Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
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Coccidiosis in lambs: treatment and control

Ane Odden

COCCIDIOSIS IN LAMBS:
TREATMENT AND CONTROL

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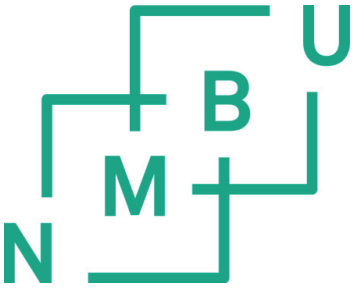
Thesis for the degree of *Philosophiae Doctor* (PhD)



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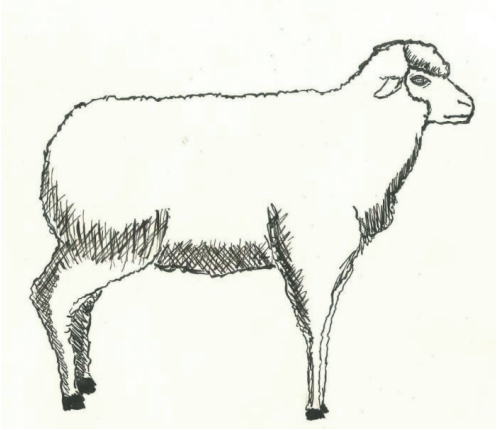

"Careful. We don't want to learn from this."

"Calvin and Hobbes"
Bill Waterson

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List of abbreviations

ACE	Anticoccidial efficacy
ACR	Anticoccidial resistance
AR	Anthelmintic resistance
BCEC	Bovine colonic epithelial cells
BUVEC	Bovine umbilical vein endothelial cells
CET	Controlled efficacy test
ELISA	Enzyme-linked immunosorbent assay
FECRT	Faecal egg count reduction test
FOCRT	Faecal oocyst count reduction test
MIC	Microneme protein
NMBU	Norwegian University of Life Sciences
NSRS	The Norwegian Sheep Recording System
NST	Norwegian Short Tail Sheep ("spæl")
NWS	Norwegian White Sheep ("norsk kvit sau")
NZL	Nitromezuril
OPG	Oocysts per gram
OSIA	Oocyst sporulation inhibition assay
PCR	Polymerase chain reaction
PT	Pilot trial
SIDA	Sporozoite invasion and development assay
ULPGC	University of Las Palmas, Gran Canaria
WAAVP	World Association for the Advancement of Veterinary Parasitology
WHO	World Health Organisation

Glossary

Anticoccidial drug	A pharmaceutical compound able to kill the coccidia, or interfere with their ability to stay in the host, thereby reducing or eradicating the coccidial infection
Antiparasitic resistance	WHO definition: "*the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within tolerance of the subject*" (Bloland 2001).
CET	Controlled efficacy test: experimental infection of naïve animals with a suspected resistant parasite isolate. Evaluation of drug efficacy is based on egg/oocyst excretion, clinical symptoms, growth rates, and necropsy findings in animals treated with the antiparasitic drug in question and untreated as controls. In some cases uninfected controls are also included in the test.
Coccidiosis vs eimeriosis	Both coccidiosis and eimeriosis are used to describe an infection with *Eimeria* spp. While eimeriosis is more accurate, coccidiosis is more commonly used. Coccidia also includes parasites such as *Cystoisospora* spp., *Toxoplasma gondii,* and *Sarcocystis* spp.
FECRT	WAAVP approved method for field detection of anthelmintic resistance in nematodes, by assessment of reduction in egg counts following treatment. Includes post-treatment (± pre-treatment) egg counts from treated (± control) animals, and calculations of drug efficacy.
FOCRT	A novel test, first described in the present project, for evaluation of anticoccidial efficacy in the field, by assessing the reduction in oocyst excretion post treatment. The FOCRT is based on oocysts counts in pre- and post-treatment faecal samples of treated and untreated lambs.
Metaphylactic treatment	Treatment during the prepatent period to prevent clinical signs of coccidiosis, i.e. treatment after infection, but before clinical signs.
Prophylactic treatment	Treatment to prevent infection, i.e. treatment prior to infection.

Summary

Coccidiosis or eimeriosis, i.e., infection with *Eimeria* spp., is important in sheep and other livestock, and may lead to both reduced animal welfare and economic losses. Clinical signs of coccidiosis include diarrhoea (± haemorrhagic), dehydration, weight loss/reduced weight gain, and occasionally death. Clinical disease is most commonly seen in young animals, prior to the development of protective immunity. The infection can be controlled by management, including good hygiene, and by chemoprophylaxis. Toltrazuril is widely used against ovine coccidiosis, and is the only anticoccidial registered for use in sheep in the Nordic countries; Denmark Norway and Sweden. At the start of this study, resistance of ovine *Eimeria* to toltrazuril had been suspected in Norway, but never investigated properly or proven. This work was therefore aimed at investigating possible anticoccidial resistance (ACR) in ovine *Eimeria* spp., identifying risk behaviour for the development of ACR, developing evaluation tools for assessment of anticoccidial efficacy (ACE), and evaluating a possible alternative treatment strategy.

The use of anticoccidials in Norwegian sheep farms was investigated in a questionnaire-based study (Paper I), sent to all members of the Norwegian Sheep Recording System. The study showed high frequency of treatment, predominantly with toltrazuril, and often without a laboratory-confirmed diagnosis (12.3 %). In addition, almost 40 % of the farmers reported having experienced clinical signs, presumably related to coccidiosis, in lambs already treated with an anticoccidial (Paper I).

For paper II, farms with a suspected high risk of ACR, i.e., farms with clinical signs of coccidiosis observed in treated lambs, continuous treatment with anticoccidials for a minimum of four years, and a flock size of ≥ 60 ewes, were selected for a field trial. The farmers collected faecal samples twice, approximately one and two weeks after turnout, from eight twin pairs, of which one twin was treated with toltrazuril (Baycox® Sheep vet, Bayer Animal Health). Based on these faecal samples, a novel method for field assessment of ACE was developed. This method, the faecal oocyst count reduction test (FOCRT), first determines whether treatment and sampling had been performed at the correct time, and subsequently assesses the ACE, by comparing oocyst counts in post-treatment faecal samples from treated and untreated lambs. Of the 36 farms complying with the protocol, timing of faecal sampling and treatment were not correct in 16 flocks and therefore the ACE could not be evaluated in those farms. Good efficacy of toltrazuril was detected in 13 farms, 2 farms had reduced efficacy, and inconclusive results were obtained in 5 farms. Of the 16 farms with incorrect timing of treatment and sampling, most treated too late,

i.e. lambs in these farms had been infected prior to turnout, indicating that infection in the housed period may be more common than previously thought.

Based on the results in paper II, a controlled efficacy trial was performed (paper III), in order to verify ACR. Lambs that had been raised coccidia-free, were infected with a suspected resistant isolate (NMBU ID 35). Then half the lambs were treated with 20 mg/kg toltrazuril (Baycox® Sheep vet., Bayer Animal Health), while the others were controls. Oocyst excretion, species composition, weight gain, clinical signs, and macro- and microscopic pathological changes were evaluated. No differences were observed between treated lambs and untreated controls. Resistance against toltrazuril was documented in all *Eimeria* species from the field isolate, including the highly pathogenic *E. ovinoidalis*.

One aim of article IV was to investigate an alternative control strategy, based on attempting to reduce ingestion of oocysts on pasture, by ensuring that iron levels remained high. Housed lambs are often iron deficient, which may lead to geophagia after turnout, and subsequent increased uptake of *Eimeria* oocysts. A trial investigating the effect of iron injection on oocyst excretion (paper IV) was therefore performed, and for that purpose, ten or eleven twin pairs from five farms located in Rogaland County were used. From each twin pair one lamb was injected with gleptoferron (Gleptosil vet, Ceva Santé Animale) within the first three days of life, while the other twin (control) was injected with sterile saline. Lambs with their dam were housed for a minimum of 14 days before turnout to spring pastures. The results showed no effect of iron supplementation on oocyst excretion or weight gain.

Based on the results from this project, more research and testing of ACE seems to be important, which is now possible due to the FOCRT (article II). The CET (article III) showed toltrazuril resistance in several *Eimeria* spp., including the highly pathogenic *E. ovinoidalis*. Resistance in such a pathogenic species, may lead to severe welfare challenges, and non-chemical measures should be implemented, such as improved pasture management, shortening of the lambing period, earlier turnout to spring pastures, and hygienic measures. This is especially important as the alternative control strategy supplementing young lambs with iron (article IV) did not seem to reduce the uptake and excretion of oocysts.

Sammendrag (Norwegian summary)

Koksidiose er forårsaket av en gruppe encellede parasitter som kalles *Eimeria* spp. og er en viktig produksjonsbegrensende sjukdom hos unge dyr blant flere arter, som sau, ku og fjørfe. Disse parasittene gir skader i tarmveggen, og kliniske tegn ved koksidiose inkluderer diaré som kan være blodig, dehydrering, vekttap/redusert tilvekst og eventuelt død. Infeksjonen holdes vanligvis i sjakk av hygienetiltak, beitebruk, og ved forebyggende bruk av legemidler. Mistanke om redusert effekt av det vanligst brukte middelet mot koksidiose hos lam, toltrazuril, kan derfor gi store velferdsmessige utfordringer, og er bakgrunnen for dette prosjektet. I dette doktorgradsarbeidet ønsket vi å undersøke bruken av antikoksidiemidler til sau (artikkel I), finne metoder for å evaluere behandlingseffekten (artikkel II), verifisere tidligere mistanke om resistens (artikkel III), og å undersøke om jerninjeksjon kan være en alternativ metode for behandling av koksidiose (artikkel IV).

I artikkel I ble norske sauebønders behandlingsmønster mot koksidiose undersøkt ved hjelp av en spørreundersøkelse. Denne undersøkelsen viste at alle lam i en flokk blir behandlet på et bestemt tidspunkt (ved utslipp (38.6 %) eller ca en uke etter utslipp (32.4 %)), ofte uten en definitiv laboratoriediagnose (87.7 %). Toltrazuril var det preparatet som oftest ble brukt, og det er også det eneste koksidiostatika som nå er registrert til sau i Norge. Nesten 40 % av bøndene opplevde at lam utviklet kliniske tegn på koksidiose etter behandling, noe som kan indikere resistens.

I artikkel II ble et utvalg bønder med stor risiko for resistens plukket ut for å delta i et feltforsøk. Risikoflokker inkluderte besetninger med ≥60 vinterfôrede søyer som hadde lam med kliniske tegn etter behandling forenlig med koksidiose. Det andre inklusjonskriteriet var at besetningene hadde behandlet mot koksidiose kontinuerlig i fire eller flere år. Bøndene tok avføringsprøver av åtte tvillingpar ca en uke etter utslipp på vårbeite, samtidig som en tvilling ble behandlet med toltrazuril (Baycox® Sheep vet). Oppfølgende avføringsprøver ble tatt av alle lam ca en uke etter den første prøven. Med dette datamaterialet i bunn, ble det utviklet en statistisk modell for evaluering av behandlingseffekt i felt kalt "Faecal Oocyst Count Reduction Test" (FOCRT). Denne modellen indikerer om behandling og prøvetakning blir utført på riktig tidspunkt, før den sammenligner oocyste-utskillelsen hos behandlede og ubehandlede lam i den oppfølgende prøven. Av de 36 flokkene som deltok, hadde 13 god effekt av behandling, to hadde redusert effekt, fem flokker hadde usikker effekt, og 16 flokker behandlet på feil tidspunkt og evaluering av behandlingseffekten kunne derfor ikke gjennomføres. Disse 16 flokkene ble i stor grad

behandlet for seint, og avføringsprøvene viste høy oocyste-utskillese allerede en uke etter utslipp, noe som indikerer at lammene ble infisert i inneperioden.

Med bakgrunn i funnet av redusert effekt av toltrazuril i feltforsøket, ble det utført et kontrollert behandlingsforsøk (artikkel III) der 20 koksidie-frie lam ble infisert med et mistenkt resistent koksidie-isolat. Halvparten av lammene ble behandlet med toltrazuril og resten med saltvann syv dager etter infeksjon. Det var ingen forskjell i oocyste-utskillelse, kliniske funn og obduksjonsresultater mellom behandlede lam og kontroller. Den manglende effekten av behandling verifiserte for første gang resistens mot toltrazuril hos sauens koksidier, inkludert den patogene *E. ovinoidalis*.

Det er behov for alternative behandlingsmåter siden toltrazuril er det eneste registrerte koksidiostatika tilgjengelig til sau i Norge. I artikkel IV ble det derfor undersøkt om jerninjeksjon av unge lam kunne påvirke opptaket og utskillelsen av oocyster. Det er tidligere vist at lam som oppstalles lenge inne utvikler jernmangelanemi, som igjen kan gi utslag i blant annet jordspising, som kan inneholde smitte, på vårbeite. Både en spørreundersøkelse om bruken av jern til lam og et feltforsøk ble gjennomført. Tvillinglam (20-22 lam per flokk) fra fem flokker i Rogaland ble inkludert i studien. En av tvillingene ble injisert med gleptoferron (Gleptosil vet, Ceva Santé Animale), mens den andre ble injisert med sterilt saltvann i løpet av de tre første levedøgnene. Lammene ble oppstallet inne i minimum 14 dager, og det ble tatt avføringsprøver, blodprøver, og vektmålinger i denne perioden. Det var ingen effekt av jerninjeksjon på utskillelsen av oocyster tre uker etter utslipp, men det ble sett en reduksjon i oocyste-utskillelsen to uker etter utslipp i en av flokkene. Resultatene så langt tyder på at jerninjeksjon ikke vil redusere behovet for behandling med koksidiostatika, men ytterligere undersøkelser er nødvendig.

Med bakgrunn i funnene fra disse artiklene vil videre kartlegging av redusert koksidiostatikaeffekt være viktig, noe som nå er mulig med den nye feltmetoden, FOCRT-modellen (artikkel II). Denne metoden er ny, og trenger videre testing. Resistens hos *E. ovinoidalis* som er en av de mest patogene koksidiene kan gi store velferdsmessige utfordringer. I besetninger med påvist resistens vil ikke-medikmentelle tiltak være viktige. Dette inkluder et større fokus på 'rene' beiter, hygienetiltak både inne og på beite, og omlegging til kortere og senere lammingsperiode, slik at lammene kan slippes ut på vårbeite når de er yngre enn det som er vanlig i dag. Dette er særlig viktig siden det per i dag ikke er noe godt alternativ til toltrazurilbehandling.

List of papers

Paper I

Odden A, Enemark HL, Robertson LJ, Ruiz A, Hektoen L, & Stuen S (2017). Treatment against coccidiosis in Norwegian lambs and potential risk factors for development of anticoccidial resistance – a questionnaire-based study. Parasitol Res 116, 1237-45.

Paper II

Odden A, Denwood MJ, Stuen S, Robertson LJ, Ruiz A, Hamnes IS, Hektoen L, & Enemark HL (2018). Field evaluation of anticoccidial efficacy: A novel approach demonstrates reduced efficacy of toltrazuril against ovine *Eimeria* spp. in Norway. Int J Parasitol Drugs Drug Resist 8(2): 304-11

Paper III

Odden A, Enemark HL, Ruiz A, Robertson LJ, Ersdal C, Nes SK, Tømmerberg V, Stuen S. Controlled efficacy trial confirming toltrazuril resistance in a field isolate of ovine *Eimeria* spp. *Parasites & Vectors, in press*

Paper IV

Odden A, Vatn S, Ruiz A, Robertson LJ, Enemark HL, Nes SK, Tømmerberg V & Stuen S. Excretion of *Eimeria* spp. oocysts in young lambs following iron supplementation. *Submitted to Acta Veterinaria Scandinavica*

Introduction

Coccidiosis or eimeriosis, i.e. infection with *Eimeria* spp., is an important parasitosis in sheep and other farm animals, and may lead to both reduced animal welfare, and economic losses for the farmer (Daugschies and Najdrowski 2005; Chartier and Paraud 2012). Infections with *Eimeria* spp. might be peracute, acute, subclinical and chronic, with symptoms ranging from diarrhoea, dehydration, weight loss/reduced growth, to death (occasionally prior to clinical symptoms and oocyst excretion) (Taylor 1995; Chartier and Paraud 2012). Coccidiosis has a worldwide occurrence and has been described in many hosts and production systems including, e.g., poultry, swine, sheep, goats and cattle (Amarante and Barbosa 1992; Svensson et al. 1993; Matjila and Penzhorn 2002; Agyei et al. 2004; Daugschies and Najdrowski 2005; Cai and Bai 2009; Saratsis et al. 2011; Skampardonis et al. 2012; Chapman 2014), as well as Norwegian sheep (Helle 1964, 1970; Gjerde and Helle 1986; Gjerde et al. 2009).

Sheep production in Norway

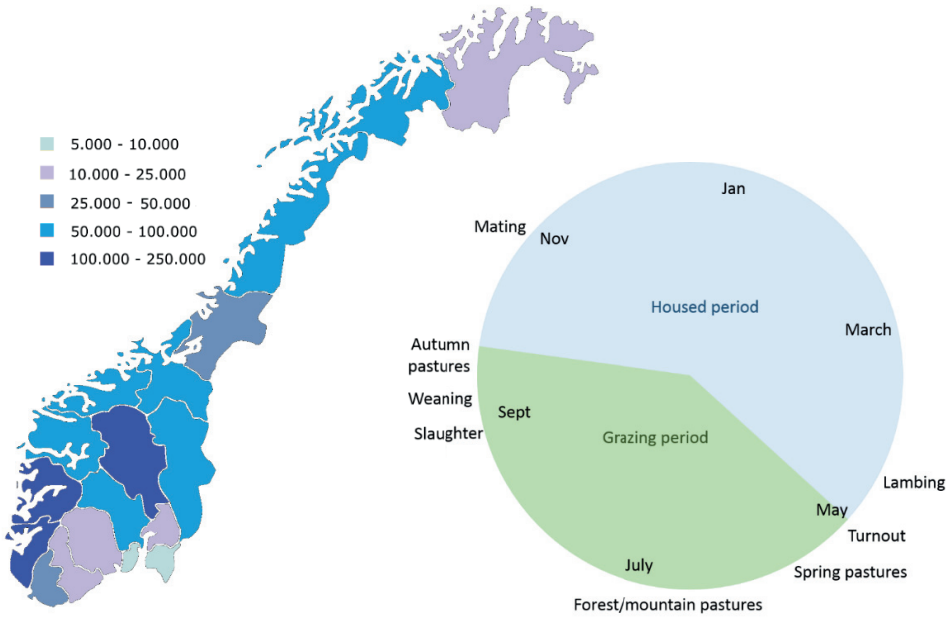

Figure 1. Left: map of Norway showing the number of ewes (one year or older) in each county per January 1st 2017 (Statistics Norway 2017). Right: Norwegian sheep farming during the year (modified from Aunsmo et al. 1998).

Due to climatic and geographical conditions, most of Norway's areas are best suited for forage-based animal production. Livestock grazing on mountain/forest pastures has a long tradition;

however, only 40 % of the available areas for mountain/forest grazing are utilised today (Austrheim et al. 2011; Arnoldussen et al. 2014). Sheep production is therefore both a wanted, and a suitable production, and can be found in all mainland Norwegian counties (Fig. 1), with Vestlandet (west), Østlandet (east) and Trøndelag (mid) as the most sheep-dense areas (Aunsmo et al. 1998; Norwegian Ministry of Foreign Affairs 2015; Statistics Norway 2016b). Sheep are important farm animals in Norway, and the average flock size has increased over the last decade, from 65.2 ewes per flock in 2006 to 75.8 ewes per flock in 2016. In the same period, the number of farms has decreased, while the total number of ewes has stayed around 1 million (Statistics Norway 2016a, b). Common breeds in Norway include the Norwegian White Sheep ("norsk kvit sau"), the Norwegian Short Tail White Landrace ("kvit spælsau") and the Old Norwegian Short Tail Landrace ("gammalnorsk spælsau") (National Sheep Recording System 2016).

Sheep are seasonal breeders, kept mainly for meat and wool production. The lambing season is in March – May, depending on the geographical region. Most ewes are housed during winter, with turnout to spring pastures after lambing (Fig. 1). During summer, ewes and lambs are moved to mountain/forest/uncultivated pastures, where the stocking densities usually are low (Mysterud et al. 2001; Vatn 2009). Lambs are weaned and slaughtered in the autumn, at around 4-5 months of age (Vatn 2009).

The Norwegian sheep recording system

The Norwegian sheep recording system (NSRS) is a national database for sheep flocks run by the Norwegian Meat and Poultry Research Centre, Animalia, and membership is voluntary (National Sheep Recording System 2016). Data from 2016 showed that 5.199 flocks with 360.982 ewes were members, corresponding to 36.5 % of all Norwegian sheep flocks or 47.9 % of all Norwegian ewes. The database fulfils all criteria for mandatory reporting and regulations on traceability of sheep, sheep products and the use of veterinary drugs.

Common pathogens of sheep

Common diseases and pathogens of sheep and lambs in Norway include, among other, arthritis, ectoparasites, endoparasites, mastitis, metritis, and pneumonia (National Sheep Recording System 2016). Bacterial infections are often dominated by *Staphylococcus aureus*, *Streptococcus* spp., *Mannheimia haemolytica, Bibersteinia trihalosi,* or *Escherichia coli* (Mørk et al. 2007; Holmøy et al. 2017). Important ectoparasites includes *Ixodes ricinus* (potentially acting as vectors for *Anaplasma phagocytophilum*), *Bovicola ovis* (chewing lice), *Linognathus ovillus* (sucking lice), and *Lucilia* spp. (Stuen 2007; Gjerde 2011). Prevalence studies on the most common endoparasites have shown the presence of *Teladorsagia circumcinta, Nematodirus* spp., *Haemonchus contortus, Fasciola hepatica,*

Cryptosporidium spp., and *Eimeria* spp., among others, with some geographical differences in the distribution (Helle and Hilali 1973; Robertson et al. 2010; Domke et al. 2013).

Ovine *Eimeria* spp.

Eimeria spp. are found within the phylum Alveolata, subphylum Apicomplexa, class Coccidea, order Eimeriida, and family Eimeriidae (Deplazes et al. 2016). Apicomplexan parasites are obligate intracellular parasites of invertebrates and vertebrates. Most of these parasites grow and replicate within a parasitophorous vacuole, a membrane bound compartment that separates the parasite from the host cell cytoplasm (Morrissette and Sibley 2002). Different characteristic morphological traits are shared by these parasites, like the apical complex; containing rhoptries, micronemes, and an apical polar ring. These organelles are important for identification and infection of suitable host cells, and formation of the parasitophorous vacuole (Dubremetz et al. 1998; Morrissette and Sibley 2002). The genus *Eimeria* consists of at least 1800 species of obligate intracellular parasites, which infect fish, reptiles, birds and mammals (Cowper et al. 2012; Walker et al. 2013). Infections with *Eimeria* spp. have been reported worldwide as a major livestock health problem in multiple production systems, e.g. cattle (Daugschies and Najdrowski 2005), poultry (Blake and Tomley 2014; Chapman 2014), and small ruminants (Chartier and Paraud 2012). In sheep, *Eimeria* spp. is a common cause of clinical disease and reduced growth in lambs (Taylor 1995; Chartier and Paraud 2012). *Eimeria* spp. are generally considered to be host specific, with some rare cases of *Eimeria* spp. crossing genus or family boundaries. However, the last statement is mainly based on morphological similarity, and not on cross-transmission studies (Vrba and Pakandl 2015). As an example, caprine and ovine *Eimeria* spp. were considered identical until attempts of infecting lambs with *E. ninakohlyakimovae* from goats failed (McDougald 1979). Today, there are 15 known species infecting sheep and 13 species infecting goats (Rommel 2000). The 11 most common *Eimeria* spp. found in sheep in Europe (Helle and Hilali 1973; Catchpole et al. 1975; Barutzki et al. 1990; Kaya 2004; Reeg et al. 2005; Dittmar et al. 2010) are listed in Table 1.

Table 1. Morphological and biological particulars about the 11 most common *Eimeria* species of sheep in Europe

Species First description[1, 2]	Main localisation[3]	Sporulation time (days)[4]	Prepatent period (days)[5]	Size (µm) Oocyst[5]	Size (µm) Sporocyst[2, 5]	Micropyle[5]	Polar cap[4]	Residual body[5] Oocyst	Residual body[5] Sporocyst
E. ashata Honess, 1942	Small intestine	2-3	18-30	29-37 x 17-28 (33.4 x 22.6)	18-20 x 7-10	+	+	-	+
E. bakuensis Levine and Ivens, 1970/ Musaev, 1970	Small intestine	2-4	18-29	23-36 x 15-24 (31 x 20)	11-17 x 6-9	+	+	-	+
E. crandallis Honess, 1942	Small intestine, caecum	1-3	15-20	17-23 x 17-22 (21.9 x 19.4)	8-11 x 5-8	+	+	-	+
E. faurei Moussu and Marotel, 1902/ Martin, 1909	Small intestine	1-3	13-15	28-37 x 21-27 (32 x 23)	14-16 x 8-9	+	-	-	-
E. granulosa Christensen, 1938	Unknown	3-4	Unknown	22-35 x 17-25 (29.4 x 20.9)	13-16 x 8-9	+	+	-	+
E. intricata Spiegel, 1925	Distal small intestine	3-7	23-27	40-56 x 30-41 (48 x 34)	16-18 x 8-10	+	+	-	+
E. marsica Restani, 1971	Unknown	3	14-16	15-22 x 11-14 (19 x 13)	8-11 x 4-6	+	+	-	-
E. ovinoidalis McDougald, 1979	Distal small intestine, caecum and colon	1-3	12-15	17-25 x 13-20 (23 x 18)	5-6 x 3-4	+	-	-	+
E. pallida Christensen, 1938	Unknown	1-3	Unknown	12-20 x 8-15 (14 x 10)	6-9 x 4-6	-	-	-	+
E. parva Kotlan et al., 1929	Small intestine	3-5	12-14	13-22 x 11-13 (16.5 x 14)	6-13 x 5-8	-	-	-	+ (few granules)
E. weybridgensis Norton et al., 1974	Jejunum	1-3	23-33	17-30 x 14-19 (24 x 17)	13-15 x 6-8	+	+	-	-

[1)]Levine (1985), [2)]Rommel (2000), [3)]Deplazes et al. (2016), [4)]Eckert et al. (1995), and [5)]Taylor et al. (1995)

Morphology

Most of the *Eimeria* spp. found in sheep can be differentiated based on oocyst morphology: size, shape and presence or not of micropyle and/or polar cap (Fig. 2 and 7, and Table 1) (Eckert et al. 1995b).

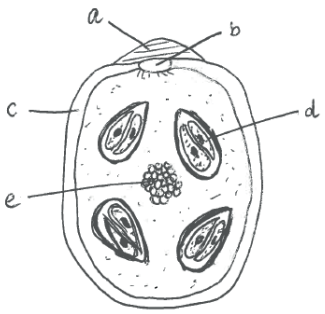

Figure 2. Schematic illustration of a sporulated oocyst: a) polar cap, b) micropyle, c) oocyst wall, d) sporocyste containing two sporozites and e) oocyst residual body

The oocyst wall consists of a rigid bilayer comprising of glucan and acid-fast lipids (Bushkin et al. 2013), which protects from physical and chemical threats. Oocysts are therefore very resistant when shed in faeces (Belli et al. 2006), and can remain viable even after treatment with sodium hypochlorite or freezing (Landers 1953; Stotish et al. 1978). However, the oocysts are sensitive to high temperatures and low humidity, and usually do not survive temperatures below -30ºC or above 40ºC (Foreyt 1990).

Life cycle

Eimeria spp. have a monoxenous lifecycle, and are transmitted via the faecal-oral route (Fayer 1980). Infection begins with the ingestion of sporulated oocysts (Urquhart et al. 1996; Walker et al. 2013). Each sporulated oocyst contains four sporocysts, which each contain two haploid sporozoites (Fayer 1980). After ingestion the oocyst wall is broken down in the host, by mechanical and chemical action, such as trypsin, bile and CO_2 (Jackson 1962; Fayer and Hammond 1967). Excystation results in the release of sporozoites from sporocysts through the anterior cap of the sporocyst. Released sporozoites invade intestinal cells, where they undergo asexual reproduction (merogony or schizogony) and produce merozoites (Fig. 3) (Wacha et al. 1971). This asexual reproduction produces vast amounts of merozoites, which complete several merogonic generations by reinvading intestinal cells. The number of generations depends on the species (Fayer 1980). Furthermore, the number of merozoites produced by the different meront generation varies. The number of excreted oocysts produced from one ingested sporulated oocyst differ, but production of more than one million oocysts is highly possible. As an example, the first-generation meronts of *E. ovinoidalis* contains several thousand merozoites, while the second-generation meront contains on average 24 merozoites (Taylor et al. 2016b). This high number of merozoites produced from one sporozoite explains why oocyst excretion follows an exponential

pattern, which has been observed in both natural and experimental infections (Chapman 1974a; Gregory et al. 1989b).

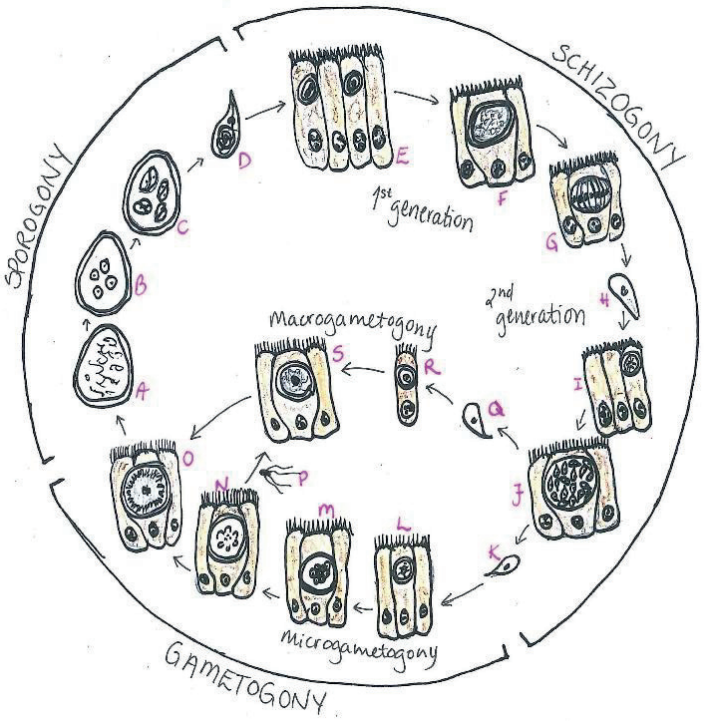

Figure 3. General life cycle of *Eimeria* spp. A-C: oocyst sporulation, D: sporozoite, E-F: formation and development of first generation meront, G: first generation meront containing merozoites, H: first generation merozoite, I-J: formation and development of second generation meront, K and Q: second generation merozoite, L-N: formation of microgametes, P: microgamete, R-S formation of macrogamete. Modified from Levine (1985).

Merogony is followed by a sexual phase, which can be divided in three: 1) gametocytogenesis (producing gametocytes from merozoites), 2) gametogenesis (producing haploid microgametes and macrogametes from gametocytes), and 3) fertilization of macrogametes by microgametes, producing diploid zygotes (Walker et al. 2013). The wall-forming bodies are mobilized to produce the oocyst wall, which protects the oocysts as they exit their host via faeces (Chapman et al. 2013). Outside the host, the oocysts undergo meiosis to produce infectious sporozoites (sporulation). The prepatent period, i.e., the time between ingestion of sporulated oocysts and excretion of unsporulated oocysts, varies between different *Eimeria* spp. (Table 1), and depends on several factors, such as the number of meront generations, and depth in the tissue where merogony, gamogony, and fertilization occur (Gjerde 2011).

It has been shown *in vivo* that avian *Eimeria* spp. exhibit a high degree of site specificity, with most species only invading narrowly defined areas within the intestine. This is also seen in other hosts, with different *Eimeria* species having varying predilection sites (Table 1) (Augustine and Danforth 1990; Deplazes et al. 2016). At the onset of invasion, exocystosis of micronemes and other secretory organelles are seen from the apical complex of the parasite. Microneme proteins (MICs) are discharged onto the parasite surface, binding to receptors on the host cell surface (Carruthers and Tomley 2008; Cowper et al. 2012). The tissue, cell, and host tropism of different apicomplexan species are therefore likely related to the range and specificity of the expressed MICs (Cowper et al. 2012).

Transmission

There are several possible routes by which ovine *Eimeria* spp. might reach a new host: 1) previous environmental faecal contamination, 2) oocysts passed by ewes, 3) oocysts passed by lambs, and 4) contaminated ewe udders and fleeces (Pout 1973; Gregory et al. 1983; Catchpole and Devonshire 1989; Dittmar et al. 2010). Oocysts excreted by ewes and/or environmental contamination might be the main source of infection for lambs initially. Nevertheless, due to the enormous multiplication rate of the parasite, infected lambs are likely to excrete several million oocysts into the environment. Thus, lamb excretion of oocysts rapidly becomes the main source of infection for younger lambs (Taylor 1995), and coccidiosis spreads rapidly within a flock of susceptible animals (Gauly et al. 2001; Reeg et al. 2005; Dittmar et al. 2010).

In Norway, lambs are usually housed indoors for two to three weeks or longer (Domke et al. 2011), and lambs infected early may contaminate the indoor environment with oocysts. Lambs may also become infected on pasture after turnout, either by oocysts excreted by already infected lambs or by oocysts that have overwintered from the previous grazing season (Helle 1970). Therefore, the main challenge with coccidiosis in Norway is seen during the spring pasture period. On the other hand, if lambs are kept in a clean indoor environment and are turned directly out onto summer pastures with low stocking densities, lambs may develop clinical coccidiosis in the autumn, if they are then put to graze contaminated pastures. Such autumn coccidiosis has been reported on Iceland (Skirnisson 2007), and may also occur in northern parts of Norway, due to similar grazing routines, although, to our knowledge, has not been reported as a challenge.

Immunity

Following an *Eimeria* spp. infection, lambs develop protective immunity to subsequent infections, and coccidiosis is therefore primarily a disease of young animals (Chapman 1974b; Gregory and

Catchpole 1989). Development of immunity is often seen as a reduction in oocyst excretion after the initial infection (Gregory and Catchpole 1989; Daugschies and Najdrowski 2005). Although the immunity is protective, it is not absolute, as a low level of infection may continue and some oocysts may still be excreted (Gregory and Catchpole 1989; Daugschies and Najdrowski 2005). Stress, such as adverse weather conditions, transport, lack of feeding/dietary changes, increased stocking densities, or severe concurrent infection, may however hamper the development and maintenance of immunity (Taylor 1995; Deplazes et al. 2016).

The immune response to an *Eimeria* spp. infection is mainly thought to be cellular, but humoral responses have also been shown (Hermosilla et al. 1999; Daugschies and Najdrowski 2005; Matos et al. 2017). The importance of passive immunity has been questioned: Gregory and Catchpole (1989) demonstrated significantly increased growth rates in lambs born from hyperimmunized ewes (ewes inoculated with high doses of *E. ovinoidalis* and *E. crandallis* during pregnancy) compared with lambs born from unimmunized ewes. However, although maternal antibodies against *Eimeria* spp. have been demonstrated in lambs fed colostrum, the antibodies were not thought to be protective (Nolan et al. 1987; Fiege et al. 1992; Reeg et al. 2005).

Different species-specific immunological responses in the host have been shown, e.g., between *E. weybridgensis* and *E. bakuensis* (Norton et al. 1974), and between pathogenic and non-pathogenic species (Reeg et al. 2005). The reason for this difference is unknown, but it has been proposed that this may reflect different antigenicities among the different species (Reeg et al. 2005). Although immunity is mainly species associated, some cross-reactivity between species has been shown by enzyme-linked immunosorbent assay (ELISA) (Nolan et al. 1987).

