nur wenige Berichte über das Vorkommen von AR in Schafen, darunter AR gegen Levamisol, Ivermectin und Benzimidazole (Geurden et al. 2014, Lambertz et al. 2019, Traversa et al. 2007).

In Süditalien scheinen konkrete Maßnahmen sich positiv auf die Sicherung der Wirksamkeit von Antiparasitika auszuwirken und die Entstehung von Resistenzen zu verlangsamen. Dazu

gehören die Überwachung von MDS-Infektionen durch regelmäßige Kotuntersuchungen, die Anwendung von „targeted treatment“ (TT), die abwechselnde Anwendung verschiedener Anthelmintika, sowie deren korrekte Applikation und ein geringer Tierwechsel zwischen Schafbetrieben (Cringoli et al. 2008, Rinaldi et al. 2019, Rinaldi, Morgan et al. 2014).

Dennoch ist die Bildung von AR unausweichlich und deren Auftreten nur eine Frage der Zeit. Daher ist die erste Hypothese der vorliegenden Arbeit, dass auf mindestens einem der untersuchten Schafbetriebe verminderte Wirksamkeit einer Anthelmintika-Klasse detektiert wird. Das Ziel dieser Studie ist die Ermittlung der derzeitigen Resistenzlage gegen Benzimidazole und makrozyklische Laktone in Süditalien auf Grundlage eines standardisierten Eizahlreduktionstests (EZRT), der sich in der Durchführung an den aktuellen Richtlinien der Europäischen COST Action “COMBAR – COMBatting Anthelmintic Resistance in Ruminants” (<https://www.combar-ca.eu>) orientiert.

Der EZRT wurde unter Verwendung von Mini-FLOTAC (Cringoli et al. 2017) für Einzel- und Sammelkotproben durchgeführt (Kenyon et al. 2016, Rinaldi, Levecke et al. 2014). Mini-FLOTAC ist eine in der Anwendung unkomplizierte, dennoch sensible und genaue diagnostische Methode für den EZRT, welche in Kombination mit *in-vitro*-Tests und molekularbiologischen Methoden das Potenzial bietet, AR präzise zu detektieren (Paras et al. 2018).

Weiters wurde in verdächtigen Schafbetrieben, in denen im Rahmen des EZRT eine verminderte Wirksamkeit für applizierte Anthelmintika festgestellt worden ist, ein *in-vitro* Larven-Schlupf-Hemmtest (Egg hatch test, EHT) und ein *in-vivo* Folgeversuch zur Bestätigung der Befunde durchgeführt. Als zweite Hypothese wird angenommen, dass im *in-vitro* EHT und im *in-vivo* Folgeversuch Funde verminderter Anthelmintika-Wirksamkeit aus dem Hauptversuch bestätigt werden.

Der Versuchsaufbau dieser Studie entspricht aktuellen Empfehlungen (Levecke et al. 2018) und setzt ein Zeichen für einen standardisierten Ansatz zur Ermittlung der Wirksamkeit von Anthelmintika und Resistenzen in Wiederkäuern mit Weidegang.

Dieses Projekt wurde im Rahmen von COMBAR während eines ERASMUS-Aufenthaltes im parasitologischen Institut CREMOPAR – Centro REgionale per il MONitoraggio delle PARassitosi, umgesetzt.

2 Angaben zum Eigenanteil

2.1 Versuchsteil

- Hauptversuch: Rektale Kotprobenentnahmen an Tag 0 und Tag 14, sowie weitere Kotprobennahme auf Betrieb 1 für EHT, Tieridentifikation und Probenzuordnung, Behandlung der Schafe mit Anthelmintika an Tag 0.
- Laborarbeit: Ansatz der gesättigten Flotationslösung, Verarbeitung und Analyse von Einzel- und Sammelkotproben mit Mini-FLOTAC (Eiauszählungen, EZ), Mitarbeit am Ansatz des EHT für Betrieb 1, Verarbeitung von Koprokulturen bis zum Schritt der Larvenextraktion.
- Datenverarbeitung: Dokumentation der EZ für Einzel- und Sammelkotproben in Excel, Kalkulation der EZRT für Einzel- und Sammelkotproben, Berechnung der Konfidenzintervalle mit Hilfe von 'eggCounts-2.3' der R Version 3.6.1.
[<http://shiny.math.uzh.ch/user/furrer/shinyas/shiny-eggCounts/> (Zugriff 20.08.2019)]

2.2 Manuskript

- Hauptverantwortlich für 'Abstract, graphical Abstract (Figure 1), Discussion, Conclusion' und Anteile in 'Background'.

3 Mitteilung zur Annahme der Arbeit

Das Manuskript wurde am 04. April 2020 bei *Parasites & Vectors* eingereicht, am 30. August 2020 akzeptiert und am 09. September 2020 publiziert. (Anhang 1)

4 Publikation

The threat of reduced efficacy of anthelmintics against gastrointestinal nematodes in sheep from an area considered anthelmintic resistance-free

Antonio Bosco¹, Jan Kießler², Alessandra Amadesi¹, Marian Varady³, Barbara Hinney², Davide Ianniello¹, Maria Paola Maurelli¹, Giuseppe Cringoli¹, Laura Rinaldi¹

¹ *Department of Veterinary Medicine and Animal Production, University of Naples Federico II, CREMOPAR, Naples, Italy*

² *Institute of Parasitology, Department of Pathobiology, University of Veterinary Medicine Vienna, 1210 Vienna, Austria*

³ *Institute of Parasitology of Slovak Academy of Sciences, 040 01 Košice, Slovakia*

4.1 Abstract

Background: The worldwide increased difficulty to combat gastrointestinal nematode (GIN) infection in sheep, due to progressing anthelmintic resistance (AR), calls for an enhanced and standardized implementation of early detection of AR. This study provides a snapshot of the current AR status against benzimidazoles and macrocyclic lactones in southern Italy, generated with standardized techniques.

Methods: On 10 sheep farms, the efficacy of albendazole (ALB) and either eprinomectin (EPR) or ivermectin (IVM) was evaluated based on the faecal egg count reduction test (FECRT) performed with the Mini-FLOTAC. For each tested drug, 40 sheep were rectally sampled at D0 and sampled again 14 days after the treatment (D14). The FECRT was calculated from individual samples and pooled samples which consist of 5 individual samples. Efficacy was classified as ‘reduced’, ‘suspected’ and ‘normal’. Coprocultures were set for D0 and D14 faecal samples of each group. From farms with FECR<95%, an *in vitro* egg hatch test (EHT) and a follow-up FECRT using fenbendazole (FBZ) were conducted.

Results: Based on the FECR, high efficacy (from 95.7% to 100%) was observed for ALB and IVM in eight farms (Farms 3-10). On Farm 1 and Farm 2, the efficacy for the macrocyclic lactones was classified as ‘normal’, but ‘reduced’ efficacy was observed for ALB on Farm 1 (FECR = 75%) and ‘suspected’ efficacy on Farm 2 (FECR = 93.3%) with the predominant GIN genus *Trichostrongylus* following by *Haemonchus* at D14. The FEC results of pooled samples strongly correlated with those of individual samples, for FEC at D0 ($r_s=0.984$; $P < 0.0001$) and at D14 ($r_s= 0.913$; $P < 0.0001$). The classifications of efficacy in Farm 1 (FECR = 86.0%) and Farm 2 (FECR = 93.0%) in the follow-up FECRT with FBZ coincide with the main FECRT trial. The *in vitro* EHT confirmed the resistance in both farms (Farm 1 = 89%; Farm 2 = 74%).

Conclusions: In regions like southern Italy, where the negative impacts from AR have played a minor role, efficient monitoring of AR is important in order to evaluate potential risks and being able to promptly respond with countermeasures.

Keywords: Sheep, Gastrointestinal nematodes, Anthelmintic resistance, Pooling faecal samples, Faecal egg count reduction test, Egg hatch test.

4.2 Background

Gastrointestinal nematode (GIN) infection endangers livestock health and welfare and is commonly associated with economic losses mostly through subclinical diseases impairing weight gain and milk yields [1–4]. GIN parasites are considered endemic and have no major regulatory or trade implications, thus their control has largely remained the responsibility of the farmers and/or the veterinarians [4].

Management and control of GIN infections is a challenging task and currently dependent almost only on efficient anthelmintic drugs. However, the improper use (over- and mis-use) of anthelmintics has led to development of anthelmintic resistance (AR) which is now reported worldwide in multiple nematode species, especially in sheep, against multiple anthelmintic classes, e.g. benzimidazoles (BZ) and macrocyclic lactones (ML) ([3, 5–8], Rose et al., in preparation).

Due to the costs of anthelmintic-resistant nematode infections, there is a wide consensus on the need to enhance and implement early detection of AR based on active monitoring of the efficacy of anthelmintics in order to promptly respond to the development of AR. There are

indications that some actions are able to slow down the development and spread of AR, e.g. promote “best practice” parasite management programmes based on sustainable use of anthelmintics through targeted treatment (TT) and targeted selective treatment (TST) based on easy-to-use diagnostics to inform treatment decisions [7, 9].

In Italy, few reports of AR in sheep against levamisole, ivermectin and benzimidazoles have been published but mainly in northern and central regions [10–12]. On the contrary, in southern Italy some concrete actions appear to be effective in maintaining the efficacy of anthelmintics and slowing the development of AR, e.g. the monitoring of GIN infection in sheep and other livestock by regular diagnosis, use of targeted treatments, rotation of different drugs, correct drenching, and low movement of animals between farms [13–15]. However, given that the development of AR is inevitable and its occurrence is not a matter of “if” but “when” (R. Kaplan, personal communication), the aim of this study was to investigate the current levels of efficacy of benzimidazoles and macrocyclic lactones in sheep in the Campania region (southern Italy) by performing a standardized survey by the faecal egg count reduction test (FECRT) in accordance with the guidelines established in the framework of the European COST Action “COMBAR - COMBatting Anthelmintic Resistance in Ruminants” (<https://www.combar-ca.eu>).

In particular, the FECRT was performed by the Mini-FLOTAC technique [16] on individual and pooled samples [17, 18]. Mini-FLOTAC is an easy-to-use yet sensitive and accurate diagnostic method for FECRT which, complemented with *in vitro* and molecular tools, is able to give a precise measure of AR [19]. In the farms where BZ resistance was suspected (i.e. reduced efficacy of the drug), confirmation was done by *in vitro* tests and a follow-up *in vivo* trial.

In doing so, the study met current recommendations in the experimental setup [20] and made a step towards a standardized approach in evaluating anthelmintic efficacy and AR in grazing ruminants.

4.3 Methods

4.3.1 Study area

The study was conducted in the Campania region of southern Italy where the climate is characterized by dry summers and rainy autumns/winters. The area is mainly used for cereal production but small pastures occur on upland areas that are unsuitable for cropping. Small ruminant production systems are a major component of the dairy and meat sector in region and each sheep farm is approximately 50 ha.

4.3.2 Study farms and animals

Trials were conducted between July and October 2019 on 10 sheep farms. Dairy sheep farms were selected throughout the region and the selection was mainly driven by the availability of the farmers, the presence of GIN positive sheep. The average flock size of the selected ten farms was of 250 sheep (range 100–700 animals). All farmers bred the sheep for milk production. Three flocks were composed of Lacaune mixed-breed dairy sheep, five of Bagnolese mixed-breed dairy sheep and two Lacaune/Bagnolese/Sarda/Comisana mixed-breed dairy sheep. Animals of all farms had access to pasture for the entire year. All farmers conducted whole-flock anthelmintic treatments twice per year, first during the dry-off period i.e. in the peripartum period (October / November or February / March) and second in May/June.

In each farm the animals were divided into 2 groups of 40 sheep randomly chosen, one group treated with macrocyclic lactones (ivermectin/eprinomectin) and one group with benzimidazoles (albendazole), without using an untreated control group [21]. On each farm the enrolled animals were individually weighed and the correct dose of drugs was administered using an appropriate equipment, calibrated to deliver the dose accurately. Specifically, in Farm 1, 40 sheep were treated with an oral suspension of albendazole (ALB, Valbazen® Zoetis, Rome, Italy; 3.8 mg/kg of body weight) and 40 with a pour on solution of eprinomectin (EPR, Eprinex Multi® Boehringer Ingelheim Animal Health, Milan, Italy; 1.0 mg/kg of body weight). In the other 9 farms 40 sheep were treated with ALB (ALB, Valbazen® Zoetis; 3.8 mg/kg of body weight) and 40 with an oral solution of ivermectin (IVM, Oramec® Boehringer Ingelheim Animal Health; 0.2 mg/kg of body weight).

Individual faecal samples were collected rectally on the day of treatment (D0) and after 14 days (D14), stored shortly thereafter at 4°C and further laboratory processed as individual

and pooled samples as soon as possible. The number of farms, individual and pooled faecal samples used in this study are provided in Fig. 1.

4.3.3 Laboratory procedures

At D0 and D14, the ovine faecal samples were analysed both individually and as pooled samples using the Mini-FLOTAC technique with a detection limit of 5 eggs per gram (EPG) of faeces, using a sodium chloride flotation solution (FS2, specific gravity=1.200) [16]. The pool size consisted of 5 individual samples according to the protocol described in Rinaldi et al. [17]. Each sample was labelled, thoroughly homogenized, individually examined and then composite (pooled) samples were prepared taking 5 g of each sample with the collector of the Fill-FLOTAC. It should be noted that the predefined pool sizes of 5 could not always be met at D14 due to insufficient amount of faeces to perform the analysis of each pool.

