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Confirmation of *Fasciola hepatica* resistant to triclabendazole in naturally infected Australian beef and dairy cattle ☆

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ABSTRACT

Triclabendazole (TCBZ) is the drug of choice for *Fasciola hepatica* control and reports of *F. hepatica* resistant to this drug from a wide range of geographic regions are very concerning. This study investigated the presence of TCBZ resistance in *F. hepatica* in naturally infected Australian beef and dairy cattle herds and evaluated methods of measuring the levels of resistance. Faecal egg count and coproantigen reduction tests (FECRT and CRT, respectively) were conducted on 6 South-eastern Australian beef properties and one dairy property where treatment failure by triclabendazole (TCBZ) was suspected. The CRT was conducted on an additional beef property. On each property 15 animals were treated with an oral preparation of TCBZ at the recommended dose and 15 animals remained as untreated controls. Fluke eggs in faeces were counted and coproantigen levels were measured before treatment and 21 days after treatment and in the untreated control animals. These data were evaluated using three different methods to calculate % reductions compared with controls. Resistance (<90% reduction) was detected on the dairy property using both FEC and CRT, and on 3/6 beef properties using FECRT and 4/7 beef properties using CRT. Using the FECRT, reductions of 6.1–14.1% were observed in dairy cattle and 25.9–65.5% in beef cattle. Using the CRT, reductions of 0.4–7.6% were observed in dairy cattle and 27.0–69.5% in beef cattle. Live flukes were recovered at slaughter following TCBZ treatment of 6 cattle from 3 of the beef properties, confirming the TCBZ resistance status of *F. hepatica* in these cattle. This is the first report of *F. hepatica* resistant to TCBZ in cattle in Australia and the results suggest that resistance is widespread in the South-eastern region. The CRT is shown to be a robust alternative to the FECRT for evaluation of TCBZ resistance in *F. hepatica* in cattle.

1. Introduction

In Australia, livestock production losses attributed to the common liver fluke *Fasciola hepatica* were estimated to be A$50 to 80 million per annum in 1999 and annual fluke treatment alone costs A$10 million (Boray, 2007). Over 6 million cattle graze at-risk pastures with most stock concentrated in South-eastern Australia where there is a suitable habitat for the intermediate snail host (MLA, 2005), especially along watercourses and in irrigation zones. The epidemiology of fasciolosis is similar to other countries.

Due to its efficacy against both immature and mature adult stages of *F. hepatica* within the mammalian host, triclabendazole (TCBZ) has been the drug of choice for parasite control. The emergence of resistance to TCBZ now threatens fluke control in livestock in several parts of Europe (Fairweather, 2009). TCBZ-resistant *F. hepatica* were first reported from sheep in Victoria, Australia, in 1995 (Overend and Bowen, 1995) and resistance has now been reported in several countries both in sheep (Mitchell et al., 1998; Moll et al., 2000; Thomas et al., 2000; Gaasenbeek et al., 2001; Mooney et al., 2009; Sargison and Scott, 2011; Gordon et al., 2012), and cattle (Moll et al., 2000; Olaechea et al., 2011; Ortiz et al., 2013). Recently, a case of TCBZ-resistant *F. hepatica* was reported in a human from the Netherlands. The patient did not respond despite several treatments with the drug, highlighting the serious zoonotic threat posed by fasciolosis especially that of resistant parasites (Winkelhagen et al., 2012).

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Anthelmintic resistance in nematode parasites is commonly detected by the use of parasite faecal egg count reduction tests (FECRT). Although FECRT have not been validated for fluke (Coles et al., 2006), this method has been applied to evaluate treatment failure and indicate the existence of possible drug resistance in *F. hepatica* populations. A commercial coproantigen ELISA is available for the detection of *F. hepatica* infection in ruminant livestock (Mezo et al., 2004). Trials using sheep (Flanagan et al., 2011a,b; Gordon et al., 2012; Novobilsky et al., 2012) and cattle (Brockwell et al., 2013) show that this coproantigen ELISA can be used to demonstrate survival of fluke following treatment and thus in identifying resistant populations. The recent work of Brockwell et al. (2013) has demonstrated that this test reflects fluke burdens in cattle and that coproantigen levels fall within 7 days after successful treatment suggesting that this test has utility as a method for measuring reductions due to treatment. This opens the way for a coproantigen reduction test (CRT) to be used for measuring the level of TCBZ resistance in *F. hepatica* in cattle.

In this study, we aimed to identify, for the first time, resistant fluke isolates in cattle in Australia and to evaluate and compare the FECRT and CRT as methods for measuring the level of drug resistance in *F. hepatica*. We used the same coproantigen ELISA test as used by others (Flanagan et al., 2011a,b; Gordon et al., 2012; Novobilsky et al., 2012) and compared the three methods described by Pook et al. (2002) for assessing reductions in FEC and coproantigen ELISA values. We show that the RESO technique, which compares post-treatment arithmetic means of treated and control groups, was favoured because its derivation generates less statistical error, relies on post treatment results only and is cheaper for field application.