Pathogenesis and pathology

The pathogenesis of ovine *Eimeria* spp. infection is dependent on several factors, such as the species of *Eimeria* involved, infective dose, and a variety of host-related factors including age, physical condition, stress, genetic susceptibility, and earlier exposure to *Eimeria* spp. (Jolley and Bardsley 2006). In sheep, two species, *E. ovinoidalis* and *E. crandallis,* are considered major pathogens (Catchpole et al. 1976; Catchpole and Gregory 1985; Joachim et al. 2018), while *E. ahsata* and *E. bakuensis* are considered minor pathogens (Mahrt and Sherrick 1965; Deplazes et al. 2016). The other species are thought to be of negligible importance under normal conditions. Natural infections are mainly mixed infections with multiple *Eimeria* spp. (Helle and Hilali 1973; Catchpole et al. 1975; Reeg et al. 2005; Dittmar et al. 2010; Nourollahi-Fard et al. 2014), but severe clinical cases are often dominated by one *Eimeria* sp. (H.L. Enemark, personal communication).

In general, most *Eimeria* spp. of sheep affect the small and/or large intestine, with the exception of *E. gilruthi* (also known as *Globidum gilruthi*). *E. gilruthi* is sporadically reported as an incidental finding in the abomasum at post mortem examination of sheep from different parts of the world, including Norway, but the importance of this species is unknown (Hilali 1973; Chineme and Njoku 1978; Hilali and Scholtyseeck 1979; Fox et al. 1991; Mahmoud 1997; Hermosilla et al. 2016).

Eimeria spp. infection of intestinal epithelial cells may result in mucosal destruction and ulceration, villus atrophy, and flattening of the mucosal surface. The function of the epithelial cells can be compromised, affecting intestinal motility and intercellular signalling. The loss of intestinal epithelium, by atrophy and necrosis, leads to a malabsorptive diarrhoea, due to the reduction in absorptive surface. As a result, electrolytes and nutrients are retained in the lumen of the intestines, along with osmotically associated fluid, which both are transferred to the large intestine (Brown et al. 2007b). Enteritis, varying in severity, will develop, and involves the lamina propria and sometimes the submucosa (Gregory and Catchpole 1987, 1990; Aleksandersen et al. 2002; Jolley and Bardsley 2006). Intestinal surfaces with damaged epithelium can only heal by hyperplasia of nearby intact epithelium. Regeneration and healing is slow, and the animal can be affected clinically for months (Gregory and Catchpole 1987). Reduced growth is therefore seen as a result of anorexia, anaemia, reduced uptake and absorption of nutrients, and loss of fluid (Chapman 1974a; Fitzgerald 1980; Gregory and Catchpole 1987).

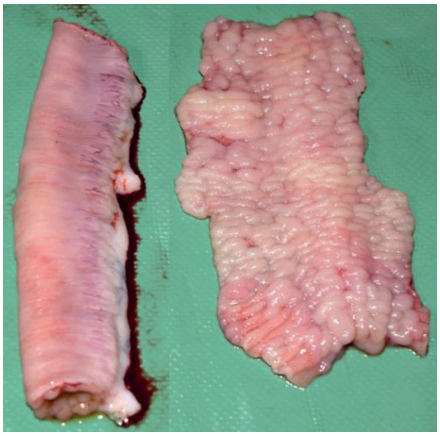

Figure 4. Thickened and edematous ileum from a lamb infected with *Eimeria* spp., including *E. ovinoidalis* and *E. crandallis/weybridgensis*. Photo: A. Odden

The highly pathogenic *E. ovinoidalis* mainly causes lesions in the terminal ileum, caecum, and proximal colon, where the affected areas can be oedematous and thickened (Brown et al. 2007a; Deplazes et al. 2016; Taylor et al. 2016b) (Fig. 4). The last meront-generation infects epithelial cells lining the colonic crypts, and the gamonts attack the remaining crypt epithelium, leading to destruction of most of the cells, including stem cells (Gregory and Catchpole 1987) (Fig. 5). Interestingly, it has been shown that the pathogenic effect of *E. ovinoidalis* can be influenced by the digestive microflora; lambs without a normal intestinal microflora (lambs delivered by aseptic caesarean section and raised sterile) showed far less clinical signs and oocyst excretion than lambs

with a normal microflora, when infected with *E. ovinoidalis* (Gouet et al. 1984).

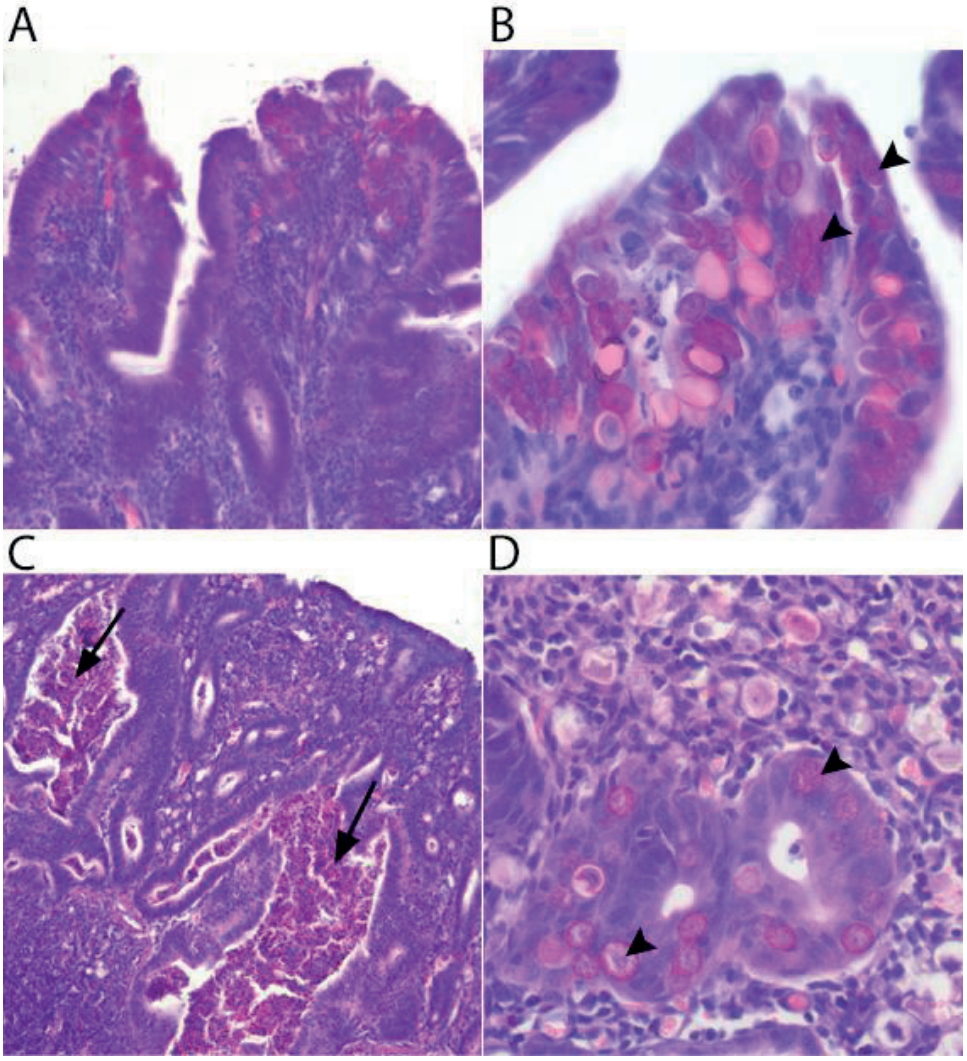

Figure 5. Examples of histopathological findings associated with experimental *Eimeria* spp. infection. (A) Blunted ileal villi with *Eimeria* in both the epithelium and superficial lamina propria. There are bleedings just below the villus epithelium. (B) Ileal villi with large amounts of *Eimeria* parasites (arrowheads) in both the epithelium and in lamina propria. (C) Giant crypt abscesses in the ileum (arrows). The surface epithelium is flattened and villi are absent. There is infiltration of inflammatory cells in lamina propria. (D) *Eimeria*-infected crypt epithelium (arrowheads) and surrounding lamina propria in the caecum. The epithelium is hypertrophic, and there is infiltration of inflammatory cells in lamina propria. Magnification: A, C: 100×, and B, D: 400×. Photo: A. Odden

Another important pathogenic ovine coccidian species, *E. crandallis,* causes lesions in the small intestine, mainly the ileum (Gregory and Catchpole 1990; Brown et al. 2007a). Changes associated with *E. crandallis* infections include villus atrophy due to first and second generation meronts, and

loss of crypts due to damaged epithelium. In addition, *E. crandallis* can cause diffuse hyperplastic lesions, leading to a thickened and folded mucosa (Pout 1974; Gregory and Catchpole 1990). *E. bakuensis* might induce similar, but focal, lesions in the small intestine, manifesting as patches and polyps. This difference may partially explain why *E. crandallis* is more pathogenic than *E. bakuensis* (Gregory and Catchpole 1990).

Clinical signs

Figure 6. Clinical signs of *Eimeria* spp. infection. (A) A group of lambs with varying degrees of diarrhoea and perianal soiling. Also note the varying body condition. Faecal examination detected *Eimeria* spp. (B) One lamb diagnosed with *Eimeria* spp. showing perianal soiling due to watery, dark green diarrhoea. Photo: A. Odden

Whether an *Eimeria* infection develops into subclinical or clinical coccidiosis may depend on factors such as infection pressure, the species involved, management system, hygiene status, nutrition of both ewe and lamb, and lamb age (Gregory and Catchpole 1989; Taylor 1995). Acute signs of coccidiosis include different degrees of yellow to dark watery diarrhoea (± blood and/or intestinal tissue), fever, abdominal pain, anorexia, emaciation, and dehydration (Gregory and Catchpole 1987; Martin and Aitken 2000; Khodakaram Tafti and Mansourian 2008) (Fig. 6). Loss of fluid and nutrients usually lead to reduced body condition score and weight loss. Recovery time is largely dependent on the severity of the intestinal damage, and especially re-

epithelialisation of lost crypt epithelium will affect the recovery time (Fitzgerald 1980; Gregory and Catchpole 1987).

Acute coccidiosis in ruminants may cause increased haematocrit (hct) due to diarrhoea and fluid loss, and decreased levels of electrolytes, such as sodium, chloride and potassium (Bangoura and Daugschies 2007; Byers and Kramer 2010; Hashemnia et al. 2014). Loss of electrolytes is commonly seen in animals with malabsorptive diarrhoea (Grove-White 2007). The loss of body weight associated with coccidiosis is mainly due to loss of nutrients as a result of parasite-induced mucosal lesions and, to a lesser degree, to alterations of intestinal digestion and absorption of nutrients (Daugschies et al. 1998).

Subclinical coccidiosis may also lead to reduced growth, uneven lamb size, and higher food conversion ratio (de la Fuente et al. 1993; Aitken 2007). Treatment of subclinically infected lambs has been shown to increase the average growth rate and improve the feed conversion rates, compared with untreated controls (Alzieu et al. 1999).

Diagnostic methods

Traditional faecal analysis

Diagnosis of coccidiosis in sheep is based on both clinical signs and coproscopic analysis. Clinical signs can occur before oocyst excretion, and the duration of the oocyst excretion period is usually around 10 - 40 days, depending on the host immunity and *Eimeria* spp. involved (Catchpole et al. 1976; Taylor 1995). Furthermore, a positive faecal sample does not automatically mean that coccidiosis is the main problem, as healthy animals may have relatively high oocyst counts (Taylor 1995).

Diagnosis by coproscopic analysis is normally performed using modifications of the McMaster technique, developed in Australia to quantify nematode eggs in faecal samples (Gordon and Whitlock 1939). McMasters are flotation methods, i.e. eggs/oocysts suspended in a liquid with a specific gravity higher than that of the egg/oocyst will float to the surface, while debris with higher specific gravity may sink. Different McMaster modifications exist, but the basis is the same: a known amount of faeces is mixed with a known volume of water or flotation fluid, often saturated sodium chloride, and the number of parasite eggs/oocysts are counted using light microscopy. Some of the methods use filtration and/or centrifugation. Filtration is used to remove large sized debris, while centrifugation may increase the sensitivity. Faecal material and flotation fluid are finally transferred to a McMaster counting chamber. These often contain 12 slots, covering a total volume of 0.3 ml, but different chambers, holding different volumes and

with different numbers of chambers, may also be used. Sensitivity varies (e.g., 5-200 oocysts or eggs per gram) based on the modification used (Vadlejch et al. 2011). Other copromicroscopical techniques include the FLOTAC, mini-FLOTAC and FECPAK, all methods without a centrifugation step (Coles 2003; Cringoli et al. 2010; Bosco et al. 2014).

Speciation

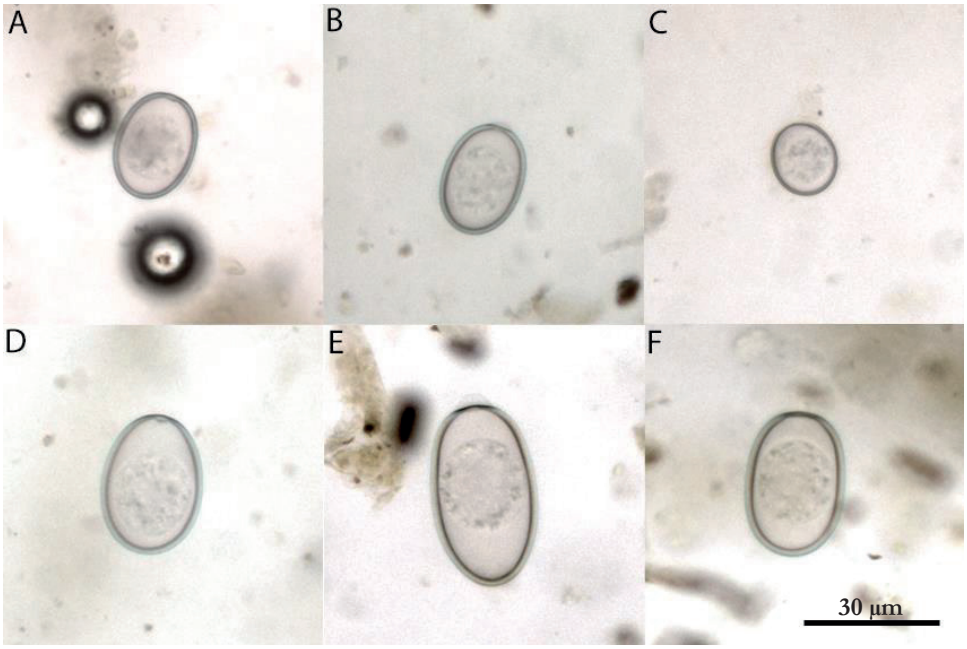

Figure 7. Different unsporulated *Eimeria* spp. (A) *E. ovinoidalis*, (B) *E. crandallis/weybridgensis*, (C) *E. parva*, (D) *E. faurei*, (E) *E. ahsata*, and (F) *E. bakuensis*. 400× magnification. Photo: A. Odden

Differentiation of the various ovine *Eimeria* spp. can be achieved to some extent by light microscopy based on morphology; size, shape and the presence of characteristic morphologic elements of the parasite (polar cap, micropyle, oocyst wall and oocystal or sporocystal residues) (Table 1, and Fig. 7) (Eckert et al. 1995b). However, microscopic evaluation cannot differentiate between all species, as some species (e.g. *E. crandallis* and *E. weybridgensis*) have morphologically similar unsporulated oocysts. To differentiate between these species, presence (*E. crandallis*) or absence (*E. weybridgensis*) of the sporozoite residual body (visible following sporulation), together with the size and shape difference of the sporozoites need to be identified by microscopy, preferably differential interference contrast microscopy (Eckert et al. 1995b).

Molecular methods

Diagnostic molecular methods, such as different polymerase chain reactions (PCR), have been established, especially for poultry *Eimeria* spp. (Fernandez et al. 2003). PCRs may be used for quantification as well as for speciation of *Eimeria* spp. By using species-specific primer sets, various species can be differentiated, including *E. crandallis* and *E. weybridgensis* (Yang et al. 2014). Furthermore, DNA-based techniques can be used to characterise the genetic diversity of *Eimeria* spp., which is important in, e.g., the development of recombinant vaccines (Beck et al. 2009; Clark et al. 2017). However, molecular methods also have major challenges, such as the removal of the oocyst and sporocyst wall, in order to obtain the nucleic acids (Berriatua et al. 1995; Haug et al. 2007; Kaya et al. 2007). Some commercial DNA extraction kits may be used to extract DNA directly from faeces, but the amount of DNA in the sample may be relatively small and/or the faecal input so large, that the method has less sensitivity than some established techniques, such as McMaster or FLOTAC (Taylor et al. 2016a). In addition, mixed infections may be difficult to detect, as amplification of DNA from the most abundant species may mask more minor species (Yang et al. 2014).

Serological methods

Serological methods (ELISA and Western blot) have been developed for the detection of antibodies to several ruminant *Eimeria* spp. However, antibody titres may remain high even after clearance of the infection, and young animals fed colostrum have maternal antibodies that may interfere with the serological results (Gregory and Catchpole 1989). In addition, there might also be a problem with cross-reaction between species (Nolan et al. 1987; Fiege et al. 1992; Faber et al. 2002). Such methods are therefore primarily useful for epidemiological and experimental studies, but not for routine diagnosis.

In vitro methods

In vitro methods are not generally considered diagnostic methods, but included here for completeness.

The pathogenesis of apicomplexan parasites is related to the intracellular life stages of the parasite, and *in vitro* culture systems should therefore include these stages (Müller and Hemphill 2013). *In vitro* assays have been used to investigate immunology, parasite-cell interactions and mechanisms of pathogenicity in ruminant *Eimeria* infections (Hermosilla et al. 2012; Hermosilla et al. 2015; Pérez et al. 2015; Ruiz et al. 2015; Carrau et al. 2016). Whereas for avian *Eimeria* spp.,

assays have also been developed for the assessment of drug efficacy (Alnassan et al. 2015; Thabet et al. 2015; Habibi et al. 2016; Jitviriyanon et al. 2016; Thabet et al. 2017).

In order to perform an *in vitro* assay looking at the intracellular development, sporozoites have to infect cells. Consequently, oocysts have to be isolated, sporulated, and excysted. Separating oocysts from faecal debris is essential, and may involve filtration and flotation (Jackson 1964) (Fig. 8). Excystation of sporulated oocysts may be accomplished by using different digestion mixes, which disrupts the oocyst and sporocyst wall and releases sporozoites. Excystation can also be mechanically induced, by using glass beads to break the oocyst wall, a method commonly used for avian *Eimeria* spp. (Haug et al. 2007; Cha et al. 2014).

Selection of the cell line used for infection is important, as not all cell lines are infected by *Eimeria* spp. due to the parasite's strict host and site specificity. Ovine *Eimeria* has been shown to infect embryonic ovine kidney, trachea, thymus and thyroid cells, Madin-Darby bovine kidney cells, as well as permanent bovine colonic epithelial cells (BCEC) and primary bovine umbilical vein endothelial cells, reaching different developmental stages depending on the host cell used (Kelley and Hammond 1970; Carrau et al. 2016). *In vitro* assays for ruminant *Eimeria* spp. are currently only used for research purposes.

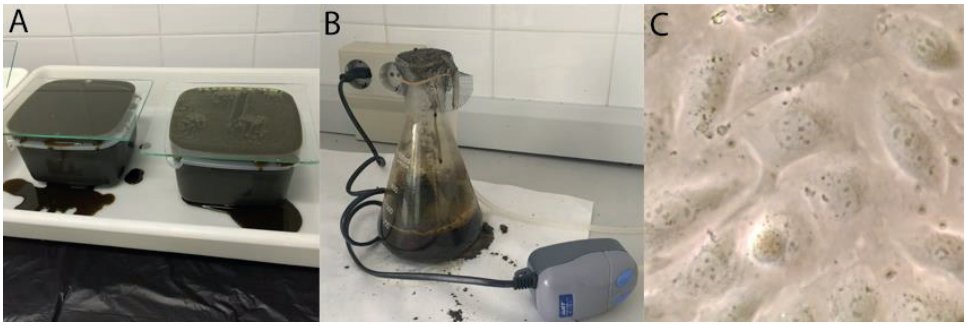

Figure 8. Methods used in the development of an *in vitro* assay for the assessment of anticoccidial efficacy. (A) Flotation using saturated sugar solution to obtain large numbers of oocysts. (B) Sporulation of *Eimeria* oocysts using continuous aeration. (C) Confluent layer of bovine colonic epithelial cells at 20× magnification (phase contrast). Photo: A. Odden

Relevant differential diagnosis

Diarrhoea in young lambs is not pathognomonic for *Eimeria* infection, as it may be caused by different infectious agents, as well as dietary problems. Infectious causes of diarrhoea include enteric viruses such as rotavirus and coronavirus, bacteria, such as enterotoxigenic *Escherichia coli,* and S*almonella* spp., protozoa such as *Cryptosporidium* spp. and helminths such as e.g. *Nematodirus battus*, and *Teladorsagia* spp. (Sargison 2004). Enteric viruses seldom cause clinical disease in

otherwise healthy animals, but might enable the establishment of other enteric infections. Similarly to coccidiosis, nematodiriosis can be seen in lambs on spring pastures, with acute-onset diarrhoea, lethargy, abdominal pain, weight loss and dehydration (Sargison 2004). *Cryptosporidium* spp. found in Norway may cause diarrhoea in young lambs depending on the species, those most commonly found in lambs (*C. xiaoi* and *C. ubiquitum*) are not usually symptom-associated, but *C. parvum* is. Infections with *Cryptosporidium* spp. is however often seen in younger animals than *Eimeria* infections, usually within the first days of life (Robertson et al. 2010).

Treatment and control

Management

Management practices for the control of coccidiosis, should be aimed at reducing the infection pressure and environmental contamination. This may be approached in several ways, e.g. through grass management, hygiene, and other management practices, such as duration of lambing season, and lamb age at turnout. Management practices focusing on hygiene have been shown to reduce the need for treatment (Lopes et al. 2014), and it has been demonstrated that goat flocks with good hygiene have lower oocyst counts than flocks with poorer hygiene (Jalila et al. 1998). Although housed lambs should be provided with clean bedding, there is some evidence that early exposure to oocysts may help in development of immunity (Gregory and Catchpole 1989; Gregory et al. 1989a). However, in practical situations, it is difficult to challenge lambs with only a small, controlled dose of oocysts, as such trickle infections will be both impractical and almost impossible to control (Catchpole et al. 1993; Gregory 1995). Thus, a clean environment, i.e., an environment with little oocyst contamination, should be the goal. Grass management may be used to reduce the infection pressure on pasture, and includes time of turnout, duration of grazing period, age composition of flocks, and frequency of pasture rotations (Thamsborg et al. 2010). As both ovine and bovine *Eimeria* spp. oocysts in the Nordic countries are able to overwinter in the pasture (Helle 1970; Svensson 1995), turnout to clean pastures, i.e. pastures not grazed by lambs the previous year, is important to control the infection pressure (Thamsborg 2001). This is, however, often not practiced on sheep farms, as many farmers use permanent pastures for spring grazing close to the farm. Although the use of clean pastures requires large areas available for grazing and seem impractical, farmers dividing their available areas into two sections, and rotating between the two halves every other year may be able to achieve this pastures with reduced oocyst contamination.

Chemoprophylaxis

In sheep production, control of coccidiosis is often based on chemoprophylaxis with anticoccidial drugs (Gjerde and Helle 1991; Alzieu et al. 1999; Platzer et al. 2005; Gjerde et al. 2009; Le Sueur et al. 2009; Mundt et al. 2009; Taylor et al. 2011; Odden et al. 2017b).

Reports on preventive medication against ovine coccidiosis were first published in the 1940s using different drugs, such as copper or ferric sulphate, sulphaguanidine, or sulphur powder (Gregory et al. 1981). Two outbreaks of coccidiosis on a sheep farm in Great Britain in 1951 and 1952 showed that administration of sulphamezathine, orally for three successive days, improved the clinical symptoms (Robertson 1953). Sulphamezathine or sulphadimidine was therefore used to prevent coccidiosis throughout the 1950s-70s in Norway, administered at days 12, 13 and 14 or days 12, 14 and 16 after turn out, just before the anticipated clinical signs (Helle 1981; Gjerde et al. 2009). Today there are no sulpha-preparations registered for coccidiosis in ruminants in Norway (Felleskatalogen 2017a).

Introduction of monensin, a polyether ionophore, in the late 1960s had a profound effect on the control of coccidiosis, especially in poultry (Chapman 2014), but also in the control of coccidiosis in feedlot lambs, when fed continuously (McDougald and Dunn 1978). The successful use of monensin led to the discovery of other ionophores, like lasalocid, narasin, and salinomycin, all broad spectrum anticoccidials with activity against different *Eimeria* spp. (McDougald and Dunn 1978). In addition to the ionophores, there are several synthetic anticoccidials, which are used in poultry production, like nicarbazin, amprolium and quinolones (Chapman 2014). During the 1980s, new drugs were developed and marketed for use in both mammals and poultry, like ponazuril, clazuril, toltrazuril, and diclazuril. These drugs belong to the triazines, also known as benzene-aceto-nitrile compounds (Stock et al. 2018). Triazines have been used to control different intestinal protozoal infections in multiple host species, including cattle, sheep, rabbits, hoses, dogs, cats, pigs and poultry (Lloyd and Smith 2001; Furr et al. 2006; Redrobe et al. 2010; Kreiner et al. 2011; Veronesi et al. 2011; Alnassan et al. 2013).

Toltrazuril and diclazuril

Toltrazuril and diclazuril (Fig. 9) are commonly used anticoccidials in sheep in Europe, but today only toltrazuril is registered in the Nordic countries: Denmark, Norway and Sweden (Felleskatalogen 2017b; Läkemedelsverket 2017; Veterinærmedicinsk Industriforening 2017).

Toltrazuril was marketed in the beginning of the 1980s in Europe, with several studies supporting its effect against coccidiosis in different hosts (Mehlhorn et al. 1984; Gjerde and Helle 1986; Peeters and Geeroms 1986; Taylor and Kenny 1988; Gjerde and Helle 1991; Le Sueur et al. 2009;

Diaferia et al. 2013). After ingestion, toltrazuril is rapidly transformed into two major metabolites: toltrazuril sulphoxide and toltrazuril sulphone, of which toltrazuril sulphoxide is a transient metabolite (Lim et al. 2010). The proposed mode of action of toltrazuril is thought to be directed against the first and second generation schizonts, microgamonts, and macrogamonts (Mehlhorn 2008). The action is probably achieved by inhibiting mitochondrial respiration and nuclear pyrimidine synthesis in the parasite, possibly by inhibiting dihydroorotate dehydrogenase (Harder and Haberkorn 1989). This enzyme is involved in the *de novo* pyrimidine biosynthesis, in, among other species, *Plasmodium* spp. (Munier-Lehmann et al. 2013). In addition, in macrogamonts, destruction of the wall-forming bodies II, can be observed (Harder and Haberkorn 1989). Although the previously mentioned mechanisms of action of toltrazuril have been published, its distribution in different intestinal segments is still unknown and several parts of the mechanisms of action have not yet been described.

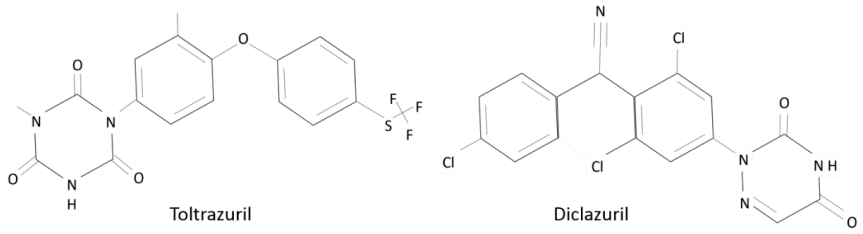

Figure 9. Chemical structures of toltrazuril and diclazuril.

Diclazuril was marketed in the beginning of the 1990s (EMA 1996), and was shown to be effective against all major *Eimeria* spp. in chickens, turkeys, rabbits, and ruminants (Maes et al. 1989). The mechanisms of action for diclazuril are unknown, but the activity is only directed against specific endogenous stages of *Eimeria* spp. (Mehlhorn 2008). A study from poultry showed action against different developmental stages, attacking all intracellular stages in some species, while only one or two stages in others (Maes et al. 1989).

Several studies have been performed in sheep looking at the effect of either toltrazuril or diclazuril in reducing oocyst excretion and clinical signs of coccidiosis (Taylor and Kenny 1988; Gjerde and Helle 1991; Alzieu et al. 1999; Taylor et al. 2003; Le Sueur et al. 2009; Taylor et al. 2011). Treatment studies showed better efficacy of toltrazuril than diclazuril in reducing oocyst counts and clinical signs (Gjerde et al. 2009; Diaferia et al. 2013; Scala et al. 2014). In addition, toltrazuril has a longer elimination half-life in plasma, and is therefore thought to have a prolonged effect against the parasite (Veronesi et al. 2011).

Treatment with both diclazuril and toltrazuril should be performed metaphylactically, i.e. in the parasite's prepatent period, after infection, but before the development of clinical signs (Dittmar

et al. 2009). Furthermore, treating too early in the prepatent period, and with too high doses, may interrupt the development of protective immunity, as demonstrated for diclazuril (Taylor et al. 2011).

Evaluation of anticoccidial efficacy (ACE) and anticoccidial drug resistance (ACR)

Anticoccidial efficacy (ACE)

Since chemoprophylaxis is a common way of controlling coccidiosis in several hosts, including sheep (Odden et al. 2017b), and because ACR may be the result of intensive long-term use of anticoccidial drugs (Peek and Landman 2011), methods for testing ACE are necessary to ensure a successful treatment outcome. Although a guideline for various techniques for research and experimental infections of *Eimeria* spp. exists (Eckert et al. 1995a), it contains no information on procedures for ACE testing in mammals. The new WAAVP guideline for evaluation of ACE in mammals therefore aims at providing recommendations on how to perform efficacy studies (Joachim et al. 2018).

In vitro evaluation of ACE

The use of *in vitro* methods may reduce the need for animal testing. Affordable and rapid assessment of ACE is essential when doing flock health planning. *In vitro* assays have already been used to test ACE of both commercial drugs and plant extracts in poultry production. Although *in vitro* culture systems have been described for several *Eimeria* spp., such testing is currently only described for avian *Eimeria* spp., and not for routine analysis (Thabet et al. 2015; Habibi et al. 2016; Thabet et al. 2017).

In vivo evaluation of ACE

As already mentioned, there are no verified *in vitro* methods for the evaluation of ACE in ruminants. Thus, *in vivo* tests have to be performed, either controlled efficacy trials (CET) or field trials. For avian *Eimeria* spp., several indexes can be used in the *in vivo* evaluation of ACE (Jeffers 1974; Chapman 1998; Fei et al. 2013), but no such indexes are available for evaluation of drug efficacy against ruminant *Eimeria* infections. Therefore, it has been recommended to perform experimental infections with both suspected resistant and known sensitive *Eimeria* spp. isolates, for comparison of efficacy (Joachim et al. 2018).

Evaluation of drug efficacy has been extensively investigated for helminths, and is often performed as a part of flock health planning. Moreover, field test protocols are available (Coles et al. 1992). Thus, evaluation of anthelmintic efficacy in animals is routinely assessed by the faecal egg count reduction test (FECRT), currently recommended by the World Association for the

Advancement of Veterinary Parasitology (WAAVP). The FECRT involves comparison of faecal egg counts pre- and post-treatment (Coles et al. 1992), and has the ability to assess a range of drugs under field conditions. A similar field method for the evaluation of ACE would be beneficial, especially since one practical requirement for a method to be useful in field situations is that it should not include euthanasia of large numbers of animals, such as is necessary for CET.

Anticoccidial drug resistance (ACR)

The World Health Organization describes antiparasitic resistance as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within tolerance of the subject" (Bloland 2001).

Poultry

In poultry production, ACR has been demonstrated against all introduced drugs, often within one year after release (Chapman 1997, 2014). Testing for ACR in poultry can be done either by *in vivo* or *in vitro* assays (Chapman 1998; Thabet et al. 2015; Thabet et al. 2017). Different *in vivo* test are available for drug testing in poultry, such as: dose determination, dose confirmation, or field effectiveness studies, usually performed in commercial husbandry (Holdsworth et al. 2004). Efficacy studies include the use of histopathological observations and the combination of different indices, such as oocyst index, body weight gain, relative weight gain, lesion scores (macroscopic intestinal pathology), and/or anticoccidial index (ACI, a combination of different parameters) (Jeffers 1974; Chapman 1998; Fei et al. 2013). Due to severe problems with ACR in poultry, shuttle programs have been applied, where two or more drugs, usually with different mechanisms of action, are used in different feeds throughout the life of a flock (Chapman 2014). In addition, rotation systems, where different drugs are used in successive flocks, have been utilised. These systems are still widely used in the broiler industry to prolong the efficacy of available drugs (Chapman 2001, 2011; Lan et al. 2017).

In addition to the development of ACR, ionophore coccidiostats may also inhibit or kill some bacterial species. Thus, some level of narasin-resistance (the ionophore registered for broiler chickens in Norway) has been seen in faecal enterococci (NORM/NORM-VET 2013). Therefore, based on a report from the Norwegian Scientific Committee for Food Safety (VKM 2015), the Norwegian government published a strategy document in which it was decided that the use of narasin and other anticoccidials with antibacterial activity should be discontinued, without increasing the usage of therapeutic antibiotics or compromising the animal welfare (Norwegian Ministry of Health and Care Services 2015).

Swine

Infection with *Cystoisospora suis* in piglets can impact morbidity and mortality, with clinical signs of diarrhoea and reduced growth (Stuart et al. 1982). A single oral metaphylactic treatment with toltrazuril has been shown to provide effective and sustained suppression of oocyst shedding and diarrhoea in piglets in both experimental and field situations (Mundt et al. 2007; Joachim and Mundt 2011; Rypula et al. 2012). Toltrazuril resistance was recently confirmed in a field isolate of *C. suis* after experimentally infecting piglets with the suspected isolate, and treating with both the recommended, and increased dose of toltrazuril, and comparing the results with piglets infected with a known susceptible isolate (Shrestha et al. 2017a). In addition, toltrazuril-treated piglets infected with the resistant isolate, showed prolonged diarrhoea in comparison with piglets infected with the sensitive isolate (Shrestha et al. 2017a).

Sheep

Control of coccidiosis in sheep often includes the use of chemoprophylaxis. As far as we know, ACR has not previously been confirmed in sheep, but there have been unverified reports of reduced anticoccidial efficacy in Norwegian lambs (Gjerde et al. 2009; Gjerde et al. 2010). One major difference between poultry, swine, and sheep production systems is the way these animals are kept. In conventional production, poultry and swine are usually housed throughout their lives, and the production is preferably managed in batches; "all in, all out" (Giner Santonja et al. 2017). In contrast, sheep have periods on pasture, where an untreated parasite refugium may be available, as seen with helminths (van Wyk 2001). The importance of such management differences for the development of ACR is, however, unknown.

Vaccines

The first commercially successful anticoccidial vaccine in poultry was marketed in 1952 as Coccivac, containing live, non-attenuated *E. tenella* oocysts (Williams 2002b; Chapman 2014). This vaccine is still widely used today, together with several other vaccines available for poultry, comprising mixes of species of non-attenuated or attenuated parasites (Williams 2002a). Non-attenuated vaccines are currently not licenced for use in Europe, due to the risks of vaccine-induced disease, but attenuated vaccines are available. Compared with non-attenuated *Eimeria* vaccines, attenuated *Eimeria* vaccines replicate more slowly, have a higher cost of production, and limitations in the possible number of doses produced (McDonald and Shirley 2009; Blake and Tomley 2014). For mammals, Ruiz et al. (2014) succeeded in immunizing goat kids against *E. ninakohlyakimovae* by oral dosing with live, attenuated oocysts. These findings have however, not yet led to the development of a commercial vaccine.

