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REVIEW ARTICLE

Drug resistance in parasitic helminths of veterinary importance in Chile: status review and research needs

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ABSTRACT. The increasing development of anthelmintic resistance (AR) in parasites of livestock is threatening animal health and production worldwide. In Chile, studies evaluating the field efficacy of anthelmintics and the detection of AR have been performed since the 2000s, but until now, no previous attempt has tried to systematise the available information. This article reviews general concepts about AR in helminths of veterinary importance, methods for diagnosis of AR and summarises the published reports of AR in Chile. Anthelmintic resistance in Chile has been reported in gastrointestinal nematodes of horses (benzimidazole resistance) and ruminants (sheep and cattle, macrocyclic lactone and benzimidazole resistance). However, these cases involved a limited number of selected farms and no further conclusions can be made of the status of parasite drug resistance at a regional or national level. No published cases of AR in *Fasciola hepatica* have been reported in Chilean livestock, but human infections with triclabendazole-resistant *F. hepatica* have been described in patients with previous consumption of watercress or untreated water from marshes grazed by livestock. Given the zoonotic potential and endemic nature of *F. hepatica* in Chile, it is urgent to determine the extent of liver fluke resistance. Current research gaps of the situation of AR in Chile and suggestions for the performance of laboratory and field studies are further discussed.

Key words: anthelmintic resistance, nematodes, livestock, Chile.

INTRODUCTION

Helminth parasitism remains one of the most prevalent infections in grazing animals and outdoor-reared livestock worldwide, inducing subclinical and clinical disease that threatens animal health, welfare and food production (Fitzpatrick 2013, Charlier *et al* 2017). Parasitic nematodes and trematodes can induce severe pathophysiological damage in infected livestock, leading to lower animal growth rate, poor body condition, dull hair coat, loss of carcass and wool quality, reduced milk yield, decreased pregnancy rate and longer calving to conception interval, anorexia, diarrhoea, anaemia, colic and in severe cases, even death of the animal (Vercruysse and Claerebout 2001, Waller 2004, Roepstorff *et al* 2011, Charlier *et al* 2014[b], Woodgate *et al* 2017). Furthermore, the effects of climate change are expected to affect parasite biology, grassland growth, length of the grazing season and animal husbandry management in very specific and complex interplays, which may have profound impacts on the infection dynamics of parasitic helminths (Morgan and Wall 2009, Dijk et *al* 2010, Phelan *et al* 2016). As a result, effective parasite control strategies are critical to sustain animal health and production, and therefore, to ensure the sustainability of veterinary interventions and livestock economies (Perry and Randolph 1999, Rist *et al* 2015, Nielsen 2015). In Chile, livestock farming is predominantly pasture-based, and animals of all ages are ubiquitously exposed to pathogenic parasitic worms, mainly to gastrointestinal (GI) nematodes and to the liver fluke *Fasciola hepatica*. As an example, liver infections with *F. hepatica* remain the main cause of organ condemnation in Chilean abattoirs, with 69.92% of all condemnations in 2016 with 435.784 animals affected nationally (~98% cattle and ~2% horses; SAG 2017).

Since the release of the benzimidazole drugs in the 1960's and the advent of ivermectin and the macrocyclic lactones in the 1980's (Egerton *et al* 1981), the use of synthetic anthelmintics has become the basis for the control of parasitic helminths of livestock worldwide (Waller 2006[a], Sutherland and Bullen 2015). The widespread success of anthelmintics for parasite control was the result of their broad therapeutic activity against different parasites species, high anti-parasitic efficacy (when released) and very low toxicity in treated animals. Farmers also rapidly adopted anthelmintics because they could be used in different production systems without requiring major changes in the husbandry practices (Nansen 1993). Despite significant research efforts on vaccine development (Matthews *et al* 2016), no commercially available broad-spectrum vaccines against parasitic helminths are expected to be released in the foreseeable future. Until novel, practical alternative control methods are available, it can be expected that parasite management in livestock will still be dominated by the use of synthetic anthelmintics. In Chile, three broad-spectrum anthelmintic classes with distinct modes of action are currently registered for use in livestock; the benzimidazoles (BZ, e.g. fenbendazole, albendazole), imidazothiazoles/tetrahydro-pyrimidines (LEV, e.g. levamisole) and the macrocyclic lactones (ML, e.g. ivermectin, moxidectin, eprinomectin, etc.). Additionally, two novel anthelmintic groups have been described: the amino-acetonitrile derivatives (ADD; Kaminsky *et al* 2008) and the spiroindoles (Little *et al* 2011), but these drugs are not yet available in Chile. In most countries, including Chile, the ML are the

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most widely used anti-parasitics in ruminants and horses, mainly due their high efficacy against adult and larval stages of parasitic nematodes and their persistent efficacy against re-infections, but also because of their effect against ectoparasites (Vercruysse and Rew 2002, Peña-Espinoza 2009, Márquez *et al* 2010). However, the sustainability of chemically-based parasite control is severely threatened by the development of drug resistance in parasitic nematode and trematode populations worldwide (Waller 2006[b], Kaplan and Vidyashankar 2012, Woodgate *et al* 2017) The situation is reaching alarming levels particularly in small ruminants, in which the therapeutic failure to all available drugs has been reported (Waller 2004, Sargison *et al* 2005). In Chile, studies evaluating the field efficacy of anthelmintics and the detection of drug resistance in livestock parasites have been performed since the 2000s, but until now, no previous attempt has tried to systematise the available information.