4.3.4 FECRT

When examining individual samples and pooled samples, the FECR (%) and 95% CI were calculated using the “eggCounts-2.3” on R version 3.6.1. [22] considering individual FECs before and after treatment (two paired samples) for each group, correction factor of 5 (Mini-FLOTAC analytical sensitivity) and no zero inflation.

Drug efficacy was classified as ‘reduced’ when $\text{FECR} < 95\%$ and the lower limit (LL) of the 95% confidence interval (CI) $< 90\%$, as ‘suspected’ when either $\text{FECR} < 95\%$ or $\text{LL} < 90\%$ and as ‘normal’ when $\text{FECR} \geq 95\%$ and $\text{LL} \geq 90\%$ [21, 23].

4.3.5 Coprocoltures

Before the storage at 4°C, the same quantity of faeces was collected from each sample to create a pool for each faecal culture group at D0 and D14, following the protocol described by the Ministry of Agriculture, Fisheries and Food [24]. Developed third-stage larvae (L3) were identified using the morphological keys proposed by van Wyk & Mayhew [25]. Identification and percentages of each nematode genera were conducted on 100 L3; if a sample had 100 or less L3 present, all larvae were identified. Thus, on the total number of larvae identified, it was possible to give the percentage of each genus.

4.3.6 Follow-up study

The farms, on which benzimidazole efficacy has been classified as ‘reduced’ (Farm 1) and as ‘suspected’ (Farm 2) through the *in vivo* trial (see the Results section), were revisited after a period of 2 months. Faecal samples were sampled as a follow-up to the *in vivo* study. The follow-up samples were taken from 20 individual sheep randomly chosen from each farm. The sheep were treated with a drug of the same anthelmintic class (side resistance) that showed low efficacy (fenbendazole-FBZ was used instead of ALB). Fourteen days after drug application, the same group of 20 animals were sampled a second time by collecting the faeces from the rectum. All samples were handled as described previously in the laboratory procedures.

4.3.7 Egg hatch test

To confirm the results of the FECRT an *in vitro* assay, i.e. the egg hatch test (EHT), was performed in Farm 1 and Farm 2 where BZ resistance was suspected (see the Results section). GIN eggs were recovered from follow-up samples collected directly from the rectal ampulla of 30–40 sheep on each farm. The faecal samples were processed within 2h of collection by using the egg recovery technique as described by Coles et al. [23] with some modifications.

Firstly, faecal samples were homogenized and filtered under running water through sieves with a mesh size of 125, 63 and 38 μm (CISA Sieving Technologies, Barcelona, Spain) in order to separate the eggs from the faeces. Next, the GIN eggs retained on the last sieve were washed and centrifuged for 3 min at $170\times g$ with distilled water, after which the supernatant was discarded. In the end, centrifugation was performed using 40% sugar solution to float the eggs which are then isolated in new tubes, mixed with distilled water and then centrifuged two more times in order to remove pellets and to get aqueous solution with eggs.

Eggs were inspected microscopically to record if embryonation had not begun. Each sample was at least tested in duplicate and at least two negative control samples were used (eggs with DMSO). A stock solution of thiabendazole (TBZ) was prepared by dissolving the pure compound in dimethyl sulfoxide (DMSO) and following the protocol described by [26].

The final concentrations in the EHT were prepared by adding 10 μl of each TBZ solution into 1.99 ml of a suspension with approximately 150 eggs/ml in water. The final TBZ concentrations used were 0.5, 0.3, 0.2, 0.1, 0.05, 0.025 and 0.01 $\mu\text{g/ml}$. A control (0.5% DMSO) without anthelmintic was also included in the test. The 24-well tissue culture test plates, (Corning

Incorporated, Life sciences, Salt Lake City, USA) were incubated for 48 h at 25°C. The incubation was then terminated by adding 10 µl of Lugol's iodine solution to each well. After 48 h, at least 100 eggs (dead, embryonated) and hatched first-stage larvae in each well were counted. The test was performed with two replicates.

4.3.8 Statistical analysis

The statistical analysis was performed with the *egg-Counts*-package in R [22]. The paired model was used to calculate FECR and 95% confidence intervals (95% CI) using individual FECs before and after treatments for each single group.

The arithmetic mean FEC of individual and pooled samples were calculated. Correlations between the different measures of FEC were assessed by Spearman's rho correlation coefficient (r_s), the associated 95% CI and P-value. Moreover, Lin's concordance correlation coefficients (CCC) and the corresponding 95% CI were calculated to quantify the agreement between the analysis from the FEC of individual samples and the FEC of pooled samples. Spearman's r_s and Lin's CCC were calculated as above between FECR (%) from individual and pooled samples. Like a correlation, CCC ranges from -1 to 1, with perfect agreement at 1. The strength of agreement was classified as poor, moderate, substantial or almost perfect for CCC values < 0.9, 0.90–0.95, 0.95–0.99 and > 0.99, respectively [27].

A four-parameter logistic equation with a variable slope was chosen for the statistical analysis of *in vitro* test results. All analyses were performed after transforming the TBZ concentrations into its logarithm ($X = \log X$) and defining the bottom value 0. The EHT protocol recommended as method for the detection of BZ resistance with cut off of > 0.1 µg TBZ per ml for 50% egg hatch inhibition (EC₅₀) [23].

4.4 Results

4.4.1 History of parasite management on farms

Only two farms (Farm1 and Farm 2), having had a known history of fasciolosis [28], used a strategic schemes of anthelmintic treatments based only on the use of ALB (four times a year in February/March, May/June, August/September and November/December) over six years to reduce the prevalence and intensity of *Fasciola hepatica* to a level, at which there were no longer any clinical symptoms of the disease.

Seven farmers used to practice regular parasitological monitoring before the anthelmintic treatment and all performed a rotation of anthelmintics using benzimidazoles and macrocyclic lactones once per year; one class was used in spring, the other class in autumn and *viceversa*. Only four farmers used coprological analysis for efficacy control.

4.4.2 FECRT

The frequency of FEC distribution of individual samples at D0, described in Fig. 2, showed that 70% of animals are responsible for shedding GIN eggs from 1 to 600 EPG while 30% of the animals are responsible for shedding most of GIN eggs (from 601 to 35,520 EPG). Furthermore, the mean EPG values and the standard error for each farm involved in this study, described in Fig. 3, showed a great variability among the different farms used in this study, with a mean of GIN EPG per farm from 188 to 3590.

The efficacies of the anthelmintic treatments are given in Table 1. Very high efficacy was obtained with the two classes of anthelmintics tested in 8 farms as follows (farm average FECR, min and max): IVM 99.1% (95.7–100%) and ALB 99.4% (97.4–100%). On all 8 farms lower confidence limits (LL) were generally high and always above 95.1% for IVM and 98.0% for ALB.

In Farm 1 and Farm 2 two macrocyclic lactones used (EPR and IVM) showed an efficacy of 97.9 % and 99.9 %, respectively. While on both farms a low efficacy of ALB was observed (in Farm 1 of 75.0% with LL of 71.2% and in Farm 2 of 93.3% with LL of 93.0%) and AR was present in Farm 1 (reduced) and suspected in Farm 2. In addition, the follow-up trial using FBZ also showed low efficacy of benzimidazoles in both farms (Table 2).

When calculated from individual samples, the GIN FEC mean values at D0 ranged from 7 to 10,819 EPG and at D14 ranged from 0 to 261 EPG, whilst when calculated from pooled

samples the GIN FEC mean values at D0 ranged from 5 to 9,485 EPG and at D14 ranged from 0 to 165 EPG. The correlation between FEC results from individual means and pool means is reported in Fig. 4. When considering results separately for D0 or D14, the FEC results of pooled samples strongly correlated with those of individual samples, for FEC at D0 ($r_s = 0.984$, 95% CI = 0.978–0.987, $P < 0.0001$) and at D14 ($r_s = 0.913$, 95% CI: 0.879–0.937, $P < 0.0001$). The level of agreement between the FEC from individual and pool means was substantial for FEC at D0 (CCC = 0.973, 95% CI: 0.963–0.980) and poor for FEC at D14 (CCC = 0.873, 95% CI: 0.830–0.905). Overall, when considering results including FEC at D0 and FEC at D14 results showed a high correlation ($r_s = 0.987$, 95% CI: 0.984–0.990, $P < 0.0001$) and a substantial level of agreement (CCC = 0.978, 95% CI: 0.972–0.982, $P < 0.001$).

The FECR (%) considering individual samples ranged between 75 and 100%, whilst considering pooled samples ranged between 73.7 and 100% and the correlation between FECRs resulting from individual and pooled samples showed an high r_s value ($r_s = 0.924$, 95% CI: 0.809–0.976, $P < 0.0001$) and a substantial CCC value (CCC = 0.995, 95% CI: 0.989–0.998).

4.4.3 Coprocultures

The genera of nematodes present (minimum and maximum percentages in each treatment group) at D0 were: *Haemonchus* (21–83%), *Trichostrongylus* (2–59%), *Teladorsagia* (0–25%), *Chabertia* (0–48%) and *Cooperia* (0–5%), whilst very few numbers of nematode third-stage larvae were found at D14 on groups of other farms (Table 3) at D14. In the two farms where AR for ALB was suspected, the following GIN genera were detected: *Haemonchus* (7–29%) and *Trichostrongylus* (93–71%).

4.4.4 Egg hatch test

The hatching in the discriminating dose of 0.1 µg TBZ/ml were 89% and 74% in Farm 1 and Farm 2, respectively (Table 4). The EC_{50} value was 0.59 µg/ml for Farm 1 and 0.75 µg/ml for Farm 2.

4.5 Discussion

Currently, control of GIN still relies heavily on regular treatments with anthelmintic drugs, but worm control is threatened by AR [6]. To enable sustainable parasite control in the future, it is important to use the available anthelmintics prudently. This study was undertaken to provide an evidence-based and a state-of-the-art picture of the AR situation for benzimidazoles and macrocyclic lactones in the Campania region (southern Italy) in ten dairy sheep farms chosen in the study area. Levamisole was not investigated in this study since no drugs are available in Italy containing only this molecule to control infections by gastrointestinal nematodes in sheep.

The distribution of egg counts between different animals of the same farm is well known to be overdispersed among hosts [29–31]. In particular, our study showed that a range from 1 to 600 EPG was seen in 70% of the sheep (Fig. 2). At animal level, our findings showed that a small number of sheep is responsible for shedding the majority of GIN eggs into the environment (range from 601 to 35,520 EPG). Such levels of overdispersion, likely due to multiple factors (e.g. based on parasite biology/epidemiology, farm management, treatment strategies, etc.), may provide an opportunity for using targeted selective treatments (TST) based on FEC [32].

On each farm, both classes of anthelmintics were tested on group sizes of 40 sheep/drug performing the FECRT according to pre- and post-treatment using the sensitive and accurate Mini-FLOTAC technique (detection limit = 5 EPG) on both individual and pooled samples. Previous studies [6, 14, 33, 34] have suggested that anthelmintic efficacy was high in southern Italy and supports the idea that with a correct management (the use of about two anthelmintic treatments in sheep per year) the development of resistance can be greatly reduced [14]. However, based on the FECRT results, AR was now detected in one and suspected in another farm (in Farm 1 efficacy of 75.0% with LL of 71.2% and in Farm 2 efficacy of 93.3% with LL of 93.0% were found) with the predominant GIN genus *Trichostrongylus* followed by *Haemonchus* at D14 for ALB. It should be noted that the results from the larval cultures may not accurately reflect actual worm burden or genera composition due to the influence of culture conditions. Therefore, molecular studies would be needed in future studies.

To confirm these results, the *in vitro* EHT was performed on GIN eggs from follow-up samples in Farm 1 and Farm 2 of the sheep treated with ALB. On both farms, the egg hatch inhibition at $> 0.1 \mu\text{g/ml}$ TBZ was lower than 50% (Farm 1: 21%; Farm 2: 26%), which implies

benzimidazole resistance [26, 35]. Additionally, to further confirm these findings, another *in vivo* trial for the FECRT on both suspicious farms was conducted two months after the first field trial with the similar outcome of reduced efficacy for administered fenbendazole with FECR values below 95% (Farm 1: 86.0%; Farm 2: 93.0%).

This approach of a follow-up trial using a different anthelmintic of the same class also excludes the phenomenon of present side resistance [36-40] and completes therefore the picture of present AR to the whole anthelmintic class. Finally, molecular detection of BZ-resistance associated polymorphisms in trichostrongyloids is a highly sensitive technique that allows to detect the manifestation of resistance alleles in the worm population and thus would be a further option to reassert resistance findings [26, 41].

The frequent, indiscriminate or inappropriate use of anthelmintic drugs to control GIN infection has resulted in selection of drug-resistant helminth populations. First, faecal examination should be done before anthelmintic treatment to assess the extent of parasite infection. FEC is rarely done in practice, which likely impacts many redundant anthelmintic treatments. Considering the treatment itself, underdosing is a common problem as weighing the sheep before deworming is far from being the routine practice. Dosage is usually calculated according to the weight estimated by farmers or veterinarians, which likely portrays the average weight of the flock rather than the weight of the heaviest animal. Therefore, underdosing is a great risk factor for the development of AR.

