First report of closantel treatment failure against *Fasciola hepatica* in cattle

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**Abstract**

Control of *Fasciola hepatica* infection in livestock is based on annual treatment using flukicides such as triclabendazole, albendazole and closantel. However, triclabendazole resistant *F. hepatica* populations are emerging worldwide and resistance is emerging to albendazole, whereas it has until now never been described for closantel. In Sweden, a topical formulation containing a combination of closantel and ivermectin (Closamectin Pour On) has been registered for use in cattle only since 2011. This study evaluated the efficacy of closantel against *F. hepatica* in naturally infected beef cattle using both coproantigen and faecal egg count reduction tests.

Faecal egg counts (FEC) and coproantigen ELISA examinations were conducted in February 2014 in three beef cattle herds (A, B, C) in south-western Sweden. On each farm, 10 *F. hepatica* coproantigen-positive and *F. hepatica* egg-positive animals were allocated after 12–16 weeks of housing into groups and treated topically with a minimum of 20 mg closantel per kg body weight. Faecal samples were collected from selected animals on 0, 7 and 21 day post-treatment (PT).

Based on FEC, closantel efficacy 21 days PT was 72% (95% CI: 65–77%) and 97% (95% CI: 95–98%) on farms A and B, respectively. No FEC reduction at all was observed on farm C. In total, 4, 1 and 6 animals remained coproantigen-positive at 21 days PT on farms A, B and C, respectively.

Closantel treatment failure was confirmed on two of the farms. As the animals were housed 12–16 weeks before treatment and thereafter during the entire study, failure due to the presence of juvenile flukes was excluded. Although the cause of closantel failure currently remains unclear, development of resistance or/and absorption failure of topical administration should be considered. To our knowledge, this is the first report of closantel treatment failure against *F. hepatica* in cattle.

1. Introduction

Control of fasciolosis in cattle and sheep is primarily based on anthelmintic treatment using flukicides. Triclabendazole (TCBZ) is considered the drug of choice against *Fasciola hepatica* due to its high efficacy against both juvenile and adult stages in grazing livestock (Fairweather, 2011a). Other flukicides, such as closantel (CLS), albendazole (ABZ) and nitroxynil, are also available on the market, often in countries where TCBZ is not registered. However, increasing numbers of cases of TCBZ resistance have recently been reported in Australia, Europe and South America (Olaechea et al., 2011; Gordon et al., 2012a; Ortiz et al., 2013; Brockwell et al., 2014), indicating that flukicide resistance is a significant concern worldwide. However, it has also been proven that lack of flukicide efficacy does not necessarily mean resistance (Fairweather, 2011b). Under-dosing, inadequate storage of the anthelmintic, metabolic changes, improperly applied anthelmintic and co-infection with rumen flukes may also explain observed treatment failure (Fairweather, 2011b; Skuce and Zadoks, 2013; Hanna et al., 2015). Lack of standardised guidelines, including treatment thresholds, for resistance in *F. hepatica* makes identification of ‘true resistance’ and ‘treatment failure’ controversial.

Closantel is an anthelmintic drug belonging to the salicylanilide derivate group and was first discovered in the 1970s (Janssen and Sipido, 1977). It is effective against immature *F. hepatica* aged 6 weeks and older, but not against juvenile flukes (Fairweather and Boray, 1999). Closantel uncouples ions involved in oxidative
phosphorylation in mitochondria, but the exact mechanism of action is not fully understood (Skuce and Fairweather, 1990). To date, there have been no reports of closantel resistance against liver flukes and only one report of resistance against Haemonchus contortus (Rofe et al., 1990). The drug shows high efficacy (95–100%) against liver flukes when applied/administered as an oral formulation or by injection (Borgsteede et al., 2005, 2008; Mooney et al., 2009). Furthermore, closantel treatment has repeatedly been shown to be effective in sheep and cattle herds infected with TCBZ-resistant F. hepatica populations (Coles et al., 2000; Gordon et al., 2012b; Hanna et al., 2015). Thus, it has become an essential second-line drug against TCBZ-resistant liver flukes.