The objective of this article is to review general concepts about drug resistance in helminths of veterinary importance and to summarise the published reports describing cases of reduced drug efficacy and anthelmintic resistance in Chile. Finally, current knowledge gaps of the situation in Chile will be identified and suggestions for future research will be discussed.

ANTHELMINTIC RESISTANCE

Anthelmintic resistance (AR) has been defined as the capacity of a parasite population (or individual parasites within the population) to tolerate doses of an anthelmintic that would have otherwise killed a normal population from the same species, and to transmit this resistant fitness to their progeny (Prichard *et al* 1980). The accumulation of resistant genes in a helminth population is an evolutionary process depending on: a) the genetic diversity of the parasite populations under selection for AR, b) the selection pressure (i.e. anthelmintic treatment) and c) time (Prichard 2002). Therefore, it can be expected that parasite populations exposed to anthelmintic drugs will evolve gradually from fully susceptible to fully resistant, and at different speeds under distinct circumstances (Kaplan *et al* 2007).

The mechanisms of AR are intrinsically related with the mode of action of a drug against a particular helminth species and the ability of the parasite to overcome the drug activity. Moreover, the detailed understanding of these drug-resistance mechanisms provides essential insights towards the development of precise diagnostic techniques such as molecular methods. A comprehensive description of the mechanisms of drug activity and AR is not intended here and the interested reader is referred to the review by Kotze *et al* (2014).

Anthelmintic resistance is now a widespread problem for the control of GI nematodes of small ruminants in almost every region of the world, and is of increasing concern for the control of nematode infections in cattle, horses and pigs (Roepstorff *et al* 2011, Sutherland and Leathwick 2011, Nielsen *et al* 2014, Sutherland and Bullen 2015, Woodgate *et al* 2017). Drug resistance in trematodes, mainly triclabendazole-resistant *F. hepatica*, have also been reported (Kelley *et al* 2016; Greenberg and Doenhoff 2017). In Latin America, the first cases of BZ resistance in sheep nematodes were described in the 1960's and AR was considered a major problem for the small ruminant industry already 20 years ago (Waller *et al* 1996). In a series of reviews by Torres-Acosta *et al* (2012[a], 2012[b]), AR was found to be widely distributed, particularly in sheep nematodes but also in cattle worms. However, no cases from Chile were included in the mentioned article. In order to characterise the extent of drug resistance the first step is to diagnose the presence of AR in helminth populations, which will be discussed in the next section.

DIAGNOSTIC METHODS FOR ANTHELMINTIC RESISTANCE

A range of methods have been developed for the diagnosis of AR in veterinary parasites, mainly for GI nematodes. These techniques can be classified as *in vivo* and *in vitro* methods and have been extensively reviewed elsewhere (Taylor *et al* 2002, Coles *et al* 2006). Guidelines for the detection of AR were released by the World Association for the Advancement of Veterinary Parasitology (WAAVP, Coles *et al* 1992). To date, no new recommendations based on international scientific consensus have been elaborated yet. Updated guidelines for the diagnosis of AR in different animal species are needed to standardise the studies conducted in different regions of the world, aiming to compare their results, to include the novel methodologies developed since the early 1990's and to facilitate the performance of such studies at the farm and laboratory levels.

In vivo DIAGNOSTIC METHODS

Controlled efficacy test (CET). The CET is considered the 'gold standard' and the most reliable method to assess the efficacy of anti-parasitic drugs and to diagnose AR (Taylor *et al* 2002, Coles *et al* 2006). The method involves the anthelmintic treatment of naturally or experimentally infected animals and the following post-mortem worm recovery and identification of surviving (resistant) parasites. Anthelmintic efficacy is then calculated by comparing the number of parasites between treated and untreated control animals (Wood *et al* 1995). Detailed guidelines for the implementation and evaluation of CET in domestic animals have been published by the WAAVP (Jacobs *et al* 1994, Wood *et al* 1995, Duncan *et al* 2002, Yazwinski *et al* 2003, Hennessy *et al* 2006). However, the high costs of the CET in terms of labour, equipment and slaughter of animals, makes this technique unpractical for routine use in commercial farms and is almost completely restricted for research purposes.

