A SYSTEMATIC REVIEW AND META-ANALYSIS OF FACTORS ASSOCIATED WITH ANTHELMINTIC RESISTANCE IN SHEEP

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SUMMARY

Anthelmintic resistance (AR) in sheep gastro-intestinal nematodes has been reported worldwide. In this study, frequency of treatment, drench-and-shift pasture management, and use of long-acting formulations were positively associated with AR, while there was conflicting evidence on mixed-species grazing. Moreover, scant evidence was found on the association between AR and other commonly recommended practices, such as rotation of drug classes or use of combination anthelmintics, suggesting the need for further research. Findings of this study also highlight the need to improve the study design and/or reporting of research done in the field of veterinary parasitology, so results can be used to support evidence-based decisions regarding AR management.

INTRODUCTION

Disease due to gastro-intestinal nematodes (GINs) is a major economic constraint to grazing sheep production worldwide (Knox et al., 2012). Traditionally, GINs have been controlled with broad-spectrum anthelmintic drugs, which are inexpensive and easy to use (Sargison, 2011). However, the routine use of anthelmintic drugs has led to the development of Anthelmintic Resistance (AR) whereby anthelmintic drugs have a reduced or no effectiveness against GINs present within the animal (Morgan, 2013).

Anthelmintic resistance is now considered the *status quo* in most sheep-rearing countries (Kaplan and Vidyahsnakar, 2012), and repeated cross-sectional studies in Europe and South America have shown a worsening situation, with both multi-drug and multi-species resistance increasingly more common (Papadopoulos et al., 2012; Torres-Acosta et al., 2012). A recent study by Scott et al. (2013) documented the first field case of monepantel resistance, a novel anthelmintic drug which has only been commercially available since 2009. All this highlights the urgent need to identify risk factors associated with AR development, to inform future recommendations on sustainable parasite control (Morgan, 2013).

Current recommendations to delay further development of AR are based either on evidence regarding key molecular processes involved in the selection for resistance (Dobson et al., 2001), or simulation models (Leathwick et al., 1995). More recently, several

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observational studies and clinical trials have been described (Suter et al., 2004; Leathwick et al., 2008; Waghorn et al., 2009; Calvete et al., 2012). However, these studies are often based on single populations, and sometimes provide conflicting evidence. By way of example, mixed-species grazing has been described as both a protective and risk factor for AR in different studies (Eddi et al., 1996; Lawrence et al., 2006). Thus, there is a need for better evidence regarding these practices.

Systematic Reviews (SRs) and Meta-Analyses (MAs) are considered the best studies for providing high-quality evidence across published literature. By this process, the current literature on a particular topic can be summarized using a standardized scientific approach and so is more informative and truthful than a subjective review of the published literature. Systematic reviews are guided by a pre-defined research question and search strategy, thus ensuring the repeatability and transparency of the process, while the quantitative synthesis of results through the MA improves the precision and external validity of the pooled estimate (Sargeant et al., 2006).

The objective of this study was to perform a SR-MA on factors associated with AR in sheep. Specifically, the objectives were: (i) to review; (ii) critically appraise; and (iii) provide a qualitative and quantitative synthesis of all currently available literature on the topic.

MATERIALS AND METHODS

Research question, search terms and literature search

A protocol was developed, and the research question and search terms were defined following consultation with experts in the field. The population of interest was sheep; the outcome was AR in GINs of economic interest (noted below); while the intervention of interest was factors associated with AR previously described in the literature. The full PubMed search string was: ["small ruminants" OR ovine OR sheep OR ewe* OR ram OR lamb OR wether] and [anthelmintic OR drench OR "macrocyclic lactone*" OR benzimidazole* OR levamisole OR ivermectin OR thiabendazole] AND [[[gastrointestinal OR internal] AND [parasite*]] OR nematode* OR roundworm* OR haemonchus OR trichostrongylus OR teladorsagia OR ostertagia OR oesophagostomum OR chabertia OR nematodirus OR cooperia] AND [resistance OR resistant] AND [breed OR age OR gender OR refugia OR “risk factor” OR association OR exposure OR prevalence OR incidence OR survey OR management OR “anthelmintic dosage” OR drench OR bolus OR “under dosing” OR "route of administration" OR “oral formulation” OR “persistent” OR "long-acting" OR “timing of treatment” OR “pre-lambing treatment” OR “treatment of adult ewes” OR “quarantine” OR “mixed species grazing” OR “rotational grazing” OR "strategic treatment" OR “targeted selective treatment” OR “pasture management” OR “flock size” OR rotation].