Furthermore, improper homogenization of the anthelmintic suspension and improper calibration of dosing equipment during treatment might result in underdosing. Due to the low treatment costs of benzimidazoles and the broad spectrum of action combatting also other helminths such as *F. hepatica*, *Dicrocoelium dendriticum*, lungworm and *Moniezia* spp., it is often used for several consecutive years. Comparing all tested farms, Farm 1 and Farm 2 having had a known history of fasciolosis relying therefore on the exclusive use of ALB, administered three to four times per year, between 2008 and 2014. High frequency of anthelmintic treatment has a major impact on speeding up the development of AR. While susceptible GIN are killed during treatment, species being resistant to the agent survive. With frequent deworming, using the same anthelmintic, resistant GIN have a selective advantage and make up an increasing proportion in the parasitic gene pool [42]. It can be assumed that the intensive use of ALB on Farm 1 and Farm 2 has contributed to the development of AR on both farms.

Moreover, the present study provides further insights into standardization of FEC and FECRT on pooled faecal samples in sheep. High correlation coefficients (Spearman) were found between the mean of individual samples and the mean of pooled samples when considering FEC at D0 ($r_s = 0.984$) and D14 ($r_s = 0.913$), whilst the Lin's concordance correlation coefficients showed a substantial agreement at D0 (CCC = 0.973) and a poor agreement at D14 (CCC = 0.873) due a lot of zero data, since only two farms (Farm 1 and 2) showed AR. Despite this, high correlation and agreement coefficients (Spearman and Lin) were found between the mean EPG of individual samples and the mean EPG of pooled samples when considering overall FEC (D0 and D14) and FECR% according to [15, 17], which found that the pooling for both FEC and FECR test was a valid alternative of reducing labour and costs involved in both assessing infection intensity and diagnosing AR in sheep and cattle, respectively. Therefore, as there is evidence that the classifications of AR on pooled samples coincide with the results on individual samples, with particularly low variance when performed with Mini-FLOTAC, this study confirms this correlation and reasserts that pooled samples are cost-effective, time-saving alternatives [17, 18]. There is the need for a standardized procedure in order to be able to recognize resistance at an early stage. Since 1992, the FECRT has been the method of choice as an *in vivo* assay [6, 23, 35]. Its quality of performance is dependent on experimental setup, egg excretion at baseline, sample size and type of diagnostic tool but no definite recommendations can be made so far [18, 20, 43]. In this respect, the study unifies current recommendations and develops improvements towards a standardized protocol of AR detection.

When carrying out this study, care was taken to exclude common sources of error and avoided possible confounding factors unrelated to AR: the compilation of sheep for the treatment group happened randomly; high quality anthelmintics from renowned producers were used; correct dosage for each enrolled animal was assured through calculation based on individually weighing of the animals; and anthelmintics were administered carefully by experienced and trained veterinary practitioners following the instructions of the manufactures. For the FEC, the Mini-FLOTAC technique was used in this trial as it has been shown beneficial diagnostic performance combining sensitivity, accuracy and easy usage [19, 44].

Therefore, it is a convenient method for detecting the presence of low level of AR in ovine nematodes [18]. The evaluation of FECR with the *eggCounts-2.3* in R version 3.6.1.

showed a higher diagnostic performance and less susceptibility to error sources compared to the evaluation considering a control group [22]. Furthermore, the chosen sample size of 40 sheep per tested drug is large compared to the recommended sample size of $n = 10\text{--}15$ suggested by Coles et al. [23]. This larger sample size implies a high probability of sufficient baseline egg excretions, measured through an extrapolation of the model designed by Levecke et al. [20].

4.6 Conclusions

The continuous monitoring of AR in sheep farms in southern Italy is essential, as anthelmintic efficacy is still high [14] and the development of AR needs to be detected early. In southern Italy the maintaining of *refugia* for susceptible nematode populations is the result of applying of methods for FECRT monitoring. Farmers should be properly informed about circumstances boosting the development of AR and receive appropriate training for responsible deworming management. The comparison of studies in Europe shows that the reflection of AR on farms is distorted by inadequate monitoring through biased choices of farms, insufficient number of tested drugs, or prevalent treatment failure [6]. Hence, the integration of a standardized, accurate and practical approach to monitor anthelmintic efficacy is required in order to compile a precise picture of the AR status worldwide. Likewise, further research is needed to develop alternative approaches to minimize the use of anthelmintics.

4.7 Abbreviations

ALB: albendazole; AR: anthelmintic resistance; BZ: benzimidazoles; CCC: Lin's concordance correlation coefficients; CI: confidence interval; COMBAR: COMBatting Anthelmintic Resistance in Ruminants; D0: pre-treatment; D14: post-treatment; DMSO: dimethyl sulfoxide; EC_{50} : 50% egg hatch inhibition; EHT: egg hatch test; EPG: egg per gram of faeces; EPR: eprinomectin; FBZ: fenbendazoles; FS: Flotation solution; FEC: faecal egg count; FECRT: faecal egg count reduction test; GIN: gastrointestinal nematode; IVM: ivermectin; L3: third-stage larvae; LL: lower limit; ML: macrocyclic lactones; r_s : Spearman's rho correlation coefficient; TBZ: thiabendazole; TST: targeted selective treatment; TT: targeted treatment.

4.8

Acknowledgements

The authors would like to express sincere appreciation to Mario Parrilla and Mario Iorio (Department of Veterinary Medicine and Animal Production, University of Naples Federico II, CREMOPAR, Naples, Italy) for their technical collaboration. This article is based upon work from COST Action COMBAR CA16230, supported by COST (European Cooperation in Science and Technology).

Authors contributions

LR, GC, BH, MV and MPM conceived, designed and coordinated the study. AB, JK, AA and DI performed sampling and laboratory analyses. All authors contributed to data analysis and preparation of the manuscript. All authors read and approved the final manuscript.

Funding

This research received no external funding.

Availability of data and materials

All data generated or analysed during this study are included in this published article. The datasets used and/or analysed during the present study available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

We obtained verbal informed consent from the owners of farms to collect the faecal samples from animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Author details

¹Department of Veterinary Medicine and Animal Production, University of Naples Federico II, CREMOPAR, Naples, Italy. ²Institute of Parasitology, Department of Pathobiology, University of Veterinary Medicine Vienna, 1210 Vienna, Austria. ³Institute of Parasitology of Slovak Academy of Sciences, 040 01 Košice, Slovakia.

4.9 References

1. Mavrot F. Livestock nematode infection in a changing world: investigating the European situation. Publisher City: University of Zurich; 2016.
2. Mavrot F, Hertzberg H, Torgerson P. Effect of gastro-intestinal nematode infection on sheep performance: a systematic review and meta-analysis. *Parasit Vectors*. 2015;8:557.
3. Glover M, Clarke C, Nabb L, Schmidt J. Anthelmintic efficacy on sheep farms in south-west England. *Vet Rec*. 2017;180:378.
4. Charlier J, Rinaldi L, Musella V, Ploeger HW, Chartier C, Rose Vineer H, et al. Initial assessment of the burden of parasitic helminth infections to the ruminant livestock industry in Europe. *Prev Vet Med*. 2020;182:105103.
5. Kaplan RM, Vidyashankar AN. An inconvenient truth: global worming and anthelmintic resistance. *Vet Parasitol*. 2012;186:70–8.
6. Rose H, Rinaldi L, Bosco A, Mavrot F, de Waal T, Skuce P, et al. Widespread anthelmintic resistance in European farmed ruminants: a systematic review. *Vet Rec*. 2015;176:546.
7. Charlier J, Thamsborg SM, Bartley DJ, Skuce PJ, Kenyon F, Geurden T, et al. Mind the gaps in research on the control of gastrointestinal nematodes of farmed ruminants and pigs. *Transbound Emerg Dis*. 2018;65(Suppl. 1):217–34.
8. Sangster NC, Cowling A, Woodgate RG. Ten events that defined anthelmintic resistance research. *Trends Parasitol*. 2018;34:553–63.
9. Easton S, Pinchbeck GL, Bartley DJ, Hodgkinson JE, Matthews JB. A survey of experiences of UK cattle and sheep farmers with anthelmintic prescribers; are best practice principles being deployed at farm level? *Prev Vet Med*. 2018;155:27–37.

10. Traversa D, Paoletti B, Otranto D, Miller J. First report of multiple drug resistance in trichostrongyles affecting sheep under field conditions in Italy. *Parasitol Res.* 2007;101:1713–6.
11. Geurden T, Chartier C, Fanke J, di Regalbono AF, Traversa D, von Samson-Himmelstjerna G, et al. Anthelmintic resistance to ivermectin and moxidectin in gastrointestinal nematodes of cattle in Europe. *Int J Parasitol Drugs Drug Resist.* 2015;5:163–71.
12. Lambertz C, Pouloupoulou I, Wuthijaree K, Gauly M. Anthelmintic resistance in gastrointestinal nematodes in sheep raised under mountain farming conditions in northern Italy. *Vet Rec.* 2019;6;:e000332.
13. Cringoli G, Veneziano V, Jackson F, Vercruysse J, Greer AW, Fedele V, et al. Effects of strategic anthelmintic treatments on the milk production of dairy sheep naturally infected by gastrointestinal strongyles. *Vet Parasitol.* 2008;156:340–5.
14. Rinaldi L, Morgan ER, Bosco A, Coles GC, Cringoli G. The maintenance of anthelmintic efficacy in sheep in a Mediterranean climate. *Vet Parasitol.* 2014;203:139–43.
15. Rinaldi L, Amadesi A, Dufourd E, Bosco A, Gadanho M, Lehebel A, et al. Rapid assessment of faecal egg count and faecal egg count reduction through composite sampling in cattle. *Parasit Vectors.* 2019;12:353.
16. Cringoli G, Maurelli MP, Levecke B, Bosco A, Vercruysse J, Utzinger J, et al. The Mini-FLOTAC technique for the diagnosis of helminth and protozoan infections in humans and animals. *Nat Protoc.* 2017;12:1723–32.
17. Rinaldi L, Levecke B, Bosco A, Ianniello D, Pepe P, Charlier J, et al. Comparison of individual and pooled faecal samples in sheep for the assessment of gastrointestinal strongyle infection intensity and anthelmintic drug efficacy using McMaster and Mini-FLOTAC. *Vet Parasitol.* 2014;205:216–23.

18. Kenyon F, Rinaldi L, McBean D, Pepe P, Bosco A, Melville L, et al. Pooling sheep faecal samples for the assessment of anthelmintic drug efficacy using McMaster and Mini-FLOTAC in gastrointestinal strongyle and *Nematodirus* infection. *Vet Parasitol.* 2016;225:53–60.
19. Paras KL, George MM, Vidyashankar AN, Kaplan RM. Comparison of fecal egg counting methods in four livestock species. *Vet Parasitol.* 2018;257:21–7.
20. Levecke B, Kaplan RM, Thamsborg SM, Torgerson PR, Vercruysse J, Dobson RJ. How to improve the standardization and the diagnostic performance of the fecal egg count reduction test? *Vet Parasitol.* 2018;253:71–8.
21. Dobson RJ, Hosking BC, Jacobson CL, Cotter JL, Besier RB, Stein PA, et al. Preserving new anthelmintics: a simple method for estimating faecal egg count reduction test (FECRT) confidence limits when efficacy and/or nematode aggregation is high. *Vet Parasitol.* 2012;186:79–92.
22. Torgerson PR, Paul M, Furrer R. Evaluating faecal egg count reduction using a specifically designed package “eggCounts in R and a user friendly web interface. *Int J Parasitol.* 2014;44:299–303.
23. Coles GC, Bauer C, Borgsteede FHM, Geerts S, Klei TR, Taylor MA, et al. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Vet Parasitol.* 1992;44:35–44.
24. Great Britain, Manual of veterinary parasitological laboratory techniques. London: H.M. Stationery Office; 1986.
25. van Wyk JA, Mayhew E. Morphological identification of parasitic nematode infective larvae of small ruminants and cattle: a practical lab guide. *Onderstepoort J Vet.* 2013;80: 539.