Faecal egg count reduction test (FECRT). The FECRT is a clinical trial that estimates the efficacy of an anti-parasitic drug against patent helminth infections by comparing the parasite faecal egg counts (FEC) of treated animals before and after treatment or the FEC between treated and untreated animals (Coles *et al* 1992, Taylor *et al* 2002). Current guidelines for the design and analysis of the FECRT in livestock derived from recommendations developed for the detection of AR in sheep nematodes (Coles *et al* 1992). Based on this guidelines, AR is declared if a) the mean FEC reduction after treatment is lower than 95% and b) the 95% lower confidence interval is less than 90%; if only one criteria is met, resistance is only suspected (Coles *et al* 1992). Due to its feasibility and relatively lower cost compared with the CET, the FECRT is the most widely used method for detection of AR in the field and the only readily-available technique to diagnose drug resistance on farms (Coles *et al* 2006, Levecke *et al* 2012[a], George *et al* 2017). A well-designed and appropriately analysed FECRT can provide valuable information about the AR status in an animal group or farm. Moreover, a comparison of FECRT and CET results in the same studies reported a specificity (detection of truly susceptible worms) of 100% and sensitivity (detection of truly resistant worms) of 90-95% for the FECRT in relation with the 'gold standard' CET in sheep (McKenna 2006).

However, several considerations need to be bear in mind when conducting a FECRT. Anthelmintic drugs may cause the temporary suppression of egg output by resistant female nematodes that survived treatment; hence, the FECRT can give a false negative result if FEC are analysed during this period. In general, the temporary worm egg suppression lasts for 3 days after LEV treatment, 8 days after BZ treatment and 10-14 days after ML treatment (Coles *et al* 2006). If several anthelmintics are tested, a general rule is to perform the FEC 14 days post-treatment (Coles *et al* 1992). Yet, animals treated with LEV (which has no effect on immature nematode stages) should be sampled 7-10 days post-treatment to avoid the detection of eggs from females not affected by the drug during their larval development (De Graef *et al* 2012). Whereas for long-acting ML such as moxidectin, which induces a longer suppression of egg excretion than other ML drugs (De Graef *et al* 2012), FEC should be analysed 17-21 days post-treatment to avoid false negative results (Condi *et al* 2009, Kaplan and Vidyashankar 2012). In addition, the fecundity of some nematode species (e.g. *Ostertagia ostertagi* in cattle) can be influenced by density-dependent mechanisms, resulting in a reduced egg excretion following an increase in parasite burden, while an increased and/or highly variable fecundity could occur when low worm burdens are present (Michel 1969, Kotze and Kopp 2008). This density-dependent regulation of parasite fecundity has been related to intraspecific competition for limiting resources (e.g. food) and to enhanced host immune responses following an increased antigenic stimulation by high worm burdens (Keymer 1982). In the context of a FECRT, these may result in a reduced egg output by female nematodes when new infections are acquired between pre- and post-treatment sampling, whereas nematodes surviving a treatment could increase their fecundity due to lower worm crowding or/and competition (Dobson *et al* 2012, Bellaw *et al* 2018). Furthermore, the lack of specific guidelines to conduct and interpret a FECRT in animal species other than sheep have prompted warnings from researchers. For example, several possible biases have been highlighted concerning the use of FECRT in cattle, mainly due to the poor correlation between FEC and actual worm burden (due to density-dependent mechanisms as discussed above), the lower faecal egg excretion commonly detected in cattle as compared with sheep and the highly aggregated (over-dispersed) distribution of FEC between animals (Coles 2002, Coles *et al* 2006, Demeler *et al* 2010, El-Abdellati *et al* 2010, Sutherland and Leathwick 2011, Levecke *et al* 2012[a]). Caveats have also been raised for the FECRT in horses, particularly regarding the not linear correlation between worm burdens and FEC (Nielsen et *al* 2010) and the variability of FEC data (Vidyashankar *et al* 2012).

A reduced anthelmintic efficacy in a FECRT, or high variability in drug efficacies within an animal group, may be caused by reasons other than AR. A reduced *in vivo* efficacy in a FECRT could be consequence of under dosing (e.g. inexact weighing), variable fat reserves in different animals which may affect the persistent efficacy of some anthelmintics (such as ML), erratic absorption of compounds from the injection site and/or interactions with co-administered drugs (González Canga *et al* 2008, El-Abdellati *et al* 2010; Areskog *et al* 2012; De Graef *et al* 2013; Areskog *et al* 2014). Therefore, an essential requirement of the FECRT is that anthelmintic treatments should be administered at the recommended dose for the species and based on a precise weighing of the animals to avoid under-dosing. This consideration is also of particular importance when using the FECRT in goats due to the general lack of recommended anthelmintic doses for this species and the common use of the sheep dosages, which result in a lower bioavailability of anthelmintic drugs in goats and potentially leading to selection of AR due to exposure of parasites to sub-therapeutic doses (Hoste *et al* 2010, Paraud and Chartier 2017).