2. Materials and methods

2.1. Tests

2.1.1. FEC

Pre and post treatment *F. hepatica* faecal egg counts (FEC) were performed by Para-Site Diagnostic Service, Benalla, Victoria on fresh faeces sent by overnight courier, using the Sedimentation test for Liver Fluke©, Western Australia Department of Agriculture and Food (WADAF). The procedure was to homogenize 10 g of faeces with 100 mL of water and pour the slurry through a sieve stack with sequentially smaller aperture sizes (150, 90 and 45 μm). The homogenate was washed through the sieves with a stream of tap water followed by washing of each of the lower two sieves after removing the sieve above. The filtrate collected on the 45 μm sieve was washed into a graduated flask and diluted to 100 mL with water and allowed to settle for 6 min. The supernatant was reduced to 20 mL using a vacuum pump, diluted again to 100 mL with water and allowed to settle for 6 min. The sediment was suspended in 10 mL and one drop of methylene blue added. After 5 min, the material was transferred to a viewing chamber and eggs counted under an inverted microscope using 40× magnification.

2.1.2. Coproantigen test

To measure faecal antigen levels, aliquots of 2 g of the same faecal samples were stored at −20 °C for up to 3 days, and for several months in the case of the Nimmitabel samples, until analysis with a commercial ELISA kit for the detection of *F. hepatica* faecal antigen (BIO K 201, BIO-X Diagnostics, Belgium). The protocol was optimised for use in our laboratory as described (Brockwell et al., 2013). Coproantigen values are expressed as a percentage of the positive control antigen and corrected to allow for a zero value by subtracting the negative cut-off value of 1.3%. This negative cut-off value was determined by the mean plus 3 times the standard deviation taken from 103 FEC negative field samples. The specificity of the coproantigen ELISA has been established in several studies against natural infections of gastrointestinal nematodes, *Moniezia, Dicrocoelium* and *Echinococcus* (Mezo et al., 2004) and *Paramphistomum cervi* (Kajugu et al., 2012; Brockwell et al., 2013).

2.2. On-farm trials

This research was conducted with approval by Charles Sturt University's Animal Care and Ethics Committee. The beef cattle properties were selected for preliminary screening following reports of suspicion of treatment failure by veterinarians in the Livestock Health and Pest Authority (NSW) and the Department of Primary Industries (Victoria). The dairy property was selected on the advice of a local veterinarian. The properties identified in this trial were located at Parrots Nest, Irvington and Caniaba in North-eastern NSW; at Numbugga, near Bega in the far South-eastern area of NSW; Nimmitabel in the Monaro region of South-eastern NSW; Gireke near Berrigan in the southern Riverina irrigation area of NSW; and in the Tallangatta Valley region of North-eastern Victoria. The dairy property was located near Maffra in the Gippsland region of eastern Victoria (see Fig. 1). The brief history of fluke control on these properties is as follows. All properties surveyed had been using TCBZ exclusively for longer than 5 years and treated animals orally. Only the Caniaba property indicated that cattle were treated using a pour on as well. None indicated using what could be considered excessive treatments (>3 treatments per year). Most treated cattle once annually, with only the Numbugga property manager stating he treated when he thought the animals showed evidence of disease. Nimmitabel reported no treatment of cattle but twice yearly oral treatment of sheep co-grazing with cattle. Parrots Nest and Irvington reported 2–3 treatments per year. Scales to weigh animals were used only on the Numbugga and Tallangatta Valley properties with all animals dosed at the rate applicable for the heaviest weight obtained. On all other properties the animal's weight was estimated for dose calculation.

On each property 30 animals of no specific age or gender were enrolled in the trial. Animals were randomly allocated to either a treatment or control group (n = 15/group). The mean body weights (kg ± SD) of the control and treated animals, respectively, on each property were: Parrots Nest: 396 ± 34; 444 ± 23; Irvington: 435 ± 31; 387 ± 32; Caniaba: 552 ± 18; 502 ± 13; Nimmitabel: 314 ± 8; 298 ± 9; Numbugga: 375 ± 9; 375. ± 11; Gireke: 447 ± 15; 421 ± 19; Tallangatta Valley: 252. ± 7; 242 ± 4; Maffra: 211 ± 6; 200 ± 5. Fifteen treated cattle were dosed orally with a commercial TCBZ drench (Flukare C®, Virbac Animal Health) at the manufacturer's recommended dose rates (12 mg/kg based on individual body weight) using a drenching hook. A second group of 15 animals remained untreated as controls. Per rectum faecal collection for FEC and coproantigen ELISA were performed on each animal prior to treatment and 21 days post-treatment. The exceptions to this protocol were: (i) for the Nimmitabel property poor weather delayed the post-treatment sample collection until day 24; (ii) on the Maffra property the 'untreated control' group were subsequently treated on day 21 and then retested for coproantigen on day 42. There was no untreated control group for comparison with this treated group. On some properties only 13 or 14 animals were available on the day of testing as shown in Tables 1 and 2.

2.3. Statistical analysis

Anthelmintic resistance was declared when the calculated TCBZ efficacy was <90% (APVMA, 2001). FEC were determined as