26. von Samson-Himmelstjerna, G, Coles GC, Jackson F, Bauer C, Borgsteede F, Cirak VY, et al. Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. *Parasitol Res.* 2009;105:825–34.
27. McBride GB. A proposal for strength-of-agreement criteria for Lin'S concordance correlation coefficient. Hamilton: National Institute of Water and Atmospheric Research; 2005. p. 10.
28. Bosco A, Rinaldi L, Musella V, Amadesi A, Cringoli G. Outbreak of acute fasciolosis in sheep farms in a Mediterranean area arising as a possible consequence of climate change. *Geospat Health.* 2015;9:319–24.
29. Grenfell BT, Wilson K, Isham VS, Boyd HEG, Dietz K. Modelling patterns of parasite aggregation in natural populations: trichostrongylid nematode-ruminant interactions as a case study. *Parasitology.* 1995;111:135–51.
30. Morgan ER, Cavill L, Curry GE, Wood RM, Mitchell ESE. Effects of aggregation and sample size on composite faecal egg counts in sheep. *Vet Parasitol.* 2005;131:79–87.
31. Torgerson PR, Paul M, Lewis FI. The contribution of simple random sampling to observed variations in faecal egg counts. *Vet Parasitol.* 2012;188:397–401.
32. Charlier J, Morgan ER, Rinaldi L, van Dijk J, Demeler J, Höglund J, et al. Practices to optimise gastrointestinal nematode control on sheep, goat and cattle farms in Europe using targeted (selective) treatments. *Vet Rec.* 2014;175:25.
33. Hamel D, Bosco A, Rinaldi L, Cringoli G, Kaulfuß K-H, Kellermann M, et al.. Eprinomectin pour-on (EPRINEX® Pour-on, Merial): efficacy against gastrointestinal and pulmonary nematodes and pharmacokinetics in sheep. *BMC Vet Res.* 2017;13:148.
34. Castagna F, Palma E, Cringoli G, Bosco A, Nisticò N, Caligiuri G, et al. Use of complementary natural feed for gastrointestinal nematodes control in sheep: effectiveness and benefits for animals. *Animals (Basel).* 2019;9:1037.

35. Coles GC, Jackson F, Pomroy WE, Prichard RK, von Samson-Himmelstjerna, G, Silvestre, A et al. The detection of anthelmintic resistance in nematodes of veterinary importance. *Vet Parasitol.* 2006;136:167–85.
36. Gasbarre LC, Ballweber LR, Stromberg BE, Dargatz DA, Rodriguez JM, Koprak CA, Zarlenga DS. Effectiveness of current anthelmintic treatment programs on reducing fecal egg counts in United States cow-calf operations. *Can J Vet Res.* 2015;79:296–302.
37. Prichard RK, Hall CA, Kelly JD, Martin IC, Donald AD. The problem of anthelmintic resistance in nematodes. *Aust Vet J.* 1980;56:239–51.
38. Sangster NC. 1999. Anthelmintic resistance: past, present and future. *Int J Parasitol.* 1999;29:115–24.
39. Ramos F, Portella LP, Rodrigues FdS, Reginato CZ, Pötter L, Cezar AS, Sangioni LA, Vogel FSF. Anthelmintic resistance in gastrointestinal nematodes of beef cattle in the state of Rio Grande do Sul, Brazil. *Int. J. Parasitol. Drugs Drug Resist.* 2016;6:93–101.
40. Kebede A. Review on anthelmintic drug resistance nematodes and its methods of detection in Ethiopia. *J Vet Med Animal Sci.* 2019;2:1013.
41. Redman E, Whitelaw F, Tait A, Burgess C, Bartley Y, Skuce PJ, Jackson F, Gilleard JS. The emergence of resistance to the benzimidazole anthelmintics in parasitic nematodes of livestock is characterised by multiple independent hard and soft selective sweeps. *PLoS Negl Trop Dis.* 2015;9:e0003494.
42. Falzon LC, O'Neill TJ, Menzies PI, Peregrine AS, Jones-Bitton A, vanLeeuwen J, Mederos A. A systematic review and meta-analysis of factors associated with anthelmintic resistance in sheep. *Prev Vet Med.* 2014;117:388–402.

43. Levecke B, Rinaldi L, Charlier J, Maurelli MP, Bosco A, Vercruysse J, et al. The bias, accuracy and precision of faecal egg count reduction test results in cattle using McMaster, Cornell-Wisconsin and FLOTAC egg counting methods. *Vet Parasitol.* 2012;188:194–9.
44. Bosco A, Maurelli MP, Ianniello D, Morgoglione ME, Amadesi A, Coles GC, et al. The recovery of added nematode eggs from horse and sheep faeces by three methods. *BMC Vet Res.* 2018;14:7.

4.10

Graphical Abstract

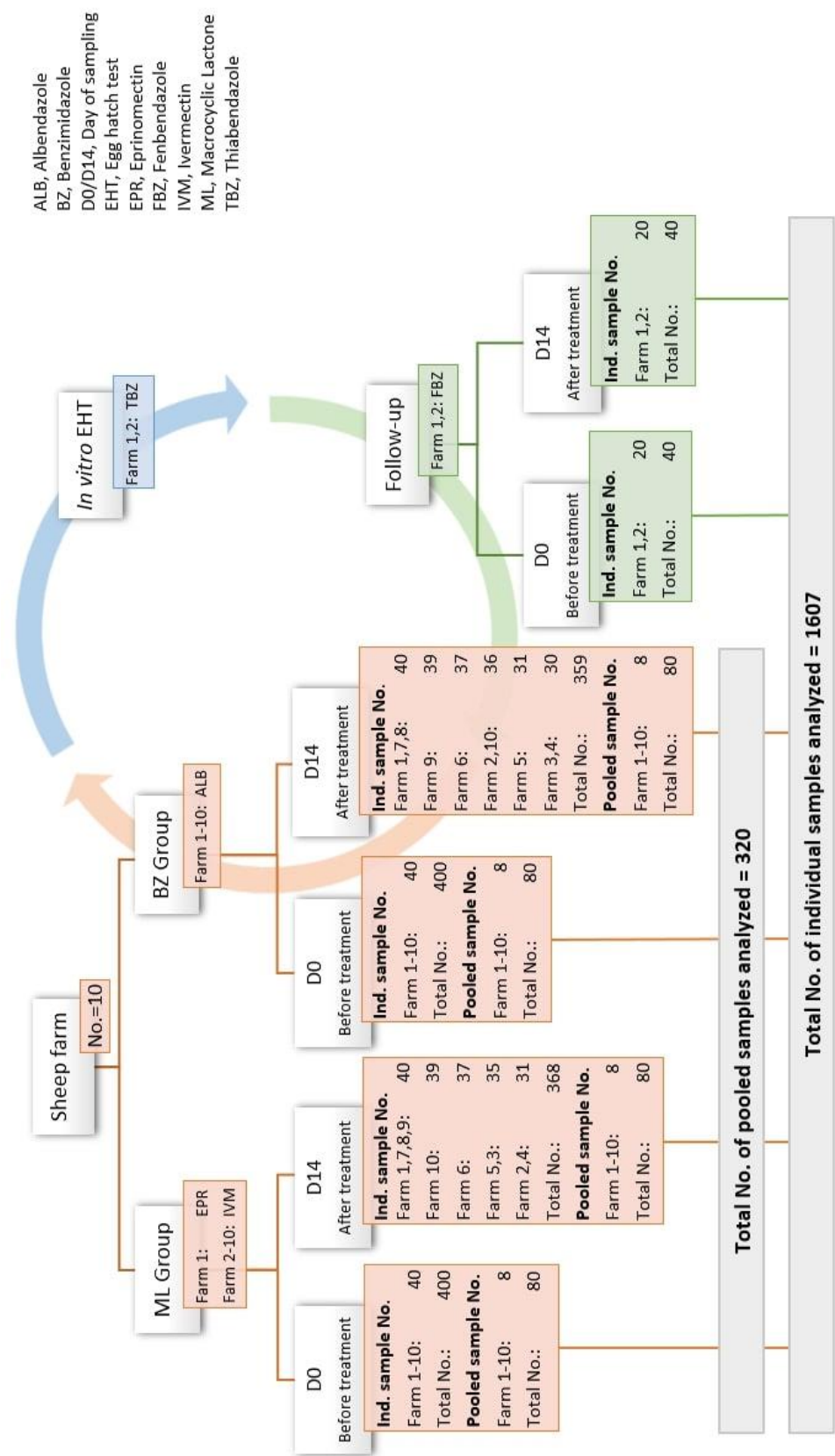


Fig. 1 The number of sheep farms, anthelmintics used, sampling time and number of samples per farm used for the field trial setup, follow-up trial and *in vitro* egg hatch tests.

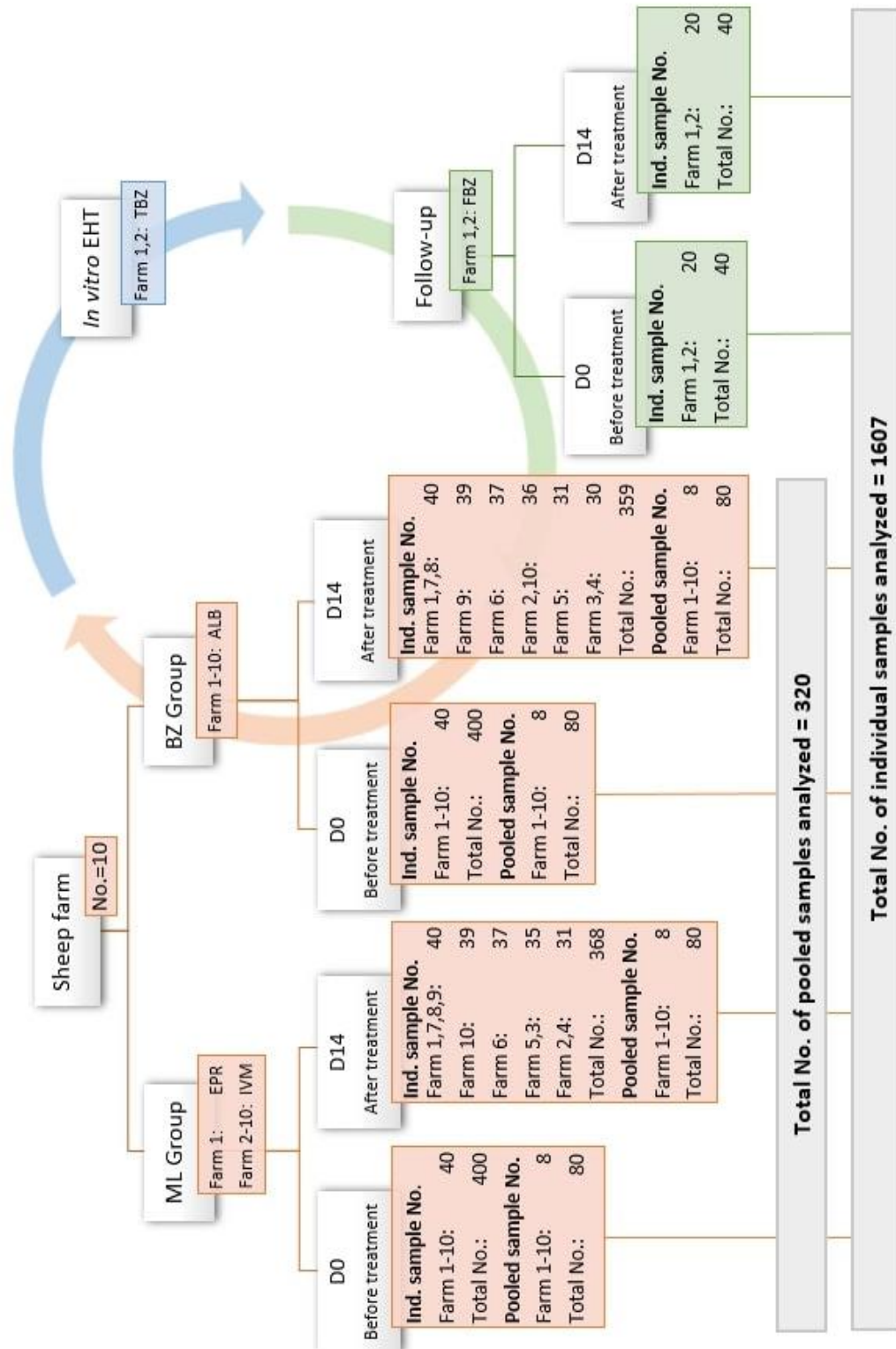


Fig. 2 Frequency distribution of gastrointestinal nematode (GIN) egg counts among different individual animals of all examined farms sampled at D0.