Another issue of the FECRT is the unreliable detection of AR when the drug efficacy is still high (80-95%), which prevents implementation of measures to avoid the progression of AR into treatment failure. To reduce the uncertainty of the FECRT, much information on the true drug efficacy can be gained when studying the 95% confidence interval of a treatment (Levecke *et al* 2012[a]). Robust statistical analyses using Bayesian modelling have been advocated to cope with aggregated and low FEC data and to increase the accuracy in detecting uncertainty intervals (Denwood *et al* 2010, Torgerson *et al* 2014). These powerful statistical methods

are been increasingly used for monitoring AR, particularly in cattle nematodes (Neves *et al* 2014, O'Shaughnessy *et al* 2014, Geurden *et al* 2015, Peña-Espinoza *et al* 2016[a], Ramos *et al* 2016). In addition, eggs from trichostrongyle GI nematodes are similar in size and shape (except the eggs from *Nematodirus* spp.), hence, the FECRT need to be combined with the identification of the resistant parasites surviving treatment. The most common procedure is the culturing of faeces to isolate infective third-stage (L3) larvae for morphological differentiation at the genus level (Coles *et al* 2006). However, different nematode species have distinct temperature and humidity requirements for hatching and larval development in faecal cultures, and this may result in the increased development and overrepresentation of some species over others if only one common culture procedure is used (Dobson *et al* 1992, Roeber and Kahn 2014). As alternative, novel molecular techniques have been developed for the sensitive and species-specific detection of the major parasitic nematodes of ruminants using eggs or larvae, and although some methods have already been evaluated in field samples (Höglund *et al* 2013, Peña-Espinoza *et al* 2016[a], Roeber *et al* 2017), these are yet to be adapted for routine use.

The FECRT has also been used to detect AR in *F. hepatica* (Alvarez-Sanchez *et al* 2006, Flanagan *et al* 2011, Novobilsky and Höglund 2015), although at present no guidelines have been adapted to detect drug resistance in this important zoonotic trematode (Coles *et al* 2006). However, if the efficacy of flukicidal drugs is being evaluated using FEC, it is recommended to compare the level of egg excretion in treated animals between the day of treatment and 21 days post-treatment (Wood *et al* 1995). A three-week period allows the removal of adult *F. hepatica* exposed to the treatment and the release of eggs concentrated in the gall bladder (Brockwell *et al* 2014). Detection of *F. hepatica*-coproantigens has also been evaluated in AR studies (Hanna *et al* 2015, Novobilsky *et al* 2016) and may provide an interesting alternative to FEC, particularly to detect immature stages of *F. hepatica* (Beesley *et al* 2017).

In vitro DIAGNOSTIC METHODS

Several *in vitro* tests have been developed for the assessment of phenotypic AR in GI nematodes of livestock (Taylor *et al* 2002, Demeler *et al* 2010, Matthews *et al* 2012). These methods involve the incubation of free-living stages (i.e. nematode eggs or larvae) in a range of different drug concentrations and the subsequent measurement of a vital characteristic (e.g. embryonation, larval development/motility). Results from these tests are then used to calculate the effective concentration of the drug able to induce an effect on 50% of the parasite population (EC_{50}). The EC_{50} values are then compared with results obtained in control assays using known susceptible/resistant strains or with established values from the literature. Some of the main *in vitro* diagnostic tests of AR are the egg hatch assay (EHA, for detection of BZ resistance), the larval development assay (LDA), the larval feeding inhibition assay (LFIA) and the larval motility inhibition assay (LMIA) (Coles *et al* 1992, Gill *et al* 1995, Alvarez-Sánchez *et al* 2005, von Samson-Himmelstjerna *et al* 2009[a], Demeler *et al* 2010). In comparison with the FECRT, the *in vitro* tests do not require the treatment of animals and only one faecal sample per individual is needed (from which different nematode stages can be cultured and isolated), reducing the costs of field sampling. However, and despite the *in vitro* tests are extensively used in parasitology research, these assays are still not widely available for routine use in diagnostic laboratories, mainly due to the need of advanced technical equipment and expertise.

At the moment, only the EHA can be reliably used in field samples due to the consistent EC_{50} threshold value related with BZ resistance observed in different GI nematode species from small ruminants, cattle and horses (>0.1 µg thiabendazole [TBZ]/ml; Coles *et al* 1992, 2006, von Samson-Himmelstjerna *et al* 2009[a], Demeler *et al* 2012). The EHA has also good correlation with molecular methods and the FECRT to detect BZ-resistant and susceptible nematode strains and has a high reproducibility between laboratories (von Samson-Himmelstjerna *et al* 2009[a], Demeler *et al* 2012). In addition, the EHA has been evaluated to detect triclabendazole-resistant in *F. hepatica* eggs (Fairweather *et al* 2012). One of the main limitations of the EHA is the need of unembryonated eggs still in the phase of anaerobic development for the test. As soon as eggs start embryonation, aerobic metabolism predominates and eggs become refractory to BZ drugs (Coles *et al* 2006). Therefore, eggs for the EHA should be isolated within 3 hours from rectal collection or stored in anaerobic conditions. Currently, the *in vitro* diagnosis of ML resistance (with LDA, LFIA or LMIA) in the field (involving mixed nematode species) is limited due to the distinct susceptibility of different parasite species to ML drugs (Gill and Lacey 1998), hindering the definition of general cut-off values related with ML resistance and requiring further standardisation (Demeler *et al* 2010).