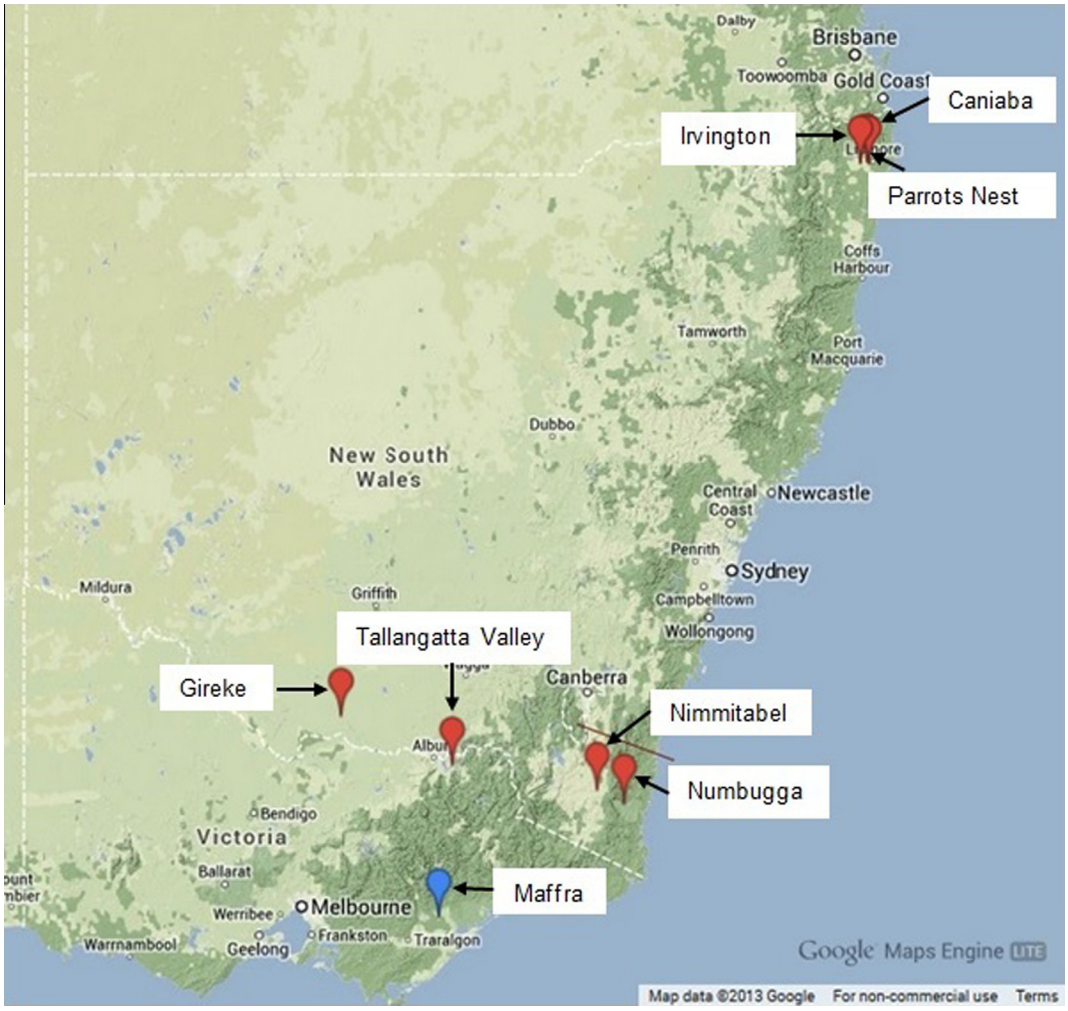

Fig. 1. Map of South-eastern Australia showing location of properties involved in *Fasciola hepatica* resistance studies. Red: beef properties; blue: dairy property. Map data ©2013 Google. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Faecal egg count reduction test: FECRT. Faecal egg count (FEC = eggs per 10 g faeces) data from 6 South-eastern Australian beef cattle properties and one dairy property at Maffra which was tested twice: *Maffra is data from the second test. FEC Untreated controls: Initial: FEC in untreated control cattle on day of treatment; Final: FEC in untreated control cattle on days 21–24 following treatment. Those properties identified as having TCBZ-resistant flukes (reduction in FEC <90%) are indicated in bold red type; na = not applicable. See text for explanation of methods. Method 1 = Coles et al. (1992); Method 2 = Pook et al. (2002); Method 3 = Dash et al. (1988).

LOCATION	n	FEC Untreated controls: Initial	FEC Untreated controls: Final	FEC pre-treatment	FEC post-treatment	FECRT (%)		
						Method 1	Method 2	Method 3
Parrots Nest (NSW)	14	74 ± 28	76 ± 39	50 ± 23	5 ± 5	93.4	**48.6**	90.3
Irvington (NSW)	15	47 ± 26	61 ± 34	42 ± 16	1 ± 1	99.9	**63.1**	99.9
Caniaba (NSW)	13	292 ± 83	248 ± 63	187 ± 55	2 ± 1	99.1	96.6	98.6
Numbugga (NSW)	15	28 ± 9	55 ± 13	48 ± 10	11 ± 2	**88.4**	**69.5**	**88.3**
Gireke (NSW)	15	33 ± 8	33 ± 8	28 ± 8	26 ± 9	**19.9**	**29.7**	**12.9**
Tallangatta Valley (Vic)	15	36 ± 11	76 ± 18	27 ± 6	16 ± 4	**78.9**	**27.0**	**72.2**
Maffra (Vic)	11	27 ± 14	28 ± 9	39 ± 38	23 ± 23	**18.7**	**0.4**	**43.6**
*Maffra (Vic)	14	na	na	28 ± 9	31 ± 10	na	**7.6**	na

described above on samples obtained before and after treatment with TCBZ. Reductions in faecal egg count (FECR) and in coproantigen levels (CR) were calculated using the Excel procedure triFECTA version 4 (Charles Sturt University) designed originally for FECR. Results are obtained using 3 methods described by Pook et al. (2002): Method 1 is the RESO technique as recommended by the

Table 2
Coproantigen reduction test: CRT. Coproantigen levels (Background corrected and presented as % positive of control wells) from 7 South-eastern Australian beef cattle properties and one dairy property at Maffra which was tested twice: *Maffra is data from the second test. FEC Untreated controls: Initial: FEC in untreated control cattle on day of treatment; Final: FEC in untreated control cattle on days 21–24 following treatment. Those properties identified as having TCBZ-resistant flukes (reduction in % positive control antigen < 90%) are indicated in bold red type, na = not applicable. Methods as in Table 1.