A systematic literature search for all publications published between 1st January 1975 to 8th February 2013 was conducted in four electronic databases (PubMed [Medline], Agricola [EBSCO Host], CAB Direct, and Web of Science). Additionally, recent literature reviews and conference proceedings on the topic were hand-searched for relevant studies. Finally, search verification was performed by consulting several experts in the field, and through solicitation of information on two list-serves dedicated to the discussion of small ruminant clinical problems (American Association of Small Ruminant Practitioners®, United States and

SheepVets®, United Kingdom). All citations were imported into Refworks© (ProQuest, LLC, Cambrdige Information Group; Betheseda, MD, USA) and de-duplicated.

Abstract and full-text screening

References were screened at the abstract level, and, if they met the inclusion criteria defined below, at the full-text level. Screening was conducted by two independent investigators (LCF and AM) using pre-defined screening tools in an electronic SRS nexus review format (Möbius Analytics, Ottawa, Ontario, Canada). Cohen's Kappa (Cohen, 1960) agreement between reviewers was computed, and a $\kappa \geq 0.8$ was considered acceptable.

References were considered eligible if they: (i) described primary research on risk or protective factors of interest associated with AR in GINs of interest in sheep; and (ii) reported observational, randomized controlled or challenge trial designs; there were no language restrictions. All other references were excluded, and the reasons for exclusion were noted. Any discrepancy regarding a study's eligibility was resolved by discussion between the two investigators; if consensus was not reached, further adjudication by a third investigator (TJO) was conducted.

Risk of systematic bias and data extraction

Two independent investigators (LCF and TJO) assessed all relevant publications for risk of systematic bias using two instruments for experimental (Higgins et al., 2011) and observational studies (Kim et al., 2013), respectively. Domains considered for potential bias included: selection, performance, detection, attrition and reporting bias. Publications were then classified as having a high, low or unclear (not reported or unable to assess) risk of bias in each domain based on pre-set definitions.

Qualitative data extraction, also performed by two independent investigators (LCF and TJO), was carried out for information on: (i) study characteristics (language and year of publication, study design and geographical location); (ii) animal characteristics (number of animals in each treatment and control group and number of animals per farm); (iii) type of protective or risk factor; and (iv) type of outcome measurement (anthelmintic drug(s) investigated, diagnostic test used to define AR, and GIN species investigated). Publications reporting more than one independent study were duplicated and extracted as separate studies, while multiple publications reporting on the same study were extracted as a single study.

Quantitative data extraction and meta-analysis

Full-text publications were included in the quantitative synthesis if they: (i) measured AR using a methodology defined *a priori* in the protocol; (ii) investigated a factor of interest; and (iii) reported quantitative results in sufficient detail to estimate the odds of AR in sheep compared to a control group.

If controlling for confounding was performed, adjusted odds ratios (ORs) and covariates were extracted as reported in a study. Otherwise, unadjusted estimates were extracted. If the OR was not reported it was estimated using raw data. Authors were not contacted to add any additional data or offer clarification due to time limitations of the present study.

When factors were reported in more than one independent study, a random effects MA to account for heterogeneity was used to estimate a pooled effect size (ES) for each factor. Statistical heterogeneity (i.e. how much variation between studies that is not due to chance, but rather to other unmeasured factors like differences in the study design or study population) was assessed using the I^2 statistic, and a χ^2 test was used to assess statistical significance of heterogeneity within each analysis ($p \leq 0.05$) (Borenstein et al., 2009). Sensitivity analyses through step-wise removal of the publications were performed to determine whether certain publications had substantial impact on the ES estimated in each analysis. Funnel plots were not assessed for publication bias determination as there were fewer than 10 studies for each included factor (Higgins and Green, 2011). Analyses were conducted in STATA version 12.0 (StataCorp, College Station, Texas, USA) and RevMan (Version 5.2; The Cochrane Collaboration, Oxford, UK).