Although guidelines for diagnosis of anthelmintic resistance in F. hepatica are lacking, there is agreement in the literature that the faecal egg reduction test (FERCT) combined with other methods such as coproantigen reduction (Gordon et al., 2012b; Novobilský et al., 2012; Brockwell et al., 2013; Hanna et al., 2015), detection of fluke DNA by polymerase chain reaction (PCR) (Robles-Perez et al., 2013) and/or in vitro egg hatch test (Alvarez et al., 2009; Fairweather et al., 2012; Canevari et al., 2014) provide reliable information about flukicide efficacy in naturally infected animals. Furthermore, post-treatment fluke histology (Hanna et al., 2010) can show direct morphological damage in flukes, but is not applicable in common veterinary practice.

In Sweden, control of liver flukes in sheep and cattle has for decades been based solely on ABZ (Novobilský et al., 2012, 2015). However, a new combination of closantel and ivermectin (marketed as Closamectin, Norbrook, UK) as a pour-on formulation was launched on the Swedish market in 2011. Immediately after registration, Closamectin Pour On became the key anthelmintic for Swedish beef cattle herds infected with F. hepatica. However, the Swedish Animal Health Service recently expressed concerns about Swedish beef cattle herds infected with F. hepatica. Thus, it has become an essential second-line drug against TCBZ-resistant liver flukes.

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### 2. Materials and methods

#### 2.1. Animals and study design

Three beef cattle farms (Farm A, Farm B, Farm C) located in Västra Götaland in south-west Sweden were selected for the study. Beef herds on farm A (coordinates: 57°20’57.3”N, 12°26’29.8”E), B (coordinates: 58°15’58.2”N, 11°36’01.6”E) and C (coordinates: 58°13’49.0”N, 11°36’13.7”E) consisted of 220, 75 and 120 Charolaise and crossbred cattle, respectively. All three farms have had a long documented history with F. hepatica confirmed by meat inspection. On all three farms, Closamectin Pour On (ivermectin + closantel) had been routinely applied at housing during January or February in the three previous years. As the in-herd prevalence at meat inspection varied from 50 to 80%, only F. hepatica-positive animals were selected using pre-screening sampling. In pre-screening, faecal samples were collected from a randomly selected subsample of 25% of cattle in each herd. Samples were examined both by coproscopy and coproantigen ELISA, as described below. Ten positive individuals identified both by sedimentation and coproantigen ELISA were selected for treatment on each farm.

The study was performed as part of the routine control programme by licenced veterinarians from Swedish Animal Health, Sweden, and no ethical permission was required according to Animal Welfare Act 2009:021. Deworming was performed on all farms in the second half of February 2014. At that time, the cattle had been housed for 12–16 weeks and had no access to outdoor pasture. All animals were last treated for liver fluke 12 months before the study. Beef cows selected on a pre-screening basis were dewormed with Closamectin Pour On Solution for Cattle® (Norbrook, UK) at the recommended dose, which corresponds to 20 mg closantel per kg body weight. The drug used on all three farms originated from the same commercial batch and it was stored in a cool, dry, dark place in accordance with the manufacturer’s recommendation. All animals except those on farm A were weighed prior to drug application. Thus, dosing was accurate except on farm A, where animals were dosed according to corresponding highest cow weight obtained at the last scaling. All treated animals were kept inside during the entire study, including drug application. At the time of drug application, the hair on the spinal area of the animals was dry, with no dermal injuries or contaminants.

Efficacy of closantel was evaluated by faecal egg count (FEC) and coproantigen ELISA. Faecal samples were collected at day 0 (just before application), day 7 and day 21 post-treatment (PT). The 7- and 21-day intervals were chosen based on previous studies (Moll et al., 2000; Daniel et al., 2012; Brockwell et al., 2013, 2014; Hanna et al., 2015).