LOCATION	n	% positive Untreated controls: Initial	% positive Untreated controls: Final	% positive pre-treatment	% positive post-treatment	CRT(%)		
						Method 1	Method 2	Method 3
Parrots Nest (NSW)	14	20 ± 5.9	19 ± 5.8	11.3 ± 3.3	0.0 ± 0.0	100	**65.5**	100
Irvington (NSW)	15	13.2 ± 5.1	19.5 ± 6.8	12.7 ± 4.7	0.0 ± 0.0	100	**55.2**	100
Caniaba (NSW)	13	67.4 ± 8.0	71.7 ± 8.5	64.4 ± 9.0	0.2 ± 0.2	99.8	98.4	99.8
Nimmitabel (NSW)	15	12.8 ± 4.1	27.1 ± 7.4	11.5 ± 4.2	2.7 ± 1.3	**89.9**	**52.1**	**88.7**
Numbugga (NSW)	15	42.5 ± 4.5	44.7 ± 5.2	47.3 ± 5.3	28.0 ± 5.8	**37.4**	**44.1**	**43.8**
Gireke (NSW)	15	4.7 ± 1.3	4.4 ± 2.0	3.3 ± 1.2	1.5 ± 0.7	**64.6**	**25.9**	**49.4**
Tallangatta Valley (Vic)	15	75.5 ± 14.9	48.5 ± 7.0	76.4 ± 13.9	21.1 ± 3.5	**56.4**	**63.2**	**56.9**
Maffra A (Vic)	14	16.6 ± 5.5	14.4 ± 5.6	10.3 ± 6.4	5.2 ± 4.5	**63.7**	**14.1**	**41.6**
*Maffra B (Vic)	15	na	na	14.4 ± 5.6	18.7 ± 5.7	na	**6.1**	na

World Association of the Advancement of Veterinary Parasitology which compares post-treatment arithmetic means of treated and control groups (Coles et al., 1992); Method 2 uses means of individual animal pre- and post-treatment counts to derive individual FECR reductions (Pook et al., 2002); Method 3 uses changes in the untreated control group means to correct for changes in FECR between collection dates using arithmetic means (Dash et al., 1988).

2.4. Slaughter trials

On three of the properties where TCBZ resistance was identified based on FECR and CR, confirmation was sought through treatment and slaughter trials. The animals chosen for slaughter were from the same herds but were not subjects of the original field trials. Three cows positive for coproantigen were purchased from the Numbugga property, one from the Gireke property and two from the Tallangatta Valley property. The animals were transported to CSU and kept on dryland pasture. The Numbugga cattle were treated orally with TCBZ at 12 mg/kg body weight (Fasinex 240®, Novartis) on the property of origin and then slaughtered 34 days post-treatment not the recommended 21 days, due to a delay in finalising purchase of the cattle from the farmer. The animals from the Gireke and Tallangatta Valley properties were treated orally with TCBZ at 12 mg/kg (Flukare C®, Virbac Animal Health) at CSU and slaughtered 21 days later. The livers and gall bladders were collected for dissection and fluke recovery as described (Brockwell et al., 2013). Faeces were also analysed for egg count and coproantigen levels at the time of slaughter.

3. Results

3.1. On farm resistance testing

The properties had been selected on the basis of earlier positive FEC for *F. hepatica*. Positive egg counts and coproantigen levels were detected on all properties. There was considerable variation in values within a property reflected in a relatively high coefficient of variation and the overdispersed nature of natural fluke infections. The data for FEC and FECR are presented in Table 1 and data for coproantigen levels and CR are shown in Table 2. Resistance, or TCBZ treatment failure, is defined as less than 90% reduction in FEC or coproantigen. Resistance was found on the Maffra dairy property using both methods (CRT and FECRT) and on 4/7 beef properties (Nimmitabel, Numbugga, Gireke, Tallangatta Valley) using CRT and on 3/6 beef properties (Numbugga, Gireke, Tallangatta Valley) using FECRT. Faecal egg count data was unavailable for one beef property. Both methods detected resistance but the percentage reductions varied for FEC and coproantigen on all properties.

Table 3
FEC, coproantigen levels and fluke numbers from slaughter trials. Faecal egg count (eggs/10 g faeces), coproantigen levels and number of fluke recovered from TCBZ-treated cattle obtained from animals on properties identified as having TCBZ resistant *F. hepatica* populations (N = Numbugga, TV = Tallangatta Valley, G = Gireke). Coproantigen levels are expressed as a percentage of the positive control antigen (% positive) provided with the BIOX coproantigen kit (cut-off or negativity = 1.3%).