MOLECULAR DIAGNOSTIC METHODS

Molecular methods for detection and quantification of resistant alleles or identification of genetic markers linked to drug resistance would be extraordinary tools to diagnose AR before it reaches therapeutic failure or becomes clinically noticeable in the FECRT. However, the molecular diagnosis of AR is linked to the comprehensive understanding of the mechanisms of action of each anthelmintic class and the genetic basis of AR, and this knowledge is currently restricted to the BZ drugs (Woodgate *et al* 2017). The BZ are known to selectively target the nematode β-tubulin, leading to inhibition of the microtubule formation and resulting in worm starvation,

inhibition of egg production and death (Martin 1997). A single nucleotide polymorphism (SNP) at the codon 200 of the nematode β-tubulin isotype 1 gene, resulting in a mutation from TTC (encoding phenylalanine[phe]) to TAC (encoding tyrosine[tyr]), confers resistance to BZ drugs in several nematode species (reviewed by Kotze *et al* 2014). Other SNPs at codon 167 and 198 in the same β-tubulin gene have been linked with BZ resistance (reviewed by Whittaker *et al* 2017). The SNP at codon 200 has allowed the development of allele-specific PCR and pyrosequencing assays for diagnosis of BZ resistance (Coles *et al* 2006, von Samson-Himmelstjerna *et al* 2009[b]). These methods have been successfully tested in the confirmation of BZ-resistant status of nematode strains selected in the field (e.g. Höglund *et al* 2009, Peña-Espinoza *et al* 2014). Despite vast research efforts have been made to detect markers for resistance to ML drugs, to date, no SNPs can be related with resistant phenotypes of parasitic nematodes, which could indicate that changes in specific target sites are not related with ML resistance (Kotze *et al* 2014). Recent studies have investigated the multigenic nature of ML resistance in nematodes, exploring the role of ATP-binding-cassette (ABC) transporters like P-glycoproteins in the active efflux of ML drugs by the nematode (Lespine *et al* 2012, Godoy *et al* 2016) and using genome-wide detection of SNPs (Luo *et al* 2017). However, once molecular diagnostic methods for ML are available, it will be necessary to correlate these assays with the phenotypic expression of AR in order to recommend farmers when to stop using a drug to prevent the further selection of resistance. This may be challenging if, for example, a molecular method indicates emerging levels of AR based in genetic markers to a drug with 95% of clinical efficacy (Kaplan and Vidyashankar 2012).

ANTHELMINTIC RESISTANCE IN CHILE: REPORTED CASES

A literature search was performed to identify peer-reviewed studies of drug resistance in veterinary helminths in Chile using common database sources (PubMed-NCBI, Web of Science, Google Scholar and Scielo). For the search, the keyword *Chile* was combined with the following terms (in English and Spanish): *anthelmintic resistance, parasite drug resistance*, *helminth resistance*, *nematode resistance*, *strongyle resistance*, *trematode resistance*, *Fasciola resistance*, *cestode resistance*. A total number of 5 publications from the period 2002-2014 were identified, but two of these articles reported the same results (von Samson-Himmelstjerna *et al* 2002[b], von Witzendorff *et al* 2003). A summary of the studies reporting original cases of AR in veterinary parasites in Chile are presented in table 1. Reduced drug efficacy and AR in veterinary helminths in Chile have been reported in sheep, cattle and horses. No cases of AR have been reported in other domestic animal species in Chile, but neither have been investigated. A detailed analysis of the published reports of AR in Chilean livestock will be presented here based on the host species.