#### 2.2. Diagnostic procedures

Faecal samples were collected at the specified intervals directly from the rectum of individual animals. Care was taken to ensure that a clean rectal glove was used for every animal. Samples were identified with animal ID on ear tags and immediately sent by mail to our laboratory in Uppsala. The samples were stored at 4 °C during delivery and storage and processed immediately after arrival. The maximum interval between sampling on the farm and sample processing in the laboratory was 48 h. FEC were carried out after sedimentation according to Thienpont et al. (1979) with some modifications. In brief, 10 g of faeces were homogenised in ~100 ml tap water and filtered through 300 and 150 μm metal sieves. The filtered suspension was left to settle for 5 min in a 100-ml beaker and then the supernatant was removed using a vacuum pump. This procedure was repeated several times. The entire sediment was observed and eggs were counted under a microscope at 40× magnification.

Coproantigen in faeces was determined using the commercial BIO-X ELISA (BIO K 201, BIO-X Diagnostics, Belgium) with slight modifications to the manufacturer’s protocol as described by Brockwell et al. (2013). The cut-off was optimised in our laboratory by prior coproantigen ELISA examination of truly F. hepatica-positive and F. hepatica-negative faecal samples. These comprised 40 positive samples originating from three different beef F. hepatica-positive herds where the infection status was confirmed by serology (Novobilský et al., 2014) and meat inspection data, and 40 negative faeces samples taken from the liver of a fluke-free herd at the SLU university farm in Lövsta, Uppsala, Sweden. Infection-free status of this herd was also verified by serology and sedimentation.

#### 2.3. Molecular confirmation of F. hepatica eggs in faecal samples

To confirm the presence of F. hepatica eggs and exclude the presence of paramphistomes (Paramphistomum cervi, Calicophoron daubneyi) in faeces, polymerase chain reaction (PCR) was carried out on DNA isolated from eggs concentrated during the sedimentation process. DNA was obtained from the eggs both by chemical disintegration used for Echinococcus eggs (Mathis et al., 1996) and mechanical disintegration using a plastic pestle (Oberhauserová et al., 2010). DNA from chemically and mechanically disrupted eggs was isolated using DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer’s instructions. Egg DNA samples were amplified using Ampli Taq Gold kit according to Rassolovicsavá et al. (2010) with two pairs of internal transcribed spacer 2 (ITS-2) primers specific for F. hepatica (Králóvá-
Hromadová et al., 2008) and P. cervi (Bazsalovicsová et al., 2010). Further, a mitochondrial fragment of cytochrome oxidase-I specific for C. daubneyi was amplified according to Martínez-Ibeas et al. (2013). The amplification products were separated on 1% agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, USA). F. hepatica and P. cervi adult DNA and DNA extracted from Galba truncatula experimentally infected with C. daubneyi obtained from previous studies (Bazsalovicsová et al., 2010; Novoborský et al., 2013; Titi et al., 2014) were used as positive controls.

2.4. Statistical analysis

Calculation of closantel efficacy based on FEC was determined by two different methods: 1) FEC reduction was expressed as the difference in arithmetic means between pre-treatment and post-treatment levels with 95% confidence limit, as recommended by the World Association for the Advancement of Veterinary Parasitology (Coles et al., 1992); 2) the percentage FEC reduction was determined using Bayesian hierarchical models in the platform ‘eggCounts’ (http://www.math.uzh.ch/as/index.php?id=254) (Torgerson et al., 2014). Coproantigen levels were expressed as per cent of positivity according to the manufacturer’s protocol and reduction in coproantigen levels was determined by the same approach as for FEC (Brockwell et al., 2014; Coles et al., 1992). The cut-off value in optimised coproantigen ELISA was identified by receiver operating characteristic (ROC) curve analysis, using Graph Pad Prism 5.02 (GraphPad Software, USA). The differences in FEC and coproantigen values between pre- and post-treatment were assessed by nonparametric Mann–Whitney test with significance P < 0.05. Non-parametric Spearman correlation analysis was applied to evaluate the relationship between FEC and coproantigen values in the corresponding day’s collection. P < 0.05 was considered significant.