Animal	FEC	% Positive	Fluke #
N 1	26	15.3	20
N 2	19	5.6	23
N 3	12	13.5	24
TV 1	7	22.7	20
TV 2	27	34.1	31
G 1	30	15.0	24

3.2. Recovery of flukes in slaughter trials

Live adult flukes were recovered from all 6 animals following TCBZ treatment demonstrating that a TCBZ dose that is known to remove susceptible fluke was ineffective on animals from all 3 properties that were tested (Numbugga, Gireke and Tallangatta Valley). These results also eliminated the possibility that positive FEC and coproantigen measures were false positives. Table 3 shows the number of fluke recovered, FEC and coproantigen level on the day of slaughter. The correlation between FEC and fluke number was $R^2 = 0.1801$ and for coproantigen $R^2 = 0.3542$. Although the sample size is small, the results confirm the TCBZ resistant status of the fluke in these 6 cattle derived from the herds on the Numbugga, Gireke and Tallangatta Valley properties.

4. Discussion

4.1. Optimisation of the coproantigen ELISA test

The coproantigen protocols used here were based on our previous evaluation of the BIOX coproantigen ELISA in a trial using an artificial infection in cattle (Brockwell et al., 2013). Analysis of

frozen faecal samples has been shown in sheep (Flanagan et al., 2011a) and cattle (Brockwell et al., 2013) to not affect coproantigen quantitation. Several studies using the BIOX coproantigen kit have adhered to the manufacturer's instructions using a cut-off for positivity of 0.15 OD or equivalent when expressed as a percentage of the supplied control antigen (Martínez-Valladares et al., 2010; Flanagan et al., 2011b; Bernardo et al., 2012; Novobilsky et al., 2012; Robles-Pérez et al., 2013). However, Novobilsky et al. (2012) expressed concerns as to the high rate of false negatives when using the BIOX kit's recommended negative cut-off point for positivity and called for further verification of the ELISA's sensitivity. The revised cut off used in our present study was based on earlier studies in cattle (Brockwell et al., 2013), which improved the test's sensitivity by removing false negatives. In a recent evaluation of the coproantigen ELISA kit for routine use, Palmer et al. (2013) also established lower custom cut-offs to improve sensitivity.

This is the first study to evaluate the BIOX coproantigen test for assessing *F. hepatica* infection status in field trials in naturally infected animals maintained on pasture in commercial settings. The test proved to be robust in measuring infection and also delivers advantages in allowing the delayed analysis of fecal samples through freezing which provides efficiencies in cost of analysis by allowing batching of samples (Brockwell et al., 2013). The test reflects parasite burdens and provides the opportunity to estimate intensity of infection (Mezo et al., 2004; Brockwell et al., 2013) and thus an assessment whether the burdens exceed the threshold for production losses in cattle of about 30–40 flukes (Hope-Cawdery et al., 1977; Vercruysse and Claerebout, 2001). The ELISA platform is universal in diagnostic laboratories and so this test is widely applicable. On the other hand, microscopy skills required for egg counting and identification require specialist skills.

4.2. Evaluation of TCBZ resistance in cattle

Our study investigated suspected cases of reduced efficacy of TCBZ on 8 cattle properties in South-eastern Australia. Our studies confirm the widespread existence of TCBZ resistance in cattle herds using FECRT, CRT and through the recovery of live adult flukes post-TCBZ treatment from three independent cattle herds. Resistance (<90% reduction in FECRT or CRT) was detected on 4/7 properties using FECRT and 5/8 properties using CRT. It is possible that TCBZ resistance has arisen independently on these farms; however, given the climate and management systems in the southern region of Australia, and the fact that sheep and cattle co-graze and can share *F. hepatica*, the presence of resistance in cattle is not surprising given the occurrence of resistant parasites in sheep (Overend and Bowen, 1995). In support of this, live fluke were also recovered post-treatment with TCBZ from sheep on both the Tallangatta Valley and Gireke properties (Brockwell et al., unpublished data). Movement of stock between farms can contribute to migration of resistant fluke and cannot be eliminated here as a means of spread. Genetic tests may help describe the origins of the fluke populations and this is the subject of further study (Elliott et al., 2013). The lack of resistance in the northern cluster of beef properties may reflect different genetic backgrounds in fluke populations or variation in selection pressures (Sangster and Dobson, 2002). For example, the lack of sheep in this environment may be significant if resistant flukes develop in sheep and are shared with cattle. However, cases of resistance in fluke in cattle such as those reported in the Netherlands (Moll et al., 2000), or on the dairy cattle in the present study, suggest that direct selection has occurred in fluke populations in cattle. Further testing is required to establish the dynamics of selection.

Failure of anthelmintic treatment may be due to factors other than resistance; examples are: poor drug delivery through faulty drench guns; under-dosing of animals due to inaccurate weighing and dose calculation; poor storage and handling of drenches and therefore reduced drug quality; or reduced drug metabolism in the host animal due to earlier liver damage. For example, the Leon isolate was first reported to be resistant (Alvarez-Sanchez et al., 2006) but FECRT and CRT and confirmation at necropsy showed it to be susceptible (Flanagan et al., 2011b). In the current study, we followed recommended guidelines for treatment on a number of properties and the subsequent slaughter study provides strong evidence of resistance in these fluke populations.

4.3. Evaluation of methods for determining resistance using FECRT and CRT

There are no standardised tests or guidelines for the determination of drug resistance in *F. hepatica*. For nematodes, standardised methods (Coles et al., 2006) are available but are currently under review as new approaches are explored. The characteristics of a suitable test are that the methods are technically achievable, that the values they generate are repeatable and that they reflect parasite burdens, including burdens present following treatment. Previous data in artificially infected cattle using the modifications described by Brockwell et al. (2013) and in sheep (Flanagan et al., 2011a) show that the FEC and coproantigen ELISA have suitable characteristics for resistance tests.