HORSES

To date, only one study has reported AR in equine parasites in Chile, characterising BZ resistance in horse cyathostomins by FECRT, EHA and genotyping of resistant nematodes from three stud farms in the Valdivia and Llanquihue provinces (von Samson-Himmelstjerna *et al* 2002[b]). Results from the same study were also published elsewhere (von Witzendorff *et al* 2003). Unpublished theses have also described other potential cases of reduced field drug efficacy and AR in nematodes of Chilean horses. von Samson-Himmelstjerna *et al* (2002[b]) investigated the efficacy of oral fenbendazole (FBZ) treatments in naturally-infected horses (1-14 years-old) from three farms in Frutillar (n=24), Riñihue (n=28) and Valdivia (n=31). Pre-treatment larval cultures detected cyathostomin (small strongyle) L3 larvae in all farms and large strongyles L3 larvae only in the Frutillar farm. All farms had previous use of BZ drugs for parasite control, particularly in the Riñihue and Valdivia farms with an extensive use of these compounds for over 30 years. Efficacy of FBZ treatments (7.5 mg FBZ/kg) were evaluated with the FECRT by comparing the individual FEC of treated animals 7 days pre- and 7-11 days post-treatment. A poor drug efficacy was observed in the three farms, with the mean of the individual faecal egg count reduction (FECR) of 26.5%, 27% and 83.9% for the Riñihue, Valdivia and Frutillar farm, respectively. All horses in the Riñihue and Valdivia farms had an individual FECR below the minimum expected efficacy (<90%, Coles *et al* 1992), whereas 13 of 31 horses in the Frutillar farm had a FECR<90%, strongly indicating the presence of highly BZ resistant nematode populations, particularly in the first two farms. Post-treatment larval cultures revealed that only cyathostomin L3 larvae survived FBZ treatment in the three farms (von Samson-Himmelstjerna *et al* 2002[b]). The authors also investigated BZ resistance by the EHA with strongyle eggs from faecal samples with > 150 epg collected before and after treatment. Isolated eggs were incubated with decreasing concentrations of TBZ and resulted in EC_{50} values related with BZ resistance (> 0.1 µg TBZ/mL; Coles *et al* 1992) only in the Riñihue farm pre-treatment, and in the Valdivia and Riñihue farms post-treatment. Furthermore, DNA from single L3 cyathostomin larvae were isolated and used in an allele-specific PCR method for genotyping codon 200 of the β-tubulin (isotype 1 gene) to detect the mutation TTC (phe) to TAC (tyr). Genotyping of larvae from horses with 0% FECR after FBZ treatment revealed frequencies of 20.4% for tyr/tyr, 25.2% for phe/phe and 54.4% for phe/tyr (von Samson-Himmelstjerna *et al* 2002[a]). These results suggested that the SNP at codon 200 is not the only mutation related with BZ resistance in

Table 1. Reported cases of reduced field efficacy and anthelmintic resistance in gastrointestinal nematodes of livestock in Chile.

Host species	Farms	Provinces of Chile	Anthelmintics	Detection method	Treatment efficacy (FECR%)	Species surviving treatment (L3 larvae)	Reference
Horse	3	Valdivia, Llanquihue	FBZ	FECRT; EHA	FECRT: 27% (Valdivia); 26.5% (Riñihue); 83.9% (Frutillar) EHA (EC_{50} µg TBZ/mL) pre/post-treatment = 0.09/0.15 (Valdivia); 0.14/0.16 (Riñihue); 0.06/0.09 (Frutillar)	Cyathostomins (species n.r.)	von Samson-Himmelstjerna *et al* 2002[b]
Cattle	1	Osorno	IVM, MOX, ABM, DOR	FECRT	81-88% (IVM); 89% (MOX); 85% (ABM); 93% (DOR)	GI strongyle nematodes (species n.r.)	Sievers and Fuentealba 2003
Cattle	2	Osorno	IVM	FECRT	73.5-90.3%	*Cooperia spp.*	Sievers and Alocilla 2007
Sheep	1	Ñuble	IVM, FBZ	FECRT	77% (IVM); 74% (FBZ)	*Teladorsagia spp.* and *Trichostrongylus spp.* (FBZ group)	Toro *et al* 2014

FECR = faecal egg count reduction; FECRT = Faecal egg count reduction test; EHA = egg hatch assay; GI = gastrointestinal; L3 = third-stage larvae; n.r. = not reported; FBZ = fenbendazole; IVM = ivermectin; MOX = moxidectin; ABM = abamectin; DOR = doramectin; TBZ = thiabendazole.

horse cyathostomins, further demonstrated by Pape *et al* (2003). However, a lower frequency of expected fully susceptible genotypes (phe/phe) was detected in larvae from pre-treatment samples in the Valdivia and Riñihue farms (24.1% and 10.6%, respectively), in comparison with the Frutillar farm (47.7%), which correlates with the lower FECR% of FBZ and the history of heavy reliance on BZ drugs in the first two farms compared with the latter (von Samson-Himmelstjerna *et al* 2002[b]).

RUMINANTS

Gastrointestinal nematodes. Three published studies have declared the presence of AR in GI nematodes infecting ruminants in Chile (Sievers and Fuentealba 2003, Sievers and Alocilla 2007, Toro et *al* 2014). Unpublished theses have also explored the field efficacy of anthelmintic drugs and AR in cattle farms in Southern Chile. All these studies have investigated the presence of AR only by the FECRT. To date, no research has confirmed the presence of drug-resistant ruminant nematodes by CET or genotyping of resistant isolated from Chilean farms.