RESULTS

Literature search, abstract and full-text screening

The search provided a total of 2,914 citations, while another 5 citations were identified through the search verification process. After de-duplication, 1,712 titles and abstracts were screened, of which 131 full-text publications were further evaluated for relevance. Thirty publications were included in the risk of bias assessment and qualitative data extraction, while the remaining 101 full-text publications were excluded because they: (i) only described factors and not AR prevalence (n=30); (ii) only described AR prevalence and not factors of interest (n=23); (iii) measured a non-relevant factor or outcome (n=23); or (iv) reported on a non-relevant study design (n=16). Three additional publications were excluded because there were no resources for translation (one Arab and two Slovak publications), while another six publications could not be retrieved.

Risk of systematic bias and data extraction

Thirty publications describing 25 individual studies were included in the methodological assessment for risk of bias; of these, 15 were observational (14 cross-sectional and 1 cohort study), while 10 were experimental (3 controlled and 7 challenge trials). Unclear (not reported, or unable to assess) and high risk of selection bias based on selection of participants was found in 20% (3/15) and 73% (11/15) of the observational studies, respectively, while 60% (9/15) had a high risk of selection bias based on assessment of confounding variables. Unclear and high risk of performance bias was identified in 40% (6/15) and 13% (2/15) of the observational studies, respectively. All observational studies were found to have a low risk of detection bias, while an unclear or high risk of attrition bias was identified in 7% (1/15) and 13% (2/15) of the studies, respectively. Unclear risk of reporting bias was identified in one observational study. All trials were found to have unclear risk of selection bias and a low risk of detection, attrition and reporting bias.

The descriptive characteristics of the observational and experimental studies included in the qualitative synthesis of this systematic review are presented in Table 1.

Table 1. Descriptive characteristics of the 15 observational studies and 10 trials included in a systematic review of factors associated with anthelmintic resistance in sheep

Study (Author and year)	Study design	Country	Protective and risk factors reported	Anthelmintic drug	Outcome measurement	Type of GIN[a] recovered
OBSERVATIONAL STUDIES:						
Bartley et al. (2003)	Cross-sectional	Scotland	Farm type	Benzimidazole	EHA[b]	Not specified
Calvete et al. (2012)	Cross-sectional	Spain	Frequency of treatment; mixed-species grazing; rotation of anthelmintic drug-classes; under-dosing	Benzimidazole	EHA[b]	Not specified
Chartier et al. (1998)	Cross-sectional	France	Frequency of treatment	Fenbendazole	FECRT[c]	Not specified
Echevarria et al. (1996)	Cross-sectional	Brazil	Flock size; frequency of treatment	Ivermectin, Benzimidazole, Levamisole, Closantel, Combination (Albendazole+ Levamisole)	FECRT[c]	Not specified
Eddi et al. (1996)	Cross-sectional	Argentina	Flock size; frequency of treatment; mixed-species grazing	Benzimidazole, Ivermectin, Levamisole, Combination (Albendazole+ Levamisole)	FECRT[c]	Not specified
Hughes et al. (2007)	Cross-sectional	New Zealand	Quarantine; drench gun calibration; drench-and-shift; mixed-species grazing; long-acting formulations; frequency of treatment	Ivermectin	FECRT[c]	Not specified
Kettle et al. (1981 & 1982)	Cross-sectional	New Zealand	Frequency of treatment	Thiabendazole, Levamisole	FECRT[c]	Not specified

Kumar and Yadav (1994)	Cross-sectional	India	Frequency of treatment	Benzimidazole	FECRT[c]	Not specified
Lawrence et al. (2006) & Waghorn et al. (2006)	Cross-sectional	New Zealand	Long-acting formulations; mixed-species grazing; quarantine	Ivermectin	FECRT[c]	Not specified
Mitchell et al. (2010)	Cross-sectional	Wales	Farm type	Benzimidazole, Levamisole	LDA[d]	Not specified
Nari et al. (1996)	Cross-sectional	Uruguay	Frequency of treatment; flock size	Albendazole, Levamisole, Ivermectin	FECRT[c]	Not specified
Niciura et al. (2012) & Verissimo et al. (2012)	Cross-sectional	Brazil	Quarantine; rotational grazing; Farm type; drench-and-shift; mixed-species grazing; targeted treatment; rotation of anthelmintic drug classes; combination drug formulations; under-dosing	Benzimidazole	Genotyping of F200Y polymorphism	*Haemonchus* sp.
Rendell et al. (2006)	Retrospective cohort	Australia	Long-acting formulations	Ivermectin	FECRT[c]	*Ostertagia* sp.
Suter et al. (2004 & 2005)	Cross-sectional	Australia	Mixed-species grazing; quarantine; frequency of treatment; rotation of anthelmintic drug classes	Ivermectin	FECRT[c]	Not specified
Swarnkar & Singh (2010)	Cross-sectional	India	Flock size; frequency of treatment	Benzimidazole, Tetramisole	FECRT[c]	Not specified
TRIALS:						
Leathwick et al. (2006)	Controlled trial	New Zealand	Timing of treatment; targeted selective treatment; long-acting formulations	Albendazole	FECRT[c]; LDA[d]	*T. circumcincta; T. colubriformis*
Leathwick et al. (2012)	Controlled trial	New Zealand	Targeted selective treatment; Combination drug formulations	Ivermectin; Levamisole	FECRT[c]; LDA[d]	*T. circumcincta; T. colubriformis*