Due to the cost of live vaccines, recombinant subunit vaccines have been considered a potential alternative. The development of such recombinant vaccines is dependent on low genetic variability in the target antigen, in order to ensure a good protective immunity (Clark et al. 2017). Selection of antigens for vaccine development has proved to be a significant barrier in other apicomplexan parasites such as *Toxoplasma gondii* and *Plasmodium* spp. (Liu et al. 2012; Stanisic et al. 2013). However, some antigens from avian *Eimeria* spp. have been shown to induce protection and serve as good candidates for further vaccine development (Song et al. 2015; Blake et al. 2017). In swine, the genome of *Cystoisospora suis* has been sequenced, an important step towards finding potential vaccine candidates (Shrestha et al. 2017b). Although recombinant subunit vaccines are successful, the small number of antigens involved (< 20) may require fewer mutations in the parasite to achieve immune escape, compared with live vaccines expressing between 6000 and 9000 antigens (Blake et al. 2017).

Previous coccidiosis research in Norway

Only scattered information is available concerning coccidiosis in lambs in Norway. During the 1960s and 70s, the presence of different *Eimeria* species and the winter survival of oocysts on pasture were studied (Helle 1964, 1970; Helle and Hilali 1973). In the 1980s and 90s, several treatment studies using toltrazuril and diclazuril were performed (Gjerde and Helle 1986, 1991), followed by unverified reports of reduced toltrazuril efficacy in ovine *Eimeria* spp. (Gjerde et al. 2009; Gjerde et al. 2010). In addition, experimental *Eimeria* trials in lambs was performed in order to describe pathologic lesions related to lymphocytes during an active *Eimeria* infection (Aleksandersen et al. 1995; Aleksandersen et al. 2002). Thus, prior to the current studies, overall knowledge of ovine coccidiosis in Norway was relatively limited.

Aims of the thesis

The overall aim of this thesis was to investigate the use of anticoccidial drugs, determine risk behaviour for reduced anticoccidial efficacy (ACE) in Norway, create tools for the evaluation of ACE, and investigate ACE and anticoccidial drug resistance (ACR) in ovine *Eimeria* spp. In addition, an alternative control strategy to reduce the uptake and excretion of *Eimeria* oocysts was investigated. The overall aim was approached by pursuing the following objectives:

I. Develop a questionnaire to assess how and why Norwegian farmers use anticoccidial drugs, and investigate potential risk factors for the development of reduced ACE (Paper I)
II. Develop a method for field evaluation of ACE based on oocyst excretion, and determine the level of reduced ACE in selected Norwegian flocks (Paper II)
III. Perform a controlled efficacy test (CET) to assess ACR *in vivo*, by infecting lambs with a suspected resistant field isolate of ovine *Eimeria* spp. (Paper III)
IV. Perform a field trial to assess the effect of iron supplementation of young lambs on the uptake and excretion of oocysts, and lamb growth rates (Paper IV)
V. Develop an *in vitro* assay for the evaluation of ACE, by looking at oocyst sporulation, infection of cells, and intracellular development (ongoing work)

Synopsis of papers

Paper I

Treatment against coccidiosis in Norwegian lambs and potential risk factors for development of anticoccidial resistance – a questionnaire-based study

The objectives of this study were to investigate the use of anticoccidials in Norwegian sheep flocks and identify farms with management procedures likely to select for drug resistance. Data were obtained by a questionnaire sent to all members of the Norwegian Sheep Recording System in October 2015. The data set consisted of 1215 answers, corresponding to 8.5% of Norwegian sheep flocks. Anticoccidials were used in 82.7 % of flocks. Main treatment was at turn-out (38.6 % of treated flocks) or 1 week after turn-out (32.4 %). Interestingly, clinical signs, possibly related to coccidiosis were observed by almost 40 % of the farmers after treatment, which might be an indication of drug resistance. Correlations between the apparently reduced anticoccidial efficacy and management conditions, such as the size of the farms, were found, as larger farms were more likely to use an anticoccidial than smaller farms. From the farmers' perspective, metaphylactic treatment was used in 88.5 % of treated flocks, of which approximately one third had no history of clinical coccidiosis. Although the farmers seemed aware of the importance of good drenching routines based on reliable estimates of weights and calibration of drench guns, drench guns used for anticoccidial administration were never calibrated in 12.1 % of the flocks. Finally, dose estimation was made by visual appraisal of lamb weight in 27.5 % of the flocks, which can lead to incorrect dosing. Based on the present study, it cannot be determined whether the apparent treatment failure was related to management practises, incorrect administration of the drug, other infections, or actual ACR.

Paper II

Field evaluation of anticoccidial efficacy: a novel approach demonstrates reduced efficacy of toltrazuril against ovine *Eimeria* spp. in Norway

Ovine *Eimeria* spp. infections cause reduced welfare, increased mortality, and substantial economic losses, and anticoccidials are crucial for their control. Recent reports of toltrazuril resistance in pigs, and anecdotal reports of reduced anticoccidial efficacy in lambs, necessitate evaluation of anticoccidial efficacy. Due to the substantial lifecycle differences between nematodes and coccidia, current WAAVP methods for assessing anthelmintic efficacy are not suitable for such evaluations. The aim of our study was therefore to develop a tool for field evaluation of anticoccidial efficacy (ACE), the faecal oocyst count reduction test (FOCRT), based on oocyst counts in lambs. The FOCRT was then used in a preliminary investigation of ACE in Norwegian sheep farms. Faecal samples were collected from 8 pairs of twin lambs from 36 Norwegian sheep farms 6-8 days after turnout. One twin of each pair was then treated with 20 mg/kg toltrazuril and a second faecal sample from all lambs was collected 7-11 days later. Oocyst excretion rate in all samples was determined using McMasters. Suitability of treatment timing was investigated by evaluating the increase in mean log oocyst excretion in untreated lambs ("the slope"). Based on comparisons between groups, a threshold of ≥0.75 (13 farms) was used to identify farms where drug efficacy could be assessed with confidence, while drug efficacy was evaluated with caution on farms with increases of ≥0.5 but <0.75 (7 farms). Drug efficacy on farms with increases of <0.5 (16 farms) was not estimated. Reduction in oocyst excretion between samples from treated lambs compared with controls from the 20 farms with a threshold of ≥0.5 were then analysed using a generalised linear mixed model. The results were classified based on 95 % CI obtained using parametric bootstrapping. Among these 20 farms, two exhibited reduced drug efficacy (upper 95 % CI <95 %), 13 had good efficacy (lower 95 % CI >90 %), and for 5 the results were inconclusive. This is the first evidence-based report of reduced ACE in ovine *Eimeria* spp. Additionally, we highlight the problem of sub-optimal timing of treatment (16/36 farms), as seen when evaluating the slope thresholds, which could potentially result in incorrect conclusions being reached regarding lack of drug efficacy.

Paper III

Controlled efficacy trial confirming toltrazuril resistance in a field isolate of ovine *Eimeria* spp.

Anticoccidial resistance has been reported in poultry and swine, and reduced toltrazuril efficacy was recently described in ovine *Eimeria* spp. in some Norwegian sheep farms using a newly developed faecal oocyst count reduction test (FOCRT). The aim of the present study was to use a controlled efficacy trial (CET) to assess the efficacy of toltrazuril against a field isolate suspected of being resistant. Twenty lambs, 17-22 days old and raised with no exposure to coccidia, were infected with a field isolate of *Eimeria* spp. This isolate was obtained from a farm previously classified as having inconclusive FOCRT-results, with a calculated drug efficacy of 56 % (95 % confidence interval: -433.9 to 96.6 %). At day 7 post infection with 100,000 oocysts, 10 of the lambs were orally treated with 20 mg/kg toltrazuril (Baycox Sheep vet., Bayer Animal Health), while the other 10 lambs (controls) were given physiological saline. Clinical examinations were conducted, and weight gains recorded. Daily faecal samples were scored for diarrhoea on a scale from 1-5, and oocyst excretion was determined using a modified McMaster technique. Oocysts were identified to species level on the basis of morphology. At 17-24 days post infection the lambs were euthanized and necropsied. Our results demonstrated that faecal score, growth rates, gross pathology or histological changes were approximately the same in treated and control lambs. In addition, no differences in oocyst excretion were identified in both pathogenic and non-pathogenic species. The pathogenic *E. ovinoidalis* was the dominant species, and other species identified included *E. crandallis/weybridgensis, E. parva, E. marsica, E. faurei, E. pallida, E. ahsata* and *E. bakuensis*. The results from this CET confirm toltrazuril resistance in ovine *Eimeria* spp. for the first time. In addition, the data support the use of the FOCRT as an appropriate tool for field evaluation of anticoccidial efficacy. Due to limited anticoccidial treatment alternatives, these findings may have important implications for the sheep industry, particularly in Northern Europe.

Paper IV

Excretion of *Eimeria* spp. oocysts in young lambs following iron supplementation

Iron is an essential nutrient, and iron supplementation has been shown to reduce the incidence of abomasal bloat in lambs. Additionally, iron deficiency has been linked to pica, which may increase the uptake, and thus the subsequent excretion, of *Eimeria* oocysts. Coccidiosis in sheep, caused by *Eimeria* spp., is an important parasitic infection, leading to reduced welfare and economic losses. The aims of our study were to investigate: 1) the use of iron supplementation in Norwegian sheep flocks using a questionnaire survey, and 2) whether iron supplementation reduced excretion of *Eimeria* oocysts and increased growth rates of young lambs. The questionnaire was sent to all members of the Norwegian Sheep Recording System (n = 4993) and showed that 152/1823 farmers iron-supplemented lambs, either orally (56.7 %) or by injection (43.3 %). The main purpose of supplementation was to prevent abomasal bloat (38.4 %), coccidiosis (9.3 %), or both (27.8 %). In the field study, 102 twin lambs from five flocks were included: one twin (treated) received 600 mg of gleptoferron subcutaneously within three days of birth, whereas the control was given saline. McMaster analysis of individual faecal samples obtained at weekly intervals (n = 4 per lamb, starting at turnout) showed no significant difference in oocyst excretion between treatment groups at any sampling, except for one flock 14 days after turnout. Mean growth rates, measured at iron injection, 21 days after turnout, and in the autumn, differed significantly between treated and untreated lambs from iron injection to 21 days after turnout, however, no difference in growth rates was observed in the overall period from iron injection to autumn. Blood analysis suggested that the controls were at risk of developing iron deficiency anaemia during the housed period, but signs of anaemia were not observed. From this study we can conclude that iron supplementation of lambs is a relatively frequent practice in Norwegian sheep farms, sometimes used for the prevention of coccidiosis. However, the field trial results indicate that iron supplementation of young lambs did not reduce oocyst excretion and only induced a transitory increase in weight gain. Further studies, including more flocks and possibly repeated iron injections would provide more definitive information.

Methodological considerations

The material and methods applied for this thesis are primarily described in **papers I-IV** (Table 2), but some additional considerations are discussed in the following sections.

Table 2. A summary of the most important methods used in this thesis.

Methods	**Paper I**	**Paper II**	**Paper III**	**Paper IV**	***In vitro* assay** (ongoing work)
Field faecal sampling		X	X	X	
Modified McMaster		X	X	X	
Haematology and biochemistry			X	X	
Eimeria spp. identification		(X)	X		
Questionnaire	X			X	
Experimental *Eimeria* spp. infection			X		
Oocyst isolation and purification			X		X
Gross pathology and histology			X		
Excystation of oocysts					X
Cell culture					X

Ethical considerations

The animal experiments were performed in compliance with ethical guidelines and approved by the Norwegian Research Authority with regards to the Norwegian regulation on animal experimentation (FOR-2015-06-18-761). The three Rs (replace, reduce and refine) are guiding principles for ethical use of animals in research (Russell and Burch 1959; NC3Rs 2017), and were for instance implemented in the CET (**Paper III**) as follows: a replacement for the live animals was not possible since we aimed to study the *in vivo* efficacy of toltrazuril, and there are currently no validated *in vitro* assays for the assessment of anticoccidial efficacy in ovine *Eimeria* spp. However, to reduce the total number of animals, a limited number of lambs were included in each group, but at the same time, the experimental groups had to be large enough to overcome normal biological variation. In addition, development of the FOCRT (a non-invasive method for testing of ACE), makes CETs less necessary, and thereby contributes to the three Rs.

Pilot trials

In order to plan the field trials, two pilot trials were performed. Pilot trial 1 (PT1) included 6 twin pairs from four commercial flocks, all with a known problem of coccidiosis in previous years. From these farms, one lamb from each twin pair was orally treated with toltrazuril (20 mg/kg Baycox® Sheep vet., Bayer Animal Health), and faecal samples were performed weekly after

turnout (n = 6). PT1 later influenced the FOCRT-protocol regarding timing of treatment and sampling (**Paper II**).

A pilot for the iron trial (**Paper IV**) was also performed (PT2). In PT2, twin lambs (n = 10) from the research farm at NMBU Sandnes were included. This farm has been diagnosed with coccidiosis repeatedly over several years. One twin lamb was supplemented with 300 mg gleptoferron (Gleptosil vet, Ceva Santé Animale) subcutaneously during the first two days of life. The other twin served as an untreated control. Oocyst excretion was then recorded weekly after turnout (n = 4). The results showed no effect on oocyst excretion, or growth rates. Calculations of iron requirements of growing lambs showed however, that an increased dose might have been beneficial, and the dosage of iron was doubled for the actual field trial.

Animals

Lambs included in this thesis were either part of the pilot trials, the two field trials, or the CET (Table 3). Lambs for the field trials were recruited from commercial flocks (**Paper II** and **IV**). In these trials, the farmers and their veterinarian handled cases of infection with pathogens other than *Eimeria* spp., e.g. *Mannheimia haemolytica*-pneumonia, to ensure good animal welfare.

Table 3. Number of farms and lambs included in the different studies.

Trial	Number of farms	Selection of farms	Number of lambs per farm
PT1	4	History of coccidiosis* Proximity to the research facility	12
PT2	1	History of coccidiosis* NMBU's experimental flock	20
FOCRT (**Paper II**)	36	Flock size ≥ 60 ewes Continuous use of anticoccidials for > 4 years Diarrhoea in lambs after treatment with an anticoccidial	10-16
CET (**Paper III**)	1	NMBU's experimental flock	20
Iron trial (**Paper IV**)	5	Participation in PT1 (farm C) or FOCRT (farm: A, B, D and E) Proximity to the research facility	20-22

*A history of diarrhoea with confirmatory faecal analysis

Lambs for the CET (**Paper III**) originated from NMBUs experimental flock, and were housed, fed, and handled by the same people throughout the study period to avoid confounding environmental factors. All lambs included in the study were considered healthy based on clinical examinations, haematology and biochemical analysis prior to the *Eimeria*-infection. In addition, gross pathology at euthanasia did not reveal any signs of disease other than that associated with an *Eimeria* infection.

Questionnaire studies

Paper I and **IV** included the use of questionnaires, which may be a challenge (Dohoo et al. 2009). As some of the questions in **Paper I** required farmers to have detailed information concerning their farm, this might have contributed to some of the missing data seen in this study, with 119 incomplete responses (9.8 %). By reducing the need for farmers to gather up information, the number of incomplete responses might have been reduced, but we might also have lost valuable information.

In the design of the actual questionnaires, closed questions were preferred, as they are far easier to assign a value, and thus facilitate statistical analysis (Dohoo et al. 2009). In addition, the questionnaire for **Paper I** was pre-tested on to two sheep farmers (study population) in order to ensure that the questions were easily understood, and if the categories in the multiple choice section were meaningful. The shorter questionnaire for **Paper IV** was however not pre-tested on the study population, due to its brevity and pre-testing of the previous questionnaire.

As publication of the actual questionnaires might ultimately improve the quality of other studies, translated copies of the questionnaires were attached as additional material in the respective publications (**Paper I** and **IV**) (Rosen and Olsen 2006; Schilling et al. 2006).

Selection of farmers/flocks

Farmers selected for the questionnaire study (**Paper I**) were all members of the NSRS with a registered email address. As already mentioned, members in the NSRS represented 36.5 and 47.9 % of all Norwegian flocks or ewes, respectively (National Sheep Recording System 2016). The NSRS-members had a larger mean flock size, higher average slaughter weights and better quality classification of carcasses than non-members (National Sheep Recording System 2016). Our analysis may therefore be biased by including better managed flocks than the average national flocks.

In order to investigate coccidiosis and treatment efficacy of anticoccidials, only flocks with known or suspected problems with *Eimeria* spp. were included in the field trials. The FOCRT-

flocks **(Paper II)** were chosen from the questionnaire-participants **(Paper I)**, and additional criteria were applied (Table 3). These criteria are recognised as being correlated with increased risk of ACR in poultry (Chapman et al. 2010; Peek and Landman 2011; Lan et al. 2017). Thus, the farms were not randomly selected, and therefore the results could not be used to estimate the prevalence of ACR in Norwegian sheep farms.

In order to verify the presence of resistance in *Eimeria* spp. detected by the FOCRT, a CET was performed. According to the FOCRT, two flocks were resistant (NMBU ID 10 and 22), but these flocks were unavailable due to practical and geographical reasons. The chosen flock (NMBU ID 35), although classified as inconclusive, showed low efficacy (56.0 %), but was not classified with reduced efficacy due to the wide CI. Given that none of the farms classified with reduced efficacy (**Paper II**) were available, this flock was the next option. In addition, the chosen flock was located one hour from the research facility, which made repeated sampling of faeces possible.

For the iron trial (**Paper IV**), selected farmers had either been part of PT1, or the FOCRT study (Table 3). In addition, the flocks were located less than an hour drive from the laboratory. The geographical distribution of the flocks was restricted to the Southwest of Norway, therefore the results may be biased due to the narrow geographical origin.

Analysis of faecal samples

Eimeria oocysts were quantified for **Papers II, III** and **IV**. Faecal samples were collected per rectum using a "faecal spoon" and analysed by a modified McMaster method with a theoretical sensitivity of 5 oocysts per gram (OPG) (Henriksen and Aagaard 1976; Henriksen and Korsholm 1984) (Fig. 10). The low sensitivity was achieved by adding less flotation fluid (i.e. less dilution) and by counting a larger volume of the sample (0.6 ml), compared to traditional McMasters, using 0.3 ml sample (MAFF 1986). Briefly, water was added to 1-4 g of faeces, which was homogenised, filtered, concentrated by centrifugation (110× g) and mixed with flotation fluid (saturated sodium chloride with glucose; density: 1.27 g/ml) at a sample/flotation fluid ratio of 1:1 to 1:2 depending on sediment. A subsample (0.6 ml) was then transferred to a disposable counting chamber fitted with a thin coverglass, and the oocysts were enumerated at 200/400 x magnification. In samples with few oocysts (OPG < 10,000, which equals to <2,000 oocysts/counting chamber) the whole chamber was evaluated, whereas one row (≈ 1/20) or three fields of vision (≈ 1/200) of the chamber was counted in samples with higher numbers of oocysts (Fig. 10c) (Henriksen and Aagaard 1976; Henriksen and Korsholm 1984).

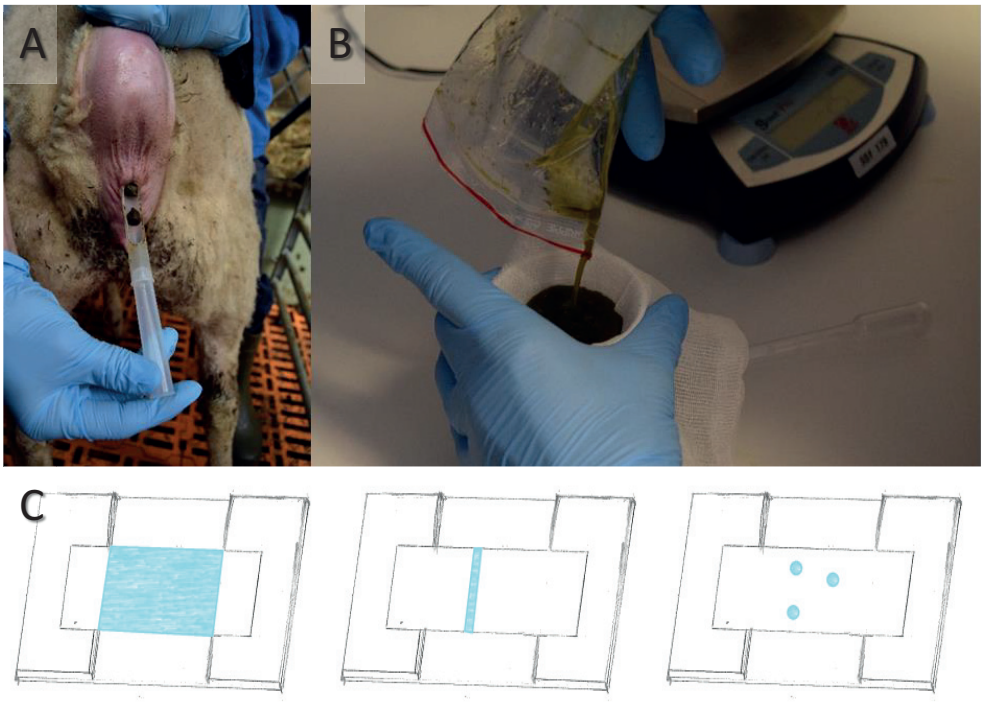

Figure 10. (A) The use of a "faecal spoon" to obtain rectal faecal samples. (B) Preparing faecal samples for analysis: the filtration step is shown. (C) Modified McMaster counting chamber used for faecal analysis, counting areas (blue): total chamber, one random line and three random fields of view (Henriksen and Korsholm 1984). Photo: A. Odden.

Speciation

Speciation was performed for **Paper III**, and on samples from the FOCRT (**Paper II**), but complete details were not included in the last publication, and will be prepared for publication later. Differentiation of species was performed by light microscopy based on morphological characteristics of the oocysts (Eckert et al. 1995b), except for *E. crandallis* and *E. weybridgensis*. Only samples with ≥1,000 OPG were speciated. This cut off was chosen based on a pragmatic approach, as samples with ≥1,000 OPG had at least 200 oocysts in the counting chamber due to the 5 OPG sensitivity.

In contrast to the morphological characteristics, molecular methods can be used for both species differentiation and quantification, although not all species have yet been detected by this method (Berriatua et al. 1995; Yang et al. 2014). Furthermore, as it may be difficult to detect mixed infections (Yang et al. 2014), which were highly present throughout the present work, valuable information concerning the species composition of the samples might have been lost.

In vitro assay

As toltrazuril is directed against the intracellular stages of *Eimeria* spp., *in vitro* assays for investigating the effect of anticoccidials on the intracellular development of ruminant *Eimeria* spp. have not been developed, but there are several *in vitro* assays for avian *Eimeria* spp. (Alnassan et al. 2015; Thabet et al. 2015; Thabet et al. 2017). In these assays the infection and development rate of sporozites treated with anticoccidials have been investigated (Alnassan et al. 2015; Thabet et al. 2015).

In the present project, a sporozoite intracellular development assay (SIDA) was used to evaluate the effect of anticoccidials on the intracellular development of ovine *Eimeria* spp. Relevant recordings for the SIDA include invasion rate, and degree of development from sporozoite to both immature and mature schizont. The work concerning the SIDA is currently not completed, but a short presentation of the techniques used is provided here, and preliminary data are provided in the results section.

The SIDA investigates whether parasites are able to complete their development *in vitro* in the presence of an anticoccidial. In order to perform such an assay, a monolayer of cells must be cultured, and sporozoites from the oocysts must be able to infect and develop in these cells in appropriate conditions. Ruminant *Eimeria* sporozoites have been shown to infect a range of cells, such as bovine colonic epithelial cells (BCEC), bovine, human, and caprine umbilical vein endothelial cells (BUVEC, HUVEC, and CUVEC), bovine foetal gastrointestinal cells (BFGC), African green monkey kidney cells (VERO), and Madin-Darby bovine kidney (MDBK). However, further development from sporozoites to schizonts and merozoites has predominantly been reported from ruminant intestinal or primary cell lines (Hermosilla et al. 2002; Ruiz et al. 2010; Hermosilla et al. 2015; Carrau et al. 2016).

In addition to the SIDA, an oocyst sporulation inhibition assay (OSIA) can be applied, investigating the sporulation rate after treatment with anticoccidials. Different OSIAs have been used previously for both avian and ovine *Eimeria* spp. (Saratsis et al. 2012; Habibi et al. 2016).

To establish the SIDA, a caprine monostrain of *E. ninakholyakimovae* was used. Originally, the assay was tested using a field isolate of ovine *Eimeria* spp. However, purification of oocysts proved difficult, predominantly due to the large variation in size between the different *Eimeria* spp., from *E. parva* (24 x 14 μm) to *E. intricata* (48 x 34 μm) (Eckert et al. 1995b), with either smaller oocysts, larger oocysts, or both being lost in the process. Following a series of pilot trials, *E. ninakholyakimovae* was finally selected for further studies, as it is similar in size to *E. ovinoidalis* (Eckert et al. 1995b), and was available in monoculture. The *E. ninakholyakimovae* isolate was

initially isolated from Gran Canaria, Spain, and has been propagated in goat kids since 2006 (Ruiz et al. 2013). Toltrazuril had not been used in the goat flock of origin, and we therefore considered this isolate to be sensitive. Sporozoites were obtained by purification, sporulation, and excystation of oocysts. Excystation was performed with the aid of cysteine digestion, followed by digestion in a solution containing a mix of Hank's balanced salt solution, trypsin, and bile (Fayer and Hammond 1967; Pérez et al. 2015).

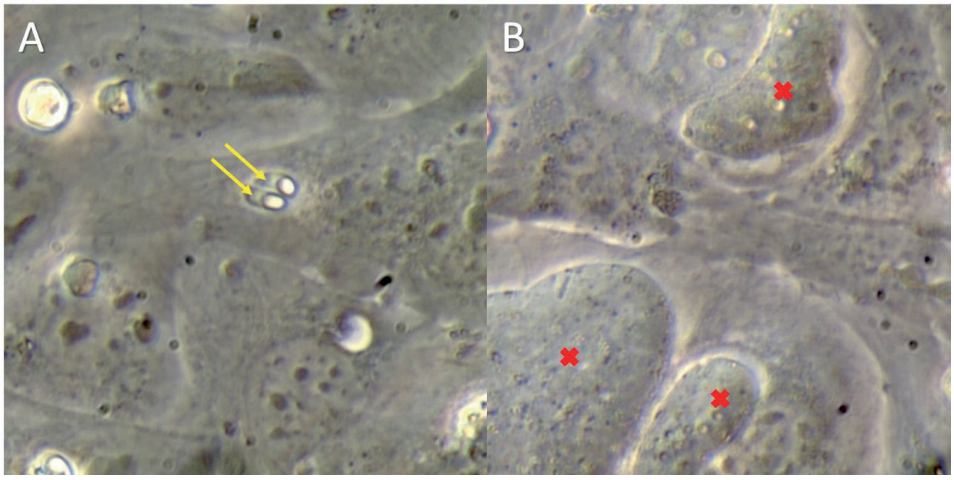

Figure 11. Examples of recordings for the sporozoite intracellular development assay (SIDA) showing (A) intracellular sporozoites (yellow arrows) 24 hours after infection, and (B) immature schizonts (red X) 8 days after infection. Phase contrast, 400× magnification. Photo: A. Odden.

Free sporozoites infected a monolayer of bovine colonic epithelial cells (BCEC), with continuous inclusion of an anticoccidial drug. The invasion rate was determined 24 hours after infection, and intracellular development was evaluated at 8 and 15 days post infection (Fig. 11). At day 8 after infection, the number of immature schizonts was evaluated, while at day 15, the number, size and appearance of mature schizonts were assessed. Both commercial anticoccidials (toltrazuril, diclazuril, decoquinate, and sulpha) and pure/derivate anticoccidials (pure toltrazuril, toltrazuril sulphone, and toltrazuril sulphoxide) were tested in this assay and the parasite development was compared with control cultures to which had been added dimethyl sulphoxide (DMSO) or dimethyl formamide (DMF).

Results and general discussion

The present work indicates that coccidiosis is an important parasitic infection in Norwegian sheep production, and that treatment with anticoccidials plays an important role in the control (**Paper I**). To evaluate anticoccidial efficacy (ACE), a model for the field evaluation of ACE, the faecal oocyst count reduction test (FOCRT, **Paper II**), was developed. Subsequently, anticoccidial drug resistance (ACR) was experimentally confirmed in *E. ovinoidalis* by a controlled efficacy trial (CET, **Paper III**), which also supported the findings of the FOCRT (**Paper II**). As an alternative control strategy, iron injection of young lambs naturally infected with *Eimeria* spp. was investigated. However, iron supplementation did not increase the weight gain of the lambs or reduce the oocyst excretion in the present study (**Paper IV**).

Use of anticoccidials and potential risk factors for reduced ACE

The use of anticoccidials by Norwegian sheep farmers was studied in **Paper I**. In total, 31.3 % of the farmers responded to the questionnaire (n = 1215), representing all 19 counties with sheep production in Norway. Management practices potentially linked to the development of reduced anticoccidial efficacy included flock size, treatment without a diagnosis and incorrect dosing due to inaccurate weight estimation and lack of drench gun calibration. An important finding was that more than 80 % of the farmers were treating their lambs with anticoccidials, mainly without a laboratory-based diagnosis, as only 12.3 % of the farmers submitted faeces for a laboratory analysis. In addition, as many as 37.9 % of the farmers reported clinical signs that were possibly related to coccidiosis in lambs treated with an anticoccidial.

Treatments with anticoccidials by farmers in **Paper I** was mainly done at a set time point, either at turnout (38.6 %), or 7-10 days post turnout (32.4 %). In addition, almost one third of the treated flocks had no problem with coccidiosis in previous years. The absence of a laboratory diagnosis, or without previous clinical signs related to coccidiosis, may lead to unnecessary treatment. Although treatment of subclinical coccidiosis has been shown to be effective in increasing weight gain (Alzieu et al. 1999; Le Sueur et al. 2009; Scala et al. 2014), such excessive use of anticoccidials may be a risk factor for the development of ACR, as reported for anthelmintics (Jackson and Coop 2000; Domke et al. 2011; Kotze and Prichard 2016).

The high number of farmers treating with an anticoccidial stands in contrast to a survey performed in the UK, showing that routine use of coccidiostats was used in < 40 % of flocks (Binns et al. 2002). However, it must be noted that sales of toltrazuril has increased in the UK during the last years, as 510,388 kg and 787,300 kg toltrazuril from products authorised for use

in farm animals were sold in 2014 and 2015, respectively (Dr Gillian Diesel, Head of the Pharmacovigilance Team, Veterinary Medicines Directorate, personal communication). If the toltrazuril use was related to pig, cattle or sheep production is however unknown.

Anthelmintic resistance (AR) was first diagnosed by faecal egg count reduction tests (FECRT) in *Haemonchus* and *Teladorsagia/Trichostrongylus* type parasites in Norway from samples obtained in 2008 and 2009 (Domke et al. 2012). Coinciding investigations into management practices possibly related to development of AR showed the farmers' use of visual appraisal (78.6 %) for estimating lamb weight and lack of drench gun calibrations (27.1 %) as two contributing factors leading to AR (Domke et al. 2011; Domke et al. 2012). Therefore, the focus from Animalia (Norwegian Sheep Health Service) over the last decade has been aimed at increasing farmers' awareness of good drenching practices. In **Paper I**, the number of farmers never calibrating their drench gun had decreased to 12.1 %, and weighing of the animals prior to drenching have become more common (67.4 %). Although an improvement was seen, some farmers are apparently still unaware of the importance of correct drenching practices.

ACE testing in sheep

Paper I showed that several farmers experienced lambs with diarrhoea possibly related to coccidiosis in treated lambs. However, due to lack of laboratory-confirmed diagnosis, the reason for this apparent lack of drug efficacy was unknown. In order to detect the presence of reduced ACE, appropriate field evaluation tools are needed. At the beginning of this project, established methods to investigate ACR in ruminant coccidian were lacking. Thus, one focus was aimed at creating a field evaluation tool that could be used for monitoring ACE.

Field testing of ACE

The field tool evaluating anthelmintic efficacy suggested by the World Association for the Advancement of Veterinary Parasitology (WAAVP), the faecal egg count reduction test (FECRT) (Coles et al. 1992), was used as a template for the development of the FOCRT (**Paper II**). Due to major biological differences between *Eimeria* and helminths, in particular, differences in *Eimeria* oocyst and helminth egg excretion (Chapman 1974a; Sréter et al. 1994; Zaros et al. 2014), the FECRT had to be modified.

The proposed FOCRT for evaluation of ACE (**Paper II**) has two steps. It first determines whether treatment and sampling have been timed correctly, and subsequently assesses the ACE, by comparing oocyst counts in post-treatment faecal samples from treated and untreated lambs.

The FOCRT was built on the assumption that ovine *Eimeria* oocysts are excreted in an exponential pattern, as has previously been observed (Chapman 1974a; Gregory et al. 1989b). The initial exponential growth phase of oocyst excretion in lambs gives a linear relationship (here referred to as a "slope") between time and the logarithm of the oocyst excretion. Thus, it is possible to evaluate when on the excretion curve the samples were obtained. However, due to the large variation in oocyst excretion, statistically significant differences may be difficult to find. The sampling should be done at the time with the potentially largest difference between treated and untreated lambs, which can be found at the end of the exponential growth curve. Therefore, the first step of the FOCRT is focused on determining the timing of treatment and sampling, i.e. the increase in oocyst excretion in the control lambs.

The defined cut-offs for the slope are not based on previous studies, but on an evaluation of which threshold of the slope that appeared to be consistent with that obtained from those farms with the greatest slope, as these farms were assumed to represent the exponential phase. A threshold of 0.75 was chosen, over which farms were assumed to be in the exponential phase. A second threshold of 0.5 was identified, above which the timing was broadly consistent with the required OPG increase in untreated lambs, but this threshold may also include farms with sub-optimal timing. Due to the degree of subjectivity in evaluation of these slopes, these thresholds may have been different if they had been based on another dataset, and pose an important limitation for the FOCRT. However, as there currently are no other verified methods for the determination of the timing of treatment and sampling, this is a starting point for further studies, and the threshold may therefore be modified following evaluation of more farms and larger datasets.

The second step was a faecal oocyst count reduction (FOCR) analysis (i.e. ACE evaluation), and was only performed in farms with a slope above the chosen cut-off values. Post-treatment oocyst counts from treated and control lambs in those farms were then analysed.

The successful and practical use of the FOCRT depends on several factors: the farmer's participation and compliance with the protocol, reliable estimates of oocyst excretion, and, in particular, a correct interpretation of reduced efficacy. Due to the wide 95 % CI observed in many of the inconclusive flocks, the sample size should have been increased in order to reduce the number of inconclusive farms. In addition, the FOCRT only takes into account the total oocyst count, without considering the different species. As only two *Eimeria* spp. are regarded major pathogens (Joachim et al. 2018), resistance would only result in increased clinical signs and reduced productivity, if these species were resistant to treatment. Based on current knowledge it is questionable if ACR in non-pathogenic *Eimeria* spp. will be of any clinical relevance. Therefore,

the present FOCRT may be considered a starting point for further investigations into ovine ACR, and further development should specifically take into account the efficacy of anticoccidial drugs against the pathogenic *Eimeria* spp. However, while little is known about the interaction between *Eimeria* spp. in multiple infections, these species should not be ruled out entirely as they may have a synergistic effect (Enemark et al. 2013).

Variation in ovine *Eimeria* spp. after treatment

All faecal samples with an OPG of ≥1000 analyzed in **Paper II** were speciated (Odden et al. 2017a). The dataset consisted of 171 and 332 speciated samples from sample date 1 and 2, respectively. The most frequently detected species were *E. ovinoidalis, E. parva* and *E. crandallis/weybridgensis*. Due to lack of normality in the oocyst counts, the non-parametric Mann-Whitney U test was used to compare the oocyst counts of different species pre- and post-treatment. Non-pathogenic *Eimeria* spp., *E. ovinoidalis,* and *E. crandallis/weybridgensis* were considered as three different groups, and all flocks were grouped together.