3. Results and discussion

The cut-off value for coproantigen ELISA obtained by ROC analysis of 40 positive and 40 negative control samples was 1.6% positivity. On farm A, one animal was slaughtered during the study due to unknown health problems. The changes in FEC and coproantigen levels during the study are summarised in Table 1. A highly significant (P < 0.001) decrease in FEC was observed on farm B both 7 and 21 days PT. The faecal egg count reduction (FECR) obtained by both methods was higher than 95%. Still, two cows in herd B contained residual eggs 21 days PT. On farm A, FEC decreased after application and at 21 days PT differed significantly (P < 0.05) from that obtained before treatment. However, the FECR achieved was approx. 52% and 72% at days 7 and 21 PT, respectively. FEC on farm C showed an increasing trend and no reduction at all was observed. Only 3 of 10 animals were egg-negative 21 days PT. On farm A, FEC decreased after application and at 21 days PT differed significantly (P < 0.05) from that obtained before treatment. However, the FECR achieved was approx. 52% and 72% at days 7 and 21 PT, respectively. FEC on farm C showed an increasing trend and no reduction at all was observed. Only 3 of 10 animals were egg-negative 21 days PT. On farm A, FEC decreased after application and at 21 days PT differed significantly (P < 0.05) from that obtained before treatment. However, the FECR achieved was approx. 52% and 72% at days 7 and 21 PT, respectively. FEC on farm C showed an increasing trend and no reduction at all was observed. Only 3 of 10 animals were egg-negative 21 days PT.

In two faecal samples from farm C, high variability in egg size

| Faecal egg counts (FEC) and coproantigen values on three Swedish beef cattle farms on days 0, 7 and 21 after pour-on administration of closantel according to the recommended dose rate of 20 mg per kg body weight. Significant differences between pre-treatment and post-treatment values were assessed by Mann–Whitney test (P < 0.05; ***P < 0.001). |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Farm A                         | Farm B          | Farm C          |
| Pre-treatment                  | Post-treatment  | Pre-treatment   | Post-treatment  | Pre-treatment   | Post-treatment  |
| Mean FEC (eggs/10 g faeces)    | Mean % coproantigen positivity | Mean FEC (eggs/10 g faeces) | Mean % coproantigen positivity | Mean FEC (eggs/10 g faeces) | Mean % coproantigen positivity |
| 20.1 ± 5.7                     | 5.4 ± 0.8       | 37.5 ± 7.2      | 2.2 ± 0.7       | 31.3 ± 5.5      | 2.9 ± 0.9       |
| Standard deviation             | Standard deviation | Standard deviation | Standard deviation | Standard deviation | Standard deviation |
| 5.5 ± 0.8                      | 0.9 ± 0.3       | 5.4 ± 0.8       | 0.7 ± 0.3       | 5.5 ± 0.8       | 0.9 ± 0.3       |
| Reduction of coproantigen in % (Ef f) | Reduction of coproantigen in % (Ef f) | Reduction of coproantigen in % (Ef f) |
| 50                             | 67              | 71              | 67              | 71              | 67              |
| n.a.                           | n.a.            | n.a.            | n.a.            | n.a.            | n.a.            |
was observed. Nevertheless, PCR testing confirmed presence of *F. hepatica* and lack of *P. cervi* and *C. daubneyi* (Fig. 1). Only samples with mechanical disintegration of eggs tested positive for *F. hepatica*. Isolation of DNA using chemical disintegration for tae-niidi eggs failed. In addition, cross-reaction between *P. cervi* and *C. daubneyi* DNA samples was observed for *P. cervi* ITS-2 primers. Rumen flukes are sensitive to different spectrum of anthelmintics than liver flukes. Thus, co-infection of *F. hepatica* with rumen flukes might affect FECR for *F. hepatica* (Skuce and Zadoks, 2013). From the results it appears that rumen fluke eggs were not present in herd C and therefore could not compromise our FEC data.

Our data showed that closantel treatment against adult live flukes in naturally infected beef cattle failed in two out of three herds investigated. To our knowledge, this is the first report of closantel failure against *F. hepatica*. Isolation of DNA using chemical disintegration for tae-niidi eggs failed. In addition, cross-reaction between *P. cervi* and *C. daubneyi* DNA samples was observed for *P. cervi* ITS-2 primers. Rumen flukes are sensitive to different spectrum of anthelmintics than liver flukes. Thus, co-infection of *F. hepatica* with rumen flukes might affect FECR for *F. hepatica* (Skuce and Zadoks, 2013). From the results it appears that rumen fluke eggs were not present in herd C and therefore could not compromise our FEC data.