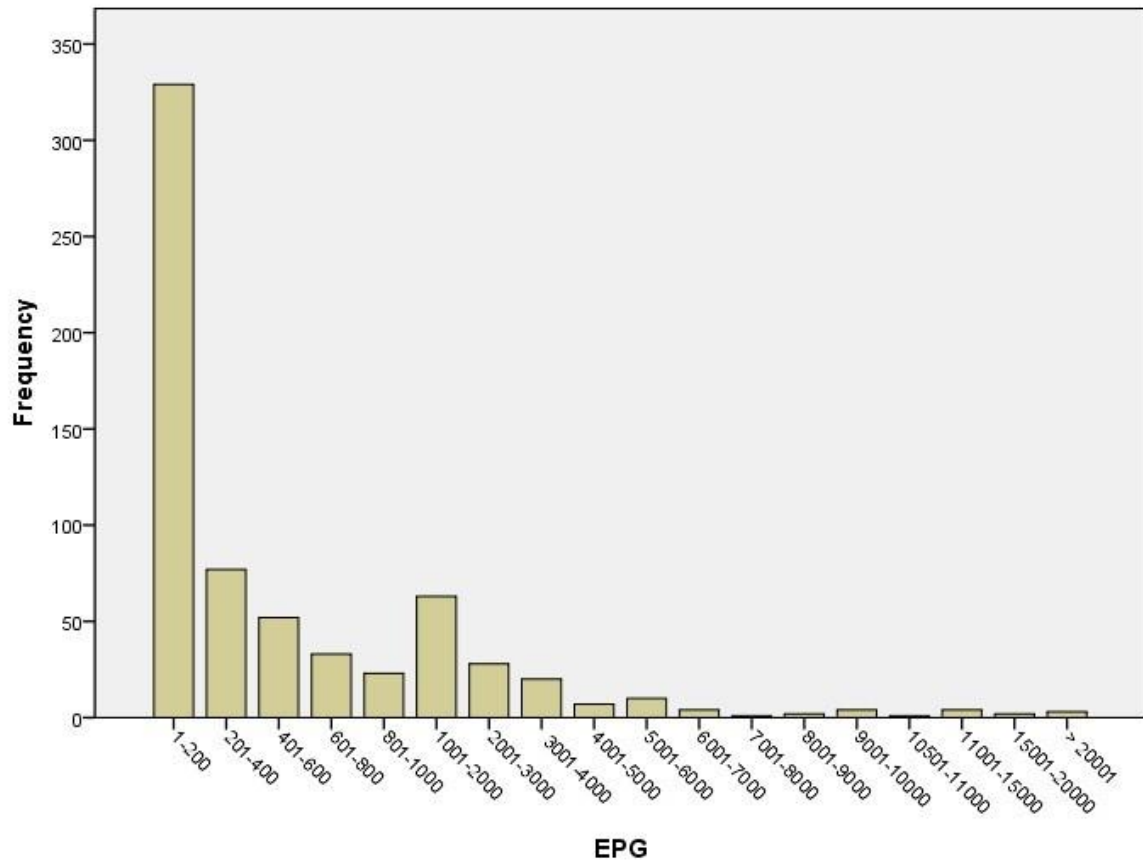


Fig. 3 Variability of gastrointestinal nematode (GIN) egg counts in the different sheep farms sampled at D0: mean eggs per gram (EPG) of faeces and standard errors (SE).

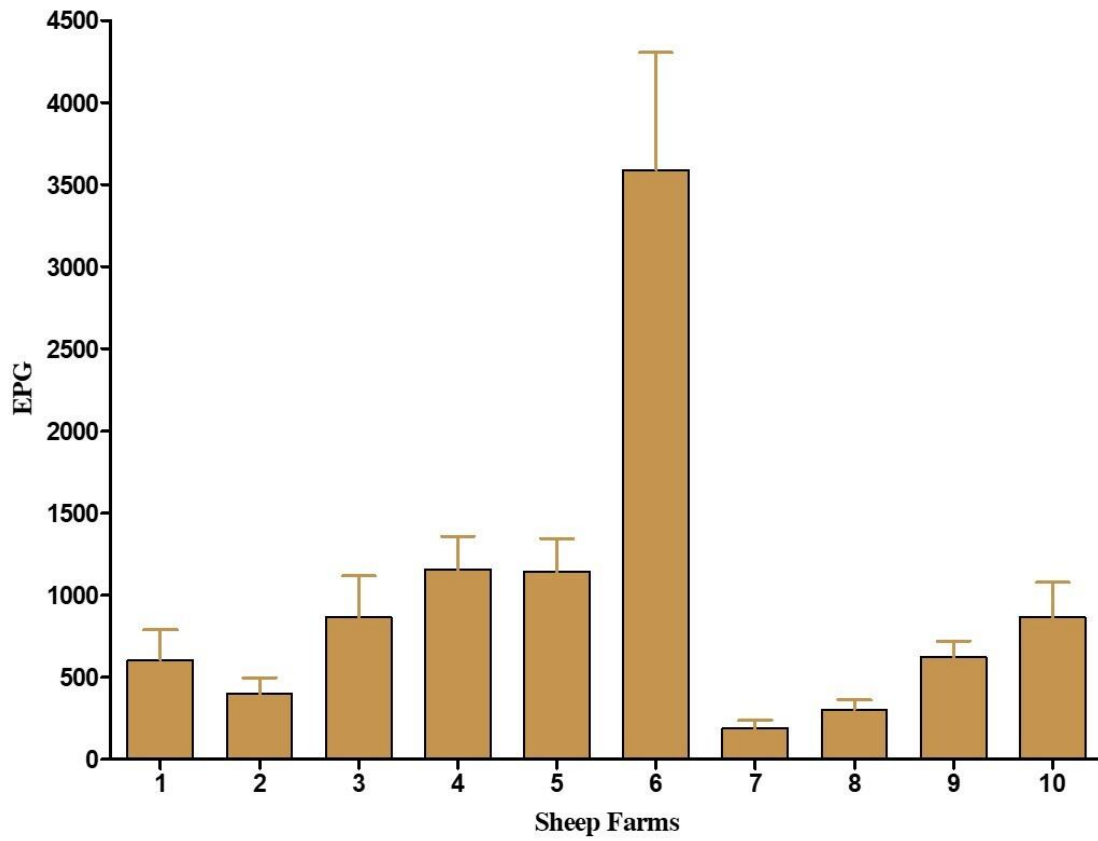
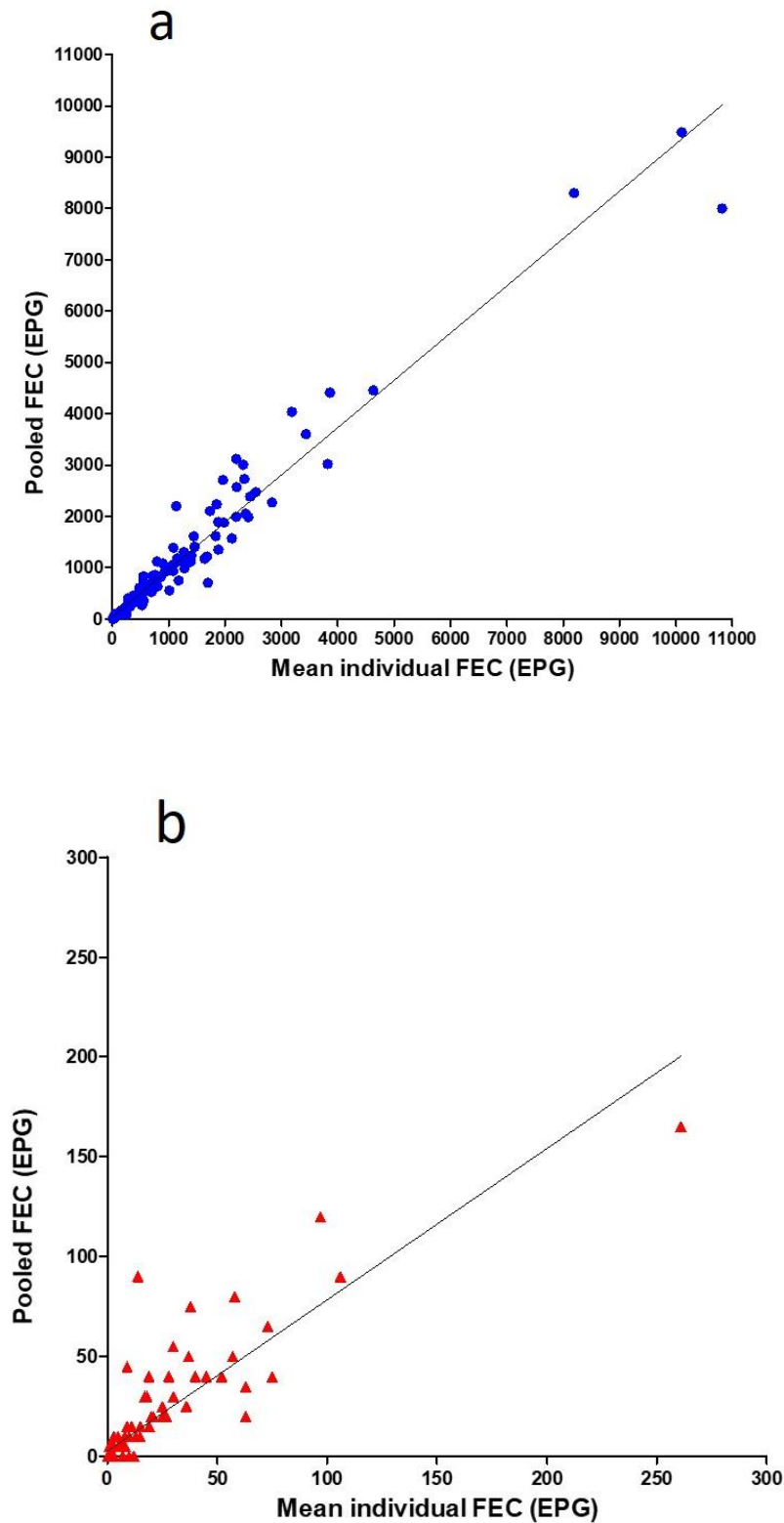


Fig. 4 The correlation in faecal egg counts at D0 (a) and at D14 (b) based on the examination of individual and pooled faecal samples of all examined farms.



4.11

Table 1 The anthelmintics (molecules and dosages) used on each group of 10 sheep farms against gastrointestinal nematodes (GIN), mean GIN eggs per gram (EPG) of faeces (day0 and day14), results of the faecal egg count reduction (FECR%), limits of the 95% confidence interval (CI) of individual and pooled faecal samples and anthelmintic efficacy classified as reduced (R), suspected (S) and normal (N).

Farn ID	Group	Molecule	Dosage (mg/kg)	Individual samples				Pooled samples				Efficacy
				D0 FEC Mean EPG	D14 FEC Mean EPG	FECR %	95% CI	D0 FEC Mean EPG	D14 FEC Mean EPG	FECR %	95% CI %	
1	EPR	Eprinomectin	1.0	1086	25	97.7	97.4–98.0	1100	23	97.9	97.1–98.5	N
	ALB	Albendazole	3.8	124	31	75.0	71.2–78.2	116	30	73.7	64.3–81.0	R
2	IVM	Ivermectin	0.2	417	0.5	99.9	99.7–100	380	0	99.9	99.4–100	N
	ALB	Albendazole	3.8	375	25	93.3	93.0–94.8	344	25	92.6	89.9–94.7	S
3	IVM	Ivermectin	0.2	540	3	99.4	99.2–99.6	478	3	99.1	98.3–99.6	N
	ALB	Albendazole	3.8	1195	31	97.4	99.5–99.6	1189	35	97.0	96.2–97.7	N
4	IVM	Ivermectin	0.2	965	1	99.9	99.7–99.9	885	1	99.7	99.3–99.9	N
	ALB	Albendazole	3.8	1348	0	100	99.9–100.0	1116	0	100	99.6–100	N
5	IVM	Ivermectin	0.2	1863	42	97.7	97.3–97.9	1816	40	97.8	97.2–98.3	N
	ALB	Albendazole	3.8	420	1	99.8	99.6–99.9	401	0	99.6	98.9–99.9	N
6	IVM	Ivermectin	0.2	3437	28	99.2	99.1–99.3	3609	40	97.8	97.4–98.2	N
	ALB	Albendazole	3.8	3742	16	99.6	99.5–99.6	3799	13	99.6	99.5–99.8	N
7	IVM	Ivermectin	0.2	102	0	100	99.9–100.0	88	0	99.5	97.3–100	N
	ALB	Albendazole	3.8	274	0	100	99.9–100.0	202	0	99.8	98.8–100	N
8	IVM	Ivermectin	0.2	272	0	100	99.9–100.0	272	0	99.8	99.2–100	N
	ALB	Albendazole	3.8	334	0	100	99.9–100.0	334	0	100	99.3–100	N
9	IVM	Ivermectin	0.2	454	0	100	99.9–100.0	434	0	100	99.5–100	N
	ALB	Albendazole	3.8	796	13	98.4	98.0–98.6	636	14	97.7	96.6–98.5	N
10	IVM	Ivermectin	0.2	1059	46	95.7	95.1–96.3	1023	33	96.7	95.8–97.5	N
	ALB	Albendazole	3.8	669	0	100	99.9–100.0	605	0	100	99.6–100	N

Table 2 Fenbendazole dosage, mean gastrointestinal nematode (GIN) eggs per gram (EPG) of faeces (Day 0 and Day14), results of the faecal egg count reduction (FECR, %), limits of the 95% Confidence Interval (CI) and anthelmintic efficacy classified as reduced (R), suspected (S) and normal (N) in the follow-up study.

Farm ID	Group	Molecule	Dosage (mg/kg)	D0 FEC (Mean EPG)	D14 FEC (Mean EPG)	FECR%	95%CI	Efficacy
1	FBZ	Fenbendazole	5.0	194	27	86.0	82.9–88.6	R
2	FBZ	Fenbendazole	5.0	151	11	93.0	90.4–94.9	S

Table 3 Numbers and percentage of sheep nematode third-stage larvae (L3) for each group of 10 sheep farms at D0 and at D14.