Martin et al. (1982 & 1984)	Controlled trial	Australia	Drench-and-shift	Thiabendazole	EHA[b]; FECRT[c]	Not specified
Leignel et al. (2010)	Challenge trial	France	Rotation of anthelmintic drug classes	Levamisole; Benzimidazole	FECRT[c]	*T. circumcincta*
Le Jambre et al. (1999)	Challenge trial	Australia	Long-acting formulations	Ivermectin; Moxidectin	Counting of resistant parasite strains	Not specified
Martin (1989)	Challenge trial	Australia	Drench-and-shift	Thiabendazole	EHA[b]; FEC[e]	*Teladorsagia* sp.
Sutherland et al. (2000)	Challenge trial	New Zealand	Long-acting formulations	Ivermectin; Albendazole	EHA[b]; FEC[e]	*T. circumcincta; T. colubriformis*
Waghorn *et al*. (2008)	Challenge trial	New Zealand	Drench-and-shift	Albendazole	EHA[b]; LDA[d]; FEC[e]	*T. circumcincta; T. colubriformis*
Waghorn et al. (2009)	Challenge trial	New Zealand	Targeted selective treatment; combination drug formulations	Ivermectin; Levamisole	FECRT[c]; LDA[d]	*T. circumcincta; T. colubriformis*
Waller et al. (1989)	Challenge trial	Australia	Frequency of treatment; drench-and-shift; rotation of anthelmintic drug classes	Thiabendazole; Albendazole; Levamisole; Ivermectin	EHA[b] LDA[d]; TBA[f]	*H. contortus; T. colubriformis*

GIN[a]=Gastro-Intestinal Nematode; EHA[b]=Egg Hatch Assay; FECRT[c]=Fecal Egg Count Reduction Test; LDA[d]=Larval Development Assay; FEC[e]=Fecal Egg Counts; TBA[f]=Tubulin Binding Assay

Meta-analysis

Publications reporting observational study designs (n=13) describing 10 individual studies were included in the quantitative synthesis, of which only one (Lawrence et al., 2006) provided adjusted estimates, controlling for the season in which AR was measured and the use of purposive sampling. All 10 studies reported observations at the aggregated (farm) level.

The 10 studies included described 8 factors; however, 3 of these factors were each described in one study only, and could therefore not be synthesized. These included: calibration of the drench gun (OR=3.11; 95% CI= 0.95, 10.15) (Hughes et al., 2007), use of quarantine strategies (OR=0.26; 95% CI=0.09, 0.66) (Suter et al., 2004 & 2005), and annual rotation of anthelmintic drug classes (OR=1.48; 95% CI=0.88, 4.26) (Suter et al., 2004 & 2005).

Random-effects MA were performed for the other five factors: frequency of anthelmintic treatment; mixed-species grazing; flock size; use of long-acting formulations; and drench-and-shift pasture management. Figure 1(a-e) below shows the individual and pooled estimates of the studies included (OR and 95% CI), and the heterogeneity of the estimate (I^2 and associated p-value), for each of these factors. In each forest plot, the center of the square represents the individual estimate for that study and the area of the square is proportional to the weight assigned to that study (based on (i) the number of observation units in the study and (ii) confidence intervals). The solid vertical line marks the value at which the factor has no effect on AR levels, while the diamond (◊) at the bottom shows the 95% CI for the pooled estimate.