Our data showed that closantel treatment against adult live flukes in naturally infected beef cattle failed in two out of three herds investigated. To our knowledge, this is the first report of closantel failure against *F. hepatica* in the world. Although the cause of the failure is unclear, it raises serious concerns for beef cattle producers world-wide.

Besides development of anthelmintic resistance, several other, often neglected, factors may be responsible for treatment failure. Underdosing, faulty drenching equipment, inadequate parasite control programmes, inappropriate dosing, product failure, age of product, reduced metabolism as a result of liver damage and inadequate and incorrect diagnostic tests are important confounding causes of flukicide failure (Fairweather, 2011a). In this study, three different herds were treated using closantel originating from the same product batch, by the same veterinarian and the same drenching equipment and in agreement with WAAVP guidelines (Coles et al., 1992). Except farm A, where animals were dosed according to the highest animal weight from previous scaling, all animals on farm B and C were dosed according to their specific weight recorded immediately before drug application. Thus, incorrect dosing cannot be completely excluded as the cause of failure on farm A. However, the clear difference in efficacy between farms B and C suggests that all alternative factors listed, including incorrect dosing, product failure and faulty drenching, are very unlikely. The most probable explanation for failure on farm C is resistance or unknown problems with dermal absorption of the drug. Thus, the cause of anthelmintic failure remains speculative at this stage. However, standardised tests for detection of flukicide resistance in live animals are still lacking.

In previous studies, closantel efficacy has been tested using per oral (PO), intramuscular (IM) and/or subcutaneous (SC) administration in sheep and cattle. The efficacy of closantel in these studies varied from 95 to 100% (Guerrero, 1984; Coles et al., 2000; Moll et al., 2000; Borgsteede et al., 2008; Gordon et al., 2012b; Hanna et al., 2015). While PO closantel administration at a dose of 10 mg/kg body weight is available for sheep, subcutaneous injection at a dose of 5 mg/kg body weight is available for cattle. In the present study, a pour-on formulation containing closantel and ivermectin was used, with doses of 20 mg and 500 µg per kg body weight, respectively. This could mean that closantel failure in the study is associated with the topical administration of the drug. Nevertheless, recent data showed that closantel administered topically has 68%, 90% and 99% efficacy against 6-, 8- and 12-week-old flukes, respectively (Geurden et al., 2012). In our study, drug application was performed in the second half of February, when all animals had been housed for 12–16 weeks before drug application. Thus, only adult flukes were present in these animals. This clearly confirms that closantel failure on the Swedish farms studied was not due to lower efficacy against immature flukes, which has been shown in cattle and sheep in previous studies (Fairweather and Boray, 1999; Geurden et al., 2012).

Closantel binds strongly to albumin in the blood and does not metabolise and thus the closantel concentration in blood remains high in comparison to that of benzimidazoles (Michiels et al., 1987). Thus, exposure of flukes to closantel is prolonged due to long half-life and this provides activity against developing immature flukes, even though the drug acts intrinsically on the mature liver fluke (Cromie et al., 2006). According to pharmacokinetic data, closantel in topical administration (Closamectin Pour On, closantel dose 20 mg/kg) (Cmax = 68.5 µg/ml; Tmax = 120 h, AUC = 35 207 µg h/ml) achieves higher concentrations in plasma (Norbrook, 2015) than when administered orally (Cmax = 59.8 µg/ml; Tmax = 67 h, AUC = 21 564 µg h/ml) or subcutaneously (Cmax = 63.4 µg/ml; Tmax = 54 h, AUC = 21 996 µg h/ml) (Cromie et al., 2006). This further confirms that administration route cannot be associated with closantel failure in this study. On the other hand, PO applications of TCBO are often superior to pour-on applications (Hutchinson et al., 2009; Martin et al., 2009). For closantel, no comparison between the efficacy of per oral, subcutaneous and topical formulations has yet been available.