Farm ID	Group	Day	<i>Haemonchus</i>		<i>Trichostrongylus</i>		<i>Teladorsagia</i>		<i>Cooperia</i>		<i>Chabertia</i>	
			n	%	n	%	n	%	n	%	n	%
1	EPR	0	78	78	19	19	1	1	0	0	2	2
		14	7	7	93	93	0	0	0	0	0	0
	ALB	0	79	79	21	21	0	0	0	0	0	0
		14	29	29	71	71	0	0	0	0	0	0
2	IVM	0	77	77	20	20	1	1	0	0	2	2
		14	6	24	19	76	0	0	0	0	0	0
	ALB	0	78	78	17	17	2	2	0	0	3	3
		14	6	6	94	94	0	0	0	0	0	0
3	IVM	0	67	67	15	15	18	18	0	0	0	0
		14	10	50	7	35	3	15	0	0	0	0
	ALB	0	72	72	8	8	20	20	0	0	0	0
		14	59	59	29	29	7	7	0	0	5	5
4	IVM	0	67	67	23	23	6	6	0	0	4	4
		14	0	0	11	61	7	39	0	0	0	0
	ALB	0	68	68	15	15	8	8	0	0	9	9
		14	9	82	2	18	0	0	0	0	0	0
5	IVM	0	73	73	15	15	12	12	0	0	0	0
		14	73	73	19	19	8	8	0	0	0	0
	ALB	0	21	21	54	54	25	25	0	0	0	0
		14	11	11	78	78	11	11	0	0	0	0
6	IVM	0	93	93	2	2	3	3	0	0	2	2
		14	11	11	72	72	17	17	0	0	0	0
	ALB	0	32	32	59	59	7	7	0	0	2	2
		14	30	30	45	45	25	25	0	0	0	0
7	IVM	0	28	28	11	11	13	13	0	0	48	48
		14	nd									
	ALB	0	37	37	29	29	12	12	0	0	22	22
		14	nd									
8	IVM	0	65	65	26	26	8	8	0	0	1	1
		14	nd									
	ALB	0	61	61	29	29	9	9	0	0	1	1
		14	nd									
9	IVM	0	83	83	10	10	5	5	0	0	2	2
		14	nd									
	ALB	0	81	81	10	10	6	6	0	0	3	3
		14	nd									
10	IVM	0	38	38	38	38	15	15	5	5	4	4
		14	nd									
	ALB	0	29	29	44	44	23	23	2	2	2	2
		14	nd									

Abbreviation: nd = no larvae were detected

Table 4 The results of the egg hatch test based on counting 100 eggs/larvae per well for each concentration of thiabendazole in Farm 1 and Farm 2.

Thiabendazole concentration (µg/ml)	Farm 1		Farm 2	
	Average number of hatched eggs (larvae)	inhibited (dead) eggs	Average number of hatched eggs (larvae)	Inhibited (dead) eggs
0	94.0	6.0	95.5	4.5
0.01	84.0	16.0	78.5	21.5
0.025	88.0	12.0	76.5	23.5
0.05	87.5	12.5	75.5	24.5
0.1	89.0	21.0	74.0	26.0
0.2	77.0	23.0	70.0	30.0
0.3	63.5	36.5	62.5	37.5
0.5	49.5	50.5	44.5	55.5

5 Diskussion

Nach wie vor ist die Bekämpfung von MDS-Infektionen abhängig von regelmäßigen Behandlungen der Bestände mit wirksamen Anthelmintika. Jedoch ist das Entwurmungsmanagement, so wie es heute betrieben wird, gefährdet durch die Bildung von Resistenzen in MDS gegenüber den Wirkstoffen (Rose et al. 2015). Um auch in Zukunft ein effektives und nachhaltiges Entwurmungsregime führen zu können, ist es besonders wichtig, die verfügbaren Anthelmintika wohlüberlegt einzusetzen.

Diese Studie wurde durchgeführt, um nach aktuellen Erkenntnissen der Wissenschaft ein evidenzbasiertes Bild der derzeitigen Resistenzlage gegenüber Benzimidazolen und makrozyklischen Laktonen in zehn Schafbetrieben der Region Kampanien (Süditalien) zu erheben. Auf jedem Schafbetrieb wurden beide Anthelmintika-Klassen an einer Gruppengröße von jeweils 40 Tieren getestet. Die Auswahl der Schafbetriebe erfolgte grundsätzlich zufällig, jedoch abhängig von der Bereitschaft der LandwirtInnen an der Teilnahme der Studie. Die Gruppengrößen von 40 Tieren wurde willkürlich, jedoch bewusst in dieser Größenordnung ausgewählt. Der EZRT wurde für Einzel- und Sammelkotproben mittels der Mini-FLOTAC-Technik [Nachweisgrenze = 5 Eier pro Gramm (EPG)] ermittelt, basierend auf Eiauszählungen (EZ) aus Stuhlproben unmittelbar vor der Behandlung und 14 Tage danach. Bisherige Studien (Castagna et al. 2019, Hamel et al. 2017, Rinaldi, Morgan et al. 2014, Rose et al. 2015) haben gezeigt, dass die Wirksamkeit der Anthelmintika in Süditalien bis dato hoch ist. Jedoch wurden auf Basis des EZRT in einem der getesteten Betriebe Albendazol-Resistenz nachgewiesen und in einem weiteren Betrieb vermutet (in Betrieb 1 wurde eine Wirksamkeit von 75.0% bei LL von 71.2% und in Betrieb 2 eine Wirksamkeit von 93.3% bei LL von 93.0% ermittelt). Die vorherrschenden MDS-Genera waren dabei *Trichostrongylus*, gefolgt von *Haemonchus* in den für Tag 14 angesetzten Stuhlkulturen. Um die Befunde des EZRT von Betrieb 1 und Betrieb 2 *in-vitro* zu bestätigen, wurden Betrieb 1 und Betrieb 2 erneut beprobt (Sammelkotproben von 20-40 Tieren der mit Albendazol behandelten Schafe) und jeweils ein EHT mit akkumulierten MDS-Eiern aus den frischen Kotproben durchgeführt. Für beide Betriebe betrug die Larvenschlupfhemmung bei der kritischen Dosis von $> 0.1 \mu\text{g/ml}$ TBZ weniger als 50% (Betrieb 1: 21%; Betrieb 2: 26%), was auf Benzimidazol-Resistenz schließen lässt (Coles et al. 2006, Samson-Himmelstjerna et al. 2009).

Zur weiteren Überprüfung der verminderten Wirksamkeit von Benzimidazolen in Betrieb 1 und Betrieb 2 wurde zwei Monate nach der ersten Feldstudie ein *in-vivo* Folgeversuch auf beiden Betrieben unter Verwendung von Fenbendazol durchgeführt. Wie im EZRT für das im Hauptversuch verwendete Albendazol, zeigte sich für das applizierte Fenbendazol im EZRT ebenfalls eine verminderte Wirksamkeit auf beiden Betrieben unter 95% (Betrieb 1: 86.0%, Betrieb 2: 93.0%). Dieser Ansatz eines *in-vivo* Folgeversuches mit einem Anthelmintikum gleicher Klasse schließt das Phänomen möglicher Nebenresistenzen aus (Gasbarre et al. 2015, Kebede 2019, Prichard et al. 1980, Ramos et al. 2016, Sangster 1999) und vervollständigt somit den Nachweis von AR gegen eine ganze Anthelmintika-Klasse. Zur Bestätigung von Resistenzbefunden ist die Identifizierung von Benzimidazol-Resistenz-assoziierten Polymorphismen in MDS auf molekularbiologischer Ebene eine weitere Möglichkeit, Resistenz in einer Wurmpopulation zu erkennen (Redman et al. 2015, Samson-Himmelstjerna et al. 2009).

Der häufige und unsachgemäße Einsatz von Anthelmintika zur Kontrolle von MDS-Infektionen führt zur Selektion von resistenten Stämmen in Wurmpopulationen (Falzon et al. 2014). Zunächst einmal sollte das Ausmaß des Wurmbefalls der Herde anhand von Kotproben ermittelt werden, bevor die Behandlung mit einem Anthelmintikum erfolgt. EZ finden selten Anwendung in der Praxis und Wurmbehandlungen werden routinemäßig durchgeführt. Folglich finden Entwurmungen auch dann statt, wenn ein niedriger Erregerdruck vorherrscht und keine Indikation vorliegt. Bei der Behandlung selbst ist die Unterdosierung des Medikaments ein häufiger Risikofaktor für die Entwicklung von AR, da das Gewicht der Schafe zur Ermittlung der korrekten Dosierung im Praxisalltag selten erhoben wird (Cernanská et al. 2008, Claerebout et al. 2020, Domke et al. 2011). Stattdessen ist es üblich, die Dosis auf Basis von Gewichtsschätzungen zu kalkulieren, was im Regelfall das Durchschnittsgewicht der Herde und nicht das Gewicht des schwersten Tieres abbildet. Daneben führt unzureichendes Homogenisieren der Arzneimittel-Suspension vor der Applikation zur Unterdosierung.

Benzimidazole werden in vielen Fällen über mehrere Jahre kontinuierlich angewendet, da deren Einsatz zum einen kostengünstig ist und sie zum anderen ein breites Wirkungsspektrum aufweisen. So wirken sie z.B. auch gegen *Fasciola hepatica*, *Dicrocoelium dendriticum*, Lungenwürmer und *Moniezia spp.*.

Im Vergleich aller Schafbetriebe fällt auf, dass nur Betrieb 1 und Betrieb 2 eine bekannte Krankengeschichte mit Fasciolose hatten und daher zwischen 2008 und 2014 ausschließlich Albendazol 3–4-mal jährlich zur Wurmbehandlung eingesetzt wurde. Eine hohe Behandlungsfrequenz trägt maßgeblich dazu bei, die Entstehung von AR zu beschleunigen (Falzon et al. 2014). Während für das Anthelmintikum empfindliche MDS durch die Behandlung abgetötet werden, überleben resistente MDS. Wird nun in hoher Behandlungsfrequenz das gleiche Anthelmintikum verwendet, so haben MDS mit Resistenz gegenüber dem Wirkstoff einen klaren Überlebensvorteil und der Genpool der Wurmpopulation verschiebt sich in Richtung resistenter MDS-Stämme (Falzon et al. 2014). Es ist anzunehmen, dass der intensive Einsatz von Albendazol auf Betrieb 1 und Betrieb 2 zur Entstehung der gefundenen Resistenzen beigetragen hat.

Während in Süditalien im Allgemeinen eine hohe Wirksamkeit von Benzimidazolen in MDS angenommen wird und dies die beiden ersten Funde von Benzimidazol-Resistenz in MDS von Schafen in Süditalien sind, gibt es in den österreichischen Bundesländern Salzburg und Steiermark zahlreiche Berichte von Behandlungsversagen durch Benzimidazole. Durch molekularbiologische Untersuchungen konnte in 16 Schafbetrieben dieser Regionen eine hohe Frequenz von Benzimidazol-Resistenz-assoziierten Einzelnukleotid-Polymorphismen (Single Nucleotide Polymorphism, SNP) detektiert werden, während simultan auf drei der untersuchten Betriebe verminderte Wirksamkeit des makrozyklischen Laktone Moxidectin durch EZRT nachgewiesen wurde. Als Hauptfaktor für die verbreitete Resistenzlage beider Bundesländer wird die langjährig praktizierte „Dose and move“ Strategie der Betriebe mit Wandertierhaltung angesehen (Hinney et al. 2020). Da das in der österreichischen Untersuchung angewendete molekularbiologische Verfahren zur Testung auf Benzimidazol-Resistenz im Frühstadium sensitiver und spezifischer ist als der EZRT, ist der direkte Vergleich zur Resistenzlage der vorliegenden süditalienischen Studie kritisch zu betrachten (Ramünke et al. 2016, Samson-Himmelstjerna et al. 2009). Es wäre möglich, dass die Entwicklung von Resistenzen auf den untersuchten Betrieben bereits fortgeschrittener ist, als durch EZRT detektierbar. Molekularbiologische Untersuchungen auf Benzimidazol-Resistenz-assoziierte SNPs sind für die zehn Schafbetriebe noch in Planung. Insgesamt muss darauf hingewiesen werden, dass eine Studie mit zehn getesteten Betrieben isoliert betrachtet nicht geeignet ist, um die Resistenzlage für ganz Süditalien abzubilden. Weit mehr standardisierte Untersuchungen müssten landesweit

durchgeführt werden, die als Mosaik zusammengesetzt die Resistenzlage für getestete Anthelmintika-Klassen repräsentativ abbilden können.

Ein weiteres Ziel der Studie war die Auseinandersetzung mit der standardisierten Verwendung von Sammelkotproben für EZ und EZRT in Schafen. Es ist bereits bewiesen worden, dass die Klassifizierung von AR anhand von Sammelkotproben mit den Ergebnissen aus Einzelkotproben positiv korreliert, insbesondere mit niedriger Varianz unter Verwendung von Mini-FLOTAC (Kenyon et al. 2016, Rinaldi, Levecke et al. 2014). Diese Studie bestätigt diese positive Korrelation und befürwortet den Einsatz von Sammelkotproben für den EZRT als eine kosteneffiziente und zeitsparende Alternative. Dennoch muss für die praktische Umsetzung angemerkt werden, dass es an Tag 14 nicht immer möglich war, von allen Tieren ausreichend Kot zu gewinnen, was sich besonders für Sammelkotproben als Herausforderung darstellt, da sich diese schlecht durch zusätzlich genommene Proben an Tag 0 kompensieren lassen. Ursachen hierfür waren meistens unzureichende Kotmengen im Rektum zum Zeitpunkt der Beprobung und in wenigen Fällen nicht identifizierbare Einzeltiere. Betriebe mussten daher ggf. erneut für fehlende Proben besucht werden bzw. bestehen an Tag 14 genommene Sammelkotproben teilweise aus weniger als 5 Einzelkotproben (vgl. Fig. 1).