There was no difference in pre-treatment oocyst counts for the different species between treated and control lambs. However, there were significantly higher oocyst counts post-treatment of *E. ovinoidalis* ($p < 0.01$) and *E. crandallis/weybridgensis* ($p < 0.05$) in the control group than in the treated group. In contrast, the non-pathogenic *Eimeria* spp. showed significantly higher oocyst counts in the treated group than in the control group ($p < 0.001$). In other words, there was a relative reduction in the oocyst count of the pathogenic species post-treatment, and a relative increase in the non-pathogenic species. As a result, these findings might limit the value of evaluation of ACE based only on oocyst counts, as the different species apparently have different sensitivities to treatment, possibly due to different antigenicities (Reeg et al. 2005). In addition, there may be a different selection pressure caused by treatment between species, as the mechanism of action of toltrazuril is largely unknown. Although we had no access to data regarding the development and testing of toltrazuril prior to when it was first marketed, it seems probable that only the pathogenic *Eimeria* spp. were used in that process. In addition, no adjustment for flock variation regarding ACE was made. Thus, the data should be re-analysed, as we now know that these data include both flocks with good, unknown and reduced ACE, when considering the total OPG. Grouping of *E. crandallis* and *E. weybridgensis* might also have influenced the outcome of the calculations.

In vivo testing for ACE

Although a field evaluation tool is important for practical and routine assessment of ACE, a controlled efficacy trial (CET) has to be performed in order to verify presence of ACR.

Randomized controlled trials are considered the gold standard to evaluate drug efficacy (Kabisch et al. 2011). CETs have been performed for the evaluation/verification of drug efficacy/resistance in ruminant helminths (De Graef et al. 2012; Peña-Espinoza et al. 2014). A CET is performed by infecting animals with a suspected resistant isolate; animals are then treated and subsequently euthanized for analysis of parasite burden, a procedure not applicable for routine diagnosis. In addition, CETs require a strictly parasite-free environment, and this cannot be readily established under field conditions (Taylor et al. 1995; Wood et al. 1995) (Fig. 12). While Joachim et al. (2018) suggested comparing a suspected-resistant field strain with a known sensitive laboratory strain, we chose to compare treated and untreated animals infected with the suspected isolate, by using an anticoccidial drug previously proven to be efficacious. To our knowledge, a sensitive laboratory strain of pathogenic ovine *Eimeria* spp. was not available, neither commercially nor from another research lab. In addition, there was no *in vitro* assay available for the evaluation of ACE in *E. ovinoidalis/crandallis* at the time of the study.

Figure 12. The facilities for the controlled efficacy trial. (A) Outside view with a locked door and chicken wire, (B) entering involved changing of shoes, clothes and the use of gloves, and (C) eating troughs for lambs with hay and concentrate. Photo: A. Odden.

The results of the CET (**Paper III**) indicated no difference between treated and control lambs with regards to clinical signs, oocyst excretion, or post-mortem findings, so the parasites investigated were classified as toltrazuril resistant. In order to improve the analysis of clinical parameters, and especially weight gain, a group of uninfected lambs would have been beneficial. However, additional lambs were not deemed necessary for this study, taking into account the three Rs.

As discussed earlier, timing of toltrazuril treatment, is important, as metaphylactic treatment is recommended (Dittmar et al. 2009). In a CET, metaphylactic treatment in experimental infection

is easier to achieve than in field situations, as the day of infection is known. In the CET study perfomed here, treatment at day 7 after infection was in the middle of the prepatent period (Fig. 13), and the oocyst excretion should have been reduced in treated lambs infected with sensitive parasites.

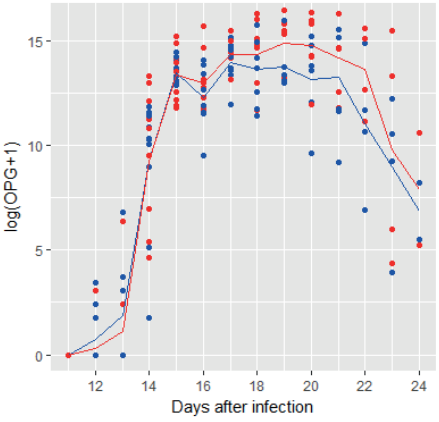

Figure 13. Mean and individual oocyst excretion [log(oocysts per gram (OPG) +1)] in the 20 *Eimeria* spp. infected lambs from the CET. Half of the lambs were treated (red) with 20 mg/kg toltrazuril or saline (blue) at day 7 after infection. (Paper III)

As discussed in the previous section, there might be a difference in sensitivity to toltrazuril treatment between *Eimeria* spp., however, the CET showed that both pathogenic and non-pathogenic species from the tested field isolate were resistant (**Paper III**).

Peek and Landman (2011) defined complete drug resistance in avian coccidiosis as ineffectiveness despite increasing the doses up to the maximum tolerated by the host. In contrast, relative resistance occurs where increased doses still show efficacy. In our study, the efficacy of an increased dose of toltrazuril was not tested. However, work done in pigs showed no effect of increasing the dose of toltrazuril in a resistant strain of *Cystoisospora suis* (Shrestha et al. 2017a).

Trichobezoars

At necropsy, a range of 1 to 12 (mean 5.5) trichobezoars (wool balls) were found in the abomasum of 19 of 20 CET lambs (**Paper III**) (Fig. 14). Trichobezoars were small (range 0.5 to 2 cm) and were not associated with pyloric obstruction. Fleece-eating was not observed during the regular daily inspections. In addition, no signs of diffuse alopecia were detected in any lambs at necropsy.

Figure 14. Trichobezoars from a lamb infected with *Eimeria* spp. in the CET. Photo: A. Odden.

In adult sheep, wool-pulling has been associated with a lack of fibre in the diet (Fraser and Broom 1990; Vasseur et al. 2006), or high protein diets in intensively housed animals (Yurtman et al. 2002). The CET-lambs were euthanized aged 35-45 days, and had been provided with hay and concentrates *ad libitum* throughout the study. Therefore, lack of dietary fibre was not considered likely

in these lambs, which were not yet fully functioning ruminants.

Wool biting and eating has also been described as a redirection of a frustrated behavioural motivation arising in housed sheep deprived of grazing activity or oral stimulation (Nowak et al. 2008). Although there is a lack of strong evidence, the wool eating of the CET lambs might have been linked to boredom, restriction of movement, redirection of sucking, or other oral behaviours, possibly due to stress. In dairy calves, trichobezoars are most commonly associated with persistent sucking of penmates (Drawer 1978). The levels of trichobezoars reported here may therefore represent a normal finding in artificially-reared young lambs.

In vitro assay for the evaluation of ACE

Previous work on *in vitro* assays investigating the intracellular development of ruminant *Eimeria* spp., has focused on immunology, parasite-cell interactions, and mechanisms of pathogenicity (Hermosilla et al. 2012; Hermosilla et al. 2015; Ruiz et al. 2015; Carrau et al. 2016; Matos et al. 2017), rather than drug efficacy. The development of an *in vitro* assay for the detection of reduced ACE would reduce the need for animal testing. However, such *in vitro* assays are currently only available in poultry production (Thabet et al. 2015; Habibi et al. 2016; Jitviriyanon et al. 2016; Thabet et al. 2017).

Challenges in developing such an *in vitro* assay are partially linked to the large size variation between different *Eimeria* spp., as purification of oocysts is easier to achieve with oocysts of similar size. In addition, it is important to choose a cell line that is suitable for infection, as the sporozoites must be able to infect and develop in the cells, and the cells must also be able to incorporate the antiococcidial drug.

For this project, work was initiated to establish an *in vitro* assay for the assessment of ACE, but as the work is still ongoing, only a short presentation of the preliminary data is provided. The proposed *in vitro* assay, the sporozoite invasion and development assay (SIDA), as briefly described in the chapter on methodological considerations, was intended to be used for assessing the intracellular development of sporozoites when treated with an anticoccidial. Preliminary results indicated no clear difference in invasion or development between any of the anticoccidials and their controls (data not shown).

It has previously been shown that *E. ninakholyakimovae* can develop into merozoites in BCECs (Ruiz et al. 2010), as was also shown in the present work. As commercial toltrazuril, pure toltrazuril, and the derivatives of toltrazuril were used, we had assumed that this would address the issue that some ingested toltrazuril is transformed *in vivo* into the metabolites toltrazuril sulphone and toltrazuril sulphoxide (Lim et al. 2010). Thus, why our study indicated no

measurable effect from the different anticoccidials on sporozoite development remains unknown, although we have some speculative theories and some suggested approaches on how to continue this work.

One possibility is that absorption of the anticoccidials included in our study differs between intestinal segments; this is relevant, as the pathogenic species mainly infect the small intestine and the caecum (Deplazes et al. 2016). Another suggestion is that the process of creating a permanent cell line (Föllmann et al. 2000), might have influenced the cells' ability to incorporate different substances. This is supported by the studies demonstrating multi-drug resistance in colonic cancer cells, which block drug activity by efflux transporters that promote metabolism, elimination, and detoxification (Chen et al. 2012). Whether the colonic cell line used here shares some of these features has to be evaluated.

In addition, to evaluate the cell line further, BCECs used here, other potentially suitable cell lines should be evaluated. The use of a different cell line, possibly of small intestinal origin or primary endothelial origin, like the bovine umbilical vein endothelial cells (BUVEC), might have provided more useful results. Infection of BUVECs by *E. ovinoidalis* sporozoites has been shown to result in merogony and macromeront I formation (Carrau et al. 2016).

Thus, although there is still considerable work to be completed to develop an *in vitro* assay for the evaluation of ACE, these preliminary data have indicated important pitfalls that must be addressed. Despite the difficulties in developing an *in vitro* assay, it should remain an important goal in ruminant *Eimeria* research as it would decrease the need for animal experiments in assessment of ACE, and would be of value in the initial assessment of ACE of new anticoccidial drugs or bioactive substances.

Control strategies

Although the prevalence of resistant ovine *Eimeria* spp. is unknown, the widespread dependence on chemoprophylaxis for the control of coccidiosis emphasises the potential consequences resistance might have for the sheep industry, especially in Northern Europe, due to limited treatment alternatives (Felleskatalogen 2017b; Läkemedelsverket 2017; Veterinærmedicinsk Industriforening 2017). Since the prevalence of anticoccidial drug resistance (ACR) in ovine *Eimeria* spp. is unknown, many flocks might still have sensitive *Eimeria* spp. Thus, focus should be directed at keeping toltrazuril efficacious, and reducing the need for anticoccidial treatment. On the other hand, flocks already diagnosed with reduced ACE need to focus on alternative treatment strategies and management practices to maintain productivity and animal welfare.

Iron supplementation

In **Paper IV** we investigated whether iron supplementation of young lambs can reduce the ingestion, subsequent excretion of *Eimeria* oocysts, and thereby decrease reliance on anticoccidial drugs. Iron is an important component or cofactor in many proteins and enzymes, such as haemoglobin and myoglobin (Weiss 2010). Housed lambs often are iron deficient (Green et al. 1993; Vatn and Framstad 2000; Radostits et al. 2007b), and iron deficiency anaemia may lead to abnormal appetite and pica, i.e. ingestion of material other than food, including soil (Radostits et al. 2007a), and thus, may cause excessive uptake of *Eimeria* oocysts. Nevertheless, the field trial found no effect of iron supplementation on the excretion of oocysts from the five included flocks three weeks after turnout. Interestingly, although only significant in one flock, there was a numeric reduction in oocyst counts in the iron-supplemented lambs in all five flocks at 14 days post turnout. This may indicate delayed uptake of oocysts in the iron supplemented lambs, which might be beneficial for development of immunity. However, as this difference was mainly non-significant and only seen at one sampling time, the importance of this finding is questionable. In addition, the apparent fall in blood haematological parameters and blood iron content in the treated lambs in the period after turnout may indicate an empty iron storage **(Paper IV)**. A repeated dose, e.g. around turnout, might have prevented this fall in blood iron, and possibly delayed the uptake of oocysts further.

Management

Hygiene

Hygiene is a key factor in lowering the infection pressure, both indoors and on pasture (Taylor 2000), and faecal contamination of food and water should be avoided, e.g. by raising the height of food and water troughs (Daugschies and Najdrowski 2005). Accordingly, it has been shown that hygienic measures, such as keeping beddings dry, gave the same reduction in oocyst excretion in sheep as treatment with sulphadoxine/trimetohoprim (Lopes et al. 2014).

To avoid environmental contamination, thorough cleaning and disinfection should be performed between separate groups of animals. However, decontamination of animal housing is often difficult owing to the robust nature of the oocysts. For *Cryptosporidium* spp., another apicomplexan parasite, it is recommended to hot wash surfaces and utensils followed by drying. This can then be followed by ammonia-based disinfectants, or hydrogen peroxide (Stuart et al. 1981; Chalmers and Giles 2010). In addition, neopredisan (chlorocresole) has been shown to inhibit the viability of avian *Eimeria* oocysts, *Crytosporidium* oocysts, and prions (Daugschies et al. 2002; Joachim et al. 2003; Riemer et al. 2006). Use of chemical compounds after cleaning may, however, be

impractical for routine implementation in sheep production, but could serve as an alternative/supplement to reduce the infection pressures in highly contaminated facilities. As oocysts are excreted with faeces, the removal of faeces by thorough cleaning and drying would in many cases decrease the infection pressure of ruminant *Eimeria* spp. (Ernst et al. 1985; Daugschies and Najdrowski 2005).

Trickle infections

While *E. crandallis* and *E. ovinoidalis* are highly pathogenic to naïve lambs infected at 4-5 weeks of age, it has been shown that lambs were resistant to infection if they were infected during the first 72 hours of life (Gregory et al. 1989b). It was also shown that repeated low doses of pathogenic *Eimeria* spp., also known as a trickle infection, may induce protective immunity (Catchpole and Gregory 1985; Gregory et al. 1989b). However, trickle infections are both impractical and almost impossible to control under natural conditions (Catchpole et al. 1993; Gregory 1995). Knowledge of trickle infections may, however, be used to encourage farmers to turn lambs out onto pasture at an earlier age than what is currently common in Norway, where > 75 % of lambs were 8 - 21 days at turnout (**Paper I**).

To turn lambs out earlier, farmers need to delay the start of the mating period in order to have later lambing, due to climatic challenges in spring time (e.g. snow, wind, and heavy rain). In addition, farmers should be encouraged to shorten the mating season, and consequently, the lambing period. The prepatent periods of ovine *Eimeria* spp. usually range from two to three weeks (Rommel 2000), and a lambing period of around three weeks might be ideal to reduce the risk of younger lambs becoming infected due to environmental contamination by older lambs. Today > 40 % of Norwegian sheep flocks have a lambing period of four weeks of more (**Paper I**), indicating that management changes to reduce the risk of coccidiosis could be implemented.

Grazing strategies

Ruminant *Eimeria* oocysts survive on pasture for at least one year in Northern parts of Europe (Helle 1970; Svensson 1995; Lassen et al. 2013). Pastures grazed by lambs the previous years (Fig. 15) are therefore considered contaminated, and an important source for infection of susceptible animals. In addition, observations from cattle suggests that oocysts shed in the fall contribute equally, or maybe even greater to the pasture infections the following grazing season (Lassen et al. 2014); this may be important when farmers choose spring pastures for their ewes and lambs.

Due to the host-specific traits of *Eimeria* spp., rotational grazing or co-grazing with other livestock may also reduce the risk of infection, as has been experienced with helminths (Waller

2006). It has also been shown that cattle farms that kept sheep on the same pastures had reduced odds of having *Eimeria* spp. positive faecal samples in their cattle (Mitchell et al. 2012).

Figure 15. Lambs and ewes on spring pasture grazed by lambs the previous year. Photo: A. Odden

Chemoprophylaxis

The large proportion (44 %) of farmers that did not time anticoccidial treatment correctly (**Paper II**) indicated that farmers may benefit from submitting samples for parasitological analyses, especially in order to determine the appropriate timing for treatment in subsequent years. Thus, high *Eimeria* spp. oocyst counts at treatment time indicates that treatment may be already too late (Mundt et al. 2003; Taylor et al. 2003).

One step towards controlling the use of coccidiostats and anticoccidials internationally is the publication of a position paper from Federation of Veterinarians of Europe (FVE) urging coccidiostats and anticoccidials to be under veterinary prescription (FVE 2016). This is currently the status in Norway, where all anticoccidials are under veterinary prescription (NMAF/NMHCS 2007). However, the lack of a laboratory diagnosis prior to treatment (**Paper I**), suggests that although anticoccidials have to be prescribed by a veterinarian, this is mostly done without a proper diagnosis.

In addition, most farmers were treating all lambs (**Paper I**), and a more selective approach to treatment could be considered in order to reduce the usage of anticoccidials. If a selective approach is considered, lambs with an increased risk of infection should be selected for treatment. These lambs may include multiparous lambs, lambs receiving small amounts of colostrum, lambs with another concurrent infection, lambs that the farmer knows will be turned out onto contaminated pastures, or lambs in mixed age groups. It might also be reasonable to believe that

lambs born early in the lambing season have a lower risk of developing coccidiosis, compared with later born lambs. However, this targeted treatment may be difficult to implement in practice, especially since one animal with clinical coccidiosis may easily contaminate the environment due to massive oocyst excretion, and thus increase the infection pressure for all lambs.

Although such a targeted treatment strategy might seem relevant, it is important to remember that practically all lambs become infected. The key factor is therefore to know the time of infection so that timing of treatment is optimal, i.e. metaphylactic. The correct timing of treatment is one of the main measures to delay development of ACR in sheep. Due to the metaphylactic treatment strategy necessary for good efficacy of toltrazuril (Dittmar et al. 2009), this requires in-depth knowledge on the farm level, including previous outbreaks of coccidiosis, and farm management, such as duration of the lambing season, time of turnout, and grazing conditions. Thus, previous years' laboratory diagnosis, combined with farm knowledge should be used when deciding timing of treatment in subsequent years.

Due to the widespread presence of coccidian oocysts and their longevity in the environment, there is a huge refugium of ovine *Eimeria* spp. Therefore, if treatment with anticoccidials is timed correctly on the sheep farms, the risk of developing resistance may not be as high as in the more intensive rearing systems, such as poultry and pig production. The current challenge seems however to be that the drugs are not used correctly (i.e. largely due to wrong timing).

Other anticoccidial drugs and bioactive substances

The most commonly used anticoccidial drugs in sheep in the Nordic countries, toltrazuril and diclazuril, have been marketed since the 1980s-90s. With the demonstration of ACR against toltrazuril (Lan et al. 2017; Shrestha et al. 2017a; Odden et al. 2018), the question arises whether there is a risk of cross-resistance with diclazuril, or if diclazuril would be effective against toltrazuril-resistant *Eimeria* spp. Decoquinate might be an alternative in flocks with uncontrolled clinical coccidiosis due to toltrazuril resistance. However, the use of decoquinate differ from the use of toltrazuril, as it should be continuously included in the feed (NOAH 2018). Thus, application might be problematic in Norwegian sheep production, as lambs are usually only a couple of weeks old at the time of infection, and therefore mainly consume milk. In addition, they are grazing with their dam on pasture, making continuous feed treatment impractical.

Few new anticoccidial drugs have recently been marketed. Nitromezuril (NZL) and ethanamizuril (EZL) are novel triazine compounds, similar to toltrazuril and diclazuril, and shown to be effective in the prevention of coccidiosis in broiler chicken (Fei et al. 2013; She et al. 2017; Cheng et al. 2018). NZL may become an important anticoccidial, as it has shown high activity against

artificially induced diclazuril-resistant strains of avian *Eimeria* spp. (Fei et al. 2013). However, it has not been investigated if these new triazines have activity against toltrazuril-resistant strains, and/or against ovine *Eimeria* spp.

In addition, different bioactive substances, and extracts from a number of plant species have been investigated for anticoccidial activities, both *in vivo* and *in vitro* in different hosts, including: sainfoin (*Onobrychis viciifolia*), carob pods (*Ceratonia siliqua*), pomegranate *(Punica granatum)* peel extract, grape seed proanthocyanidin extracts (GSPE) and antioxidants such as artemisinin (*Artemisia annua*) (Allen et al. 1998; Naidoo et al. 2008; Wang et al. 2008; Dkhil 2013; Saratsis et al. 2016). However, none of these bioactive substances have yet been marketed for the prevention/control of clinical coccidiosis.

Vaccines

Both attenuated and non-attenuated live vaccines are available for immunisation of poultry against *Eimeria* spp. (Williams 2002a; Blake and Tomley 2014; Chapman 2014). In addition, restoration of anticoccidial sensitivity in commercial poultry farms with drug-resistant *Eimeria* populations has been seen after use of live wild-type vaccine, thus extending the "life" of several important drugs (Peek and Landman 2006; Chapman and Jeffers 2014). Such non-attenuated wild-type vaccines are currently not licensed in Europe. In addition, the use of live coccidiosis vaccines, such as those used against avian *Eimeria* spp. (Williams 2002b; Chapman and Jeffers 2014) have not been tested against ruminant *Eimeria* spp., and probably never will. This is due to the production process of such vaccines, requiring the infection of healthy animals in order to propagate the parasite, which is a challenge due to ethical guidelines (Russell and Burch 1959; NC3Rs 2017).

Key findings and implications

- Toltrazuril resistance in a field isolate of ovine *Eimeria* spp., including resistance in the highly pathogenic *E. ovinoidalis,* was confirmed through a controlled efficacy trial. Resistance against common anticoccidials may lead to both reduced animal welfare, and reduced productivity in the sheep production.
- Due to the diagnosed anticoccidial resistance (ACR) in ovine *Eimeria* spp. in Norway, flocks using anticoccidials should ideally test the efficacy of treatment, or, as a minimum, flocks with a high degree of diarrhoea in lambs, despite treatment, should be tested, either to diagnose resistance, or treatment failure, e.g., due to incorrect timing of treatment.
- Reduced anticoccidial efficacy (ACE) was diagnosed by the faecal oocyst count reduction test (FOCRT) in two flocks. The FOCRT developed in this project is the only tool, to our knowledge, available for field evaluation of ACE in ovine *Eimeria* spp. However, due to the many inconclusive results from the FOCRT, the sample procedure should be improved.
- There are several potential risk factors for the development of ACR, including low degree of laboratory-confirmed diagnosis, lack of post-anticoccidial treatment testing, and unknown timing of treatment. The farmers' and veterinarians' lack of knowledge of the parasite status in the different flocks is a challenge for optimal timing of the treatment.
- One dose of iron supplementation did not significantly reduce the oocyst excretion. However, the iron-treated lambs' iron storage was mostly empty at turnout, and further research into repeated iron injections should be performed.
- Only 17.3 % of the sheep farmers included in the questionnaire study had never treated with an anticoccidial, and these flocks had significantly lower degree of diarrhoea during the spring pasture period than the farmers treating with an anticoccidial. This may indicate that management can be used to control coccidiosis. However, more information regarding the *Eimeria* status in these farms is needed in order to evaluate the apparent difference between treating and non-treating farms.
- Although the prevalence of ACR is unknown, the focus should be aimed at keeping the available anticoccidials efficacious, especially as there are limited treatment options.

Future perspectives

The present project reported the first verified case of anticoccidial resistance (ACR) in ovine *Eimeria* spp., however, further investigations are needed to investigate the prevalence and distribution of resistant strains. This could be done by running the FOCRT in randomly selected sheep farms throughout Norway. The easiest way to do this is to select from members of the Norwegian Sheep Recording System, however this would be a bias, as these farms have more ewes than the average farm, and we have shown that larger farms are more likely to treat against coccidiosis.

To improve the faecal oocyst count reduction test (FOCRT), studies into the expected efficacy of commonly used anticoccidials against 'sensitive' *Eimeria* spp. are needed to define more accurately the initial efficacy and thus the cut-off values. One way to do this would be to encourage the authors of the initial field trials during the 1980s and 90s to calculate the efficacy on these data sets, as they represent treatment against untreated *Eimeria* oocysts.

Possible cross-resistance between toltrazuril and diclazuril should be studied, for instance by testing the known toltrazuril-resistant NMBU ID 35 isolate. This could either be tested by a controlled efficacy trial (CET), or by an *in vitro* assay, if such an assay was available.

The development of an *in vitro* test system for assessment of anticoccidial efficacy (ACE) would be beneficial both for routine diagnosis, but also for research into new anticoccidial drugs or bioactive substances. The development of such an *in vitro* assay has begun, and the work continues. The next step may be to test a different cell line, possibly BUVEC, in order to find cells that are able to incorporate the anticoccidial drugs.

Investigations into bioactive substances possibly efficient against ovine *Eimeria* spp., should be strengthened. However, in order to avoid the use of lambs, this should preferably be performed *in vitro* initially, which requires an *in vitro* assay able to detect anticoccidial activity.

Repeated doses of iron as a prophylactic treatment method against clinical coccidiosis should be tested, as the present study showed the iron storage of the included iron trial lambs were almost empty at turnout. A proposed trial would be to repeat the dose prior to turnout. In addition, this trial should be performed in different geographical regions.

The questionnaire indicated that some farmers were able to avoid diarrhoea in their flocks during the spring pasture period, even without treating with an anticoccidial. Research into the *Eimeria* infection and management on these farms may shed light on possible strategies to reduce the need for treatment.

Due to the large amount of farmers treating without a confirmed diagnosis, veterinarians should be encouraged or even required to obtain a laboratory diagnosis from the given farm prior to prescribing anticoccidial drugs. Thus, to better guide veterinarians and sheep farmers, a guideline on how to obtain faecal samples, so that they can identify the ideal timing of treatment, should be published. These samples should be taken as a part of a flock diagnosis, and can be used for planning of treatment in subsequent years.

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Papers I-IV

I

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ORIGINAL PAPER

Treatment against coccidiosis in Norwegian lambs and potential risk factors for development of anticoccidial resistance—a questionnaire-based study

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Abstract The objectives of this study were to investigate the use of anticoccidials in Norwegian sheep flocks and identify farms with management procedures likely to select for drug resistance. Data were obtained by a questionnaire sent to all members of the Norwegian Sheep Recording System in October 2015. The data set consisted of 1215 answers, corresponding to 8.5% of Norwegian sheep flocks. Anticoccidials were used in 82.7% of flocks. Main treatment was at turnout (38.6% of treated flocks) or 1 week after turnout (32.4%). Interestingly, clinical signs possibly related to coccidiosis were observed by almost 40% of the farmers after treatment, which might be an indication of drug resistance. Correlations between the apparently reduced anticoccidial efficacy and management conditions, such as the size of the farms, were found. From the farmers' perspective, metaphylactic treatment was used in 88.5% of treated flocks, of which approximately one third had no history of clinical coccidiosis. Even though farmers seem aware of the importance of good drenching routines based on reliable estimates of weights and calibration of drench guns, drench gun used for anticoccidial administration was never calibrated in 12.1% of the flocks. Finally, dose estimation was made by visual appraisal in 27.5% of the flocks, which can lead to incorrect dosing. Based on the present study, it cannot be determined whether the apparent treatment failure was related to management practises, incorrect administration of the drug, other infections or actual anticoccidial drug resistance.

Keywords Ovine coccidiosis · *Eimeria* spp. · Anticoccidials · Norway · Drug resistance

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Introduction

Coccidiosis caused by *Eimeria* spp. is a common cause of clinical disease and reduced growth in lambs (Chartier and Paraud 2012). Currently, 15 species are known to occur in sheep, of which 2 are considered major pathogens: *Eimeria ovinoidalis* and *Eimeria crandallis* (Rommel 2000; Catchpole et al. 1976; Catchpole and Gregory 1985). Depending on *Eimeria* species, the prepatent period varies from 2 to 3 weeks. The clinical signs include diarrhoea (occasionally haemorrhagic), abdominal pain, anorexia and weight loss/reduced weight gain (Wright and Coop 2007). Clinical disease is usually seen in young lambs with debut of symptoms 4 to 6 weeks post-partum depending on various factors, such as management and infection pressure (Gregory et al. 1980).

The lambing season in Norway is in March–May, dependent on geographical region. Lambs are weaned in the autumn, at around 4–5 months of age (Vatn 2009). During the summer, most ewes and lambs are moved to mountain or forest pastures, where the stocking densities are low: between 10 and 80 animals per square kilometre (Mysterud et al. 2001; Vatn 2009). Clinical coccidiosis is therefore primarily related to spring pastures with symptoms appearing 2 to 3 weeks after turnout (Helle 1964; Helle 1970).

Since ovine coccidiosis can have a major economic impact due to reduced weight gain and increased mortality, controlling the infection is important (Foreyt 1990; Alzieu et al. 1999). In 1987, Baycox® Sheep vet. (toltrazuril, Bayer Animal Health) was approved in Norway for treatment of coccidiosis as a single oral dose, and in 2007, Vecoxan® vet. (diclazuril, Elanco Animal Health) was marketed in Norway (Gjerde et al. 2009). Worldwide, several other drugs are licenced for treatment of ovine coccidiosis, e.g. decoquinate (Deccox®, Zoetis UK Limited). However, none of these other drugs are licenced for use in Norway (Norwegian Institute of Public Health 2015).

Anticoccidial resistance (ACR) in poultry has been reported against several anticoccidials, such as monensin, salinomycin, nicarbazin, halofuginone, robenidine, toltrazuril and diclazuril (McDougald 1981; Stephan et al. 1997). Testing for ACR in poultry can be done either by in vivo or in vitro assays (Chapman 1998; Thabet et al. 2015, 2017). However, despite the widespread use of anticoccidials in mammals, ACR has not yet been documented and no tests are available for livestock animals except for poultry.

Gjerde et al. (2009, 2010) reported reduced efficacy of Baycox® Sheep vet. in two farms on the southwest coast of Norway, thus prompting the need for more information on the use of anticoccidials in Norway. Additionally, several farmers have experienced an apparent lack of anticoccidial efficacy during recent years (Stuen S, personal communication). The aim of this study was to collect information concerning coccidiosis in lambs in Norway and the use of anticoccidials during the 2015 lambing and grazing season, with emphasis on identification of risk factors for anticoccidial resistance.

Materials and methods

Questionnaire

In October 2015, a questionnaire was sent by email to all members of the Norwegian Sheep Recording System (NSRS) with a registered email address using the Enalyzer Survey Solution (Enalyzer A/S). Of the 4781 farmers who were members in the NSRS, representing 33.5% of all sheep farmers in Norway, 3874 had a registered email address (Statistics Norway 2016a; National Sheep Recording System 2015). Farmers not responding to the questionnaire within 3 weeks were reminded once by email. In addition, the questionnaire was advertised in the Sheep and Goat Farmers' Journal, a journal published six times a year, and subscribed to by 11,014 sheep and goat farmers (Norsk Sau og Geit 2015).

The questionnaire consisted of two sections: one concerning the general management of the flock, such as flock size, breed, housing time, age at turnout and grazing conditions. On the other hand, the second section was focused on coccidiosis and the use of anticoccidials, with questions regarding clinical signs, timing of anticoccidial treatment and reasons for use. A translation of the entire questionnaire (the original of which is in Norwegian) is provided in Online Resource 1. Additional data regarding the breed and numbers of ewes (>1 year on 1 January) reported to the Norwegian Agricultural Authority were collected via NSRS.

Statistical analysis

Statistical calculations were done in Excel 2013 (Microsoft Inc.) and Stata 14 (Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). For calculations of significance based on means, *t* tests were used. Fisher's exact test was used for categorical data, while the Pearson correlation coefficient was used for continuous data. $P < 0.05$ was regarded as significant.

Results

Questionnaire

The final data set consisted of 1096 complete and 119 incomplete questionnaires, of which 6 responded to the advertisement in the Sheep and Goat Farmer's Journal. This corresponds to a response rate among the NSRS members of 31.3%. When possible, the incomplete questionnaires were included in the analysis. Thus, *n* values vary between calculations. The respondents represent all 19 counties in Norway, with most of the respondents from the west coast and the inland mountain area (Fig. 1). The number of respondents in each county corresponded to the general geographical flock distribution in Norway (Statistics Norway 2016a) and showed a strong correlation ($r = 0.94$).

Management conditions

Average flock size (mean ± SEM) was 102.6 ± 2.3 ewes with a range of 1–755. The main sheep breed was Norwegian white sheep (Table 1). Most ewes and lambs were kept on slatted floors (wood, plastic or expanded metal) (65.3%) or on solid floor (straw bedding/wood shavings) (24.0%) (Fig. 2). There was no significant difference ($P > 0.05$) between type of floor

Fig. 1 Distribution of sheep farms included in the study, grouped by county. The size of the circle indicates the number of respondents, range 2–228 respondents per county

and the farmers' observation of diarrhoea or reduced growth. The lambing period lasted for 14–27 days in 57.2% of the flocks and for 28–41 days in 37.2% of the flocks (Fig. 2). Age at turnout was 0–7 days (10.8%), 8–14 days (34.2%), 15–21 days (41.2%) and 22 days or older (13.9%). Cultivated and uncultivated pasture was used as spring pasture for 51.4 and 40.9% of the flocks, respectively. Lambs were turned out onto pastures that had been used for grazing during the previous spring or autumn in 70.6 and 61.7% of the flocks, respectively, while only 7.9% of the lambs were grazing

Table 1 Total number of ewes and breed distribution per 1.1.2015 in the Norwegian flocks included in the study

Breed	Number of ewes (%)	Number of flocks
Norwegian white sheep ("norsk kvit sau")	89,224 (74.5)	983
Norwegian white short tail ("kvit spæl")	12,166 (10.2)	301
Norwegian coloured short tail ("farga spæl")	2676 (2.2)	157
Old Norwegian short tail ("gammelnorsk spæl")	2382 (2.0)	125
Norwegian Pelt sheep ("norsk pelssau")	1845 (1.5)	88
Dala	1455 (1.2)	137
Other breeds	10,083 (8.4)	812

Several flocks had multiple breeds

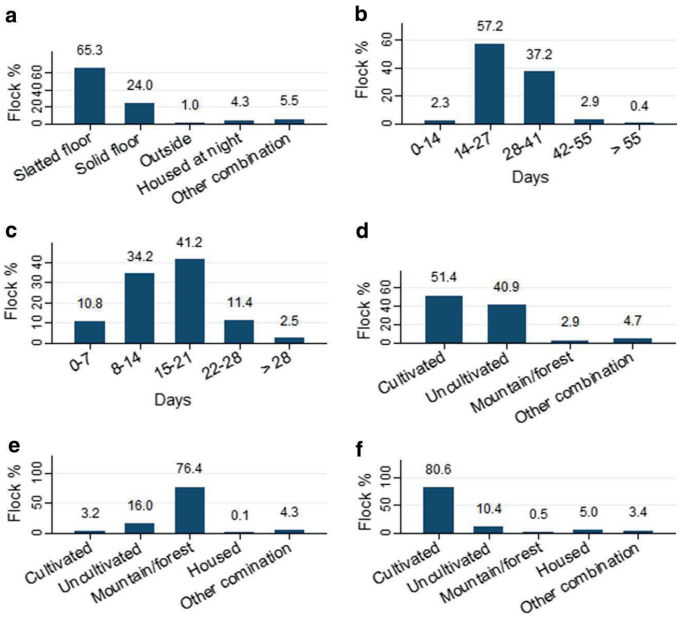

Fig. 2 Management of Norwegian sheep farms. **a** Type of housing ($n = 1152$). **b** Duration of lambing period ($n = 1154$). **c** Lamb age at turnout ($n = 1133$). **d** Type of spring pasture ($n = 1138$). **e** Type of summer pasture ($n = 1138$). **f** Type of autumn pasture for lambs ($n = 1135$). Percentages indicated above *bars*

pastures not used for sheep the previous year. Lambs and ewes were grazing spring pastures for 0–14 days (13.4%), 15–28 days (44.2%) or more than 29 days (42.4%). During summer, 76.4% of the flocks were grazing mountain or forest pastures, and in autumn, 80.6% of the flocks used cultivated pastures for the lambs.

Coccidiosis and anticoccidials

Faecal samples for parasitological analysis of gastrointestinal parasites were submitted to diagnostic laboratories from 140 (12.3%) of the flocks during 2014 and 2015. The main reasons for parasitological analyses were (a) surveillance (65.4%), (b) disease (18.4%) and (c) combinations of these (16.2%).

In response to a question on which parasites and parasitic diseases the farmers felt were of relevance in their flocks, 54.0% of farmers selected coccidiosis as being relevant. Other important parasites were nematodes (59.6%), *Fasciola hepatica* (54.0%) and *Ixodes ricinus* (34.3%).