The World Association for the Advancement of Veterinary Parasitology (WAAVP) 1995 guidelines include guidance on clinical trials with *Fasciola* (Wood et al., 1995). They recommend that efficacy should be determined by FEC three weeks after treatment. The present study followed these guidelines. Three weeks is a long delay, but was justified to allow ample time for the removal of adult fluke in large animals following treatment and allow for the clearance of any fluke eggs potentially stored in the gall bladder. It also allows time for additional immature flukes that survive treatment to mature to antigen production and elevate coproantigen levels. Three weeks is also a long delay for a farmer to hold treatment while waiting for a laboratory result. Flanagan et al. (2011b) adopted a 14 day post treatment FEC test in sheep and used the parameter of a positive coproantigen level as an indication for resistance. In their hands, all but one animal that was infected with drug-susceptible flukes and treated with TCBZ returned a zero antigen level by 14 days. In our hands, in artificially infected cattle, both fluke FEC and coproantigen levels fell to negative values within 7 days after treatment (Brockwell et al., 2013). Because TCBZ kills immature fluke the issue of development of immature fluke to coproantigen producing status is not likely to be a problem. On the other hand, analysis of FEC and coproantigen at day 7 after treatment may not be a good protocol for albendazole testing as noted by Novobilsky et al. (2012). Flanagan and others used a 95% reduction in FECRT to signal resistance. We elected to use a more conservative 90% reduction in FECRT as the threshold for resistance as adopted for Australian flukicide efficacy trials (AVPMA 2001). A group size of 15 was chosen because it was the upper limit for nematode testing, is feasible for farmers in the field and would assist in reducing statistical variance.

The merits of various methods of analysis of FECRT have been debated in the literature. We employed two techniques (FECRT and CRT) as resistance tests and analysed the data using three existing methods (Dash et al., 1988; Coles et al., 1992; Pook et al., 2002). It is clear from Tables 1 and 2 that Method 2 fails to provide reliable data in our hands for both tests on two properties (Parrots Nest, Irvington). Method 2 compares pre- and post-treatment levels in the same animal and does not account for changes in control levels over time (3 weeks). While this is a useful method for nematode parasites, especially when group numbers are small, the values obtained in the tests here varied between collection

dates and so are misleading. Changes in the coproantigen values in the control groups between the pre- and post- treatments samples varied over 3 fold between properties, ranging from a 36% reduction (Tallangatta Valley) to a 212% increase (Nimmitabel) as a percentage of positive control antigen. We observed a 2–6 fold variation in coproantigen levels over a consecutive 5 day period in our previous study and such variation could be due to biological or day to day variation in antigen release (Brockwell et al., 2013) or variation in feed availability which may alter faecal volume. The relatively long 3 week period between pre and post sampling would also allow more parasites to contribute to test levels especially in the window up to 8 weeks for FEC and 5 weeks for coproantigen analysis. Animals with negative results pre-treatment also appeared to cause an underestimation of the efficacy when using Method 2 in the analysis because values can only increase or remain the same. The fact that Method 2 scores both tests similarly for the Parrots Nest and Irvington properties (% reductions of 48.6 and 63.1 vs. 65.5 and 55.2, respectively) indicates that the two tests reflect similar phenomena. This close agreement is a good measure of equivalence as statistical errors are highest nearer to 50% reduction.

Methods 1 and 3 give similar results on each property for FECRT and CRT. Method 1 remains favoured because its derivation generates less statistical error and relies on post treatment results only so is cheaper for a farmer to apply. Considering the calculations with Method 1, resistance was detected using both FECRT and CRT and the agreement between the two reduction tests is excellent. Existing methods including those used here presume a known distribution of data. Methods that are independent of distribution are likely to provide more accurate estimates of means and hence % reduction. Future work will investigate biases in these analyses and use simulations to optimise test parameters such as study design and group size.

An additional benefit of comparing control and post-treatment values is that it is amenable to a cheap field test for resistance using a herd analysis. When monitoring herd health for parasitism in grazing animals, it is a common practice to pool a number of faecal samples from the group for diagnosis (Eysker and Ploeger, 2000). Experimental evaluation of pooling known faecal samples from cattle prior to analysis with the coproantigen ELISA demonstrated that the pooled result is a mean of the values from samples tested individually (Brockwell et al., 2013). Our recommended protocol for efficient and affordable field testing to identify fluke drug resistance (and success of treatment) is: (i) Fourteen days after treatment with the recommended dose by a farmer, faecal samples are taken from 15 animals and 3 pools of faeces from 5 animals each are subject to coproantigen testing; (ii) A value above background in any sample post-treatment gives 95% confidence that flukes have survived in at least one of the 15 animals tested in the pooled sample. Coproantigen testing in this context is forgiving with respect to transport times and the samples can be shipped frozen (Brockwell et al., 2013).

Martinez-Perez et al. (2012) compared FEC and coproantigen ELISA, with both a standard and nested faecal PCR assay, reporting that the ELISA and both PCR techniques improved the early diagnosis of fluke infection over FEC. More recently these researchers compared these three techniques for use in the detection of fluke drug resistance in sheep (Robles-Pérez et al., 2013), finding PCR to be the most sensitive. However the authors used the coproantigen ELISA kit manufacturer's recommendations of an optical density of 0.15 as the cut-off value for positivity. Our results applying this test in cattle suggests that this OD value is too high for accuracy in the detection of low fluke burdens and antigen levels in faeces (Brockwell et al., 2013). Further evaluation of these PCR techniques in the detection of TCBZ resistance is warranted.