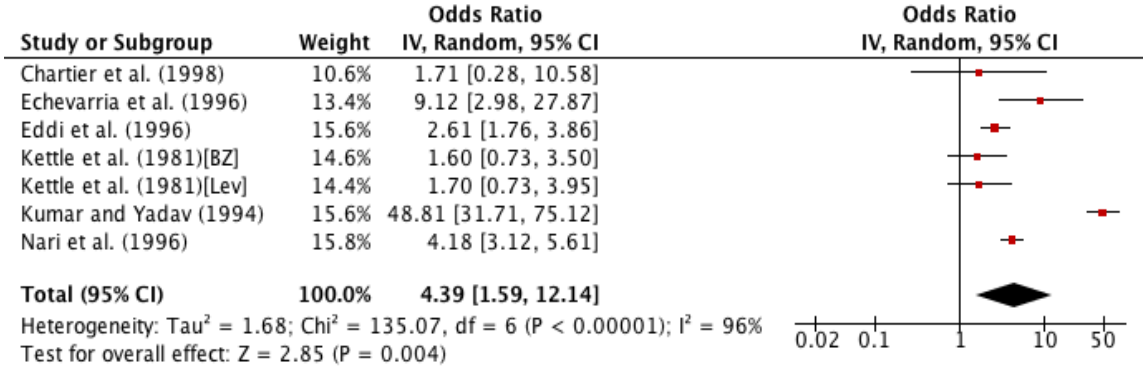

Fig. 1a Forest plot for studies included in the meta-analysis evaluating the association between anthelmintic resistance in sheep and frequency of anthelmintic treatment

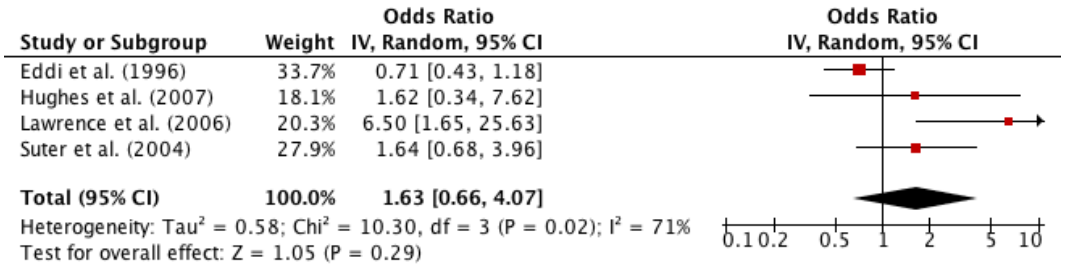

Fig. 1b Forest plot for four studies included in the meta-analysis evaluating the association between anthelmintic resistance in sheep and mixed-species grazing

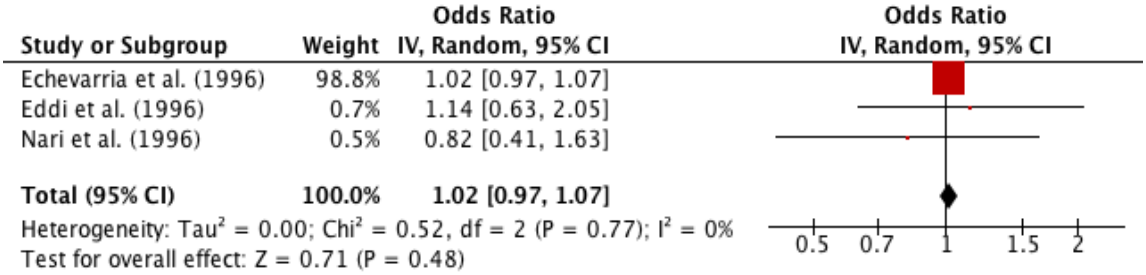

Fig. 1c Forest plot for three studies included in the meta-analysis evaluating the association between anthelmintic resistance in sheep and flock size