Toltrazuril (Baycox® Sheep vet.) and diclazuril (Vecoxan® vet.) were used in 87.4 and 5.8% of the treated flocks, respectively (Table 2). In 17.3% of the total number of flocks, anticoccidials had never been used. A significant difference in flock size was observed between the farmers that never used anticoccidials (mean flock size 73.3 ± 4.1) compared with farmers that treated with anticoccidials (109.5 ± 2.7) ($P < 0.05$).

In treated flocks, anticoccidials were administered at turnout (38.6%), 1 week after turnout (32.4%), in lambs showing clinical signs (12.4%) or by using a combination of the above (16.6%). According to the farmers, metaphylactic treatment, i.e. treatment in the prepatent period to prevent clinical signs of coccidiosis, was practised in 88.5% of the treated flocks. Of these, one third had no history of clinical outbreaks. The majority of the flocks (84.1%) treated the lambs only once with anticoccidials (Table 2).

Drench gun calibrations were usually performed once each year (49.3%). Dose estimation of anticoccidials was based on the weight of the heaviest lambs and visual appraisal of lamb weight in 24.9 and 27.5%, respectively (Fig. 3). A significant difference in flock size was seen between the farmers using visual appraisal as dose estimation (mean flock size 99.0 ± 4.8), compared with farmers weighing the heaviest animal (129.5 ± 6.7) ($P < 0.001$).

The occurrence of diarrhoea and/or impaired weight gain correlated to the use of anticoccidials is presented in Table 3. Farms with no use of anticoccidials reported significantly less diarrhoea/perineal soiling and a more normal growth rate among lambs during the spring pasture period (Table 3). Additionally, flocks with diarrhoea were significantly larger than flocks without signs of diarrhoea, both during the housing period (mean flock size 115.2 ± 3.8 vs 91.9 ± 2.9) and after turnout (110.7 ± 3.2 vs 91.1 ± 3.5) ($P < 0.01$). Flocks that were described by the farmers as having reduced growth rates were significantly larger than flocks reporting of

Table 2 Use of anticoccidial drugs in Norwegian sheep farms included in the study

		Number	Percentage
Treatment	Never	193	17.3
	Not every year	166	14.8
	Annually (last 1–4 years)	179	16.0
	Annually (>4 years)	580	51.9
	Total number of farms	1118	
Purpose	Metaphylactic (previous problems)	551	60.4
	Metaphylactic (no previous problems)	257	28.1
	Therapeutic	84	9.2
	Other	21	2.3
	Total number of farms	913	
Drug	Baycox® Sheep vet. (Bayer Animal Health)	794	87.4
	Vecoxan® vet. (Elanco Animal Health)	53	5.8
	Baycox® Sheep vet. and Vecoxan® vet.	19	2.1
	Sulpha preparations	6	0.7
	Unknown	36	4.0
	Total number of farms	908	
Time	All lambs at turnout	347	38.6
	All lambs 7–10 days after turnout	292	32.4
	Individual lambs with clinical signs	112	12.4
	Other management[a]	149	16.6
	Total number of farms	900	
Frequency	Once per year	746	84.1
	≥Twice	46	5.2
	Selected symptomatic lambs >once	95	10.7
	Total number of farms	887	

[a] Other management includes different treatment times within one flock, e.g. lambs born early were treated a week after turnout, while lambs born later were treated at turnout

apparent normal growth rates: during the housing period (mean flock size 116.0 ± 3.8 vs 91.1 ± 3.1) and after turnout (115.5 ± 3.6 vs 88.6 ± 3.3) ($P < 0.01$).

In 37.9% of the flocks, the farmers experienced lambs with clinical signs possibly related to coccidiosis after treatment with anticoccidials. These flocks were larger than the flocks not reporting this potential lack of treatment effect (122.9 ± 4.6 vs 101.9 ± 3.4) ($P < 0.001$). However, of these flocks, only 16.7% reported that they submitted faecal samples for parasitological analysis.

Discussion

In this study, we report the main management practises in Norway regarding coccidiosis in lambs and the use of anticoccidials and link them to potential risk factors for reduced anticoccidial efficacy, i.e. flock size, treatment without a confirmed diagnosis and incorrect dosing due to inaccurate weight estimation and lack of gun calibration.

One important finding of our survey was that more than 80% of the Norwegian sheep flocks were treated for coccidiosis, mainly without a laboratory-based diagnosis or presence of clinical signs. Metaphylactic treatment is recommended for both toltrazuril and diclazuril, based on the mode of action of the drugs and the intention of reducing the

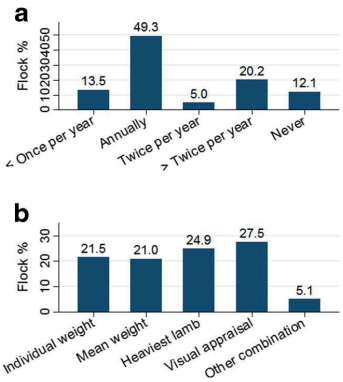

Fig. 3 **a** Drench gun calibrations per year ($n = 901$). **b** Methods used for dose estimation ($n = 903$) in Norwegian sheep farms. Percentages indicated above *bars*

Table 3 Presence of diarrhoea and/or reduced weight gain in lambs in Norwegian sheep farms during housing and spring pasture periods, respectively, depending on treatment with anticoccidials or absence of treatment

			Treatment with anticoccidials		No treatment	
			n	%	*n*	%
Indoor period	Diarrhoea/perineal soiling	Yes	428	47.2	76	39.8
		No	479	52.8	115	60.2
		Total	907		191	
	Reduced weight gain	Yes	436	51.4	86	46.7
		No	413	48.6	98	53.3
		Total	849		184	
Spring pasture period	Diarrhoea/perineal soiling	Yes	583	63.9	90	47.4**
		No	329	36.1	100	52.6
		Total	912		190	
	Reduced weight gain	Yes	499	58.5	83	45.4*
		No	354	41.5	100	54.6
		Total	853		183	

Statistical (Fisher's exact) differences between treating/non-treating and the presence or absence of diarrhoea/perineal soiling and reduced weight gain are marked: *$P < 0.05$, **$P < 0.001$

massive destruction of the intestinal epithelium, which is particularly severe when the oocysts are excreted (Gregory and Catchpole 1990, 1987). Both drugs act against all intracellular stages in the schizogony and gamogony phases (Haberkorn and Stoltefuss 1987; Harder and Haberkorn 1989; Maes et al. 1989) and have been shown to reduce oocyst excretion efficiently in lambs when administered as metaphylactic treatment (Mundt et al. 2009). During the period 2010–2015, the annual use of Vecoxan® vet. declined from 869 to 379 L, while the annual use of Baycox® Sheep vet. in the same period increased from 2933 to 4985 L (Norwegian Institute of Public Health 2015). The farmers' and veterinarians' preference for Baycox® Sheep vet. over Vecoxan® vet. may be linked to the usage of the drug, as Baycox® Sheep vet. Administered at turnout is less time-consuming than the later treatment (Gjerde et al. 2009). In addition, studies have indicated that Baycox® Sheep vet. may have a better effect against ovine coccidiosis than Vecoxan® vet. (Mundt et al. 2009; Gjerde et al. 2009). Sulpha-containing drugs were also used by the farmers although these drugs are not registered for treatment of coccidiosis in Norway.

Almost one third of the farmers treated their flocks, despite clinical coccidiosis not being considered a problem in previous years. Furthermore, the farmers apparently had little knowledge about the actual infection status of their animals, since diagnostic samples were analysed in only around 10% of the farms. These diagnostic samples were analysed for all gastrointestinal parasites, and the percentage of farmers requesting diagnostics particularly for coccidiosis was probably even lower. The potential presence of other infectious agents in young lambs with similar symptoms, such as *Nematodirus battus*, *Cryptosporidium*, *Escherichia coli* and rotavirus (Jackson and Coop 2007; Tzipori et al. 1981; Snodgrass et al. 1976; Munoz et al. 1996) emphasizes the need for a correct diagnosis. In addition, concurrent infections with other microbes can lead to increased severity of the coccidial infection (Catchpole and Harris 1989). Treatment in flocks without previous history of coccidiosis or in the absence of a diagnosis may lead to unnecessary and unsuccessful treatment. Consequently, uncontrolled and extended use of anticoccidials may be a risk factor for the development of ACR in Norwegian sheep farms, as reported for anthelmintics (Barton 1983; Jackson and Coop 2000; Domke et al. 2011).

According to the questionnaire and the widespread use of anticoccidial treatment, most Norwegian farmers are concerned about coccidiosis and consider this disease to be important in their flocks. This concurs with previous results (Gjerde and Helle 1991; Gjerde et al. 2010) in which it was reported that coccidiosis is one of the most important parasitosis affecting Norwegian lambs. Several factors, including stress, poor hygiene during housing, low availability of clean pastures, lack of pasture rotation and the capacity of pathogenic *Eimeria* spp. to overwinter, may be decisive for the widespread clinical problems (Helle 1970; Taylor 2000; Mitchell et al. 2012). For example, poor hygiene at housing, especially related to food and water troughs, has been linked to increased risk of clinical coccidiosis (Taylor 1995; Mitchell et al. 2012). In addition, bad weather during spring may lead to delayed turnout, which can increase the infection pressure during the housing period and affect the farmer's ability to treat at the optimal time.

Lambs in our study were largely turned out onto permanent pastures used for grazing during the previous spring and/or autumn, thereby increasing the risk of infection (Svensson et al. 1994). In addition, almost 60% of the farmers kept lambs

and ewes on spring pastures for more than 3 weeks, which is long enough for the parasite to complete at least one full life cycle. Consequently, the infection pressure increases, explaining why coccidiosis in Norway is usually a problem after turnout. This contrasts with countries such as Iceland, where the lambs are on spring pastures for such a short period that development of immunity is compromised, and therefore, coccidiosis can occur when the lambs are brought back to home pasture in autumn (Skirnisson 2007).

Surprisingly, farmers that treated their flock for coccidiosis reported significantly more diarrhoea and reduced weight gain than untreated flocks during both housing and spring grazing periods. However, this may indicate that farmers were treating their lambs because they were symptomatic. On the other hand, in accordance with the positive correlation between flock size and the use of anticoccidials, farmers with larger flocks reported coccidiosis-related symptoms more frequently. The reason for this observation is unknown, but it may be related to a higher animal density leading to an increased infection pressure, as described for caprine coccidiosis (Ruiz et al. 2006) and nematode infections in sheep (Thamsborg et al. 1998).

Signs related to possible clinical coccidiosis after treatment was observed by almost 40% of the farmers in our study, and the development of ACR could be one explanation. However, as only a few of these farmers had submitted faecal samples for diagnosis, clinical signs could also be related to other infections. Additionally, possible coccidiosis-related symptoms after treatment could be associated with treatment failure due to factors such as poor timing (Enemark et al. 2015) or incorrect storage of the drug (Gradwell 2000). Gjerde and Helle (1986) demonstrated that 20 mg/kg toltrazuril, administered as a single oral dose, is more effective at reducing oocyst numbers than a single oral dose of 10 or 15 mg/kg, so under-dosing is also factor that should be taken into account. Under-dosing is not only a cause of treatment failure but also a well-known risk factor for anthelmintic resistance (Smith et al. 1999; Wolstenholme et al. 2004) and probably also increases the risk of ACR development (Ryley 1980). Inaccurate estimation of animal live weight and lack of drench gun calibration might cause incorrect dosing. Accordingly, farmers are encouraged to calibrate their drench guns at least annually, preferably at each drenching, and to estimate the body weight as accurately as possible, preferably by weighing individual animals. Nevertheless, in about a quarter of the flocks of the present survey, visual appraisal of bodyweight was the basis for calculation of the dose and drench guns used for anticoccidial administration were never calibrated in 12.5% of the flocks. However, this is a marked improvement compared with a previous study by Domke et al. (2011), where almost 80% of respondents used visual appraisal for dose estimation and a quarter never calibrated their drench gun. In the present study, the use of visual appraisal was significantly more common in smaller flocks, suggesting that farmers with larger flocks may be more aware of information concerning correct treatments or consider this to be of greater importance.

High infection pressure, possibly related with herd density, could potentially be a factor promoting treatment failure and/or development of ACR, based on the biological characteristics of the parasite itself. Due to the existence of asexual haploid stages of *Eimeria* spp., resistant mutants will for instance be immediately selected in the presence of a drug at the expense of sensitive forms; this stands in contrast to diploid organisms, where the degree of dominance of resistance genes plays a role (Chapman 1993). In addition, coccidia have an enormous capacity for multiplication in the intestine, and resistant strains may rapidly become the dominant phenotype. On the other hand, there is also a huge untreated refugia consisting of oocysts in the environment. This is one main difference between poultry production and sheep production. Poultry housing is thoroughly cleaned between each batch, which is not the case for lambs due to outdoor grazing.

Apart from herd size, no other significant correlations were detected in the present study between management practises and the possible lack of treatment efficacy. Although other studies have indicated that lambs reared on straw appear to be at particular risk of coccidiosis compared with lambs raised on expanded metal (Berriatua et al. 1994; Taylor et al. 1973), our data demonstrate no apparent correlation between floor type and presence of diarrhoea/perineal soiling and/or reduced weight gain. The reason for this is unknown. However, lambs raised on solid floors with deep litter may be trickle-infected and develop effective immunity without clinical symptoms (Reeg et al. 2005; Catchpole et al. 1993).

Recruitment to the study was mainly from members of NSRS with an email address, and this may be a selection bias, perhaps excluding older farmers or those with more remote locations. However, electronic communication is widespread in Norway. Previous questionnaire-based studies of Norwegian sheep flocks have shown response rates of 12.5–50% (Simensen et al. 2014; Holmøy et al. 2012; Domke et al. 2011), compared with the response rate of 31.3% in our study. The lowest response rate was from a study in which participation was requested by regular mail and the response was via the Internet (Simensen et al. 2014). Studies with higher response rate have used email (Holmøy et al. 2012) and regular mail (Domke et al. 2011) for data collection. Thus, the route of communication seems to have no clear association with response rate.

Among members of the NSRS, the mean flock size is larger, average slaughter weights higher and quality classification of carcasses better than for non-members (National Sheep Recording System 2015). In the present study, the flock size was larger (102.6), than the average flock size in Norway in 2015 (74.1) (Statistics Norway 2016a,b). Although the geographical distribution of the respondents corresponded well

with the actual geographical distribution of Norwegian sheep farmers (Statistics Norway 2016a), our analysis may therefore be biased by including farms that were better managed than the average national flock.

Conclusion

Coccidiosis is considered by farmers to be an important parasitic disease in Norwegian sheep flocks. Accordingly, metaphylactic treatment with anticoccidials seems to be the routine practise in most farms, although it is usually performed without a definitive diagnosis. Farmers also reported lambs with possible coccidiosis-related symptoms after treatment. However, from our data, it cannot be determined whether such potential treatment failure is related to management practises, incorrect administration of the drug, other infections or actual ACR.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Supplementary data for the article: **Treatment against coccidiosis in Norwegian lambs and potential risk factors for development of anticoccidial resistance—a questionnaire-based study**

Questionnaire regarding coccidiosis

Dear sheep farmer!

This questionnaire is a part of a three-year project to investigate how Norwegian sheep farmers are treating for coccidiosis, and whether the treatment is effective. The reason for the project is that there have been several reports of reduced efficacy of the anticoccidials being used.

Coccidiosis can be suspected when young lambs, often at spring pasture, have diarrhoea and/or reduced weight gain. The cause of coccidiosis is the protozoan parasite, *Eimeria*, which infects the gut epithelium and destroys the cells. Coccidiosis can have a major economic impact, especially if many animals in the flock are affected.

Answers from this questionnaire will give us a better understanding of how we are treating coccidiosis in Norway, and whether the efficacy is as expected. Based on information obtained from this questionnaire, we will invite some farmers to participate in a sampling programme during spring 2016, to assess the efficacy of anticoccidial treatment.

We would like to combine the answers obtained in this questionnaire with data in the Norwegian Sheep Recording System, and we therefore ask your permission to access your data. All data and answers will be handled anonymously.

If you are keeping sheep both housed and outdoors, we would like you to let us know the proportion of your sheep that are housed.

The questionnaire should take 5-10 minutes to complete. All farmers completing the questionnaire will be entered into a draw with the possibility of winning a tablet computer.

Thank you for your participation!

1. Are you a member of the Norwegian sheep recording system?
(Only one answer)

- ❑ Yes
- ❑ No

2. Farm number (10 digits):

_ _ _ _ _ _ _ _ _ _

3. Number of winter-fed sheep (ewes >one year at Jan 1st):

_ _ _ _ _ _ _ _ _ _

4. Which breeds do you keep? (Specify the percentage of each breed, the total should be 100%)

Norwegian White	_____ %
Norwegian White Short Tail	_____ %
Norwegian Coloured Short Tail	_____ %
Old Norwegian Short Tail	_____ %
Old Norwegian Sheep	_____ %
Other	_____ %

5. Do you have regular health visits from a veterinarian?
(Only one answer)

- ❑ Yes
- ❑ No

6. Do you have organic production?
(Only one answer)

- ❑ Yes
- ❑ No

7. Duration of the lambing period in your flock?
(Only one answer)

- ❑ 0-14 days
- ❑ 14-27 days
- ❑ 28-41 days
- ❑ 42-55 days
- ❑ More than 55 days

8. How are your lambs housed before turnout to pasture?
(Only one answer)

- ❑ Slatted floor: plastic, wood or expanded metal
- ❑ Solid floor with straw or wood shavings
- ❑ Sheep are outside all year round
- ❑ Housed at night, outside at day
- ❑ Other

Section A, for farmers answering "Sheep are outside all year round" **at question 8.**

9. Have you submitted faecal samples from your lambs to a veterinary diagnostic laboratory during the last two years?
(Only one answer)

- ❑ Yes
- ❑ No

10. When were the samples taken?

- ❑ Before/at lambing
- ❑ Spring pasture
- ❑ Summer pasture
- ❑ Autumn pasture

11. Why were the samples taken?

- ❑ Clinical disease
- ❑ Reduced growth
- ❑ General surveillance
- ❑ To evaluate the need for treatment in otherwise healthy animals
- ❑ Other

12. Which parasites do you consider important in your flock:
(Only one answer per parasite)

	Important	Not important	Uncertain
Ixodes ricinus	❑	❑	❑
Lice	❑	❑	❑
Fasciola hepatica	❑	❑	❑
Dicrocoelium dendritticum	❑	❑	❑
Nematodirus battus	❑	❑	❑
Other nematodes	❑	❑	❑
Tapeworms	❑	❑	❑
Lung worm	❑	❑	❑
Coccidia	❑	❑	❑

13. Have you seen diarrhoea/perineal soiling or reduced weight gain in lambs up to 8 weeks old?
(Only one answer per row)

	In 0-20%	In 20-40%	In 40-60%	In 60-80%	In 80-100%	Not seen
Diarrhoea/ perineal soiling	❑	❑	❑	❑	❑	❑
Reduced weight gain	❑	❑	❑	❑	❑	❑

14. Do you treat against coccidiosis, and for how long have you treated?
(Only one answer)

- ❑ No, have never treated
- ❑ Yes, but do not treat every year
- ❑ Yes, have treated annually for 1-4 years
- ❑ Yes, have treated annually for 5-9 years
- ❑ Yes, have treated annually for 10 or more years

15. Which preparation did you use in 2015?

- ❑ Baycox® Sheep vet
- ❑ Vecoxan® vet
- ❑ Sulpha-preparations
- ❑ Other

16. For how long have you used the same preparation?
(Only one answer)

- ❑ 1 year

- ❑ 2-5 years
- ❑ 5-10 years
- ❑ More than 10 years

17. How and why do you treat?

- ❑ To prevent clinical coccidiosis: flock has never had clinical coccidiosis previously
- ❑ To prevent clinical coccidiosis; flock has had clinical coccidiosis previously
- ❑ Treatment of clinical coccidiosis
- ❑ Other

18. When do you treat against coccidiosis?

- ❑ All lambs are treated before they are two weeks old
- ❑ All lambs are treated before they are two - four weeks old
- ❑ All lambs are treated when they are older than four weeks
- ❑ Individual lambs are treated if needed, e.g. if they have diarrhoea
- ❑ All lambs are treated when some individual lambs have diarrhoea
- ❑ All lambs are treated regularly, e.g. at every change of pasture
- ❑ Only bottle fed lambs are treated
- ❑ Other

19. How many times did you treat the whole flock this spring?

- ❑ Once
- ❑ Twice
- ❑ More than twice
- ❑ Only some individual lambs were treated more than once (not the whole flock)
- ❑ No lambs were treated more than once

20. How do you estimate the dose?
(Only one answer)

- ❑ Individual weighing
- ❑ Weighing of medium-sized/average lamb
- ❑ Weighing of heaviest lamb
- ❑ Visual appraisal
- ❑ Other

21. How often do you check that your drench gun gives the correct dose?
(Only one answer)

- ❑ Less than once a year
- ❑ Once a year
- ❑ Twice a year
- ❑ > twice a year
- ❑ Never

22. Have your lambs ever experienced clinical disease/diarrhoea because of what you think is coccidia, even though the lambs have been treated with an anticoccidial?
(Only one answer)

- ❑ Yes
- ❑ No

23. Do you treat adult sheep against coccidiosis?
(Only one answer)

- ❑ Yes
- ❑ No

24. Why do you treat adult sheep against coccidiosis?

- ❑ Diarrhoea during the housed (winter) period
- ❑ Diarrhoea in the spring pasture period
- ❑ Low body condition score

❑ Other

25. When do you treat adult sheep against coccidosis?

❑ At lambing
❑ At turnout
❑ When the ewes and lambs are sent to mountain/forest pastures
❑ Other

26. Who are your advisors regarding treatments against parasitic diseases?
(Only one answer per row)

	Not important	Somewhat important	Either or	Important	The most important
Articles in farmers' journals	❑	❑	❑	❑	❑
Internet	❑	❑	❑	❑	❑
Other farmers	❑	❑	❑	❑	❑
Close family	❑	❑	❑	❑	❑
Abattoir adviser	❑	❑	❑	❑	❑
Veterinarians	❑	❑	❑	❑	❑

27. Are you interested in participating in this project? Participation includes faecal sampling of lambs and analysis without cost. The analysis will be especially concerned with coccidia and the efficacy of treatment. If you are interested in participating, we might contact you during winter 2016.
(Only one answer)

❑ Yes
❑ No

28. Thank you for participating! If we need further information, we would like to have your address, email address, telephone number and farm number.

_ _

29. Other comments?

_ _

Section B, for farmers NOT answering "Sheep are outside all year round" **at question 8.**

30. Average lamb age at turnout spring 2015?
(Only one answer)

❑ 0-7 days
❑ 8-14 days
❑ 15-21 days
❑ 21-28 days
❑ 29 days or older

31. Average spring pasture period for ewes and lambs?
(Only one answer)

❑ 0-7 days
❑ 8-14 days
❑ 15-24 days
❑ 22-28 days
❑ 29-35 days
❑ More than 35 days

32. Which type of pasture was used for spring pasturing?
(Only one answer)

- ❑ Cultivated pastures
- ❑ Uncultivated pastures
- ❑ Direct turn out to mountain/forest pastures
- ❑ Other

33. Which type of pasture did the majority of your sheep graze during summer? (Only one answer)

- ❑ Cultivated pastures
- ❑ Uncultivated pastures
- ❑ Mountain/forest pasture
- ❑ They were housed
- ❑ Other

34. What type of pasture/housing did most of your ewes have during autumn?

(Only one answer)

- ❑ Cultivated pastures
- ❑ Uncultivated pastures
- ❑ Mountain/forest pasture
- ❑ Housed
- ❑ Other

35. What type of pasture/housing did most of your lambs have during autumn?

(Only one answer)

- ❑ Cultivated pastures
- ❑ Uncultivated pastures
- ❑ Mountain/forest pasture
- ❑ Direct housing after weaning
- ❑ Other

36. What type of pasture/housing did the majority of your ewes have during winter? (Only one answer)

- ❑ Cultivated pastures
- ❑ Uncultivated pastures
- ❑ Housed
- ❑ Housed, with access to an outdoor pen
- ❑ Other

37. Did you use spring pastures that had not been used during the previous spring? (Only one answer)

- ❑ Yes
- ❑ No
- ❑ Both

38. Did you use spring pastures that had not been used during the previous autumn? (Only one answer)

- ❑ Yes
- ❑ No
- ❑ Both

39. Have you submitted faecal samples for parasitological analysis during the last two years? (Only one answer)

- ❑ Yes
- ❑ No

40. When were the samples taken?

- ❑ At turnout
- ❑ On spring pasture

- ❑ On summer pasture
- ❑ On autumn pasture
- ❑ At housing

41. Why were the samples submitted?

- ❑ Disease
- ❑ Reduced growth
- ❑ Generel surveillance
- ❑ To assess treatment needs
- ❑ Other

Parasites

42. Which parasites do you consider important in your flock:
(Only one answer per parasite)

	Important	Not important	Uncertain
Ixodes ricinus	❑	❑	❑
Lice	❑	❑	❑
Fasciola hepatica	❑	❑	❑
Dicrocoelium dendritticum	❑	❑	❑
Nematodirus battus	❑	❑	❑
Other nematodes	❑	❑	❑
Tapeworms	❑	❑	❑
Lung worm	❑	❑	❑
Coccidia	❑	❑	❑

Cocccidiosis

43. Have you noticed diarrhoea/perineal soiling or reduced weight gain in lambs in the housed period this spring (2015)?
(Only one answer per row)

	In 0-20%	In 20-40%	In 40-60%	In 60-80%	In 80-100%	Not seen
Diarrhoea/ perineal soiling	❑	❑	❑	❑	❑	❑
Reduced weight gain	❑	❑	❑	❑	❑	❑

44. Have you seen diarrhoea/perineal soiling or reduced weight gain in lambs 2 to 6 weeks after turnout this spring (2015)?
(Only one answer per row)

	In 0-20%	In 20-40%	In 40-60%	In 60-80%	In 80-100%	Not seen
Diarrhoea/ perineal soiling	❑	❑	❑	❑	❑	❑
Reduced weight gain	❑	❑	❑	❑	❑	❑

45. Do you treat against coccidiosis, and for how long have you treated?
(Only one answer)

- ❑ No, have never treated
- ❑ Yes, but do not treat every year
- ❑ Yes, have treated annually for 1-4 years

- ❑ Yes, have treated annually for 5-9 years
- ❑ Yes, have treated annually for 10 or more years

46. Which preparation did you use in 2015?

- ❑ Baycox® Sheep vet
- ❑ Vecoxan® vet
- ❑ Sulpha-preparations
- ❑ Other

47. For how long have you used the same preparation?
(Only one answer)

- ❑ 1 year
- ❑ 2-5 years
- ❑ 5-10 years
- ❑ More than 10 years

48. How and why do you treat?

- ❑ To prevent clinical coccidiosis; flock has never had clinical coccidiosis previously
- ❑ To prevent clinical coccidiosis; flock has had clinical coccidiosis previously
- ❑ Treatment of clinical coccidiosis
- ❑ Other

49. When did you treat?

- ❑ All lambs at turnout
- ❑ All lambs 7-10 days after turnout
- ❑ Individual lambs if needed, e.g. diarrhoea
- ❑ All lambs are treated when one lamb shows clinical signs
- ❑ All lambs are treated regularly, e.g. at each pasture change
- ❑ Only bottle fed are treated
- ❑ Other

50. How many times did you treat the whole flock this spring?

- ❑ Once
- ❑ Twice
- ❑ More than twice
- ❑ Only some individual lambs were treated more than once
- ❑ No lambs were treated more than once

51. How do you estimate the dose?
(Only one answer)

- ❑ Individual weighings
- ❑ Weighing of medium-sized/average lamb
- ❑ Weighing of heaviest lamb
- ❑ Visual appraisal
- ❑ Other

52. How often do you check that your drench gun gives the correct dose of anticoccidial?
(Only one answer)

- ❑ Less than once a year
- ❑ Once a year
- ❑ Twice a year
- ❑ > twice a year
- ❑ Never

53. Have sheep in your flock ever experienced clinical disease/diarrhoea because of what you think is coccidia, even though the lambs have been treated with an anticoccidial?
(Only one answer)

- ❑ Yes

❑ No

54. Do you treat adult sheep against coccidiosis?
(Only one answer)

❑ Yes
❑ No

55. Why do you treat adult sheep against coccidiosis?

❑ Diarrhoea in the housed period
❑ Diarrhoea in the spring pasture period
❑ Low body condition score
❑ Other

56. When do you treat adult sheep against coccidosis?

❑ At lambing
❑ At turnout
❑ When the ewes and lambs are sent to mountain/forest pastures
❑ Other

Advisors

57. Who are your advisors regarding treatments against parasitosis?
(Only one answer per row)

	Not important	Somewhat important	Either or	Important	The most important
Articles in farmers' journals	❑	❑	❑	❑	❑
Internet	❑	❑	❑	❑	❑
Other farmers	❑	❑	❑	❑	❑
Close family	❑	❑	❑	❑	❑
Abattoir adviser	❑	❑	❑	❑	❑
Veterinarians	❑	❑	❑	❑	❑

58. Are you interested in participating in this project during spring 2016; participation involves faecal sampling from lambs?
(Only one answer)

❑ Yes
❑ No

59. Thank you for participating. Please record your name, address, email address, and telephone number.

_ _

60. Do you have any final comments?

_ _

II

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Field evaluation of anticoccidial efficacy: A novel approach demonstrates reduced efficacy of toltrazuril against ovine *Eimeria* spp. in Norway

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ABSTRACT

Ovine *Eimeria* spp. infections cause reduced welfare, increased mortality, and substantial economic losses, and anticoccidials are crucial for their control. Recent reports of toltrazuril resistance in pigs, and anecdotal reports of reduced anticoccidial efficacy in lambs, necessitate evaluation of anticoccidial efficacy. Due to the substantial lifecycle differences between nematodes and coccidia, current WAAVP methods for assessing anthelmintic efficacy are not suitable for such evaluations. Faecal samples were collected from 8 pairs of twin lambs from 36 Norwegian sheep farms 6–8 days after turnout. One twin of each pair was then treated with 20 mg/kg toltrazuril and a second faecal sample from all lambs was collected 7–11 days later. Oocyst excretion rate in all samples was determined using McMasters. Suitability of treatment timing was investigated by evaluating the increase in mean log oocyst excretion in untreated lambs. Based on comparisons between groups, a threshold of ≥0.75 (13 farms) was used to identify farms where drug efficacy could be assessed with confidence, drug efficacy on farms with increases of ≥0.5 but < 0.75 (7 farms) were evaluated with caution, and drug efficacy on farms with increases of < 0.5 (16 farms) was not estimated. Reduction in oocyst excretion between samples from treated lambs compared with controls from the 20 farms with a threshold of ≥0.5 were then analysed using a generalised linear mixed model. The results were classified based on 95% CI obtained using parametric bootstrapping. Among these 20 farms, two exhibited reduced drug efficacy (upper 95% CI < 95%), 13 had good efficacy (lower 95% CI > 90%), and for 5 the results were inconclusive. This is the first evidence-based report of reduced anticoccidial efficacy in ovine *Eimeria* spp. Additionally, we highlight the problem of sub-optimal timing of treatment (16/36 farms), which could potentially result in incorrect conclusions being reached regarding lack of drug efficacy.

1. Introduction

Eimeria spp. are host-specific obligate intracellular protozoan parasites that infect fish, reptiles, birds and mammals (Walker et al., 2013). Of the 15 *Eimeria* spp. known to infect sheep, only two are regarded as major pathogens: *E. ovinoidalis* and *E. crandallis* (Catchpole et al., 1976; Catchpole and Gregory, 1985; Rommel, 2000; Joachim et al., 2018). *E. ahsata,* and occasionally *E. bakuensis,* are generally considered to be minor pathogens, which may cause clinical signs in heavily infected animals (Mahrt and Sherrick, 1965; Deplazes et al., 2016). In addition, infections with multiple species might also be important for the development of clinical signs, as described for calves (Enemark et al., 2013). Coccidiosis in lambs caused by pathogenic *Eimeria* spp. leads to reduced welfare, increased mortality and substantial economic losses in the sheep industry worldwide (Foreyt, 1990; Chartier and Paraud, 2012).

Pasture management and hygienic measures, e.g., cleaning water troughs and maintaining dry bedding, are considered important factors for reducing the infection pressure from *Eimeria* spp. (Taylor, 2000; Daugschies and Najdrowski, 2005). However, these measures are often

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labour intensive and can be difficult to implement, and chemoprophylaxis with anticoccidials is therefore frequently used, in addition to hygiene measures for control of clinical coccidiosis in sheep farms (Taylor and Kenny, 1988; Platzer et al., 2005; Saratsis et al., 2013; Odden et al., 2017). Metaphylactic administration of a single oral treatment with toltrazuril in the prepatent period has been shown to be effective at reducing clinical signs and maintaining adequate growth rates in different production systems (Gjerde and Helle, 1986, 1991; Taylor and Kenny, 1988; Le Sueur et al., 2009; Saratsis et al., 2013). In several European countries (e.g., Denmark, Sweden, and Norway) toltrazuril is the only anticoccidial available for use in sheep (Felleskatalogen, 2017; Läkemedelsverket, 2017; Veterinærmedicinsk Industriforening, 2017). In other countries, other treatments such as diclazuril and decoquinate are also available (Taylor, 2000; Diaferia et al., 2013). According to the Veterinary Medicines Directorate, 510,388 kg and 787,300 kg toltrazuril from products authorised for use in farm animals were sold in the UK in 2014 and 2015, respectively (Dr Gillian Diesel, Head of the Pharmacovigilance Team, personal communication). However, treatment of clinical coccidiosis is considered to be inefficient due to the extensive intestinal damage caused by the parasite (Mundt et al., 2003; Taylor et al., 2003).

Anticoccidial resistance (ACR) is a widely recognised problem in poultry production (Chapman, 1997; Stephan et al., 1997; Chapman and Jeffers, 2014; Lan et al., 2017), and has been reported for monensin, salinomycin, nicarbazin, halofuginone, robenidine, toltrazuril and diclazuril (McDougald, 1981; Chapman, 1997; Stephan et al., 1997). ACR in poultry production is generally considered to be the result of intensive use of anticoccidials, which has led to loss of sensitivity to these drugs (Peek and Landman, 2011). Testing for anticoccidial efficacy (ACE) in poultry production involves the use of histopathological observations and the combination of different indexes, such as oocyst index, body weight gain, relative weight gain, lesion scores, and anticoccidial index (Chapman, 1998). However, no such methods have been published for the evaluation of anticoccidial efficacy in other livestock, including sheep. One obvious practical requirement for a method to be useful in field situations is that it should not include euthanasia of large numbers of animals.

The controlled efficacy test (CET) is the gold standard method for the evaluation of anthelmintic efficacy (Wood et al., 1995; Coles et al., 2006). The CET is performed by infecting animals with a suspected resistant isolate, treating the animals with the drug under evaluation, and then euthanizing the animals before quantifying the parasite burden post mortem. This procedure has various difficulties for implementation, not only because of the ethical concerns associated with euthanasia of the animals, but also due to the requirement for a parasite-free environment in testing the suspected strain (Taylor et al., 1995; Wood et al., 1995). Similar problems relate to the assessment of anticoccidial efficacy against *Eimeria* spp. in poultry (Chapman, 1998) and *Cytosisospora suis* in pigs (Shrestha et al., 2017). Thus, evaluation of anthelmintic efficacy in animals is routinely assessed by the faecal egg count reduction test (FECRT), currently recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP), and involves comparison of faecal egg counts pre- and post-treatment (Coles et al., 1992). The advantage of the FECRT is its ability to assess a range of drugs under field conditions. However, analysis of the results for FECRTs can be difficult in cases where egg excretion rate is low, where the sensitivity of the counting methods is poor, for highly aggregated faecal egg counts, and when the sample size is small (Torgerson et al., 2005; Denwood et al., 2010; Dobson et al., 2012; Peña-Espinoza et al., 2014).