Es bedarf der Einführung eines standardisierten Verfahrens zur Früherkennung von AR. Seit 1992 ist der EZRT als *in-vivo*-Test die Methodik der Wahl (Coles et al. 1992, Coles et al. 2006, Rose et al. 2015). Seine Aussagekraft ist abhängig vom Versuchsaufbau, der Höhe der Eiausscheidung an Tag 0, dem Stichprobenumfang und der Empfindlichkeit des verwendeten Testsystems, wobei bisher keine definitiven Anforderungen an die Einflussgrößen gestellt werden können (Kenyon et al. 2016, Levecke et al. 2012, Levecke et al. 2018).

In diesem Sinne vereint die Studie aktuelle Empfehlungen und entwickelt Verbesserungen in Richtung eines standardisierten Protokolls zur Identifizierung von AR. Bei der Durchführung dieser Studie wurde darauf geachtet, bekannte Fehlerquellen und Störfaktoren möglichst auszuschließen: Die Behandlungsgruppen wurden zufällig zusammengesetzt, es wurden qualitativ hochwertige Anthelmintika seriöser Hersteller verwendet, die korrekte Dosierung wurde auf Grundlage des individuellen Lebendgewichts der Schafe sichergestellt und die Behandlung erfolgte sorgfältig durch geschultes wissenschaftliches Personal nach Anweisung des Herstellers. Für die EZ wurde in dieser Studie die Mini-FLOTAC -Technik eingesetzt, da

sie aufgrund ihrer Genauigkeit und Empfindlichkeit, sowie einfacher Handhabung, diagnostische Vorteile bietet (Bosco et al. 2018, Paras et al. 2018). Daher ist sie eine geeignete Methodik, um ein niedriges AR-Niveau in Nematoden von Schafen zu detektieren (Kenyon et al. 2016).

Die EZR wurde mit der Formel $EZR = [1 - (\text{arithmetisches Mittel EZ nach Behandlung} \div \text{arithmetisches Mittel EZ vor Behandlung})] \times 100\%$ berechnet, welche für die meisten Versuchsbedingungen eine hohe diagnostische Leistung zur Bestimmung von AR bietet und weniger anfällig für Fehlerquellen ist im Vergleich zu Berechnungsmodellen, die eine Kontrollgruppe berücksichtigen (Calvete und Uriarte 2013). Außerdem ist die gewählte Stichprobengröße von 40 Schafen pro Behandlungsgruppe im Vergleich zur von Coles et al. (1992) empfohlenen Stichprobengröße von $n = 10-15$ groß. Dieser größere Stichprobenumfang impliziert eine hohe Wahrscheinlichkeit ausreichender Basiseiausscheidung, gemessen an einer Hochrechnung des von Levecke et al. (2018) entworfenen Modells.

6 Fazit

Die regelmäßige aktive Überwachung der Wirksamkeit von Anthelmintika auf Schafbetrieben spielt eine wesentliche Rolle, um entstehende AR frühzeitig zu erkennen und mit entsprechenden Anpassungen des Entwurmungsregimes reagieren zu können. Dass in Süditalien Refugien mit sensiblen MDS-Populationen vorkommen und insgesamt Anthelmintika auf Schafbetrieben noch eine hohe Wirksamkeit zeigen, ist unter anderem der regelmäßigen Anwendung von EZRT zu verdanken (Rinaldi, Morgan et al. 2014). LandwirtInnen sollten außerdem fachgerecht über die Entstehung von AR informiert werden und Wissen über ein verantwortungsvolles Entwurmungsmanagement müsste aktiv zugänglich gemacht werden.

Im Vergleich europäischer Studien wird deutlich, dass das Abbild der geografischen Prävalenz von AR verzerrt ist. Gründe hierfür sind die nicht flächendeckende Überwachung von AR, voreingenommene Auswahl von landwirtschaftlichen Betrieben, Testung weniger Wirkstoffklassen oder ein generelles Behandlungsversagen (Rose et al. 2015). Daher ist die Einführung eines standardisierten, effizienten und praktikablen Protokolls zur Überwachung von AR sinnvoll, um die Wirksamkeit von AR topografisch vergleichbar abbilden zu können.

Diese Studie setzt ein wichtiges Zeichen in Richtung eines standardisierten Protokolls zur Detektion von AR. Weiterhin steht die Frage offen, welche definitiven Anforderungen im Versuchsaufbau an Stichprobenumfang und verwendetes Testsystem in Wechselwirkung mit der Eiausscheidung an Tag 0 gestellt werden können, sowie welche evidenzbasierten Protokolle für makrozyklische Laktone und andere Anthelmintika-Klassen geeignet wären.

7 Zusammenfassung

Die vorliegende Studie liefert ein standardisiertes und evidenzbasiertes Abbild der Wirksamkeit der zwei Anthelmintika-Klassen Benzimidazole (BZ) und makrozyklische Laktone (ML) gegen Magen-Darm-Strongyliden (MDS) in Schafen auf zehn zufällig ausgewählten Schafbetrieben der süditalienischen Region Kampanien. Versuchsgrundlage bildet der *in-vivo* Eizahlreduktionstest (EZRT) unter Verwendung der Mini-FLOTAC Methode, der auf allen zehn Schafbetrieben für beide Anthelmintika-Klassen durchgeführt wurde. Als Vertreter der BZ wurden auf Betrieb 1–10 Albendazol (ALB) verwendet, als Vertreter der ML auf Betrieb 1 Eprinomectin (EPR) und auf Betrieb 2–10 Ivermectin (IVM). Der EZRT erfolgte je angewendetem Anthelmintikum vergleichend für Einzel- und Sammelkotproben an Gruppengrößen von 40 Tieren, basierend auf Eiauszählungen (EZ) aus Stuhlproben unmittelbar vor (Tag 0) und 14 Tage nach der Behandlung (Tag 14) ohne Berücksichtigung einer Kontrollgruppe. Koprokulturen wurden für Tag 0 und Tag 14 pro Behandlungsgruppe angesetzt. Die Wirksamkeit der Anthelmintika wurde als ‚vermindert‘ (*reduced*), ‚vermutet‘ (*suspected*) und ‚normal‘ (*normal*) klassifiziert. In Schafbetrieben mit als ‚vermutet‘ oder ‚vermindert‘ wirksam eingestuftter Wirksamkeit, wurden zur weiteren Abklärung ein *in-vitro* Larven-Schlupf-Hemmtest (Egg hatch test, EHT) und ein *in-vivo* Folgeversuch (EZRT) unter Einsatz von Fenbendazol (FBZ) durchgeführt. Für die eingesetzten ML wurde die Wirksamkeit in allen zehn Schafbetrieben als ‚normal‘ eingestuft, für BZ in acht Betrieben als ‚normal‘, in einem Betrieb als ‚vermutet‘ (Betrieb 2) und in einem weiteren Betrieb (Betrieb 1) als ‚vermindert‘. Die Ergebnisse aus Einzel- und Sammelkotproben korrelieren dabei positiv. Die vorherrschenden MDS-Genera in den Koprokulturen von Tag 14 waren *Trichostrongylus*, gefolgt von *Haemonchus*. Die für Betrieb 1 und Betrieb 2 durchgeführten EHTs und Folgeversuche bestätigen die Resistenzlage. Im EHT betrug für beide Betriebe die Larvenschlupfhemmung bei der kritischen Dosis von $> 0.1 \mu\text{g/ml}$ TBZ weniger als 50%. Im *in-vivo* Folgeversuch wird die Wirksamkeit des BZ wie im Hauptversuch für Betrieb 1 als ‚vermindert‘ und für Betrieb 2 als ‚vermutet‘ eingestuft. Im europaweiten Vergleich ist das Level von Anthelmintika-Resistenzen (AR) in der untersuchten Region noch gering, dennoch ist sichtbar, dass auch hier AR zunehmen. TierärztInnen und LandwirtInnen sollten zur Implementierung von Strategien zur nachhaltigen Parasitenkontrolle angehalten werden.

8 Summary

The present study provides a standardized and evidence-based snapshot of the current anthelmintic resistance (AR) status against benzimidazoles (BZ) and macrocyclic lactones (ML) in gastrointestinal nematodes (GIN) of sheep on ten randomly chosen sheep farms in the southern Italian region of Campania. The trial is based on the *in vivo* faecal egg count reduction test (FECRT) performed with the Mini-FLOTAC technique on each farm for agents of both anthelmintic classes. Albendazole (ALB) was administered on Farm 1–10, eprinomectin (EPR) on Farm 1 and ivermectin (IVM) on Farm 2–10. The FECRT was conducted on individual and pooled samples for each tested drug on groups of 40 sheep based on faecal egg counts (FEC) straight before the treatment (D0) and 14 days after the treatment (D14) without considering a control group. Coprocultures were set for D0- and D14-faecal samples of each group. Efficacy was classified as 'reduced', 'suspected' and 'normal'. In sheep farms where 'suspected' or 'reduced' efficacy was observed, an *in vitro* egg hatch test (EHT) and a follow-up FECRT using fenbendazole (FBZ) were conducted.

For the agents of ML, efficacy was classified as 'normal' in all ten farms, for ALB as 'normal' in eight farms, in one farm (Farm 2) as 'suspected' and in another farm (Farm 1) as 'reduced'. The results for FECR from individual and pooled faecal samples correlated positively. The predominant GIN genera in the D14 coprocultures were *Trichostrongylus*, followed by *Haemonchus*. The EHTs and follow-up FECRT carried out on Farm 1 and Farm 2 confirmed the AR status. The egg hatch inhibition at the discriminating dose of $> 0.1 \mu\text{g} / \text{ml}$ TBZ was less than 50% for both farms. In the *in vivo* follow-up FECRT, efficacy was classified as 'reduced' for Farm 1 and as 'suspected' for Farm 2 as in the main trial.

The level of AR in the investigated region is still low compared with other European countries, nevertheless rising prevalence of AR is visible. Veterinarians and farmers should be encouraged to implement strategies for sustainable parasite control.

9 Literaturverzeichnis

- Bosco A, Maurelli MP, Ianniello D, Morgoglione ME, Amadesi A, Coles GC, Cringoli G, Rinaldi L. 2018. The recovery of added nematode eggs from horse and sheep faeces by three methods. *BMC veterinary research*, 14 (1): 7. DOI 10.1186/s12917-017-1326-7.
- Calvete C, Uriarte J. 2013. Improving the detection of anthelmintic resistance: evaluation of faecal egg count reduction test procedures suitable for farm routines. *Veterinary parasitology*, 196 (3-4): 438–452. DOI 10.1016/j.vetpar.2013.02.027.
- Castagna F, Palma E, Cringoli G, Bosco A, Nisticò N, Caligiuri G, Britti D, Musella V. 2019. Use of Complementary Natural Feed for Gastrointestinal Nematodes Control in Sheep: Effectiveness and Benefits for Animals. *Animals : an open access journal from MDPI*, 9 (12). DOI 10.3390/ani9121037.
- Cernanská D, Várady M, Cudeková P, Corba J. 2008. Worm control practices on sheep farms in the Slovak Republic. *Veterinary parasitology*, 154 (3-4): 270–276. DOI 10.1016/j.vetpar.2008.03.026.
- Charlier J, Thamsborg SM, Bartley DJ, Skuce PJ, Kenyon F, Geurden T, Hoste H, Williams AR, Sotiraki S, Höglund J, Chartier C, Geldhof P, van Dijk J, Rinaldi L, Morgan ER, Samson-Himmelstjerna G von, Vercruysse J, Claerebout E. 2018. Mind the gaps in research on the control of gastrointestinal nematodes of farmed ruminants and pigs. *Transboundary and emerging diseases*, 65 Suppl 1: 217–234. DOI 10.1111/tbed.12707.
- Charlier J, Rinaldi L, Musella V, Ploeger HW, Chartier C, Vineer HR, Hinney B, Samson-Himmelstjerna G von, Băcescu B, Mickiewicz M, Mateus TL, Martinez-Valladares M, Quealy S, Azaizeh H, Sekovska B, Akkari H, Petkevicius S, Hektoen L, Höglund J, Morgan ER, Bartley DJ, Claerebout E. 2020. Initial assessment of the economic burden of major parasitic helminth infections to the ruminant livestock industry in Europe. *Preventive veterinary medicine*, 182: 105103. DOI 10.1016/j.prevetmed.2020.105103.
- Claerebout E, Wilde N de, van Mael E, Casaert S, Velde FV, Roeber F, Veloz PV, Levecke B, Geldhof P. 2020. Anthelmintic resistance and common worm control practices in sheep farms in Flanders, Belgium. *Veterinary parasitology, regional studies and reports*, 20: 100393. DOI 10.1016/j.vprsr.2020.100393.