Sievers and Fuentealba (2003) studied the field efficacy of six formulations (subcutaneous) of either ivermectin (IVM), moxidectin (MOX), abamectin (ABM) or doramectin (DOR) against GI nematodes in naturally infected calves (~one-year old; n=84) in a farm from the Osorno province (Los Lagos Region). At day 14 post-treatment, the authors reported a mean FECR% of 94-100% for IVM, 98% for MOX and 100% for ABM and DOR treatments. Whereas at day 21 post-treatment, the authors reported mean FECR% of 81-88% for IVM, 89% for MOX, 85% for ABM and 93% for DOR treatments. In that study, the observed efficacy reductions between days 14 and 21 post-treatment correlates with the known inhibition of egg excretion by ML-exposed worm females, which results in an apparent higher efficacy at day 14 post-treatment and the resumption of egg excretion by the surviving worms at day 21 post-treatment. Despite the 95% confidence intervals of the FECR% were not described for any treatment, thus preventing the declaration of AR based on available guidelines, the reported results were generally below the threshold of mean FECR ≥ 95% expected with efficacious treatments (Sievers and Fuentealba 2003). Subsequently, Sievers and Alocilla (2007) investigated the presence of IVM-resistant GI nematodes in naturally infected calves (7-8 months-old; n=14/22) from two cattle farms in the Osorno province with distinct history of ML use. At 21 days post-treatment with IVM (subcutaneous), mean FECR% were 73.5% and 90.3% in the farms with intense and sporadic use of IVM, respectively, with identified nematodes surviving treatment from the genus *Cooperia* spp., *Trichostrongylus* spp. and *Nematodirus* spp. Although it is known that some *Nematodirus* and *Trichostrongylus* species infecting cattle are inherently more tolerant to IVM treatment (Egerton *et al* 1981, Campbell and Benz 1984), the reduced efficacy of IVM against *Cooperia* spp. in these farms may have been related with drug resistance. However, the 95% confidence interval of FECR% after ML treatments was not provided for any of the experimental groups and therefore no further conclusions can be made.

More recently, Toro *et al* (2014) evaluated the efficacy of IVM (subcutaneous) and FBZ (oral) in 6-8 months-old lambs (n=36) naturally infected with GI nematodes from a sheep herd in the Ñuble province (Bio Bio Region). At day 7 post-treatment, the authors reported a mean FECR% of 34% and 41% for IVM and FBZ treatments, respectively. Whereas at day 15 post-treatment, the authors reported a mean FECR% of 77% and 74% for IVM and FBZ treatments, accordingly (Toro et *al* 2014). Post-treatment larval cultures revealed the presence of *Teladorsagia* spp. and *Trichostrongylus* spp. L3 in faecal samples from FBZ-treated lambs, while no L3 where recovered from positive samples of IVM-dosed animals. Toro *et al* (2014) did report the 95% lower confidence interval of the mean FECR%, with values of 38% and 40% for IVM and FBZ treatments, respectively. However, the authors reported a common lower confidence interval value between two sampling dates (7 and 15 days post-treatment) instead of correctly presenting the data for each post-treatment date, as indicated in the current FECRT guidelines. Nevertheless, the study indicated the presence of nematodes resistant to IVM and FBZ in the investigated sheep farm (Toro *et al* 2014).

From the cited studies there is the indication of AR in the investigated ruminant farms, mainly towards ML. Although the findings need to be corroborated with CET or molecular methods, these studies highlight the potential poor efficacy of ML treatments in farmed ruminants in Chile. However, no further conclusions can be made of the status of drug resistance at a regional or national level. A first attempt of a regional study was a preliminary FECRT survey in 16 dairy farms in Los Rios Region (Southern Chile), which revealed that 9 out of 16 farms investigated had confirmed reduced efficacy to at least one anthelmintic drug (56.3%), with observed mean FECR% of 80.2-99.3% for benzimidazoles (FBZ and febantel), 61.9-98.8% for levamisole and 14.1-98.1% for macrocyclic lactones (IVM, ABM or DOR; Peña-Espinoza and Sievers, unpublished results).

Fasciola hepatica. Until now, no published cases of AR in *F. hepatica* have been reported in ruminants or other livestock species in Chile and only unpublished theses have suggested the presence of triclabendazole-resistance in Chilean cattle (Rodriguez 2005, Laverde 2007). However, a recent publication has reported the first human cases of triclabendazole-resistant *F. hepatica* in Chile (Gil *et al* 2014). In this case report, four patients with previous consumption of watercress ('berro') or untreated water from marshes grazed by livestock were positive to *F. hepatica* and repeatedly treated with triclabendazole (20 mg/kg),

without therapeutic effect. All the patients declared no contact with potential infection sources after the start of triclabendazole treatments, therefore the persisting parasitism post-treatment was unlikely due to reinfections. Due to the lack of drug activity, adult *F. hepatica* individuals had to be directly isolated from the bile ducts of the patients by surgery and endoscopic cholangiopancreatography (Gil *et al* 2014). Although the cited report did not provide information regarding the specific origin of the *F. hepatica*-resistant strains, it is likely that these resistant populations were selected in livestock exposed to triclabendazole in Chilean farms. Considering the potentially severe impact in human health of drug-resistant helminths of zoonotic potential and the endemic nature of *F. hepatica* infections in Chilean livestock, it is urgently needed to detect and monitor AR in farms positive to *F. hepatica*.