Different statistical models have been applied to improve the calculation of the estimated efficacy from FECRT results, including bootstrapping techniques, and Bayesian methods such as Markov chain Monte Carlo (Denwood et al., 2010; Torgerson et al., 2014; Peña-Espinoza et al., 2016). However, challenges remain regarding the use of faecal oocyst count reduction tests (FOCRT), the (coccidial) oocyst equivalent of FECRT, for the assessment of ACE, due to extreme variation in oocyst excretion rates compared with excretion of helminth eggs. This is, in general, a reflection of the more complicated biology and lifecycle of *Eimeria* spp., in which sexual reproduction of the parasite in the animal host is preceded by several rounds of intracellular asexual reproduction that occurs in waves (Walker et al., 2013). The maximum range of *Eimeria* oocyst excretion can differ from between 0 and 75,000 to between 0 and 2,000,000 oocyst per gram (OPG), with large inter-individual variation (Chapman, 1974). In contrast, helminth egg excretion usually does not exceed 20,000 eggs per gram (Sréter et al., 1994; Zaros et al., 2014). As toltrazuril acts against intracellular stages of the parasite, and extracellular stages are unaffected (Haberkorn and Stoltefuss, 1987; Harder and Haberkorn, 1989; Mehlhorn, 2008), oocyst counts immediately post treatment may not be zero. Detailed data concerning the efficacy of toltrazuril when the drug was first marketed are not available from the literature, but practical experience confirms that post-treatment oocyst counts are not always zero even when the observed clinical efficacy is good. Thus, any model for evaluation of ACE has to take into account that a reduction to zero is not always the case, even when highly efficacious anticoccidials are used (Taylor and Kenny, 1988; Gjerde and Helle, 1991).

The emergence of ACR in poultry and pig production systems (Lan et al., 2017; Shrestha et al., 2017), along with anecdotal reports of reduced ACE in Norwegian lambs (Odden et al., 2017), demonstrates the need for a FECRT-type method to evaluate drug efficacy in live animals. However, due to the reasons outlined above, the standard FECRT (Coles et al., 1992) currently recommended by WAAVP for evaluation of anthelmintic efficacy is unsuitable for use with coccidia. The aim of our study was therefore to develop a tool for field evaluation of ACE, based on oocyst counts in lambs, and use it in a preliminary investigation of ACE in Norwegian sheep farms.

2. Materials and methods

2.1. Study design

2.1.1. Inclusion criteria

Norwegian sheep farms (n = 80) were selected based on a previous questionnaire study performed in October 2015 (Odden et al., 2017). The inclusion criteria were: a) treatment with anticoccidials annually for at least four years, b) coccidiosis-related symptoms in lambs treated with an anticoccidial, and c) flock size of more than 60 winter-fed ewes. The geographical location of the farms was consistent with the population density of sheep farms in Norway (Supplementary data 1) (Statistics Norway, 2017). All 80 farmers, of whom 60 agreed to participate, were contacted via telephone during the winter of 2016. The 60 participating farmers received a detailed written sampling and treatment protocol, a 10 ml syringe for oral drenching, envelopes with pre-paid postage, and a “faecal spoon” to facilitate sampling of young lambs (Supplementary data 2). Farms with < 5 lambs per treatment group were excluded.

2.1.2. Timing of treatment and sampling

Most Norwegian ewes are winter housed, with indoor lambing in the spring i.e. March–May (Vatn, 2009). Turnout to spring pastures commonly occurs two to three weeks postpartum (Domke et al., 2011). Clinical signs due to coccidiosis are mainly seen at around turnout. Lambs may become infected before turnout, mainly due to oocyst excretion from older, already infected lambs (Taylor, 1995), or immediately after turnout, as the oocysts survive overwintering on permanent pastures (Helle, 1970; Gjerde and Helle, 1991). Current Norwegian recommendations against ovine coccidiosis consist of a single metaphylactic treatment with toltrazuril, either at turnout or around one week after turnout (Animalia, 2017).

Farmers enrolled in the study were instructed to identify 8 pairs of twin lambs from which they would twice collect faecal samples during

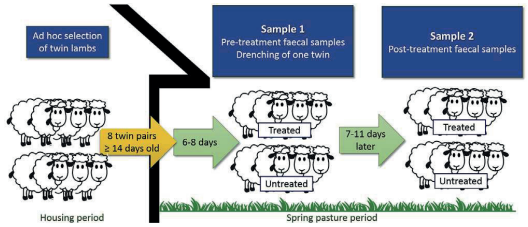

Fig. 1. Study design. Twin lambs (n = 16 per farm) from 36 Norwegian farms with signs of coccidiosis in previous grazing seasons were included in the study. The treatment group was treated with toltrazuril (20 mg/kg) whereas the control group was left untreated. Oocyst counts were based on McMaster analysis.

the 2016 spring grazing season. Age at turnout was ≥14 days for all lambs included in the study. Sample 1 was taken 6–8 days after turnout (Fig. 1). The aims were: 1) to have a common sampling protocol for all lambs, regardless of whether they became infected indoors or after turnout, and 2) to collect the first sample while oocyst excretion was either below the limit of detection, or low. At the same time as collection of the first sample, the lamb with the lowest ear tag number from each twin pair was treated with 20 mg/kg toltrazuril (Baycox® Sheep vet, Bayer Animal Health). Farmers were encouraged to weigh the lambs prior to treatment. Sample 2 was collected from the same animals 7–11 days after Sample 1; that is, before any oocysts ingested post-treatment could have resulted in additional oocyst excretion. All faecal samples were collected per rectum from individual lambs using a "faecal spoon".

2.1.3. Evaluation of faecal samples

The faecal consistency was scored on a scale from one to five (Holm et al., 2014). The faecal samples were stored for a maximum of 7 days at 4 °C and the rate of oocyst excretion was determined using a modified McMaster technique with a theoretical sensitivity of 5 oocysts per gram (OPG). Briefly, water was added to 1–4 g of faeces, which was homogenised, filtered, concentrated by centrifugation and mixed with flotation fluid (saturated sodium chloride with glucose; density: 1.27 g/ml; sample/flotation fluid ratio: 1:1 to 1:2 depending on volume of sediment). A subsample (0.6 ml) was then transferred to a disposable counting chamber fitted with a thin coverglass, which facilitated detection, and the oocysts were enumerated at 200/400× magnification. In samples with few oocysts (OPG < 10,000) the whole chamber was evaluated, whereas one row (≈1/20) or three fields of vision (≈1/200) of the chamber was counted in samples with higher numbers of oocysts (Henriksen and Aagaard, 1976; Henriksen and Korsholm, 1984). One hundred *Eimeria* oocysts from all samples with OPG ≥1000 were examined by light microscopy at 400× magnification. The oocysts were identified to species level without sporulation, using morphological criteria (Eckert et al., 1995).

2.2. Evaluation of anticoccidial efficacy

2.2.1. Statistical justification

Previously published observations of oocyst excretion in lambs have shown that excretion follows an exponential pattern initially, followed by a plateau phase (Chapman, 1974; Gregory et al., 1989). This implies that the logarithm of the expectation of oocyst excretion increases linearly during the initial phase, followed by a reduction in the rate of increase during the subsequent plateau phase (Fig. 2). This known relationship has the useful feature that an anticoccidial intervention at any time point has no effect on the slope of the linear relationship with time, so that anticoccidial efficacy can be calculated as the absolute difference in log expectation of oocyst count at any point during the exponential growth phase. However, this assumes that parasite replication also follows an exponential rise between the time of treatment and the time of oocyst quantification, and therefore that the plateau phase of oocyst excretion has not yet been reached. Thus, three conditions must be satisfied regarding timing of anticoccidial treatment and FOCRT quantification:

1. Treatment must be given after the initial infection of the lambs.
2. A minimum time must be allowed between treatment and FOCRT to allow a reduction in oocyst shedding to become detectable.
3. Both treatment and the FOCRT must be performed during the phase of exponential rise in excretion.

Additionally, although not a strict requirement for the procedure to be valid, greater statistical confidence will be obtained in the mean count estimates, and therefore FOCRT percentage, if the mean oocyst counts are relatively high (Denwood et al., 2012). Therefore, the theoretical optimal time for the FOCRT (Sample 2) to be conducted, from a statistical point of view, is as close as possible to the end of the phase of exponential rise in excretion. We note that this statistical consideration is somewhat at odds with the optimal time point for most effective control of the parasite, for which an earlier treatment is likely to be preferable.

2.2.2. Identifying the phase of exponential rise in excretion rate

There are currently no guidelines for identifying when oocyst excretion in the field is in the phase of exponential increase. We therefore followed a simple heuristic, based on oocyst counts derived from the untreated control animals at the time of the pre-treatment and post-treatment sampling. This heuristic assumes that the dynamics of parasite replication during the exponential phase are similar between farms, which is a substantially simplifying assumption, given that there are multiple species of *Eimeria* with different cycle parameters within the host animal, and that different farms may have different species.

The heuristic used was as follows. Firstly, a crude estimation of the linear effect of days between oocyst observations on the mean of the logarithmically transformed oocyst counts in the untreated animals at each farm was made (referred to as the "slope"). For this procedure, a fixed constant of 1 was added to all data before transformation to avoid numerical problems with observations of zero. Secondly, the results for all farms were compared graphically in order to identify a qualitatively appropriate threshold above which the estimated slopes were subjectively judged to be consistent with those obtained from farms with the greatest change in mean log faecal oocyst count (FOC), and therefore assumed to be representing the exponential phase. A second, lower threshold was also identified above which the slopes were deemed to be only moderately consistent with those obtained from farms with the greatest change in FOC. Data from farms for which the lower threshold for slope was not met were not included in the analyses due to the apparently poor timing of sampling and treatment.

2.2.3. Analysis of faecal oocyst count reduction

The farms for which the data from the control animals were judged to indicate sampling had been performed during the exponential phase were analysed for faecal oocyst count reduction (FOCR) using a generalised linear mixed model, implemented using the lme4 package (Bates et al., 2015) for R (R Core Team, 2017). Only the post-treatment counts from treated and control animals were used for evaluation of efficacy due to the theoretical justification of the linear increase in log FOC given in section 2.2.1, as well as empirical evidence for a lack of relationship between the pre-treatment mean and these efficacy estimates (data not shown). A Poisson distribution with log link was used as the response distribution for the number of oocysts, using an offset within the model to take into account the dilution factor applied during counting of each sample. Random effects of observation and twin pair were used to describe the extra-Poisson variation (over dispersion)

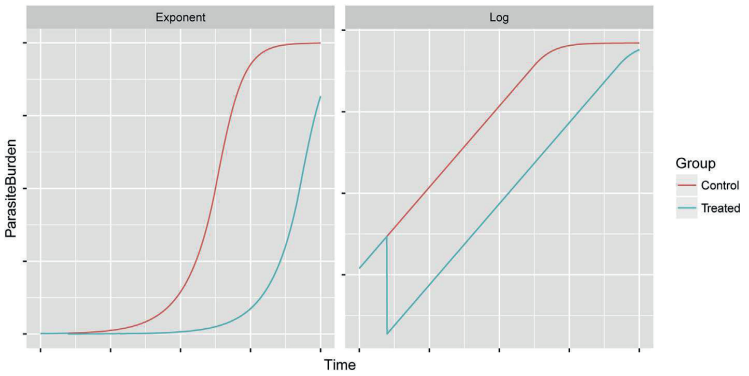

Fig. 2. Graphical illustration of the theoretical relationship between an exponentially increasing true parasite burden on the exponent scale (left) and the log of the same quantity (right) over time. A proportional reduction in the simulated 'treated' (blue) mean occurring 1/10 into the time span results in the log of the two quantities increasing in parallel during the exponential phase of the control mean (red). This exponential phase ends around 6/10 into the time span, after which a plateau can be seen on the exponent scale and the lines cease to be parallel on the log scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

within the data, and all farms were analysed separately. Confidence intervals (CIs) for the geometric mean FOCR were obtained using parametric bootstrapping with 500 iterations. The FOCRT results then enabled classification of ACE at the farm level, based on 95% CI, as "good efficacy" (lower 95% CI > 90%), "reduced efficacy" (upper 95% CI < 95%), or inconclusive (neither of the above is true), based on equivalent classifications for arithmetic mean reductions as previously used by Geurden et al. (2015) and Peña-Espinoza et al. (2016) in investigations of anthelmintic efficiency.

3. Results

3.1. Identifying the exponential growth phase

Of the 60 farms that initially agreed to participate in the study, 49 completed the sampling. However, 13 farms were excluded due to lack of compliance with the sampling protocol, leaving 36 farms for which the increase in mean log OPG could be assessed in the control animals. Based on these estimates, along with the assumption that the parasite dynamics (and therefore exponential rate of increase) should be similar between farms for which sampling was during the exponential phase (see comments regarding this assumption in section 2.2.2), a threshold of approximately 0.75 was identified. Above this threshold, the absolute increase in mean log OPG in untreated lambs seemed to be sufficiently consistent for us to be confident that both treatment and sampling were conducted at appropriate time points for the analyses (Fig. 3). A further threshold of 0.5, was identified above which the timing is broadly consistent with the required increase in OPG in untreated lambs, but also where the possibility of sub-optimal timing cannot be excluded. Farms for which the increase in control lambs was below 0.5 were deemed to represent farms where the timing of sampling and treatment were not during the phase of exponential increase. Based on these criteria, 13 farms were considered to have been sampled at the appropriate time for our purposes and could therefore provide a useful estimate of drug efficacy, and 7 farms were deemed to have possibly been sampled at a suboptimal time and could therefore yield a drug efficacy estimate that should be analysed with caution. The data from the remaining 16 farms were considered unsuitable for further analysis as treatment and/or sampling were not during the exponential phase of oocyst excretion.

3.2. Assessment of efficacy

The 20 farms for which treatment and sampling were deemed to have either been consistent with, or broadly consistent with, appropriate timing were then analysed for ACE. Of the 13 farms classified with confidence, 7 were found to have good ACE, 4 were inconclusive, and 2 had reduced treatment efficacy (Table 1). Of the 7 farms classified with caution, 6 had good ACE and 1 was inconclusive. Mean and median OPG from all the 36 farms complying with the sampling protocol can be found in Table 2, and the oocyst counts from all included lambs can be found in Supplementary data 3.

Of the 20 farms included in the assessment of drug efficacy, lamb weights at treatment were submitted for 16 farms (Table 1). The post-treatment composition of *Eimeria* spp. in treated lambs from the farms where reduced efficacy was detected was: 73.7% *E. faurei*, 15.5% *E. ovinoidalis*, and 10.8% other *Eimeria* spp. (farm 10), and 39.1% *E. parva*, 35.4% *E. ovinoidalis*, and 25.5% other *Eimeria* spp. (farm 22).

4. Discussion

Appropriate field tests are necessary in order to determine ACE and to detect potential resistance to treatment among ovine *Eimeria* isolates. However, suitable approaches for identifying ACR in farmed ruminants have not previously been developed (Joachim et al., 2018). The current work presents one approach for field evaluation of ACE against ovine *Eimeria* spp. using a method based on the WAAVP recommended FECRT for identifying resistance to anthelmintics (Coles et al., 1992), but modified to enable evaluation of drug efficacy against *Eimeria* spp., or resistance of *Eimeria* spp. against specific treatments.

One important finding of our study was that timing of anticoccidial treatment was often sub-optimal, being detected in 16 of 36 farms. Such timing could potentially result in false conclusions regarding lack of drug efficacy, as well as not providing optimal protection against clinical coccidiosis for the individual lambs.

A second important finding was an apparent reduction in the efficacy of toltrazuril against *Eimeria* spp., including the pathogenic *E. ovinoidalis,* in 2 of 20 farms for which treatment and sampling time was appropriate. It should be noted that inclusion criteria for the study included flock size, continuous use of anticoccidials for several years, and occurrence of previous episodes of diarrhoea. These are all factors that are recognised as being correlated with increased risk of ACR in poultry (Chapman et al., 2010; Peek and Landman, 2011; Lan et al., 2017). Thus, these "potential ACR farms" are not representative of Norwegian sheep farms, and further studies are needed to establish the true prevalence of ACR in Norway. However, due to the widespread dependence of sheep farmers on chemoprophylaxis to control ovine coccidiosis, the emergence of reduced efficacy of toltrazuril indicated here may have severe consequences for the sheep industry, particularly in Northern Europe, due to the limited treatment alternatives (Felleskatalogen, 2017; Läkemedelsverket, 2017; Veterinærmedicinsk Industriforening, 2017).

The main objectives of anticoccidial treatment are: 1) to decrease oocyst excretion, 2) to reduce the severity of clinical signs, and 3) to

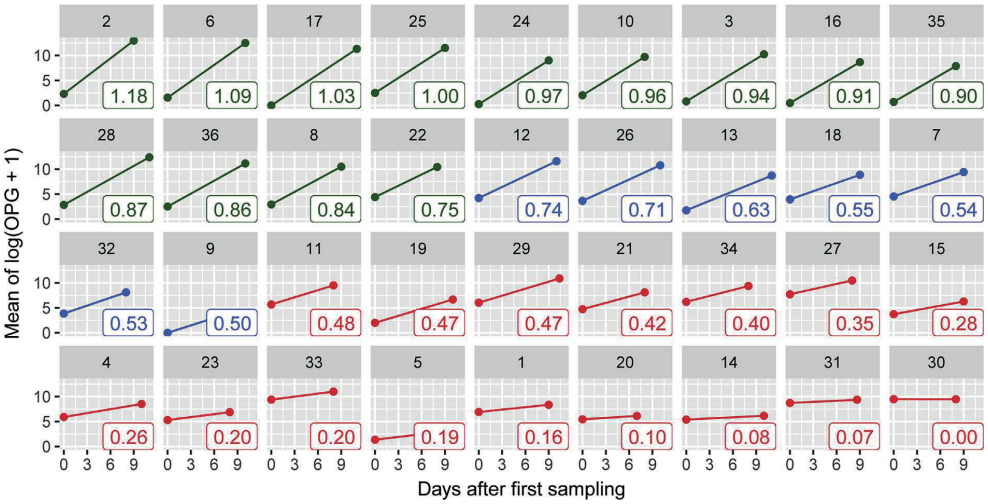

Fig. 3. Identification of the exponential growth phase of the oocyst excretion. Graphs illustrate the slope between the mean log OPG and time (pre- and post-treatment samples) for the control lambs in the 36 farms complying with the sampling protocol. Classification was performed with **confidence** in farms with a slope of ≥ 0.75 (green), and with **caution** if the slope was ≥ 0.5 and < 0.75 (blue). Data from farms for which these thresholds were not met were deemed **unclassifiable** due to the poor timing of sampling (red). The number at the bottom right of each panel indicates the estimated slope for the given flock (panel title). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Maximum likelihood estimates and 95% confidence intervals (CI) for the geometric mean efficacy based on post-treatment oocyst counts for the 20 classifiable sheep farms. The slope gives the change in mean log OPG per day in the controls, and was used to determine if drug efficacy could be calculated: 13 of the flocks could be evaluated with confidence (slope ≥ 0.75) and 7 of the flocks were evaluated with caution (0.5 ≤ slope < 0.75).

Farm	*n*		Slope	Mean efficacy (%)	Lower 95% CI	Higher 95% CI	Interpretation
	Control	Treated					
10[a]	8	8	0.96	37.8	−58.3	73.3	Reduced efficacy
22[a]	8	8	0.75	81.7	53.1	94.0	Reduced efficacy
35[a]	7	7	0.90	56.0	−433.9	96.6	Inconclusive
16[a]	7	6	0.91	81.3	−103.8	98.9	Inconclusive
6[a]	7	8	1.09	96.0	72.7	99.3	Inconclusive
28[a]	6	6	0.87	95.4	86.5	98.4	Inconclusive
8	8	8	0.84	100.0	99.7	100.0	Efficacious
3	8	8	0.94	99.3	95.0	99.9	Efficacious
17[a]	6	8	1.03	99.3	93.6	99.9	Efficacious
2	8	8	1.18	99.5	96.8	99.9	Efficacious
25[a]	7	7	1.00	99.5	96.6	99.9	Efficacious
24[a]	7	8	0.97	99.8	98.6	100.0	Efficacious
36[a]	8	8	0.86	99.8	98.4	100.0	Efficacious
9[a]	8	8	0.50	97.6	16.5	100.0	Caution: inconclusive
13[a]	8	8	0.63	100.0	100.0	100.0	Caution: efficacious
26[a]	8	8	0.71	100.0	100.0	100.0	Caution: efficacious
7	8	8	0.54	99.4	97.3	99.8	Caution: efficacious
12[a]	8	8	0.74	99.5	96.4	99.9	Caution: efficacious
32[a]	8	8	0.53	99.8	99.2	100.0	Caution: efficacious
18[a]	6	6	0.55	99.9	99.0	100.0	Caution: efficacious

[a] Farms from which body weights at treatment were available.

allow development of protective immunity (Taylor et al., 2011). Metaphylactic treatment (i.e., treatment administered during the prepatent period before oocyst excretion can be detected and prior to development of clinical signs) is therefore preferable. Due to the exponential oocyst excretion curve (Chapman, 1974; Gregory et al., 1989), the effect of treatment can only be assessed during the exponential growth phase of the parasite, which necessitates inclusion of both pre-treatment samples and untreated controls in the analyses. Identification of this growth phase in our study was based on evaluation of the "slope" (change in mean log OPG per day in the controls) for each farm that was used to determine if drug efficacy could be interpreted with confidence. However, this evaluation was based on thresholds chosen somewhat arbitrarily due to characteristics of our dataset, and may require adjustment following acquisition of data from more farms in future studies. Timing of treatment, and also timing of the FOCRT itself, which is supposed to be approximately one-week post-treatment, is thus a major challenge and should be based on knowledge of previous outbreaks of coccidiosis and farm management factors, such as duration of the lambing season, time of turnout, grazing conditions (± permanent pasture), and weather conditions. Historically, treatment practices in Norway relied on the assumption that lambs were infected after turnout, with the development of clinical symptoms 2–3 weeks later (Helle, 1970; Gjerde and Helle, 1986). However, results from the present study indicate that lambs in some farms are infected with *Eimeria* spp. shortly after lambing, while they are still housed indoors. This seems to be common, particularly in cases where turnout is delayed due to adverse weather conditions (unpublished results from our group). For the present study, we standardised the sampling protocol based on general knowledge of management procedures and treatment routines in Norwegian sheep farms (Odden et al., 2017). However, sampling based on specific knowledge of transmission dynamics in the individual farms would most likely have helped for at least some of the 44% of farms for which treatment timing was sub-optimal.

Another factor that is known to be associated with accelerating the development of drug resistance is under-dosing with the drug (Smith et al., 1999; Wolstenholme et al., 2004). In our study, the farmers were responsible for treating their animals with the correct dosage. The

Table 2
Oocyst counts pre- and post-treatment in toltrazuril treated lambs and untreated controls from the 36 farms complying with the sampling protocol. Arithmetic mean: A-mean; geometric mean: G-mean and median oocysts per gram (OPG).

Farm	Sample 1						Sample 2					
	Treated			Control			Treated			Control		
	A-mean	G-mean	Median	A-mean	G-mean	Median	A-mean	G-mean	Median	A-mean	G-mean	Median
1	1691.9	454.5	665.0	3993.1	998.6	2795.0	742.5	96.8	65.0	20373.1	4269.1	4840.0
2	5.6	12.3	0.0	175.0	220.3	0.0	31719.4	2289.1	1537.5	796962.5	434112.9	649500.0
3	6903.1	874.3	0.0	6.3	25.0	0.0	3545.0	347.6	217.5	120957.5	28274.0	38600.0
4	1178.8	580.5	20.0	33903.1	12810.4	1712.5	979.4	715.3	360.0	34691.3	4998.2	15850.0
5	40000.0	240000.0	0.0	9.5	13.8	2.5	23031.7	736.6	105.0	140.8	69.6	17.5
6	78461.4	11530.7	0.0	8.8	10.8	5.0	63010.6	10770.7	19050.0	394271.4	268180.9	364000.0
7	6991.3	473.5	370.0	141.9	92.5	34450.3	260.6	206.5	110.0	16668.1	12340.6	13100.0
8	340.6	51.4	0.0	802.5	343.6	2.5	386.3	83.6	5.0	46440.0	36473.6	58600.0
9	0.0		0.0	0.0		0.0	9.4	14.6	5.0	1831.3	186.5	10.0
10	36.9	31.5	10.0	4283.1	217.2	0.0	88583.1	10338.4	11577.5	36303.8	16651.7	21700.0
11	138855.6	293.0	195.0	13166.3	657.4	51312.5	323.8	172.9	167.5	18421.3	13427.8	14700.0
12	64060.0	689.9	5.0	19786.3	265.6	17.5	10748.8	548.8	795.0	167975.0	108179.3	165000.0
13	83828.1	5739.0	5.0	66.9	108.9	0.0	1.9	7.1	0.0	309127.5	6087.3	9880.0
14	21357.5	991.3	3065.0	18635.6	217.1	42.5	3.1	10.0	0.0	17991.9	3726.0	472.5
15	524.4	148.3	5.0	595.6	67.1	25.0	3.1	10.0	0.0	11368.6	1470.2	8695.0
16	0.0		0.0	4.3	30.0	0.0	10642.5	1072.7	1820.0	29298.6	5818.4	12800.0
17	0.0		0.0	0.0		0.0	3638.8	550.0	842.5	240960.8	83343.9	276000.0
18	42008.0	18822.7	23000.0	1012.5	374.6	2805.0	55.0	28.0	5.0	33958.3	7179.3	12550.0
19	80.0	640.0	0.0	18.8	13.1	7.5	1.4	10.0	0.0	4541.9	792.9	757.5
20	128262.5	278198.1	0.0	54519.4	54073.8	4527.5	762.5	731.8	0.0	29780.7	1267.2	2445.0
21	102764.4	6311.4	320.0	6687.9	742.5	175.0	129.4	79.9	32.5	93361.3	3297.5	7567.5
22	1915.0	110.5	7.5	8001.9	354.2	50.0	19973.8	6340.0	4885.0	57913.1	34616.6	50000.0
23	96601.3	4230.8	40.0	43156.9	1116.2	25.0	28.1	21.9	0.0	22013.1	2583.0	6232.5
24	0.0		0.0	0.7	5.0	0.0	886.3	126.6	7.5	22245.0	8182.9	17600.0
25	2117.1	270.5	10.0	1693.6	74.6	5.0	2784.3	517.2	190.0	256910.0	99538.7	221200.0
26	7186.9	1681.0	8715.0	10192.5	1482.7	47.5	9.4	18.7	0.0	225352.5	48547.0	27950.0
27	24008.8	12488.0	915.0	6543.1	2258.3	22107.5	999.4	696.2	225.0	200101.3	35781.5	32800.0
28	7295.0	374.3	15.0	260.8	69.5	15.0	24566.7	10982.1	8100.0	284233.3	240255.5	239500.0
29	85386.3	416.7	330870.0	16203.8	3131.5	52300.0	25531.4	17417.5	20400.0	103425.0	53751.7	40500.0
30	208383.1	84689.6	6100.0	171998.8	51253.1	27950.0	2828.8	783.1	697.5	27607.5	12939.1	25925.0
31	32433.8	2448.7	114242.5	106513.8	6197.6	702.5	6787.9	1829.5	897.5	77640.0	11681.4	23900.0
32	432.1	42.2	5.0	11560.6	74.8	5.0	11.3	12.0	10.0	7839.4	3298.0	5547.5
33	54625.0	11426.8	8172.5	42547.5	11990.0	17300.0	3573.8	1399.3	2097.5	141297.5	59903.4	91300.0
34	18411.9	1065.2	1370.0	8442.5	481.2	422.5	10470.0	249.4	85.0	27636.9	11697.0	9930.0
35	1.4	10.0	0.0	20.0	140.0	0.0	22897.1	1257.8	495.0	52500.7	9852.9	10100.0
36	6.9	18.2	0.0	4286.9	811.3	0.0	3581.3	220.7	112.5	163162.5	69687.7	84800.0

farmers were encouraged to weigh their lambs prior to treatment, and a dosage table and a syringe were provided to enable accurate dosages being used. Although the accuracy of dosing was not investigated, lambs from all farms where reduced efficacy or inconclusive results were found had been weighed prior to treatment. Therefore, although under-dosing cannot be excluded, it does not seem to be a likely cause of the reduced drug efficacy.

There has been extensive discussion of the statistical aspects of interpreting the data from FECRT, including the relative merits of arithmetic and geometric means. Miller et al. (2006) concluded that the results of a FECRT based on an arithmetic mean reduction in egg counts can be inconsistent, whereas Dobson et al. (2009) suggested that arithmetic means provide better estimates of parasite egg output than geometric means. However, the situation regarding the data from *Eimeria* FOCRT is quite different to that for nematode FECRT in that the distribution of oocyst counts is typically far more skewed than that of egg counts, with occasional extremely high oocyst count observations. This is partially a reflection of the multiple rounds of intracellular asexual reproduction in the *Eimeria* lifecycle, and that do not occur with nematodes. In this situation of highly over-dispersed parasite populations with inconsistent variation, the geometric mean is a more appropriate estimator of the central tendency parameter (Smothers et al., 1999). More fundamentally, the statistical justification presented in section 2.2.1 suggests that considering the change in arithmetic mean of the data on the log scale is the most appropriate course of action, and this is equivalent to considering the change in geometric mean on the exponent scale. We therefore believe that considering the geometric mean reduction for treated animals is most appropriate. This also facilitates the use of a more standard frequentist modelling approach using an over-dispersed Poisson distribution with log link and fixed effect of time interval to estimate the change in geometric mean count, with CIs generated using a relatively standard parametric bootstrapping procedure.

Other widely discussed statistical aspects of interpreting data from FECRT relate to the sample size and the sensitivity of the laboratory method. The sensitivity of commonly used McMaster methods for performing *Eimeria* oocyst counts usually range between 5 and 50 OPG (Reeg et al., 2005; Saratsis et al., 2011). The method used in our study has a theoretical sensitivity of 5 OPG, which, given the extremely high mean OPG values, is of sufficient sensitivity to ensure the oocyst count values will be high enough to minimise the proportion of false/excess zeros that may otherwise affect the distribution of counts and bias the final results (Denwood et al., 2008; Love et al., 2017). In FECRT-calculations, diagnostic accuracy can be improved by increasing the sample size and lowering the detection limit (Levecke et al., 2012). Coles et al. (1992) suggested a minimum sample size of 15 animals per treatment group. In the present study, the sample size of 8 lambs per treatment group was chosen as a pragmatic compromise between the statistical importance of a large sample size and the number of lambs that we expected the farmers to be able to sample twice. However, based on the relatively large proportion of farms that were classified as inconclusive, we recommend that this sample size should be increased

in future studies.

Previous studies investigating the effects of anticoccidials in sheep have mostly compared different drugs with respect to oocyst excretion, faecal consistency, and weight gain without calculating the efficacy of the individual drugs (Gjerde et al., 2009; Le Sueur et al., 2009; Diaferia et al., 2013). The classification thresholds used in our study were based on the figures of 90% and 95% used by Geurden et al. (2015) and Peña-Espinoza et al. (2016) in anthelmintic studies, as there is currently no published target efficacy available for toltrazuril. The same classification targets were used by de Souza Rodrigues et al. (2017) who investigated the efficacy of different toltrazuril treatment strategies in Brazil. However, we note that although these figures seem reasonable in the absence of alternatives, there is no specific evidence to support the direct translation of 90% and 95% efficacy targets from their intended context of estimating the arithmetic mean efficacy of anthelmintic compounds against nematodes to the quite different context of estimating the geometric mean efficacy of anticoccidial compounds. The lack of published target efficacy was highlighted by Joachim et al. (2018) as one of the main challenges evaluating ACE in farm animals, and accentuate the importance of performing CETs in order to diagnose ACR properly. Consequently, there is a need for additional research into the expected geometric mean efficacy of anticoccidial drugs in a 'susceptible' population, after which the currently used arbitrary thresholds may be modified on the basis of evidence.

The Federation of Veterinarians of Europe has recently highlighted the importance of coccidiostats being only available by veterinary prescription (FVE, 2016), which is an important measure to control their use, and thereby potentially extend their efficacious period. By applying the method described here, we were able to produce the first evidence-based description of reduced toltrazuril efficacy against ovine *Eimeria* spp., and to highlight the importance of ensuring that treatment timing is appropriate. However, the validity of our results requires confirmation by CET. The present results suggest that the threat of emerging ACR should be taken seriously in order to safeguard animal welfare and future productivity of the sheep industry. Additional studies to establish the true prevalence of ACR and the *Eimeria* spp. involved are warranted.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2018.05.002.

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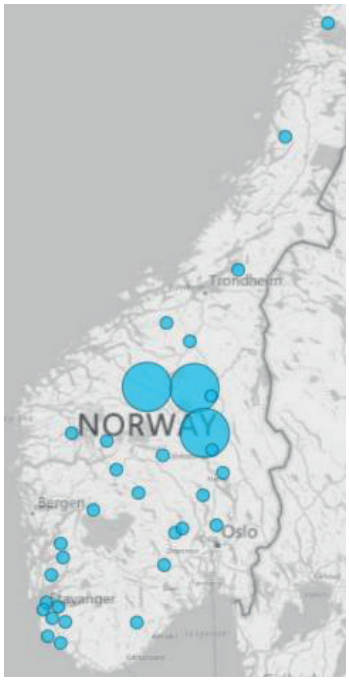
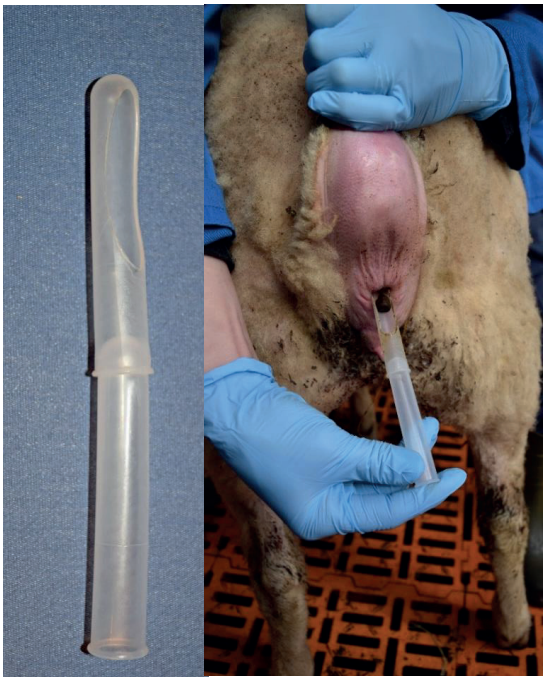


Supplementary data 1

Geographical location of the 36 sheep farms included in the study. Each dot represents the municipality of the farm, and the size illustrates the number of farms per municipality (small = 1, large = 2).



Supplementary data 2

The “faecal spoon” consists of two polypropylene tubes with rounded bottoms pushed into each other. An oval part of the wall of the distal tube is removed by scalpel, and the edges are smoothened by heat to avoid damage of the rectal mucosa.

Supplementary data 3

Oocyst counts pre- and post-treatment for the 36 farms complying with the sampling protocol.

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III

Controlled efficacy trial confirming toltrazuril resistance in a field isolate of ovine *Eimeria* spp.

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Abstract

Background: Coccidiosis due to *Eimeria* spp. infections in lambs causes increased mortality and substantial production losses, and anticoccidials are important for control of the infection. Anticoccidial resistance has been reported in poultry and swine, and we recently described reduced toltrazuril efficacy in ovine *Eimeria* spp. in some Norwegian sheep farms using a newly developed faecal oocyst count reduction test (FOCRT). The aim of the present study was to use a controlled efficacy trial to assess the efficacy of toltrazuril against a field isolate suspected of being resistant.

Methods: Twenty lambs, 17–22 days old and raised protected against exposure to coccidia, were infected with a field isolate of 100,000 *Eimeria* spp. oocysts. This isolate was obtained from a farm with a previously calculated drug efficacy of 56% (95% confidence interval: -433.9 to 96.6%). At day 7 post-infection, 10 of the lambs were orally treated with 20 mg/kg toltrazuril (Baycox Sheep vet., Bayer Animal Health), while the other 10 lambs (controls) were given physiological saline. Clinical examinations were conducted, and weight gains recorded. Daily faecal samples were scored for diarrhoea on a scale from 1 to 5, and oocyst excretion was determined using a modified McMaster technique. Oocysts were morphologically identified to species level. At 17–24 days post-infection, the lambs were euthanized and necropsied.