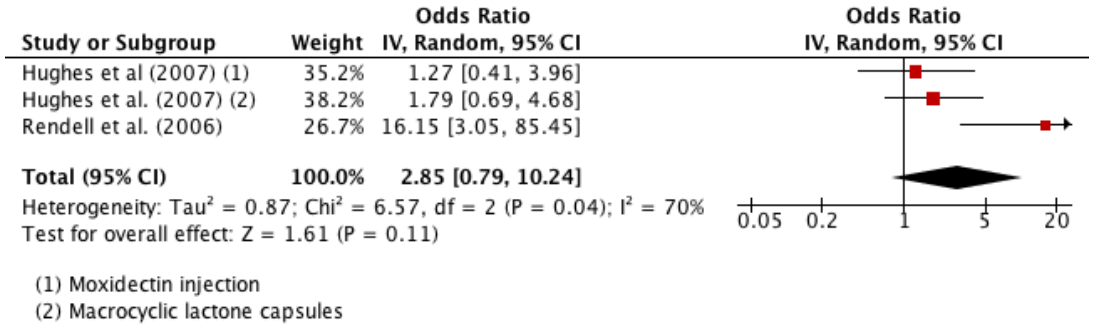

Fig. 1d Forest plot for studies included in the meta-analysis evaluating the association between anthelmintic resistance in sheep and long-acting formulations

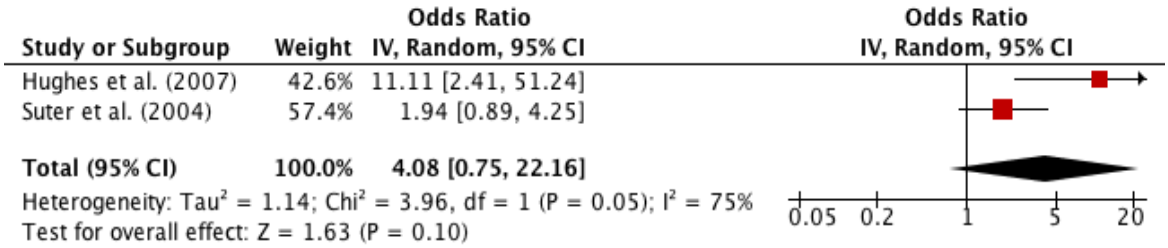

Fig. 1e Forest plot for studies included in the meta-analysis evaluating the association between anthelmintic resistance in sheep and the practice of drench-and-shift

Sensitivity Analysis

The sensitivity analysis using stepwise removal of publications for frequency of treatment showed that removal of estimates by Kumar and Yadav (1994) significantly reduced the pooled estimate (OR=3.67; 95% CI= 2.33, 5.78) and the observed heterogeneity (I^2=56%; p=0.08). Similarly, the removal of Rendell et al. (2006) from the MA for use of long-acting formulations significantly reduced the overall pooled estimate (OR=1.55; 95% CI=0.75, 3.23) and the observed heterogeneity (I^2=0%). No other significant changes were observed when the analyses for the other three factors were repeated with step-wise omission of study estimates.

DISCUSSION

This SR-MA provides further evidence on the association between certain management practices and AR in sheep, while highlighting future research needs. Despite the current relevance of the topic for the sheep industry worldwide, there were surprisingly few studies that investigated the association between putative risk factors and AR in sheep, and fewer still provided sufficient data for a quantitative synthesis.

A high level of heterogeneity in the results was observed in most of the MA results. This was to be expected, as all of the studies included were observational studies. While some authors argue that observational studies should not be included in a MA (Lean et al., 2009), others justify their inclusion since certain risk factors can only be investigated this way (Stroup et al., 2000). Given the limited number of studies included in this MA, sub-group analysis or meta-regression to further investigate the heterogeneity between the studies was not possible. Apart from heterogeneous study populations and independent research groups, other possible explanations for the observed heterogeneity are the use of different definitions for putative risk factors by the different studies, and the use of multiple diagnostic tools for AR. The latter is of particular concern, as there is currently no standardized test for AR diagnosis, greatly impeding the comparison of study results (Knox et al., 2012).

Frequency of treatment was the factor most commonly described in the included publications, which is not surprising as for a long time this has often been incriminated as one of the most important contributors of AR development (Sutherland and Scott, 2010). Higher treatment frequencies increase the selective advantage for resistant parasites, allowing for an increase in the proportion of resistant parasites over time (Sargison, 2011). In this study, it was found that flocks that were treated more frequently had higher odds of having resistance, compared to those farms that treated the sheep less often. Therefore, producers should be encouraged to use fecal egg counts or other physiological indicators of parasitism to identify and treat the more heavily parasitized animals, thus avoiding unnecessary treatments (Kenyon and Jackson, 2012).