4.4. Conclusions

In this study we have demonstrated the presence of TCBZ resistance in *F. hepatica* in cattle from a range of properties and production systems in South-eastern Australia using the FECRT and CRT. The FECRT has been applied in a similar fashion to investigations of anthelmintic resistance in nematode parasites and follows the same analysis. The CRT provides an alternative test and offers several advantages: (i) the coproantigen ELISA shows a moderate to good association with parasite burdens with R^2 values ranging from 0.239 to 0.871 in cattle (Brockwell et al., 2013) to $R = 0.889$ in lambs (Mezo et al., 2004) and reflects the intensity of infection; (ii) it can evaluate treatment efficacy in the pre-patent infection stage since the CRT detects infections within 6–8 weeks of infection in cattle; (iii) coproantigen levels fall to zero well within 14 days of successful treatment, reducing the interval required between treatment and collection from 21 to 14 days; this testing interval aligns with recommendations for nematodes (10–14 days) so it would be possible to test nematode and fluke resistance in parallel even on the same 30 animals following treatment with a combination of a nematocide and a flukicide; (iv) the coproantigen in the samples is stable and withstands freezing and an extended shelf life, so laboratory scheduling is more flexible; (v) the opportunity to pool samples and only collect and analyse post-treatment provides a cheaper option for a farmer for field resistance testing; (vi) the coproantigen test has high specificity (vii) the results parallel the FECRT. We conclude that, provided custom cut-offs are determined to improve sensitivity, the coproantigen ELISA remains the simplest quantitative technology for routine detection of fluke infection in livestock.

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References

Alvarez-Sanchez, M., Mainar-Jaime, R., Perez-Garcia, J., Rojo-Vázquez, F., 2006. Resistance of *Fasciola hepatica* to triclabendazole and albendazole in sheep in Spain. Vet. Rec. 159, 424–425.

APVMA Gazette National Registration Authority. 6, 5 June 2001. <http://www.apvma.gov.au/publications/gazette/2001/06/gazette0106p31.php>.

Bernardo, C., Avelar, B., Ignacchiti, M., Martins, I., Pereira, M., 2012. Kit commercial de ELISA® para a detecção de coproantígenos e exame coproparasitológico em bovinos com fígados condenados por fasciolose. Ciência Rural 42, 2025–2029.

Boray, J.C., revised by G.W. Hutchinson and S. Love, 2007. Liver fluke disease in sheep and cattle. Primefact 446, NSW Department of Primary Industries.

Brockwell, Y.M., Spithill, T.W., Anderson, G.R., Grillo, V., Sangster, N.C., 2013. Comparative kinetics of serological and copro-antigen ELISA and faecal egg count in cattle experimentally infected with *Fasciola hepatica* and following treatment with triclabendazole. Vet. Parasitol. 196, 417–426.

Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor, M.A., Waller, P.J., 1992. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 44, 35–44.

Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., Von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A., Vercruysse, J., 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 136, 167–185.

Dash, K.M., Hall, E., Barger, I.A., 1988. The role of arithmetic and geometric mean worm egg counts in faecal egg count reduction tests and in monitoring strategic drenching programs in sheep. Aust. Vet. J. 65, 66–68.

Elliott, T., Muller, A., Brockwell, Y., Murphy, N., Grillo, V., Toet, H.M., Anderson, G., Sangster, N., Spithill, T.W., 2013. Evidence for high genetic diversity of NAD1 and COX1 mitochondrial haplotypes among triclabendazole resistant and susceptible populations and field isolates of *Fasciola hepatica* (liver fluke) in Australia. Vet. Parasitol. (in press).

Eysker, M., Ploeger, H.W., 2000. Value of present diagnostic methods for gastrointestinal nematode infections in ruminants. Parasitology 120 (Suppl.), S109–S119.

Fairweather, I., 2009. Triclabendazole progress report, 2005–2009: an advancement of learning? J. Helminthol. 83, 139–150.

Flanagan, A.M., Edgar, H.W.J., Forster, F., Gordon, A., Hanna, R.E.B., McCoy, M., Brennan, G.P., Fairweather, I., 2011a. Standardisation of a coproantigen reduction test (CRT) protocol for the diagnosis of resistance to triclabendazole in *Fasciola hepatica*. Vet. Parasitol. 176, 34–42.

Flanagan, A.M., Edgar, H.W.J., Gordon, A., Hanna, R.E.B., Brennan, G.P., Fairweather, I., 2011b. Comparison of two assays, a faecal egg count reduction test (FECRT) and a coproantigen reduction test (CRT), for the diagnosis of resistance to triclabendazole in *Fasciola hepatica* in sheep. Vet. Parasitol. 176, 170–176.

Gaasenbeek, C.P.H., Moll, L., Cornelissen, J.B.W.J., Vellema, P., Borgsteede, F.H.M., 2001. An experimental study on triclabendazole resistance of *Fasciola hepatica* in sheep. Vet. Parasitol. 95, 37–43.

Gordon, D.K., Zadoks, R.N., Stevenson, H., Sargison, N.D., Skuce, P.J., 2012. On farm evaluation of the coproantigen ELISA and coproantigen reduction test in Scottish sheep naturally infected with *Fasciola hepatica*. Vet. Parasitol. 187, 436–444.