PERSPECTIVES AND FURTHER RESEARCH

Substantial research work is needed to advance our knowledge on the extent of AR in parasite populations of veterinary importance in Chile, particularly to the ML drugs. In the absence of quantitative molecular techniques for detection of AR to ML and the high cost of the CET, at present the only readily available technique to diagnose AR on farms is the FECRT. In sheep and cattle, AR surveys that include several farms are warranted to start elucidating the spatial distribution of drug resistance in Chile. Farmers and veterinarians need to be encouraged to perceive the FECRT as a versatile method that can help them to evaluate the efficacy of a treatment intervention and to detect the potential presence of AR in the farm. However, one of the main practical limitations for the performance of FECRT is the lack of interest by farmers and veterinarians in Chile to conduct faecal sampling and pay for individual FEC examination. An alternative to individual analyses is determine the FEC in composite (pooled) faecal samples from several animals (Daniel *et al* 2012, George *et al* 2017), and this strategy needs to be evaluated under local conditions. Meanwhile updated guidelines for the performance and evaluation of FECRT trials are made available, the outlined limitations of the test need to be addressed. For example in cattle, where FEC are usually lower than in sheep, the use of more sensitive egg counting methods such as FLOTAC and Mini-FLOTAC could be evaluated (Levecke *et al* 2012b; Godber *et al* 2015; Cringoli *et al* 2017). In cases where AR is detected, further studies can look at the direct impact of infection with resistance parasites (leading to inefficacious treatments) on animal health and farm productivity, as earlier reported (Sutherland *et al* 2010, Miller *et al* 2012, Borges *et al* 2013).

Given the zoonotic and economic potential of *F. hepatica* infections, there is an urgent need to investigate the status of flukicide resistance in Chilean livestock. A first attempt can be the detection of AR by FECRT, possibly later combined with the identification of coproantigens pre and post-treatment. Drug resistance in *F. hepatica* could also be screened in animals sent to slaughter at the abattoir right after the withdrawal period of the flukicidal treatment (e.g. 28 days for triclabendazole), with the examination for surviving adult parasites in the liver of treated animals during official meat inspection.

In horses it is imperative to evaluate the efficacy of ML treatments and the presence of ML resistance in strongyles, particularly cyathostomins. Previously, ML were reported to have a high field efficacy in naturally-infected horses in Chile (Rubilar *et al* 2001), but no field studies have been reported ever since. Another interesting research approach is the potential role of wild animals in Chile as reservoirs of drug-resistant parasites and even as vectors of resistant populations between livestock farms, as recently suggested for wild deer (Chintoan-Uta *et al* 2014). In Chile, semi-captive guanacos (*Lama guanicoe*) have been reported to share nematode species with domesticated livestock (Correa *et al* 2012) and *F. hepatica* has been identified in pudu (*Pudu puda;* Bravo Antilef 2013), stressing the importance to explore the potential transmission of drug-resistant helminths between domesticated and wild animals.

In parallel to the detection of AR, strategies to prevent the further selection of parasite drug resistance should be evaluated and adapted to local farm conditions. A first approach is the administration of anthelmintics at the recommended dose and avoiding under-dosing and further exposure of helminths to sub-therapeutic doses. Anthelmintic treatments should aim to maintain a *refugia* population, i.e. the proportion of the worm population not exposed to anthelmintics which is outside the host as free-living stages or in untreated animals (Van Wyk 2001), that can help to dilute the resistant population and hence delay the onset of AR. A recent study reported the successful introduction of susceptible parasites into a farm with a high proportion of resistant nematodes, resulting in an increased drug efficacy after two years of introducing the susceptible worms (Fiel *et al* 2017). Another strategy to ensure a *refugia* population is the targeted selective treatment (TST) of the animals in need of anthelmintic drenching, instead of treating the whole herd (Charlier *et al* 2014[a]). Evidence from research on small ruminant nematodes suggests that TST, i.e. leaving some (or most) of the animals untreated to guarantee a *refugia* of unselected worms, is perhaps the most important strategy to slow the development of AR in nematode populations (Kenyon et *al* 2009, Kenyon and Jackson 2012). The TST strategy has also been recently investigated in cattle (Jackson *et al* 2017). Still, it is critical that the anthelmintic used in a TST approach is highly efficacious (Knox *et al* 2012). Moreover, in order to further reduce the reliance on anti-parasitic drugs, future research needs to explore the efficacy of novel control strategies such as preventive grazing strategies,

feeding with anthelmintic forages and the development of vaccines (Hoste *et al* 2015, Peña-Espinoza *et al* 2016[b], Charlier *et al* 2017, Kenyon *et al* 2017).

CONCLUSIONS

Anthelmintic resistance has been reported in GI nematodes parasitising horses, sheep and cattle and in *F. hepatica* infecting humans in Chile. However, the reported cases of reduced drug efficacy in livestock involved a limited number of selected farms and there is a need to evaluate the level of AR in Chile by larger surveys. Given the zoonotic potential of *F. hepatica*, it is urgent to determine the extent of liver fluke resistance. Considering the few cases of AR reported in Chile and the widespread use of anti-parasitic drugs for helminth control, it seems timely to advance our knowledge on the status of AR at the farm, regional and national levels, before reaching alarming levels of drug resistance and to ensure a sustainable parasite control for livestock.

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