A combination of FEC and coproantigen ELISA is generally accepted as the optimal method for diagnosis of efficacy/resistance in *F. hepatica* (Planagan et al., 2011; Gordon et al., 2012b; Brockwell et al., 2013; Hanna et al., 2015). However, interpretation of data from FEC and coproantigen ELISA and their combination and particularly thresholds for both tests are unclear. According to WAAVP guidelines 95% or 90% efficacy has been applied as the threshold for FECR (Coles et al., 1992). However, WAAVP guidelines were originally designed for gastrointestinal nematode and not for flukes. In addition, disadvantages of FEC as regards irregular

**Table 2** Spearman correlation analysis *P*-values between faecal egg counts (FEC) and coproantigen (cAg) values within each group. (* *P* < 0.05; ** *P* < 0.01).

| Farm | cAg day 0 | cAg day 7 | cAg day 21 |
|------|-----------|-----------|-----------|
| A    | 0.744     | 0.050     | 0.021*    |
| B    | 0.003**   | 0.218     | 0.313     |
| C    | 0.427     | 0.060     | 0.005**   |

**Fig. 1.** Agarose gel electrophoresis of PCR products obtained by amplification of DNA samples using primers specific for (A) *Fasciola hepatica*, amplified region 112 bp (Krállová-Hromadová et al., 2008), (B) *Parafasciola cervi*, amplified region 161 bp (Bazaralovicová et al., 2010) and (C) *Calicophoron daubneyi*, amplified region 885 bp (Martínez-Ibeas et al., 2013). The eggs tested originated from farm C, where high variability in egg size was observed. (1) DNA isolated from egg suspension by chemical disintegration; (2) DNA isolated from egg suspension by mechanical disintegration; (3) DNA from *F. hepatica* adult; (4) DNA from *P. cervi* adult; (5) DNA from *Galba truncatula* experimentally infected with *C. daubneyi*. 

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excretion of eggs from the gall bladder have been reported (Flanagan et al., 2011). In our study, FECR was apparently >90% on farms A and C, but >95% on farm B. Coproantigen results for these three herds were more complex. Although percentage coproantigen positivity decreased in all three groups 21 days PT in comparison with pre-treatment values, the numbers of coproantigen-positive animals on day 21 PT differed (Table 1). Even on farm B, where FECR was >95% and coproantigen was reduced significantly (P < 0.001), two cows had a few eggs in faeces and one coproantigen-positive animal was observed 21 days PT. Similarly, Flanagan et al. (2011) detected one coproantigen-positive sheep in a group successfully treated with TCBZ where no flukes were found after treatment and FECR was 95–98%. They attributed this finding to a false-positive reaction due to slow release of disintegrated fragments of flukes from the liver.

Interestingly, FEC and coproantigen values within the test group and the corresponding sample collection were positively correlated after treatment on farms A and C, where closantel failure occurred, but before treatment on farm B. The significant correlation between FEC and coproantigen further confirms mutual and complementary application of both methods for flukicide efficacy/resistance detection, as described previously (Flanagan et al., 2011; Novobilský et al., 2012; Brockwell et al., 2013).

Limitations of FEC as regards irregular release of F. hepatica eggs and the fact that commercial coproantigen ELISA can give both false-negative (Gordon et al., 2012b; Novobilský et al., 2012; Brockwell et al., 2013) and false-positive results (Flanagan et al., 2011) make interpretation of flukicide efficacy very complicated, especially under field conditions. Thus, there is an urgent need for standardised protocols for evaluation of flukicide efficacy, especially for thresholds applied for FEC and coproantigen ELISA.

Conflict of interest statement

The authors declare that they have no competing interests.