- Coles GC, Bauer C, Borgsteede FHM, Geerts S, Klei TR, Taylor MA, Waller PJ. 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary parasitology*, 44 (1-2): 35–44. DOI 10.1016/0304-4017(92)90141-u.
- Coles GC, Jackson F, Pomroy WE, Prichard RK, Samson-Himmelstjerna G von, Silvestre A, Taylor MA, Vercruysse J. 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary parasitology*, 136 (3-4): 167–185. DOI 10.1016/j.vetpar.2005.11.019.
- Cringoli G, Maurelli MP, Levecke B, Bosco A, Vercruysse J, Utzinger J, Rinaldi L. 2017. The Mini-FLOTAC technique for the diagnosis of helminth and protozoan infections in humans and animals. *Nature protocols*, 12 (9): 1723–1732. DOI 10.1038/nprot.2017.067.
- Cringoli G, Veneziano V, Jackson F, Vercruysse J, Greer AW, Fedele V, Mezzino L, Rinaldi L. 2008. Effects of strategic anthelmintic treatments on the milk production of dairy sheep naturally infected by gastrointestinal strongyles. *Veterinary parasitology*, 156 (3-4): 340–345. DOI 10.1016/j.vetpar.2008.06.015.
- Domke AVM, Chartier C, Gjerde B, Leine N, Vatn S, Osterås O, Stuen S. 2011. Worm control practice against gastro-intestinal parasites in Norwegian sheep and goat flocks. *Acta veterinaria Scandinavica*, 53: 29. DOI 10.1186/1751-0147-53-29.
- Falzon LC, O'Neill TJ, Menzies PI, Peregrine AS, Jones-Bitton A, vanLeeuwen J, Mederos A. 2014. A systematic review and meta-analysis of factors associated with anthelmintic resistance in sheep. *Preventive veterinary medicine*, 117 (2): 388–402. DOI 10.1016/j.prevetmed.2014.07.003.
- Furgasa W, Abunna F, Yimer L, Haile G (2018) Review on Anthelmintic Resistance against Gastrointestinal Nematodes of Small Ruminants: Its Status and Future Perspective in Ethiopia. *J Vet Sci Ani Husb* 6(4): 40
- Gasbarre LC, Ballweber LR, Stromberg BE, Dargatz DA, Rodriguez JM, Kopral CA, Zarlenga DS. 2015. Effectiveness of current anthelmintic treatment programs on reducing fecal egg counts in United States cow-calf operations. *Canadian Journal of Veterinary Research*, 79 (4): 296–302.

- Geurden T, Hoste H, Jacquet P, Traversa D, Sotiraki S, Di Frangipane Regalbono A, Tzanidakis N, Kostopoulou D, Gaillac C, Privat S, Giangaspero A, Zanardello C, Noé L, Vanimisetti B, Bartram D. 2014. Anthelmintic resistance and multidrug resistance in sheep gastro-intestinal nematodes in France, Greece and Italy. *Veterinary parasitology*, 201 (1-2): 59–66. DOI 10.1016/j.vetpar.2014.01.016.
- Glover M, Clarke C, Nabb L, Schmidt J. 2017. Anthelmintic efficacy on sheep farms in south-west England. *The Veterinary record*, 180 (15): 378. DOI 10.1136/vr.104151.
- Hamel D, Bosco A, Rinaldi L, Cringoli G, Kaulfuß K-H, Kellermann M, Fischer J, Wang H, Kley K, Mayr S, Rauh R, Visser M, Wiefel T, Fankhauser B, Rehbein S. 2017. Eprinomectin pour-on (EPRINEX® Pour-on, Merial): efficacy against gastrointestinal and pulmonary nematodes and pharmacokinetics in sheep. *BMC veterinary research*, 13 (1): 148. DOI 10.1186/s12917-017-1075-7.
- Hinney B, Schoiswohl J, Melville L, Ameen VJ, Wille-Piazzai W, Bauer K, Joachim A, Krücken J, Skuce PJ, Krametter-Frötscher R. 2020. High frequency of benzimidazole resistance alleles in trichostrongyloids from Austrian sheep flocks in an alpine transhumance management system. *BMC veterinary research*, 16 (1): 132. DOI 10.1186/s12917-020-02353-z.
- Kalkal H. 2020. Anthelmintic Resistance in Livestock Parasites: Indian Scenario. *International Journal of Current Microbiology and Applied Sciences*, 9 (2): 2839–2851. DOI 10.20546/ijcmas.2020.902.323.
- Kaplan RM, Vidyashankar AN. 2012. An inconvenient truth: global worming and anthelmintic resistance. *Veterinary parasitology*, 186 (1-2): 70–78. DOI 10.1016/j.vetpar.2011.11.048.
- Kebede A. 2019. Review on anthelmintic drug resistance nematodes and its methods of detection in Ethiopia. *J Vet Med Animal Sci*.
- Kenyon F, Rinaldi L, McBean D, Pepe P, Bosco A, Melville L, Devin L, Mitchell G, Ianniello D, Charlier J, Vercruysse J, Cringoli G, Levecke B. 2016. Pooling sheep faecal samples for the assessment of anthelmintic drug efficacy using McMaster and Mini-FLOTAC in gastrointestinal strongyle and *Nematodirus* infection. *Veterinary parasitology*, 225: 53–60. DOI 10.1016/j.vetpar.2016.03.022.

- Lambertz C, Pouloupoulou I, Wuthijaree K, Gauly M. 2019. Anthelmintic resistance in gastrointestinal nematodes in sheep raised under mountain farming conditions in Northern Italy. *Veterinary record open*, 6 (1): e000332. DOI 10.1136/vetreco-2018-000332.
- Levecke B, Kaplan RM, Thamsborg SM, Torgerson PR, Vercruysse J, Dobson RJ. 2018. How to improve the standardization and the diagnostic performance of the fecal egg count reduction test? *Veterinary parasitology*, 253: 71–78. DOI 10.1016/j.vetpar.2018.02.004.
- Levecke B, Rinaldi L, Charlier J, Maurelli MP, Bosco A, Vercruysse J, Cringoli G. 2012. The bias, accuracy and precision of faecal egg count reduction test results in cattle using McMaster, Cornell-Wisconsin and FLOTAC egg counting methods. *Veterinary parasitology*, 188 (1-2): 194–199. DOI 10.1016/j.vetpar.2012.03.017.
- Mavrot F. 2016. *Livestock Nematode Infection in a Changing World: Investigating the European Situation*. University of Zurich.
- Mavrot F, Hertzberg H, Torgerson P. 2015. Effect of gastro-intestinal nematode infection on sheep performance: a systematic review and meta-analysis. *Parasites & vectors*, 8: 557. DOI 10.1186/s13071-015-1164-z.
- Paras KL, George MM, Vidyashankar AN, Kaplan RM. 2018. Comparison of fecal egg counting methods in four livestock species. *Veterinary parasitology*, 257: 21–27. DOI 10.1016/j.vetpar.2018.05.015.
- Prichard RK, Hall CA, Kelly JD, Martin IC, Donald AD. 1980. The problem of anthelmintic resistance in nematodes. *Australian veterinary journal*, 56 (5): 239–251. DOI 10.1111/j.1751-0813.1980.tb15983.x.
- Ramos F, Portella LP, Rodrigues FdS, Reginato CZ, Pötter L, Cezar AS, Sangioni LA, Vogel FSF. 2016. Anthelmintic resistance in gastrointestinal nematodes of beef cattle in the state of Rio Grande do Sul, Brazil. *International journal for parasitology. Drugs and drug resistance*, 6 (1): 93–101. DOI 10.1016/j.ijpddr.2016.02.002.

- Ramünke S, Melville L, Rinaldi L, Hertzberg H, Waal T de, Samson-Himmelstjerna G von, Cringoli G, Mavrot F, Skuce P, Krücken J, Demeler J. 2016. Benzimidazole resistance survey for *Haemonchus*, *Teladorsagia* and *Trichostrongylus* in three European countries using pyrosequencing including the development of new assays for *Trichostrongylus*. *International journal for parasitology. Drugs and drug resistance*, 6 (3): 230–240. DOI 10.1016/j.ijpddr.2016.10.002.
- Redman E, Whitelaw F, Tait A, Burgess C, Bartley Y, Skuce PJ, Jackson F, Gilleard JS. 2015. The emergence of resistance to the benzimidazole anthelmintics in parasitic nematodes of livestock is characterised by multiple independent hard and soft selective sweeps. *PLoS neglected tropical diseases*, 9 (2): e0003494. DOI 10.1371/journal.pntd.0003494.
- Rinaldi L, Amadesi A, Dufourd E, Bosco A, Gadanho M, Lehebel A, Maurelli MP, Chauvin A, Charlier J, Cringoli G, Ravinet N, Chartier C. 2019. Rapid assessment of faecal egg count and faecal egg count reduction through composite sampling in cattle. *Parasites & vectors*, 12 (1): 353. DOI 10.1186/s13071-019-3601-x.
- Rinaldi L, Levecke B, Bosco A, Ianniello D, Pepe P, Charlier J, Cringoli G, Vercruysse J. 2014. Comparison of individual and pooled faecal samples in sheep for the assessment of gastrointestinal strongyle infection intensity and anthelmintic drug efficacy using McMaster and Mini-FLOTAC. *Veterinary parasitology*, 205 (1-2): 216–223. DOI 10.1016/j.vetpar.2014.06.011.
- Rinaldi L, Morgan ER, Bosco A, Coles GC, Cringoli G. 2014. The maintenance of anthelmintic efficacy in sheep in a Mediterranean climate. *Veterinary parasitology*, 203 (1-2): 139–143. DOI 10.1016/j.vetpar.2014.02.006.
- Rose H, Rinaldi L, Bosco A, Mavrot F, Waal T de, Skuce P, Charlier J, Torgerson PR, Hertzberg H, Hendrickx G, Vercruysse J, Morgan ER. 2015. Widespread anthelmintic resistance in European farmed ruminants: a systematic review. *The Veterinary record*, 176 (21): 546. DOI 10.1136/vr.102982.
- Salgado JA, Santos CdP. 2016. Overview of anthelmintic resistance of gastrointestinal nematodes of small ruminants in Brazil. *Revista brasileira de parasitologia veterinaria = Brazilian journal of veterinary parasitology : Orgao Oficial do Colegio Brasileiro de Parasitologia Veterinaria*, 25 (1): 3–17. DOI 10.1590/S1984-29612016008.

- Samson-Himmelstjerna G von, Coles GC, Jackson F, Bauer C, Borgsteede F, Cirak VY, Demeler J, Donnan A, Dorny P, Epe C, Harder A, Höglund J, Kaminsky R, Kerboeuf D, Küttler U, Papadopoulos E, Posedi J, Small J, Várady M, Vercruysse J, Wirthlerle N. 2009. Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. *Parasitology research*, 105 (3): 825–834. DOI 10.1007/s00436-009-1466-1.
- Samson-Himmelstjerna G von, Walsh TK, Donnan AA, Carrière S, Jackson F, Skuce PJ, Rohn K, Wolstenholme AJ. 2009. Molecular detection of benzimidazole resistance in *Haemonchus contortus* using real-time PCR and pyrosequencing. *Parasitology*, 136 (3): 349–358. DOI 10.1017/S003118200800543X.
- Sangster NC. 1999. Anthelmintic resistance: past, present and future. *International journal for parasitology*, 29 (1): 115–124. DOI 10.1016/s0020-7519(98)00188-x.
- Sangster NC, Cowling A, Woodgate RG. 2018. Ten Events That Defined Anthelmintic Resistance Research. *Trends in parasitology*, 34 (7): 553–563. DOI 10.1016/j.pt.2018.05.001.
- Traversa D, Paoletti B, Otranto D, Miller J. 2007. First report of multiple drug resistance in trichostrongyles affecting sheep under field conditions in Italy. *Parasitology research*, 101 (6): 1713–1716. DOI 10.1007/s00436-007-0707-4.

Anhang 1

About this article



Check for
updates

Cite this article

Bosco, A., Kießler, J., Amadesi, A. *et al.* The threat of reduced efficacy of anthelmintics against gastrointestinal nematodes in sheep from an area considered anthelmintic resistance-free. *Parasites Vectors* **13**, 457 (2020). <https://doi.org/10.1186/s13071-020-04329-2>

[Download citation](#) 

Received
04 April 2020

Accepted
30 August 2020

Published
09 September 2020

DOI
<https://doi.org/10.1186/s13071-020-04329-2>

Danksagung

Bedanken möchte ich mich bei allen, die mir die Forschung im CREMOPAR bzw. mein gesamtes Südtalienerlebnis im Sommer 2019 ermöglicht und unvergesslich gemacht haben. Besonders bedanken möchte ich mich bei meiner Betreuerin Barbara Hinney, die sich direkt bereit erklärt hat, mich in diesem Erasmus-Projekt zu unterstützen und mich von Wien aus begleitet hat; bei Davide Ianniello, der während meines Aufenthaltes gemeinsam mit mir im Labor die Proben aufbereitet und ausgewertet hat; bei Antonio Bosco, Giuseppe Mangieri und Mario Iorio für die tolle Zusammenarbeit auf den Schafbetrieben und teils im Labor; bei Giuseppe Cringoli, dem Leiter des Forschungszentrums CREMOPAR; und ganz besonders bei meiner Betreuerin Laura Rinaldi, die sich über das Maß hinaus für mich eingesetzt hat, sowohl während meines Aufenthaltes im CREMOPAR, als auch später bei der Ausarbeitung des Papers.