Results: The tested *Eimeria* isolate was resistant against toltrazuril, and resistance was seen in both pathogenic and non-pathogenic species. In addition, no significant differences in faecal score, growth, gross pathology or histological changes were identified between the two groups. The pathogenic *E. ovinoidalis* was the dominant species, and no significant difference in the individual prevalence of *E. ovinoidalis* post-treatment was found between treated (66.9%) and control lambs (61.9%). Other species identified included *E. crandallis*/*weybridgensis*, *E. parva*, *E. marsica*, *E. faurei*, *E. pallida*, *E. ahsata* and *E. bakuensis.*

Conclusions: This study confirms toltrazuril resistance in ovine *Eimeria* spp.; in addition, the data support the use of FOCRT as an appropriate tool for field evaluation of anticoccidial efficacy. Due to limited anticoccidial treatment alternatives, these findings may have important implications for the sheep industry, particularly in northern Europe.

Keywords: Controlled efficacy test, Anticoccidial resistance, Toltrazuril, *Eimeria* spp., *Eimeria ovinoidalis*; Sheep

Background

Anticoccidial resistance (ACR), which develops mainly as a result of intensive long-term use of anticoccidial drugs, occurs widely in poultry production and has also been identified in *Cystoisospora suis* in piglets [1–5]. In addition, a field method for the evaluation of reduced anticoccidial efficacy (ACE) in ovine *Eimeria* spp., the faecal oocyst count reduction test (FOCRT), has recently been developed and indicated that the efficacy of toltrazuril is reduced in some Norwegian sheep flocks [6].

Infections with *Eimeria* spp. may impact both animal welfare and productivity in the sheep industry, and controlling the infection is important to minimise mortality and morbidity, and to ensure that lamb growth is not compromised [7–9]. Suggested strategies to control ruminant coccidiosis include pasture management, adequate nutrition, and hygienic measures [10, 11]. However, these measures are often difficult to implement in practice, and the main control approach is often metaphylaxis with anticoccidials [12–15]. Metaphylactic administration of a single oral dose of toltrazuril in the prepatent period has been shown to be effective at reducing clinical signs and maintaining adequate lamb growth rates in different production systems [13, 15–19]. In contrast, treatment of clinical coccidiosis is considered inefficient due to the extensive intestinal damage already caused by the infection [20, 21]. Loss of sensitivity to toltrazuril, the only anticoccidial registered for use in sheep in the Nordic countries [22–24], should therefore be a matter for serious concern for lamb production.

The World Association for the Advancement of Veterinary Parasitology guidelines for evaluation of ACE in mammals [25], states that there is a need for verified methods for evaluation of ACE. Field methods for assessment of drug efficacy, such as the FOCRT [6] and the faecal egg count reduction test used to evaluate anthelmintic efficacy [26], give only an indication of reduced efficacy, and need verification through controlled efficacy trials (CET) [27, 28]. In addition, due to the variation in pathogenicity between ovine *Eimeria* spp., the differentiation of species should be considered separately [25].

The aim of the present study was to perform a CET in order to determine whether different species in a field isolate of ovine *Eimeria* spp. with suspected ACR, based on the FOCRT [6], actually demonstrated resistance to toltrazuril.

Methods

Study animals

A total of 20 lambs from 8 ewes of the Norwegian White Sheep breed ("Norsk kvit sau") was included in the study, which was approved by the Norwegian Animal Research Authority (ID: 11657). The ewes were synchronised using Chronogest® CR and PMSG® (MSD Animal Health, Buckinghamshire, UK) and served by natural mating. Lambs were either snatched at birth (n = 16) or delivered by caesarean section (n = 4) over a period of 6 days, and thereafter reared artificially. Individual ear tags were used for identification. Directly after birth, all lambs were washed with Optima pH 4 soap (Optima Produkter AS, Norheimsund, Norway) and dried before being placed in boxes with expanded metal floors, in groups of four. Infrared heaters were used during the whole trial. An overview of the study groups, including lamb age, birth weight and gender can be found in Additional file 1: Table S1.

Lambs received ovine colostrum from ewes vaccinated against *Clostridium* spp. (Covexin-8, Zoetis) during the first 30 min of life, followed by colostrum from vaccinated cows (Covexin-8, Zoetis) during the next 24 h. To avoid cases of haemolytic anaemia, the cow-colostrum had previously been tested on naturally reared lambs. Lambs were then fed *ad libitum* with a commercial milk replacer (Denkamilk, Denkavit, Fiskå, Mølle, Stavanger), using an automatic feeding system (Holm & Laue, Godkalven, Figgjo, Norway). The lambs had *ad libitum* access to water, hay and commercial lamb-starter concentrate (FORMEL lam vår, Felleskjøpet, Norway). To ensure that transmission of *Eimeria* to the lambs *via* contaminated colostrum and hay could not occur, both were frozen at -75 °C for a minimum of 24 h, prior to provision to the lambs.

Field isolate of *Eimeria*

The field isolate of *Eimeria* spp. was obtained from one of the flocks (ID 35) participating in the recent FOCRT study [6]. According to the FOCRT results, toltrazuril had reduced efficacy against *Eimeria* in two flocks. However, neither of these flocks were available for the CET, due to geographical and practical reasons. Thus, treatment with toltrazuril in the selected flock had been found to have an efficacy of 56.0%, but the results were classified as inconclusive, due to the wide 95% confidence interval (CI) of -433.9 and 96.6% [6].

To obtain sufficient *Eimeria* oocysts of this mixed field isolate (named "NMBU ID 35") for the present study, faecal samples were obtained from 35 lambs in this flock 9 days after toltrazuril treatment (Baycox® Sheep vet., Bayer Animal Health, Oslo, Noray). Oocysts were isolated according to Jackson [29] with some modifications. Briefly, faeces were mixed 1:1 with

water and filtered. The faecal mix filtrate was subsequently mixed 1:1 with saturated sugar-solution (density: 1.5 g/l) in a plastic container and left to float onto a glass slide. The slide was washed every second hour with deionized water for three consecutive days, and the washings collected. The washings were centrifuged at 2300× *g* for 20 min, the supernatant discarded and the sediment mixed 1:1 with deionized water in a glass flask with constant aeration. The oocysts in the flask were left to sporulate for 7 days at room temperature. Sporulated oocysts were stored for 18 days at 4 °C. Based on morphology [30], as seen by light microscopy at 400× magnification (see also section 2.5), and classification of 300 oocysts, the field isolate consisted of *E. parva* (32%), *E. crandallis*/*weybridgensis* (25%), *E. ovinoidalis* (24%), *E. faurei* (9%), *E. marsica* (8%), *E. pallida* (1%), *E. ahsata* (<1%) and *E. bakuensis* (<1 %).

Infection and treatment of lambs

All lambs were infected (day 0) at 17–22 days of age, using an oesophageal tube. A dose of approximately 100,000 sporulated oocysts, diluted in water to a total volume of 5 ml, was given to each of the 20 lambs. Then, two randomly selected (coin toss) lambs from each group of four were orally treated (day 7) with 0.4 ml/kg toltrazuril (Baycox® Sheep vet. 50 mg/ml, Bayer Animal Health) and the remaining lambs (controls) were given 0.4 ml/kg of 0.9% NaCl (B. Braun Medical AS, Vestskogen, Norway).

Body weight, general health and blood samples

Clinical examinations were performed daily throughout the trial. Rectal temperature was measured at days 0, 1, 2 and 7, and daily from day 14, and temperatures > 40.5 °C were considered as fever. The lambs were weighed once a week using a calibrated weight (Kruuse, Drøbak Norway) with 0.1 kg sensitivity, until 14 days post-infection, and thereafter three times a week.

Two lambs (controls) were treated orally with trimethoprim/sulphamethoxasole (Bactrim, Roche, Etterstad, Norway) during the first three days of life due to suspected *Escherichia coli*-infection, from which both recovered within 48 h. Six lambs, two controls and four treated with toltrazuril, developed lameness due to interdigital abscessation, and *Streptococcus aureus* was detected in two lambs. Four lambs recovered without treatment, and two of the lambs recovered after treatment with benzylpenicillinprocaine (Penovet vet., Boehringer Ingelheim Vetmedica, Copenhagen, Denmark) administered intramuscularly for three days.

On clinical examination, special attention was paid to clinical signs associated with *Eimeria* spp. infections, i.e. dehydration, pyrexia, weakness, anorexia and, in particular, the presence of diarrhoea.

Severe haemorrhagic diarrhoea and dehydration in one lamb at day 17, led to euthanasia of that whole group of four lambs. At day 18, another lamb showed signs of haemorrhagic diarrhoea, and all lambs in this group were also euthanized. The remaining three groups were euthanized on days 21, 23, and 24.

Blood samples were drawn from *v. jugularis* using vacuette tubes (plain and EDTA-treated; BD, Franklin Lakes, USA) at 48 ± 2 h after birth and at days 0, 7 and at euthanasia. Haematology was performed using the ADVIA 120 Haematology system (Bayer Diagnostics, Leverkusen, Germany). Dehydration was considered with a haematocrit (hct) of > 45.0% [31]. Whole blood tubes were centrifuged, and the serum removed and stored at -20 °C until further analysis. Biochemical analysis was performed by ABX Pentra 400 (Horiba, Les Ulis, France), and included analysis of iron, total protein, albumin, urea, creatinine, gamma-glutamyl transferase, glutamate dehydrogenase and beta hydroxybutyric acid.

Faecal samples

Individual faecal samples from each of the lambs were obtained daily from day 10 of life until the end of the experiment. Visual scoring of faecal consistency was performed on a scale from one to five (1: normal, pelleted; 2: soft; 3: liquid; 4: watery; 5: watery with blood and/or intestinal tissue) [32]. A score ≥ 3 was considered as diarrhoea.

Samples were collected using an in-house "faecal spoon" [6] and the faecal samples were put in zip-lock bags, which were vacuum packed (Fresh'n'easy, OBH Nordica, Sundbyberg, Sweden), stored at 4 °C, and analysed within 37 days. The rate of oocyst excretion was determined using a modified McMaster technique with a theoretical sensitivity of 5 oocysts per gram (OPG) [6]. One hundred *Eimeria* oocysts from all samples ≥ 1000 OPG were examined by light microscopy at 400× magnification and identified to species level, using morphological criteria [30]. However, due to their morphological similarity, oocysts of *E. crandallis* and *E. weybridgensis* were not differentiated.

Oocyst counts were analysed by the FOCRT [6], which consists of a two-step procedure. First, timing of treatment and sampling was evaluated, followed by evaluation of treatment efficacy, by comparing post-treatment faecal samples from treated lambs with equivalent samples from untreated controls. Pre-treatment samples (sample 1) were obtained on day 7 (day of treatment), and post-treatment samples (sample 2) were obtained on days 14–18. The FOCRT was then run using the post-treatment oocyst counts for all five possible time intervals (7–11 days) between samples 1 and 2.

Differential diagnoses

Faecal samples obtained at euthanasia were analysed for rotavirus, coronavirus, *Cryptosporidium* spp. and general bacteriology. Additional testing for *Cryptosporidium* spp. was performed in diarrhoeic lambs at the time of infection (day 0, $n = 10$). Faecal smears were analysed at the Norwegian Veterinary Institute in Oslo for *Cryptosporidium* by direct immunofluorescence analysis (Crypt-a-Glo™, Waterborne Inc., New Orleans, USA), whereas presence of rotavirus and coronavirus were tested by standard diagnostic methods. Samples for bacteriological analyses were obtained from mid-jejunum and the colon spiral, spread on sheep blood agar plates, and incubated under anaerobic and aerobic conditions for 24–48 h at 37 °C and 5% CO_2. In cases of haemorrhagic diarrhoea, additional samples were grown on bromothymol-blue lactose cysteine agar (brolactin/CLED agar) for potential identification of *Salmonella* [33].

Necropsy

Lambs were euthanized at days 17–24, by intravenous injection with pentobarbital (Euthasol vet., Virbac, Sollihøgda, Norway) at 140 mg/kg. Standard necropsy was performed immediately thereafter, with emphasis on the intestines.

Histological samples were taken from mid-jejunum, proximal and distal ileum, mid and base of caecum, colon spiral, and distal colon, in addition to heart, lung, liver and kidney. The samples were immersion-fixed in 4% formaldehyde, paraffin-embedded, and stained with haematoxylin and eosin (HE). Histological evaluation was performed by light microscopy and a blinded semi-quantitative evaluation (single evaluator) was done to assess intestinal pathology. Evaluation parameters included changes in: (i) villi, (ii) surface epithelium (atrophy/attenuation), (iii) degree of *Eimeria*-infection, (iv) hyperaemia, (v) oedema, (vi) infiltration of inflammatory cells and (vii) crypt abscesses, and were scored as follows: 0 = minimal; 1 = little; 2 = moderate; 3 = severe, including half-step grading. In addition, the presence of epithelial necrosis was graded as present (1) or absent (0). A total histology score was calculated for each tissue by summation of all parameters evaluated (i-vii).

Statistical analysis

Data were managed in Excel 2013 (Microsoft Inc., Redmond, USA), and subsequently analysed in R [34] and Stata 14 (Stata Statistical Software: Release 14. StataCorp LP, College Station, TX, USA). Evaluation of efficacy was performed according to the FOCRT [6]. For calculations of significance based on means, a t-test was used. $P < 0.05$ was considered significant.

Results

Body weight, general health and blood analysis

Mean growth rates were above 300 g/day until days 14–16, whereupon mean growth rate decreased to around 0 g/day (Fig. 1). Growth rates increased again from day 21 onwards. The same pattern was observed in both treated and control lambs.

From day 15, both treated and control lambs had a mean faecal score of ≥ 3, indicating diarrhoea. The maximum mean faecal score was seen at day 17 (3.9 ± 0.2) and day 18 (4.4 ± 0.3) in the treated and control groups, respectively. Haemorrhagic diarrhoea was seen from day 14, in two treated and five control lambs, and tenesmus was observed in two control lambs (day 17).

An increase in rectal temperature was seen from day 14, with maximum temperatures measured at day 18 (40.4 ± 0.4 °C) and 16 (40.9 ± 0.4 °C) in the treated and control groups, respectively. The mean duration of fever (> 40.5 °C) was 2.3 ± 0.5 days and 1.9 ± 0.4 days for the treated and control groups, respectively. For these parameters, no significant difference between groups were seen at any time.

At euthanasia, the mean hct was 39.2 ± 1.7% and 41.4 ± 1.9% in the treated and control groups, respectively. However, dehydration (hct > 45.0%) was only seen in 3 lambs, of which one had been treated with toltrazuril. Mean total serum protein decreased in both groups from infection to euthanasia, but no significant differences between the groups were observed. Other biochemical parameters were within normal ranges (data not shown).

Faecal analysis

Oocyst excretion was first recorded in one treated lamb at day 10 (10 OPG), followed by oocyst excretion in all lambs in both groups from day 14 onwards. Peak oocyst excretion was seen in the treated group at day 20 (mean OPG: 5,438,500), and in the control group at day 21 after infection (mean OPG: 3,630,850) (Fig. 2). Thereafter, oocyst excretion decreased. There was no significant difference in oocyst excretion and species distribution between the groups at any time. All species present in the field isolate were isolated from the faecal samples of all the 20 infected lambs. *E. ovinoidalis* was the most prevalent species in both treated and control lambs (Table 1).

Efficacy, according to the FOCRT, was evaluated with confidence if the slope was ≥ 0.75, and with caution if slope was ≥ 0.5 and < 0.75 [6]. The slope ranged from 1.24 to 1.69 for the total oocyst excretion in the control lambs.

Slopes, maximum likelihood estimates, and 95% CIs for the geometric mean efficacy of all oocysts, *E. ovinoidalis*, *E. crandallis/weybridgensis*, and the non-pathogenic *Eimeria* spp. are

presented in Table 2; reduced efficacy of toltrazuril is apparent against both pathogenic and non-pathogenic species. The slope was ≥ 0.75 for all time intervals and species, except for four of the five time intervals of *E. crandallis/weybridgensis*.

Differential diagnoses

Samples analysed for *Cryptosporidium* spp., *Salmonella*, coronavirus and rotavirus were all negative. Bacteriological analyses showed a mixed flora, dominated by coliforms and *Enterococcus* spp.

Necropsy

Gross pathological findings included diffused thickened and folded ileal mucosa (7 treated and 7 controls), and fibrinous ileal content in two lambs (one treated and one control). Nodular or plaque-like foci in the ileal mucosa were seen in 4 treated and 6 control lambs (Fig. 3a). The regional distal jejunal lymph nodes were moderately increased in size and oedematous in 5 treated and 6 control lambs. Finally, watery abomasal content was seen in > 50 % of the animals in both groups.

Microscopy evaluation showed lesions, mainly in the ileum, caecum and colon, with minor lesions in the jejunum (Fig. 3b-f). However, there were no significant differences with respect to histological scores between the treated and control groups in any of the intestinal segments. The highest calculated histological score was found in the proximal ileum and at the base of caecum (Fig. 4). The mean score for each parameter can be found in Additional file 2: Table S2. Varying quantities of intracellular *Eimeria* stages were observed in all intestinal segments, except from jejunum, and they were mostly located in the villus epithelium, with fewer parasites in the crypt epithelium and lamina propria, and few in the submucosa and lymphatic vessels. In both treated and control lambs, changes in the intestinal surfaces varied from light atrophy of the jejunal epithelium and blunting of affected ileal villi (Fig. 3b), to areas of total flattening, attenuation of surface epithelium (Fig. 3e) and necrosis (Fig. 3d). Patches of epithelial necrosis were found in all lambs. Infiltration of inflammatory cells included mostly monocytes and eosinophils, but also neutrophils and macrophages, and was found in both the lamina propria and submucosa. Different degrees of oedema, hyperaemia, and haemorrhage were seen in all tissue sections examined, and in both treated and control lambs. Crypt abscesses (Fig. 3b) were found in varying degree in all lambs, and contained inflammatory cells, debris and different stages of *Eimeria* spp.

Discussion

As far as we know, this is the first report of experimentally confirmed toltrazuril resistance in a field isolate of ovine *Eimeria* spp. The results also support the use of FOCRT as a tool to evaluate ACE in the field. Although ten of the 20 lambs experimentally infected with *Eimeria* were metaphylactically treated with the recommended dose of 20 mg/kg toltrazuril (Baycox® Sheep vet., Bayer Animal Health), this treatment did not result in a significant reduction in oocyst excretion in the treated animals, compared with the controls. In addition, no significant differences were noted in clinical presentation, gross pathology, and histopathological findings. The speciation data showed that both pathogenic and non-pathogenic species of *Eimeria* in this isolate were resistant to toltrazuril.

The lambs excreted high numbers of oocysts, as has previously been recorded in experimental infections with multiple *Eimeria* spp. [35]. Although oocyst excretion decreased from around day 20 after infection, the total duration of excretion could not be determined, as the lambs were euthanized. The excretion pattern noted here, with an exponential increase, a plateau phase, and a decline, has previously been noted in experimental infections [35–37]. However, due to continuous reinfection under natural field conditions, the duration of oocyst excretion may be longer [38, 39] than observed in the present study. This might also explain why the calculated slope seen for all species in this experimental study is higher than the slopes reported from the preceding field trial [6].

Multi-species resistance, as observed here, has also been noted in field isolates of avian *Eimeria* spp. [3, 40]. Of particular importance in this study is that *E. ovinoidalis* was the dominant species excreted from infected lambs. As this species is one of the most pathogenic *Eimeria* spp. in sheep [41, 42], resistance against the most commonly used anticoccidial drug indicates that severe clinical coccidiosis may be expected to occur in resistant flocks. Although *E. ovinoidalis* was the dominant species excreted, the most prevalent species in the original field-isolate inoculum was *E. parva*. This could reflect similarities between *E. ovinoidalis* and *E. ninakholyakimovae* in goats, the latter of which develops macroschizonts in endothelial cells, resulting in the release of thousands of merozoites [42, 43]. Thus, the extent of intracellular multiplication/replication, which is presumably also related to the extent of pathogenicity associated with this species, is higher for *E. ovinoidalis* than for the other *Eimeria* species.

For *E. crandallis*/*weybridgensis*, the FOCRT calculations showed invalid results from three of the five sampling time points, probably due to the tests being performed too early in the infection. Excretion of *E. crandallis/weybridgensis* increased predominantly from day 16

onwards, and euthanasia was performed at days 17–24. Thus, the longer prepatent periods for these species compared with *E. ovinoidalis* [44] probably explain these results. This is an important finding, as the number of invalid farms tested in the FOCRT [6] might have been fewer should sample 2 have been collected 10–11 days after sample 1. These findings also highlight the fact that although *Eimeria* spp. are often considered as a relatively uniform group, they are in fact separate species with potentially important differences in biology and pathogenic potential.

Two of the lambs were treated with trimethoprim/sulpha during their first days of life, preparations that have been shown to be effective in treating ovine coccidiosis [45, 46]. However, withdrawal periods for comparable drugs licenced in cattle are 10–15 days for meat [47], and these lambs were treated > 17 days prior to the experimental infection. In addition, these treated lambs were in the control group, and therefore this treatment should not have affected the results of the study.

Similar clinical signs as observed here might be caused by *Cryptosporidium* spp., coronavirus, rotavirus, and *Salmonella* spp., but none of these pathogens were detected. In addition, the findings of coliforms and *Enterococcus* spp. may be considered as normal intestinal flora of lambs [48]. The observed clinical signs were therefore almost certainly caused by *Eimeria* spp., particularly the two major pathogenic species, *E. ovinoidalis* and *E. crandallis* [35, 36]. Thickened ileal mucosa is often seen in lambs infected with *E. ovinoidalis* [49]. In addition, the histological changes, such as blunted villi and surface necrosis, as well as the presence of coccidia, hyperaemia, oedema, infiltration of inflammatory cells and crypt abscesses, are also in accordance with previous reports [42, 50, 51].

To improve our study, an additional group of uninfected lambs might have been advantageous as this would have enabled better comparisons between weight gain and histopathological changes. However, this was not feasible at the time of the study. Furthermore, due to the lack of defined cut-off values for ACE, it might have been advantageous to include an oocyst isolate from a non-suspected farm (i.e. a susceptible isolate) [25]. This would have enabled comparisons of different parameters, such as oocyst excretion, between treated and control lambs infected with susceptible or resistant *Eimeria* spp. However, due to lack of tools for selection of such susceptible ovine *Eimeria* isolates, we therefore chose to restrict our CET to treated and control lambs infected with isolate "NMBU ID 35" as a first step in the characterisation of anticoccidial resistance in ovine *Eimeria* spp.

Although the initial efficacy values have not been provided for toltrazuril by the manufacturer, several studies have investigated its effect on oocyst excretion. For example, its efficacy has been found to be 96.9–99.9% in the period from 7 to 98 days after first treatment, in a

study in which the lambs were treated every 14 days [52]. Other studies have shown toltrazuril efficacies [either provided in the publication or calculated as 1-(mean OPG treated group)/(mean OPG control group) from data in the publication] ranging from 90.0 to 100.0% in the period from two to three weeks after treatment [13, 18, 19, 53–56]. These efficacies are far higher than that calculated in the present study, and therefore the comparative data provides a further clear indication of resistance in the "NMBU ID 35" isolate.

Toltrazuril has been marketed for anticoccidial treatment in sheep since the 1980s, and its use has increased during recent years, both in Norway [57] and in the UK (Dr Gillian Diesel, personal communication). Extensive use of a drug over time may result in decreased efficacy, possibly due to the haploid stages of *Eimeria*, which immediately select for resistance [1, 5]. Since toltrazuril is the only registered anticoccidial for sheep in several countries, development of resistance in ovine *Eimeria* species may result in there being few treatment options available for sheep farmers, especially in northern Europe [22–24]. Diclazuril is an anticoccidial that has been registered for treatment of sheep in several countries, but as it may share a common mode of action to that of toltrazuril [58], cross-resistance between these two triazine-derivates in ovine *Eimeria* spp. seems highly likely and should be investigated. Indeed, cross-resistance between diclazuril and toltrazuril was reported for an isolate of avian *Eimeria* spp. over 20 years ago [3].

Our results indicate that there is a clear need for tools for evaluating ACE, such that inefficient treatments and, thus, the potential for reduced animal welfare and productivity can be avoided. Such tools are available for poultry, using different metrics, such as oocyst index, body weight gain, relative weight gain, lesion scores and anticoccidial index [59]. However, such methods have not yet been established for use in ruminants [25], with the exception of the newly published FOCRT [6]. Although FOCRT may serve as a tool for field evaluation of ACE, there is a clear requirement for further testing of its use in different settings.

Confirmation of the spectre of resistance in ovine *Eimeria* species increases the urgency of identifying alternative treatments and optimising other control strategies. The anticoccidial effects of different plants and natural extracts, such as sainfoin (*Onobrychis viciifolia*), carob pods (*Ceratonia siliqua*), pomegranate (*Punica granatum*) peel extract, grape seed proanthocyanidin extracts, and different natural antioxidants, have been investigated *in vivo* and *in vitro* in different hosts [60–64]. However, none of these bioactive substances have, as yet, been brought to the market for the prevention of clinical coccidiosis. In addition, there are vaccines available for avian *Eimeria* spp. [65, 66], and successful immunisation of goat kids with attenuated *Eimeria* spp. oocysts has been performed [67].

Future studies are necessary in order to develop a commercial vaccine against ovine *Eimeria* spp. Therefore, current efforts should focus on identifying ACE, and maintaining the efficacy of toltrazuril in susceptible flocks. Management strategies that decrease the need for anticoccidials by reducing the infection pressure, possibly achieved by applying strict hygienic measures, and improved flock and pasture management should be actively encouraged by veterinarians and agricultural policy incentives [11]. Additionally, farmers should be informed about the importance of correct drenching techniques, including dosage estimation and drench gun calibration, as these have been shown to be inadequate in several farms [12].

Conclusions

To our knowledge, this is the first report of ACR against toltrazuril in an ovine *Eimeria* field isolate, which included the highly pathogenic species, *E. ovinoidalis*. The results also support the use of FOCRT for field evaluation of ACE. However, the distribution and prevalence of ACR is unknown and further studies are warranted. In the future, difficulties in managing coccidiosis without chemotherapy, due to few available treatment options, may severely affect both animal welfare and the economy of the sheep industry.

Additional files

Additional file 1: Table S1. Information about the 20 lambs infected with *Eimeria* spp. at day 0.
Additional file 2: Table S2. Histopathological findings from toltrazuril treated lambs and controls euthanized 17-24 days post infection with 100,000 *Eimeria* oocysts.

Abbreviations

ACE: anticoccidial efficacy, ACR: anticoccidial resistance, CET: controlled efficacy trial, FOCRT: faecal oocyst count reduction test, hct: haematocrit, OPG: oocysts per gram

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Declarations

Ethics approval

The animal experiment was performed in compliance with ethical guidelines and approved by the Norwegian Animal Research Authority (ID 11657) with reference to the Norwegian regulation on animal experimentation (FOR-2015-06-18-761).

Consent for publication

Not applicable.

Availability of data and materials

The data supporting the conclusions of this article are included within the article and its additional files. The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors participated in the planning of the study. AO and SKN performed the study. AO performed the statistical analysis and drafted the manuscript. All authors have critically read and approved the final manuscript.

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Legends to figures

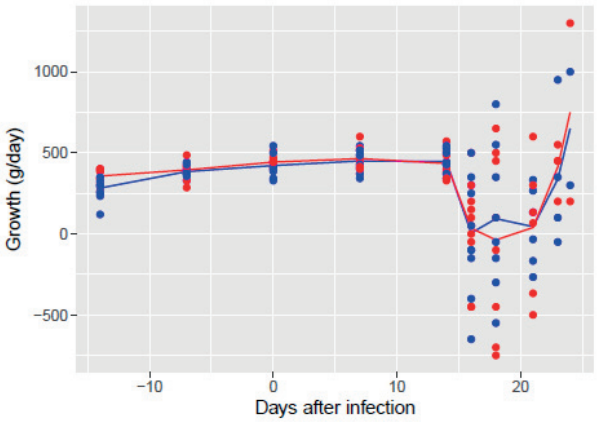

Fig. 1 Mean and individual growth (g/day) of the 20 *Eimeria* spp. infected lambs. Red: toltrazuril treated, and blue: controls. *n* varies due to euthanasia: day ≤ 17, *n* = 20; days 18–20, *n* = 16; days 21–22, *n* = 12; day 23, *n* = 8; day 24, *n* = 4

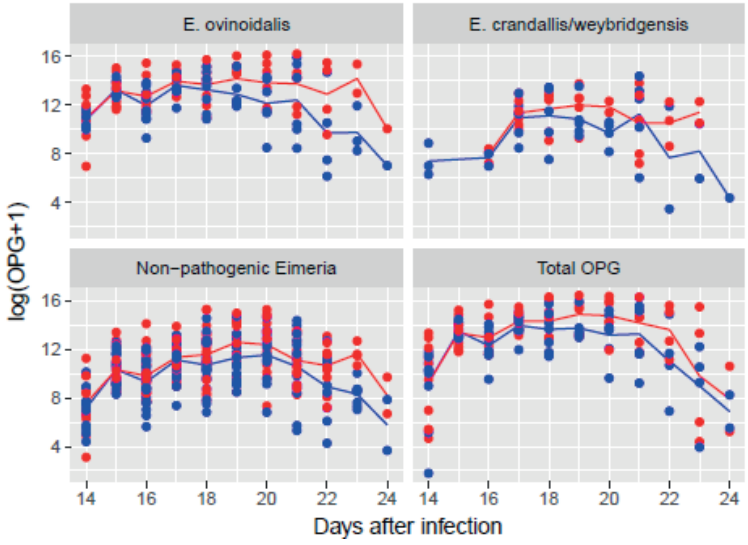

Fig. 2 Mean and individual oocyst excretion [log(OPG+1)] in 20 *Eimeria* spp. infected lambs. *E. ovinoidalis*, *E. crandallis/weybridgensis*, non-pathogenic *Eimeria* spp. and the total OPG is shown. There was no significant difference in oocyst excretion between toltrazuril treated lambs (red) and controls (blue) at any time point. *n* varies: day ≤ 17, *n* = 20; days 18–20, *n* = 16; days 21–22, *n* = 12; day 23, *n* = 8; day 24, *n* = 4

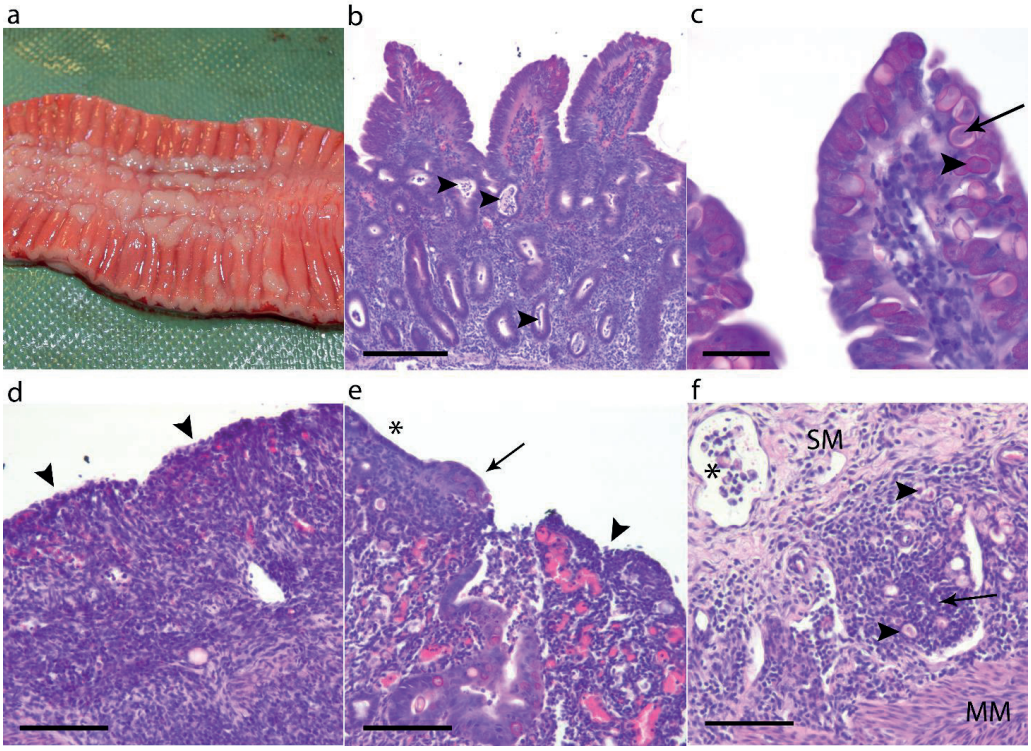

Fig. 3 Examples of gross pathology and histological findings in lambs infected with *Eimeria* spp. **a**, **b**, **d-f** were treated lambs, while **c** was a control. **a** Section from ileum with multiple, coalescing beige nodules; also note the thickened and folded intestinal wall. **b** Proximal ileum: blunted villi with large amounts of *Eimeria* spp. in the epithelium. Arrowheads point at some of the numerous crypt abscesses. There is also infiltration of inflammatory cells in lamina propria and superficial haemorrhage and hyperaemia. **c** Heavy infection of surface epithelium of the proximal ileum with both gamonts (arrowhead) and zygotes (arrow) present. **d** Proximal ileum: large area of epithelial necrosis (arrowheads) with atrophy of villi and full destruction of normal architecture. There is marked infiltration of inflammatory cells, proliferation of fibrous tissue, hyperaemia and haemorrhage. **e** Basis of caecum: The surface epithelium is flattened (*), hyperplastic (arrow) and eroded (arrowhead). There is a colonic gland with hyperplastic epithelium and debris and next to this a destructed area with hyperaemia. **f** Basis of caecum: arrow points at a marked infiltration of inflammatory cells, mostly monocytes, with some *Eimeria*-zygotes (arrowhead) in submucosa (SM). A lymph vessel (*) with degenerated *Eimeria* (MM: muscularis mucosa). **b-f** Haematoxylin and eosin staining, scale bar and magnification: **b**, 100µm, 100×; **c**, 25µm, 400×; and **d-f**, 50µm, 200×

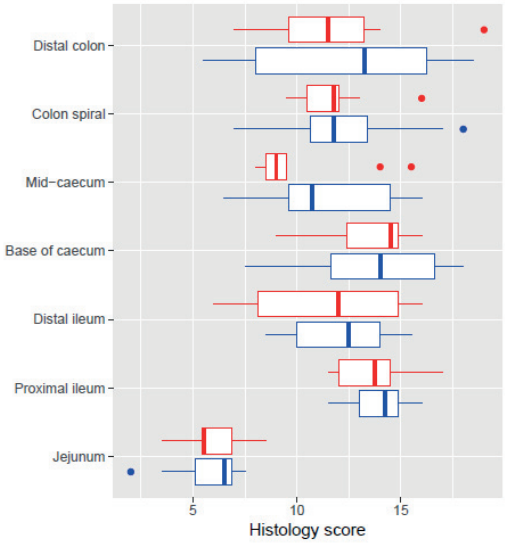

Fig. 4 Box-and-whisker plots with outliers illustrating the histology score. The score was a summation of all histological parameters evaluated (see text) in the 20 *Eimeria* spp. infected lambs, red: toltrazuril treated, and blue: controls

Table 1 *Eimeria* spp. excreted by toltrazuril treated lambs (n = 10) and controls (n = 10). The excretion is presented as percentage per species of the total number of oocysts excreted

	Treated (%)	**Control (%)**
E. ovinoidalis	66.87	61.88
E. crandallis/weybridgensis	3.61	12.11
E. faurei	0.81	1.00
E. pallida	1.54	0.98
E. parva	23.51	19.83
E. marsica	3.60	4.12
E. bakuensis	0.02	0.00
E. ahsata	0.04	0.09

Table 2 Maximum likelihood estimates and 95% confidence intervals (CI) for the geometric mean efficacy

Sample 2 (day)	*Eimeria* spp.	Slope	Mean efficacy (%)	Lower 95 % CI	Higher 95 % CI	*n*[a] Treated	Control	Interpretation
14	All species	1.32	-0.3	-1116.8	92.5	10	10	Reduced efficacy
14	*E. c/w*	0.26	–	–	–	10	10	Invalid
14	*E. ovi*	1.33	-114.8	-431.4	16.8	10	10	Reduced efficacy
14	Non-pathogenic	1.22	-48.3	-252.3	44.0	10	10	Reduced efficacy
15	All species	1.69	13.5	-90.9	61.2	10	10	Reduced efficacy
15	*E. c/w*	0	–	–	–	10	10	Invalid
15	*E. ovi*	1.47	8.7	-102.8	63.2	10	10	Reduced efficacy
15	Non-pathogenic	1.31	33.0	-73.6	70.2	10	10	Reduced efficacy
16	All species	1.37	-93.4	-395.5	29.5	10	10	Reduced efficacy
16	*E. c/w*	0.26	–	–	–	10	10	Invalid
16	*E. ovi*	1.33	-114.8	-418.7	9.5	10	10	Reduced efficacy
16	Non-pathogenic	1.22	-48.3	-265.8	37.3	10	10	Reduced efficacy
17	All species	1.40	-41.9	-139.4	16.6	10	10	Reduced efficacy
17	*E. c/w*	0.73	-202,2	-834.6	-25.6	10	10	Caution: reduced efficacy
17	*E. ovi*	1.51	-41.2	-260.1	42.8	10	10	Reduced efficacy
17	Non-pathogenic	1.38	-37.0	-241.0	44.0	10	10	Reduced efficacy
18	All species	1.24	-97.2	-684.4	45.6	8	8	Reduced efficacy
18	*E. c/w*	0.77	-198.2	-769.8	-3.6	8	8	Reduced efficacy
18	*E. ovi*	1.47	-56.1	-316.6	47.6	8	8	Reduced efficacy
18	Non-pathogenic	1.35	-228.6	-815.1	-15.1	8	8	Reduced efficacy

Notes: The estimates were based on post-treatment oocyst counts for five time intervals between sample 1 (day 7 after infection) and sample 2, and was calculated according to the FOCRT [6]. A slope ≥ 0.5 and < 0.75 was evaluated with caution, whereas a slope < 0.5 was interpreted as invalid

[a]Four lambs were euthanized at day 17

Abbreviations: *E. ovi*, *E. ovinoidalis*; *E. c/w*, *E. crandallis/weybridgensis*; Non-pathogenic, all species except *E. ovinoidalis* and *E. crandallis/weybridgensis*

Table S1

Information about the 20 lambs infected with *Eimeria* spp. at day 0.