Hope-Cawdery, M.J., Strickland, K.L., Conway, A., Crowe, P.J., 1977. Production effects of liver fluke in cattle I. The effects of infection on live weight gain, feed intake and food conversion efficiency in beef cattle. Br. Vet. J. 133, 145–159.

Kajugu, P.E., Hanna, R.E., Edgar, H.W., Forster, F.I., Malone, F.E., Brennan, G.P., Fairweather, I., 2012. Specificity of a coproantigen ELISA test for fasciolosis: lack of cross-reactivity with *Paramphistomum cervi* and *Taenia hydatigena*. Vet. Rec. 171, 502.

Martinez-Perez, J.M., Robles-Perez, D., Rojo-Vazquez, F.A., Martinez-Valladares, M., 2012. Comparison of three different techniques to diagnose *Fasciola hepatica* infection in experimentally and naturally infected sheep. Vet. Parasitol. 190, 80–86.

Martínez-Valladares, M., del Rosario Famularo, M., Fernández-Pato, N., Castañón-Ordóñez, L., Cordero-Pérez, C., Rojo-Vázquez, F.A., 2010. Efficacy of nitroxynil against Fasciola hepatica resistant to triclabendazole in a naturally infected sheep flock. Parasitol. Res. 107, 1205–1211.

Mezo, M., Gonzalez-Warleta, M., Carro, C., Ubeira, F.M., 2004. An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). J. Parasitol. 90, 845–852.

Mitchell, G.B., Maris, L., Bonniwell, M.A., 1998. Triclabendazole-resistant liver fluke in Scottish sheep. Vet. Rec. 143, 399.

MLA, 2005. The cattle parasite atlas: A regional guide to cattle parasite control in Australia. Meat & Livestock Australia. <http://www.mla.com.au/Livestock-production/Animal-health-welfare-and-biosecurity/Parasites/Cattle-parasite-atlas>.

Moll, L., Gaasenbeek, C.P.H., Vellema, P., Borgsteede, F.H.M., 2000. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in The Netherlands. Vet. Parasitol. 91, 153–158.

Mooney, L., Good, B., Hanrahan, J.P., Mulcahy, G., de Waal, T., 2009. The comparative efficacy of four anthelmintics against a natural acquired *Fasciola hepatica* infection in hill sheep flock in the west of Ireland. Vet. Parasitol. 164, 201–205.

Novobilsky, A., Averpil, H.B., Hoglund, J., 2012. The field evaluation of albendazole and triclabendazole efficacy against *Fasciola hepatica* by coproantigen ELISA in naturally infected sheep. Vet. Parasitol. 190, 272–276.

Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R., 2011. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). Vet. Parasitol. 178, 364–366.

Ortiz, P., Scarcella, S., Cerna, C., Rosales, C., Cabrera, M., Guzmán, M., Lamenza, P., Solana, H., 2013. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Cajamarca (Peru): a clinical trial and an *in vivo* efficacy test in sheep. Vet. Parasitol. 195, 118–121.

Overend, D.J., Bowen, F.L., 1995. Resistance of *Fasciola hepatica* to triclabendazole. Austr. Vet. J. 72, 275–276.

Palmer, D.G., Lyon, J., Palmer, M., Forshaw, D., 2013. Diagnostic sensitivity and specificity of a F. hepatica copro-antigen ELISA and its use for maintaining freedom of liver fluke infection in Western Australia. In: 24th International Conference of the World Association for the Advancement of Veterinary Parasitology, Perth, August.

Pook, J.F., Power, M.L., Sangster, N.C., Hodgson, J.L., Hodgson, D.R., 2002. Evaluation of tests for anthelmintic resistance in cyathostomes. Vet. Parasitol. 106, 331–343.

Robles-Pérez, D., Martínez-Pérez, J.M., Rojo-Vázquez, F.A., Martínez-Valladares, M., 2013. The diagnosis of fasciolosis in feces of sheep by means of a PCR and its application in the detection of anthelmintic resistance in sheep flocks naturally infected. Vet. Parasitol. 197, 277–282.

Sangster, N.C., Dobson, R.J., 2002. Anthelmintic resistance. In: Lee, D.L. (Ed.), The Biology of Nematodes. Taylor and Francis, London and New York, pp. 531–567.

Sargison, N.D., Scott, P.R., 2011. Diagnosis and economic consequences of triclabendazole resistance in *Fasciola hepatica* in a sheep flock in south-east Scotland. Vet. Rec. 168, 159–164.

Thomas, I., Coles, G.C., Duffus, K., 2000. Triclabendazole-resistant *Fasciola hepatic*a in southwest Wales. Vet. Rec. 146, 200.

Vercruysse, J., Claerebout, E., 2001. Treatment vs. non-treatment of helminth infections in cattle: defining the threshold. Vet. Parasitol. 98, 195–214.

Winkelhagen, A.J.S., Mank, T., de Vries, P.J., Soetekouw, R., 2012. Apparent triclabendazole-resistant human *Fasciola hepatica* infection, the Netherlands. Emerg. Infect. Dis. 18, 1028–1029.

Wood, I.B., Amaral, N.K., Bairden, K., Duncan, J.L., Kassai, T., Malone Jr., J.B., Pankavich, J.A., Reinecke, R.K., Slocombe, O., Taylor, S.M., Vercruysse, J., 1995. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). Vet. Parasitol. 58, 181–213.