Both mixed-species grazing and drench-and-shift management practices are based on the concept of *refugia*. This term refers to the proportion of susceptible parasites that are not exposed to anthelmintic treatment, either because they are found in the environment or because they are in untreated animals (van Wyk, 2001). These parasites therefore constitute a reservoir of susceptible genes. Mixed-species grazing with cattle has long been hypothesized as a protective factor for AR, as cattle are generally unaffected by sheep GINs and can therefore be used to reduce parasitic challenges on pasture (Eddi et al., 1996). However, recent evidence indicates that co-grazing sheep with cattle may actually increase AR levels, as it results in a reduction in the number of parasites in *refugia* on pasture (Lawrence et al., 2006). Results from our study also showed higher odds of AR on farms that practiced mixed-species grazing, though this estimate was not statistically significant, likely due to the limited number of studies. Further research is therefore required to establish the true association between mixed-species grazing and AR.

Drench-and-shift was commonly practiced in the 1980s and 1990s, whereby producers were encouraged to treat their animals with anthelmintic drugs immediately before moving them to clean pastures to reduce GIN re-infection. However, this practice has been found to be positively associated with AR, as resistant parasites that survive the anthelmintic treatment have a selective advantage when put on clean pastures with no susceptible parasites in *refugia*

(Abbott et al., 2009). Results from the present study show that the odds of having AR were four times higher on farms that practiced drench-and-shift, compared to those farms that did not. While the pooled estimate was not statistically significant, it does suggest that this practice is associated with AR and should therefore be discouraged.

Another factor frequently investigated in the included publications was the use of long-acting anthelmintic drugs, which delay GIN re-infection. However, as the anthelmintic concentration subsides, both homozygous and heterozygous resistant parasites have a selective advantage over homozygous susceptible parasites, thus allowing for an increase in the number of resistant parasites (Sutherland and Scott, 2010). Results from the present study showed a marginally significant positive association between the use of long-acting formulation drugs and AR, which further provides evidence that these formulations could be positively associated with AR. They should therefore only be used when the *refugia* status of the farm is known to be high through grazing management history and repeated fecal egg counts (Sargison et al., 2010).

Three studies reported the association between flock size and AR, as higher flock sizes are usually associated with higher stocking rates and, hence, more frequent treatments (Eddi et al., 1996). However, the MA pooled estimate showed no association between flock size and AR levels. Several other factors could not be investigated in the MA, either because they were only reported in one study, or because they were not reported in sufficient detail to allow for data extraction and synthesis. This highlights the need for further research and better reporting on some of these factors, such as the use of combination anthelmintics or annual rotation of anthelmintic drug classes.

There was a variable risk of systematic bias in many studies, which is similar to findings reported by other recent SR-MAs in the veterinary parasitology field (Mederos et al., 2012; Belo et al., 2013). Most of the observational studies included in this review had a high or unclear risk of selection bias as there was no justification for the choice of the study population, and confounding was rarely taken into account. An unclear risk of selection bias was also identified in all of the trials, as none adequately described how blinded randomization was achieved. The STROBE (Vandenbroucke et al., 2007) and REFLECT (O'Connor et al., 2010) reporting guidelines for observational studies and clinical trials have been published recently to help offset this deficiency in reporting. However, Cobo et al. (2011) noted that few authors currently implement these guidelines, and suggested that either the authors are unaware of their existence, or the guidelines are too hard to include in the writing stages if they have not been adequately addressed during the study design and implemenation. It is thus important that researchers are familiar with these guidelines early in the research process.

Publication bias could not be investigated in this study due to an insufficient number of studies, and this may have influenced the estimates reported in this study. Moreover, though there were no exclusion criteria based on language, three full-text articles that passed through the abstract screening were excluded since translation was not possible, and this might have introduced language bias. Since most relevant studies had an unclear to high risk of bias in many of the domains investigated, it was decided not to restrict the MA to studies with low bias; however, the high risk of bias identified in these studies may have influenced the pooled estimates. Lastly, the high levels of heterogeneity observed in the MA suggest that the pooled estimates should be interpreted with caution.

In conclusion, this SR-MA provides an important overview of the evidence currently available on factors associated with AR in sheep, while highlighting the need for further research, particularly on the impact of mixed-species grazing on AR levels as this management practice is often recommended for sustainable parasite control on both conventional and organic sheep farms. Findings from this study also underscore the need to improve methodological reporting in the field of veterinary parasitology and to standardize methods used for the detection of AR, thus allowing for a better comparison and synthesis of results from different studies.

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