Group	Lamb	Sex	Birthweight (kg)	Age at infection	Weight at infection (kg)	Treatment with toltrazuril	Euthanasia (day)
A	1	Ram	4,3	22	11,5	No	23
	2	Ram	4,3	22	13,0	Yes	23
	3	Ewe	4,7	22	13,1	Yes	23
	4	Ewe	3,8	22	11,6	No	23
B	5	Ram	3,6	22	10,5	Yes	18
	6	Ewe	4,1	22	13,1	No	18
	7	Ewe	5,1	22	14,3	Yes	18
	8	Ewe	3,6	22	11,5	No	18
C	9	Ram	4,3	21	12,4	Yes	21
	10	Ewe	4,6	21	12,7	Yes	21
	11	Ram	4,1	19	11,1	No	21
	12	Ewe	3,0	19	8,9*	No	21
D	13	Ram	4,2	20	12,0	Yes	17
	14	Ewe	4,0	20	12,8	Yes	17
	15	Ram	6,0	18	13,9	No	17
	16	Ram	3,9	18	10,2	No	17
E	17	Ewe	5,3	18	12,9	Yes	24
	18	Ram	4,8	18	11,7	No	24
	19	Ram	7,1	17	14,2	No	24
	20	Ram	7,8	17	15,5	Yes	24

*Lamb 12 had to be bottle-fed and occasionally tube fed the first five days of life. Consequently, the growth was low the first week (120.0 g/day), but improved the following week (371.4 g/day).

Table S2

Histopathological findings from toltrazuril treated lambs and controls euthanized 17-24 days post infection with 100,000 *Eimeria* oocysts.

	Jejunum		**Proximal ileum**		**Distal ileum**		**Base of caecum**		**Mid-caecum**		**Colon spiral**		**Distal colon**	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Epithelial reaction	0.7 ± 0.1	0.7 ± 0.1	2.4 ± 0.2	2.4 ± 0.1	1.6 ± 0.3	1.8 ± 0.3	2.2 ± 0.1	2.3 ± 0.2	1.8 0.2	2.1 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	2.3 ± 0.3	2.2 ± 0.3
Epithelial necrosis	0	0	0.6 ± 0.2	0.7 ± 0.2	0.2 ± 0.1	0.5 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.2	0.5 ± 0.2
Degree of *Eimeria*-infection	0	0	1.6 ± 0.4	1.7 ± 0.4	1.9 ± 0.4	1.6 ± 0.3	1.9 ± 0.2	2.0 ± 0.3	1.2 ± 0.3	1.4 ± 0.2	1.4 ± 0.1	1.5 ± 0.2	1.3 ± 0.3	1.6 ± 0.4
Hyperaemia	1.7 ± 0.2	1.7 ± 0.2	2.4 ± 0.1	2.6 ± 0.1	1.9 ± 0.2	2.2 ± 0.2	2.3 ± 0.1	2.4 ± 0.1	2.1 ± 0.1	2.2 ± 0.2	2.3 ± 0.1	2.2 ± 0.2	2.3 ± 0.1	2.2 ± 0.2
Oedema	2.3 ± 0.1	2.1 ± 0.2	2.5 ± 0.2	2.5 ± 0.2	1.9 ± 0.2	2.2 ± 0.2	2.0 ± 0.1	2.0 ± 0.2	1.3 ± 0.1	1.9 ± 0.2	1.7 ± 0.2	1.6 ± 0.3	1.9 ± 0.3	1.8 ± 0.3
Inflammatory cells	1.4 ± 0.2	1.3 ± 0.2	2.3 ± 0.2	2.4 ± 0.2	2.0 ± 0.2	2.2 ± 0.2	2.5 ± 0.2	2.4 ± 0.1	2.2 ± 0.2	2.2 ± 0.2	2.5 ± 0.1	2.4 ± 0.1	1.9 ± 0.2	2.3 ± 0.1
Crypt abscess	0.2 ± 0.1	0.1 ± 0.1	2.0 ± 00.2	1.7 ± 0.3	1.9 ± 0.2	1.9 ± 0.2	2.1 ± 0.2	2.2 ± 0.2	1.4 ± 0.4	1.7 ± 0.3	1.8 ± 0.2	2.1 ± 0.2	1.7 ± 0.2	2.0 ± 0.3
Total score	5.9 ± 0.5	5.8 ± 0.6	13.6 ± 0.6	13.8 ± 0.5	11.4 ± 1.2	12.2± 0.8	13.5 ± 0.8	13.7 ± 1.1	10.1 ± 0.9	11.5 ± 1.0	11.8 ± 0.6	12.2 ± 1.1	11.6 ± 1.1	12.4 ± 1.1
Total score range	3.5 – 8.5	2.0 – 7.5	11.5 – 17.0	11.5 – 16.0	6.0 – 16.0	8.5 – 15.5	9.0 – 16.0	7.5 – 16.0	8.0 – 15.5	6.5 – 18.0	19.5 – 16.0	7.0 – 18.0	7.0 – 19.0	5.5 – 18.5

Haematoxylin and eosin stained tissue sections were blindly scored semi-quantitatively on a scale from 0-3, with half grading, except for villus necrosis (present = 1 or absent = 0), total score was calculated by summation

IV

Excretion of *Eimeria* spp. oocysts in young lambs following iron supplementation

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Abstract:

Background: Iron is an essential nutrient, and iron supplementation has been shown to reduce the incidence of abomasal bloat in lambs. Additionally, iron deficiency is linked to pica, which may increase uptake of *Eimeria* oocysts. Coccidiosis in sheep, caused by *Eimeria* spp., is an important infection, leading to reduced welfare and economic losses. The aims of our study were to investigate: 1) the use of iron supplementation in Norwegian sheep flocks using a questionnaire survey, and 2) whether iron supplementation reduced excretion of *Eimeria* oocysts and increased the growth rates of young lambs.

Results: A questionnaire regarding the use of iron supplementation, sent to all members of the Norwegian Sheep Recording System (n=4993), showed that 152/1823 farmers iron-supplemented lambs, either orally (56.7%) or by injection (43.3%). The main purpose of supplementation was to prevent abomasal bloat (38.4%), coccidiosis (9.3%), or both (27.8%). In the field study, 102 twin lambs from five flocks were included: one twin (treated) received 600 mg of gleptoferron subcutaneously within three days of birth, whereas the control was given saline. McMaster analysis of individual faecal samples obtained at weekly intervals (n=4 per lamb, starting at turnout) showed no significant difference in oocyst excretion between treatment groups at any sampling, except for one flock 14 days after turnout. Mean growth rates, measured at iron injection, 21 days after turnout, and in the autumn, differed significantly between treated and untreated lambs from iron injection to 21 days after turnout, however, no difference in growth rates was observed in the overall period from iron injection to autumn. Blood analysis suggested that the controls were at risk of developing iron deficiency anaemia during the housed period, but signs of anaemia were not observed.

Conclusion: Iron supplementation of lambs was used by 8.3% of the farmers responding to the questionnaire, mainly with the intention to prevent abomasal bloat, coccidiosis, or both. The field trial results indicate that iron supplementation of young lambs did not reduce oocyst excretion and only induced a transitory increase in weight gain. However further studies, including more flocks and possibly repeated iron injections, would provide more definitive information.

Keywords: iron supplementation, coccidiosis, sheep, Norway, *Eimeria* spp.

1 BACKGROUND

Iron is an essential element in all living organisms, including as an important component or cofactor in many proteins and enzymes, such as haemoglobin and myoglobin [1]. Due to rapid growth, low iron content in milk, and no access to soil, which is the main source of dietary iron for farm animals [2-4], housed lambs may develop anaemia. Iron deficiency anaemia is well recognised, both in housed piglets [5-7] and in housed lambs [8-12]. Dietary deficiency in iron may lead to pica, i.e. ingestion of material other than normal food, including soil [13]. In Norway, anaemia is occasionally seen in connection with abnormal appetite and development of abomasal bloat in lambs [11, 14]. Pica in lambs on spring pasture, leading to ingestion of excessive amounts of soil, could potentially result in uptake of high numbers of *Eimeria* spp. oocysts as they can survive for at least one year in soil under Norwegian conditions [15].

In Norway, most ewes are winter housed, and lambing occurs in March-May, followed by turnout to spring pastures 1-4 weeks post-partum [16, 17]. During summer, ewes and lambs normally graze on mountain/forest/uncultivated pastures, before the lambs are weaned in the autumn, at around 4-5 months of age [16]. Lambs become infected with *Eimeria* spp. either during the housed period or immediately after turnout [15]. Coccidiosis in sheep caused by *Eimeria* spp. leads to reduced welfare, increased mortality, and substantial production losses [18-20]. Clinical signs of coccidiosis include abdominal pain, anorexia, diarrhoea (± haemorrhagic) and weight loss/reduced growth [21]. Control strategies include adequate nutrition, hygienic measures, and pasture rotation [22, 23]. However, prevention of outbreaks in Norway is largely based on chemoprophylaxis with anticoccidials, usually with toltrazuril treatment at turnout or about one week later [24, 25]. Anticoccidial resistance in poultry has been reported against several anticoccidials [26, 27]. In addition, toltrazuril resistance has been confirmed in a field isolate of *Cystoisopora suis* [28]. Widespread use of anticoccidials in Norway, combined with unverified reports of reduced anticoccidial efficacy in ovine *Eimeria* spp. [25, 29], accentuate the importance of alternative control strategies.

Previous research has indicated that iron supplementation of lambs might increase growth rates and prevent abomasal bloat [12, 14]. These results have prompted the current guidelines for iron supplementation in Norwegian sheep flocks, which recommend the use of iron supplementation for

prevention of abomasal bloat [30]. The aims of our study were therefore: 1) to map the use of iron supplementation in Norwegian sheep flocks based on a questionnaire survey, and 2) to investigate whether iron supplementation of young lambs reduces the uptake and excretion of *Eimeria* oocysts and increases lamb growth rates, thus, potentially, reducing the need for treatment with anticoccidials.

2 METHODS

2.1 QUESTIONNAIRE

A questionnaire on iron supplementation in lambs was sent by email to all members of the Norwegian Sheep Recording System (NSRS) with a registered email address, using the Enalyzer Survey Solution (Enalyzer A/S). Membership in the NSRS is voluntary, and 36.5 % of all farmers were members in 2016, representing 47.9 % of all ewes in Norway and all sheep producing counties [2]. A translated copy of the questionnaire can be found as Additional file 1. Farmers (n= 4993) who received the questionnaire, represented 32.2 % of all sheep flocks in Norway [31]. Non-responding farmers were reminded once.

2.2 IRON SUPPLEMENTATION TRIAL

The study on investigation of the effect of iron supplementation of young lambs on *Eimeria* oocyst excretion was approved by the Norwegian Animal Research Authority, ID 8535. The CONSORT statement was used as a guideline in the design of the study [32].

Five flocks (A-E) located in Rogaland County, in Southwest Norway, were included in the study. Flocks were selected based on known clinical problems with coccidiosis (unpublished data), and proximity to the laboratory (Norwegian University of Life Sciences (NMBU), Sandnes). Twin pairs born within a period of 6 days were selected from each flock. The twins were randomly allocated (coin toss) to either iron supplementation (treated) or control groups. Treated lambs were injected with 600 mg gleptoferron (Gleptosil vet., Ceva Santé Animale) subcutaneously in the inguinal fold, 0-3 days after birth. At the same time, the twin was injected with a corresponding volume (3 ml) of 9 mg/ml sterile NaCl (B. Braun Melsungen AG). Lambs were housed with their dam for 16 to 31 days before turnout, and were kept on

slatted floors (plastic in flocks A, B, and E, and expanded metal in flocks C and F). In flock C, 18 lambs (9 treated and 9 controls) were kept for about one week on solid floors with wood shavings after injection. All five flocks used cultivated pastures for spring grazing, and all pastures had been grazed by lambs during the previous year. The farmers treated against other helminths at around three weeks after turnout using either benzimidazoles or ivermectins.

Faecal samples were taken at day 0 (turnout), 7, 14 and 21. All samples were collected individually in zip-lock bags and vacuum packed (Fresh'n'easy, OBH Nordica) on the day of sampling, and stored at 4°C until analysis within 28 days. Faecal samples were analysed using a modified McMaster technique with a minimum theoretical sensitivity of 5 oocysts per gram (OPG) [33, 34]. *Eimeria* were not identified to the species level. Additionally, the faecal consistency was scored visually on a scale from one to five: 1: normal, pelleted; 2: soft; 3: liquid; 4: watery; 5: watery with blood and/or intestinal tissue [35]. Scores ≥ 3 were regarded as diarrhoeic.

Weights were recorded at iron injection, day 21, and day 101 to 165 after iron injection (autumn). Blood were drawn from *v. jugularis* using a vacutainer system (plain and EDTA-treated, BD Company, USA) at turnout and 14 days later. Haematology was performed immediately using the ADVIA 120 Haematology system (Bayer Diagnostics, Germany). The main haematological parameters evaluated were red blood cell counts (rbc), haemoglobin (hgb), and haematocrit (hct). Whole blood tubes were centrifuged within two hours, and serum was stored at -20 °C. Serum iron (Fe) was analysed by ABX Pentra 400 (Horiba, France). Internal reference limits (NMBU, Sandnes) for blood parameters were calculated based on previous results [36, 37]. In brief, blood samples had been collected from 118 clinically healthy lambs within five days of birth and one week later, and parameters measured as described above; in addition, 50 of the lambs had been sampled 2, 3, 4, 8 and 18 weeks after the first sample.

2.3 Statistical methods

Data were managed in Excel 2013 (Microsoft Inc.). Statistical analyses were performed in Stata 14 (Stata Statistical Software: Release 14. College Station, TX: StataCorp LP), and graphs were made in

R [38]. *T*-tests were used for calculations of significance based on means, except for oocyst counts where, due to lack of normality, Mann-Whitney U-tests were used. Fisher's exact tests were used to evaluate correlations. $P < 0.05$ was considered significant.

3 RESULTS

3.1 QUESTIONNAIRE

The dataset from the questionnaire consisted of 1822 complete and 36 incomplete answers, corresponding to a response rate of 38.1 %. When possible, data from the incomplete questionnaires were included in the analysis, and thus *n* varies between calculations. Iron supplementation in lambs were used by 152 of 1823 farmers (8.3%). Farmers using iron supplementation were mainly located in Oppland (40.1%), Rogaland (15.8%), or Hedmark (9.9%) counties. The mean flock size was 95.5 ± 1.9 winter-fed ewes (range: 3 – 800), with a significant difference ($p < 0.01$) between non-supplementing (90.7 ± 1.8, range: 3 – 610) and supplementing (148.9 ± 9.8, range: 29 – 800) flocks. Table 1 shows the administration route and the purpose of the treatment.

Table 1. Questionnaire data from Norwegian sheep farmers supplementing with iron

		%	n
Administration route	Oral	56.7	85
	Injection	43.3	65
	Total		150
Purpose	Abomasal bloat	38.4	58
	Abomasal bloat and coccidiosis	27.8	42
	Coccidiosis	9.3	14
	Other/uncertain*	24.5	37
	Total		151
Intend to supplement next year	Yes	93.4	142
	No	6.6	10
	Total		152

*Other purpose/uncertain includes recommendations by veterinarian, experience of pica in lambs, and focus on increasing growth rates. n = number of farms.

3.2 IRON SUPPLEMENTATION TRIAL

In total, 102 lambs were included in the trial (22 lambs from flock A and 20 lambs from each of the flocks B-E). Age at turnout of the lambs ranged from 16-31 days (Table 2). In flock B, one lamb from

the control group died 17 days after turnout, and post mortem showed pneumonia with *Mannheimia haemolytica*. In flock E, two lambs were treated for pneumonia, one around turnout and another 14 days after turnout. These lambs were excluded from evaluation of growth rates and faecal analysis. In flock D, one lamb from the treated group died of unknown reasons on summer pasture.

Table 2. Twin lambs from five flocks (A-E) located in Rogaland County, Norway, included in an iron injection field trial. Lambs were either supplemented with 600 mg gleptoferron (treated) or physiological saline (controls) subcutaneously.

Flock	Number of lambs	Treated		Control		Age at iron injection (days)	Age at turnout (days)	Breed*
		Rams	Ewes	Rams	Ewes			
A	22	3	8	9	2	1-3	16 - 18	NWS
B	20	5	5	6	4	0-2	20 - 23	NWS
C	20	5	5	5	5	1-3	29 - 31	NWS and NST
D	20	4	6	4	6	0-3	16 - 21	NWS
E	20	4	6	5	5	2-3	16 - 17	NWS and NST

*NWS: Norwegian White Sheep, NST: Norwegian Short Tail

There was a significant difference in mean growth rates (g/day) between treated and untreated lambs in the period from iron injection to 21 days post turnout ($p = 0.021$), where treated lambs had higher mean growth rates than controls. However, at the flock level, this difference was only found in flock E ($p = 0.027$) (Table 3). There were no differences in mean growth rates from day 21 after turnout to autumn or from iron injection to autumn (101-165 days later).

Table 3. Mean growth rates (g/day, mean ± SEM) of iron supplemented lambs and controls in the five flocks (A-E). Treated lambs were subcutaneously supplemented with iron within three days of birth.

	Iron injection - 21 days after turnout			21 days after turnout - autumn[1]		Iron injection - autumn[1]	
	Treated		Control	Treated	Control	Treated	Control
A	392.3 ± 19.4		356.5 ± 18.5	176.7 ± 11.6	202.9 ± 13.5	223.6 ± 11.7	238.1 ± 13.5
B	394.1 ± 13.9		370.5 ± 12.1	294.1 ± 17.1	324.0 ± 37.4	324 ± 14.4	334.0 ± 22.3
C	374.3 ± 10.9		331.7 ± 24.8	247.9 ± 28.2	250.5 ± 24.7	286.8 ± 19.1	275.6 ± 15.8
D	410.0 ± 13.2		422.8 ± 22.2	252.4 ± 12.0	249.2 ± 10.4	293.4 ± 12.1	294.6 ± 12.5
E	366.9 ± 16.9	*	311.4 ± 15.1	345.3 ± 26.8	367.0 ± 26.4	351.4 ± 20.6	351.3 ± 20.4
All flocks	387.6 ± 6.9	*	359.3 ± 10.0	261.9 ± 11.8	274.4 ± 13.1	294.8 ± 9.2	295.7 ± 9.3

*$p < 0.05$

[1]Autumn: 101-165 days after iron injection

Four of the five flocks were infected with *Eimeria* spp. during the housed period, i.e. oocysts were detected at turnout (day 0) (Fig. 1), and lambs in all five flocks excreted *Eimeria* oocysts (range 10 - 1,043,000 OPG) 14 days after turnout. Although OPG counts were lower in treated lambs than in untreated lambs at day 14 in all flocks, this difference was not statistically significant ($p > 0.05$) in any of the flocks, except flock B ($p < 0.01$). In addition, there was no statistical significant difference in OPG ($p > 0.05$) between the treated and control lambs in any of the flocks at the other sampling dates. Maximum oocyst excretion for both groups of lambs and in all five flocks was observed at day 14 or 21.

Diarrhoea was observed during the study period. At turnout, one treated lamb from flock D had diarrhoea, whereas two lambs (one treated and one control) from flock C had diarrhoea on day 14. On day 21, the mean faecal score was < 2, except for in the control group in flock A, where the mean faecal score was 2.1 ± 0.3 (mean ± SEM). However, there was no significant difference in the faecal scores between treated and control lambs in any of the flocks at any sampling time.

Two flocks were positive for *Nematodirus battus* at day 21; in flock B, 77.8 % of the lambs were positive (range: 20-310 EPG) and in flock D, 25.0 % were positive (range: 10-50 EPG). However, presence of diarrhoea was not associated with detection of *N. battus.* Diarrhoea was only seen in two of the lambs diagnosed with *N. battus* in flock B, but in none of the *N. battus*-positive lambs in flock D. No other helminths were detected.

Except for in flock C ($p = 0.36$), there was a significant difference in blood iron content ($p < 0.05$) between treated and control lambs at day 0 (Fig 2), and the mean blood iron values in the control groups of flocks A, C, and E were below the reference limit of 25.0 µmol/l (internal references, NMBU, Sandnes). However, at day 14 after turnout, there was no difference in mean blood iron concentrations between treated and control groups in any of the flocks. In addition, a significant reduction in blood iron was seen in the treated group from turnout to day 14 in flocks B ($p = 0.018$) and E ($p < 0.01$), and for the whole dataset ($p < 0.001$). A similar significant reduction between samplings in the treated group was seen for hgb in flocks D and E ($p < 0.01$), and the whole dataset ($p = 0.049$), and hct in flocks D and E ($p < 0.01$), and the whole dataset ($p < 0.001$).

4 DISCUSSION

According to the questionnaire, iron supplementation was performed in 8.3 % of the sheep flocks, amongst which more than 90 % of the farmers intended to continue this practice. Moreover, more than 30 % of the farmers that supplemented lambs with iron did so to with the intention of preventing coccidiosis. An important finding from the questionnaire was the significant difference in flock size between flocks receiving iron supplementation and flocks that did not, with larger flocks more likely to practice iron supplementation than smaller flocks. The reason for this is unknown, but might reflect a shift in focus from individual animals to the flock, especially as the average flock size has increased in Norway over the last decade [31].

Few studies have investigated the effect of iron treatment of lambs on the excretion of *Eimeria* spp. and development of clinical coccidiosis. However, unpublished data (S. Vatn, personal communication) indicated a significant reduction in *Eimeria* oocyst excretion three to five weeks after turnout that was associated with iron supplementation. These findings were not supported by the present study, in which iron supplementation of lambs did not reduce excretion of *Eimeria* oocysts three weeks after turnout. This may indicate that reduction of geophagia by iron supplementation is not an efficient way to reduce *Eimeria* oocyst uptake and excretion in lambs. Regardless of the reason for decreased oocyst excretion in the study by Vatn, a similar reduction in oocyst excretion did not occur in our study despite the larger number of animals and farms included. There was however, an apparent reduction in oocyst excretion in iron supplemented lambs two weeks after turnout. Although this was mostly non-significant, the potential that this may reflect a delay in uptake and excretion of *Eimeria* oocysts might suggest that development of immunity could be affected. However, whether this occurred and whether this could confer some protection on the lambs is unknown.

Clinical signs of coccidiosis in lambs in Norway tend to occur 2-3 weeks after turnout, and it has been assumed that the lambs are primarily infected following ingestion of oocysts on permanent spring pastures [15, 39, 40]. Nevertheless, the present study shows that indoor infection with *Eimeria* spp. may not be unusual in Norway, as oocysts were detected in the faecal samples at turnout in four of the five flocks.

All five flocks participating in the treatment trial experienced diarrhoea and perianal soiling, signs related to both coccidiosis and nematodiriosis [21, 41]. However, based on the parasitological analyses, the diarrhoea was not correlated with nematodiriosis. Other gastrointestinal pathogens, such as rotavirus, coronavirus, *Cryptosporidium* spp., and *Salmonella* are not commonly diagnosed in lambs in Norway and were not investigated in our study, and we cannot rule out that they may have had a role in the observed clinical signs.

Previous studies investigating effects of iron supplementation of lambs have used various dosages and iron preparations: e.g., Bassett et al. (1995) administered 200 mg iron dextran intramuscularly within 24 hours of birth [8], Vatn & Torsteinbø (2000) injected 300 mg iron dextran subcutaneously to lambs within one week of birth [14], and in our study we used 600 mg gleptoferron subcutaneously within the first 3 days of life. In addition, Pollmann et al. (1983) showed that there was no difference in serum Fe concentrations, serum Fe-binding capacities, rbc, hgb or hct, between piglets supplemented with iron dextran, compared to piglets supplemented with gleptoferron [42]. The dose employed might be of importance, as the need for iron is largely dependent on growth rates; i.e., rapidly growing animals require iron to maintain haematopoiesis during the first weeks of life [9, 43]. The dose used in our study, 600 mg gleptoferron, should be sufficient to cover the lambs' requirements. However, treated lambs showed significantly lower levels of iron, hgb and hct 14 days after turnout than at turnout, indicating that their iron storage was low, and that higher or repeated doses of iron might have been beneficial. Should an increased or repeated iron dose be used, then the risk of reaching toxic levels must be evaluated. Clinical signs of acute iron toxicity in ruminants include anorexia, respiratory distress, icterus and central nervous signs [1, 44]. In our study, no signs related to iron toxicity were observed.

Iron supplementation of lambs may have a variable effect on lamb growth rates [8, 11, 14, 45-47]. In the present study, weight gain was not significantly affected by iron supplementation in any of the flocks when considering the growth period from birth to autumn. The difference in growth rates in the period from iron injection to 21 days post turnout might be the result of iron supplementation, as control lambs in many cases showed blood values for iron below the reference level at turnout. However, although no significant differences were found, the control lambs grew better than the iron-supplemented lambs in

four out of five flocks during the subsequent summer grazing period. This might be explained by the lambs' capacity for compensatory growth [48]. In addition, it is important to remember that lamb growth is dependent on several other factors, such as nutrition [49], ewe mastitis [50], and gastrointestinal helminths [51].

Blood values from the field trial lambs suggest that without iron supplementation, the lambs were at risk of developing anaemia, although none of the flocks showed associated clinical signs [52]. The significant difference in blood iron content between treated and untreated lambs at turnout, was largely absent 14 days later, indicating that the control lambs ingested iron and started producing red blood cells. In one of the flocks (C), differences in blood parameters between treated and untreated lambs could not be demonstrated. The lambs from this flock were around 1.5 weeks older than lambs from the other flocks, and might have started ingesting solid feed, such as concentrates, prior to turnout. Additionally, these lambs had access to wood shavings during the indoor period, which may also have affected the blood parameters.

The farmers reporting use of iron supplementation in the questionnaire were mainly located in the inland, mountainous areas (Oppland and Hedmark), whereas the field trial was performed in the Southwest coastal area (Rogaland). This geographical difference might have affected our findings, as significant climatic variations between the regions are known [53]. Likewise, differences in the iron concentration of feed crops may vary between areas [54]. However, this is unlikely to have had a significant effect on the iron levels of the young lambs in this study.

5 Conclusion

Iron supplementation was used by less than 10 % of the sheep farmers responding to the questionnaire, and the purpose of treatment was mainly to prevent abomasal bloat, but also coccidiosis. However, in the field study, iron supplementation did not affect excretion of *Eimeria* oocysts by lambs, nor was it associated with increased growth rates. These results indicate that iron supplementation of young lambs does not provide an appropriate alternative control strategy for prevention of coccidiosis. However, further studies are needed in order to verify this statement in flocks with different parameters, by

including more flocks, preferably from different geographical regions, and using higher or repeated doses of iron.

6 DECLARATIONS

Ethics approval and consent to participate: The study was approved by the Norwegian Animal Research Authority (ID 8535).

Consent for publication: Not applicable

Availability of data and material: The datasets used and analysed during the current study is available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contribution: All authors participated in the planning of the study. AO and SKN performed the study. AO did the statistical analysis and drafted the manuscript. All authors have critically read and approved the final manuscript.

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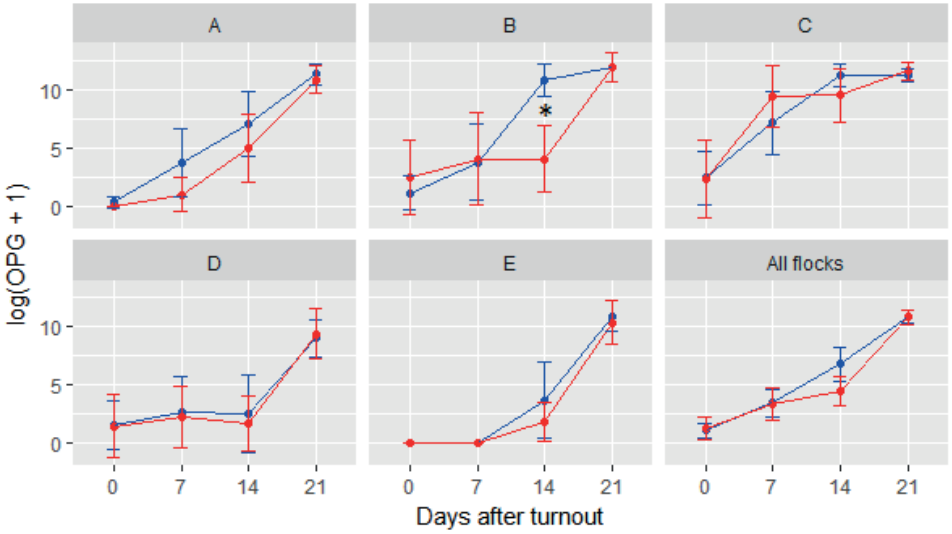

Figure 1. Mean oocyst excretion in 102 twin lambs supplemented subcutaneously with iron (red) or saline (blue). Lambs from five Norwegian sheep flocks (A-E) with known coccidiosis problems were sampled at day 0, 7, 14 and 21 after turnout. *$p < 0.05$

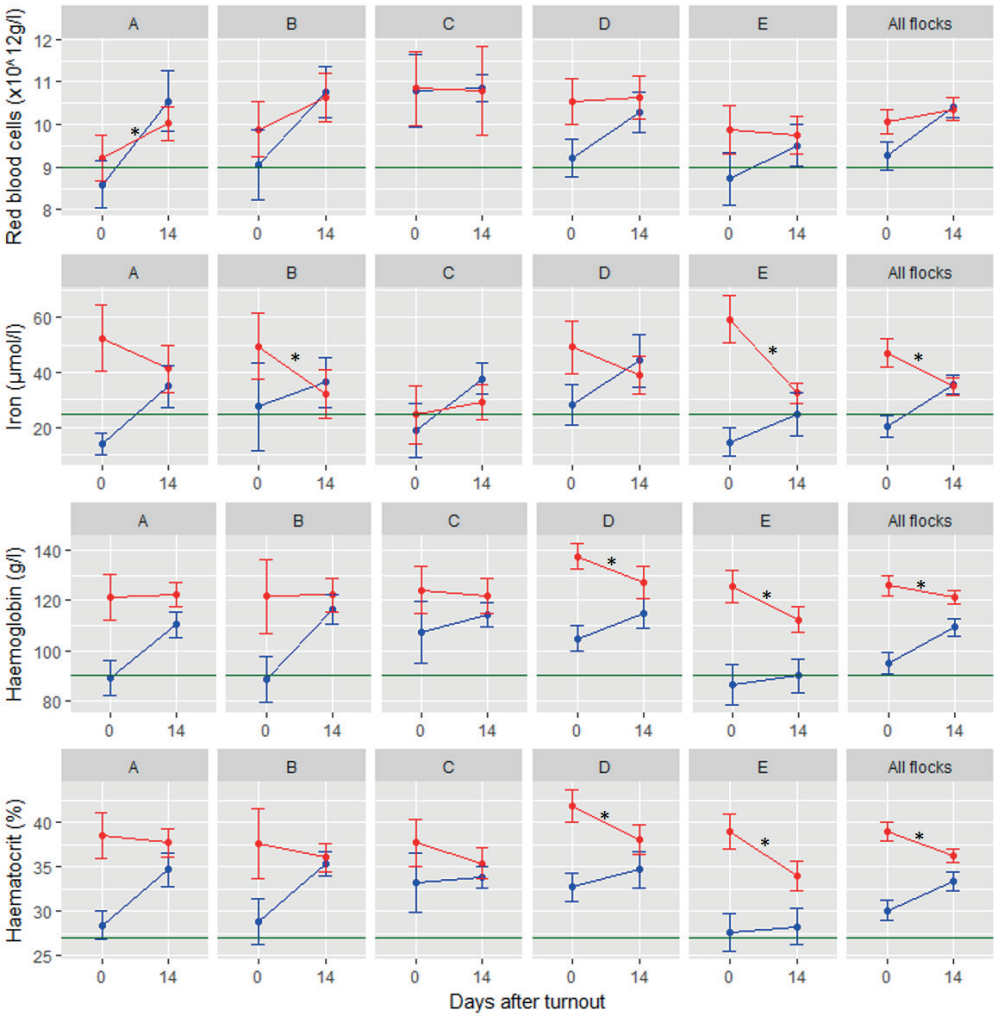

Figure 2. Mean blood levels of red blood cells, iron, haemoglobin and haematocrit with 95 % confidence intervals for twin lambs in the five included flocks (A - E) at day 0 and 14 after turnout. Half of the lambs were supplemented with iron 16-31 days before turnout. Red: iron supplemented lambs, blue: control lambs, green line: lower reference limit (internal references). * Significant difference in the treated group between samplings ($p < 0.05$).

Additional file

A translated copy of the questionnaire sent to members of the Norwegian sheep recording system (n=4993).

1. In which county is your farm located?
2. How many winter fed ewes do you have?
3. Did you supplement lambs with iron (injection/oral) in 2017?
    - Yes
    - No

If yes:

4. How was iron administered?
    - Orally
    - Injected
5. What was the purpose of the treatment?
    - Against abomasal bloat
    - Against coccidiosis
    - Against both abomasal bloat and coccidiosis
    - Other reason, please describe
6. Do you think supplementation had the effect you wanted?
    - Yes
    - No
7. Will you supplement lambs next year?
    - Yes
    - No
8. How old were the majority of your lambs at turnout?
    - 0-7 days
    - 8-14 days
    - 15-21 days
    - 22-28 days
    - 29-35 days
    - 36-42 days
    - > 42 days
9. Any additional comments regarding iron supplementation of lambs?

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