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References

Alvarez, L., Moreno, G., Moreno, L., Ceballos, L., Shaw, L., Fairweather, I., 2009. Comparative assessment of albendazole and triclabendazole ovicidal activity on Fasciola hepatica eggs. Vet. Parasitol. 164, 211–216.
Bazsalovicsová, E., Králová-Hromadová, I., Spakulová, M., Reblanová, M., Oberhauserová, K., 2010. Determination of ribosomal internal transcribed spacer 2 (ITS2) interspecific markers in Fasciola hepatica, Fascioloides magna, Dicrocoelium dendriticum and Paramphistomum cervi (Trematoda), parasites of wild and domestic ruminants. Helminthology 47, 76–82.
Borgsteede, F.H.M., Moll, L., Vellema, P., Geurden, T., Bartram, D., Van Brussel, L., Bo, L., Scott-Rand, E., Rugg, D., 2012. Evaluation of the comparative efficacy of a moxidectin plus triclabendazole pour-on solution against adult and immature liver fluke, Fasciola hepatica, in cattle. Vet. Parasitol. 189, 227–232.
Brockwell, Y.M., Zadors, R., Skuce, P., Sangster, N., 2012a. Confirmation of triclabendazole resistance in liver fluke in the UK. Vet. Rec. 171, 159.
Brockwell, Y.M., Zadors, R.N., Stevenson, H., Sangsörn, N.D., Skuce, P.J., 2012b. On farm evaluation of the coproantigen ELISA and coproantigen resistance test in Scottish sheep naturally infected with Fasciola hepatica. Vet. Parasitol. 187, 436–444.
Guerrero, J., 1984. Closantel – a review of its antiparasitic activity. Prev. Med. 2, 317–327.
Hanna, R.E., McMahon, C., Ellison, S., Edgar, H.W., Kajugu, P.E., Gordon, A., Irwin, D., 2010. Fasciola hepatica: histological changes in the reproductive structures of triclabendazole (TCBZ)-sensitive and TCBZ-resistant flukes after treatment in vivo with TCBZ and the related benzimidazole derivative,compound alpha. Vet. Parasitol. 178, 240–252.
Hutchinson, C.W., Dawson, M., White, J.A., McFarland, C.C., Martin, P.J., 2009. Efficacy of an injectable combination anthelmintic (nioxynil plus clorsulon plus ivermectin) against early immature Fasciola hepatica compared to triclabendazole combination in Fasciola hepatica (Trematoda) from naturally and experimentally infected cattle. Vet. Parasitol. 162, 278–284.
Janssen, M.A.C., Sipido, V.K., 1977. Antiparasitic salicylamine derivatives. Patent Number: US4005218 A.
Králová-Hromadová, I., Spakulová, M., Horáčková, E., Turčeková, L., Novobilský, A., Beck, R., Koudelá, R., Marinucil, A., Rajský, D., Pybus, M., 2008. Sequence analysis of ribosomal and mitochondrial genes of the giant liver fluke Fascio
coides magna (Trematoda: Fasciolidae): intraspecific variation and differentiation from Fasciola hepatica. J. Parasitol. 94, 58–67.
Martin, P.J., Chambers, M., Hennessey, D.R., 2009. Efficacy against Fasciola hepatica and the pharmacokinetics of triclabendazole administered by oral and topical routes. Aust. Vet. J. 87, 200–203.
Martínez-Ibeas, A.M., Narciso, F., Gonzalez-Warleta, M., Martinez-Valladares, M., Castro-Martínez, J.A., González-Landa, T., Núñez, C., Merlo, M., Martínez-González, M.Y., 2012. Development and validation of a mtDNA multiplex PCR for identification and discrimination of Calicophoron daubneyi and Fasciola hepatica in the Galá truncate snail. Vet. Parasitol. 195, 57–64.
Martin, A., Deplazes, P., Eckert, J., 1996. An improved test system for PCR-based specific detection of Echinococcus multilocularis eggs. J. Helminthol. 70, 219–222.
Michiels, M., Meulders, W., Heykants, J., 1987. The metabolism and fate of closantel in cattle (Flukovir) in situ in cattle. Drug. Dev. Res. 14, 235–231.
Toboli, G., Koudelá, R., Marinucil, A., Rajský, D., Pybus, M., 2008. Sequence analysis of ribosomal and mitochondrial genes of the giant liver fluke Fascio
coides magna (Trematoda: Fasciolidae): intraspecific variation and differentiation from Fasciola hepatica. J. Parasitol. 94, 58–67.
Martin, P.J., Chambers, M., Hennessey, D.R., 2009. Efficacy against Fasciola hepatica and the pharmacokinetics of triclabendazole administered by oral and topical routes. Aust. Vet. J. 87, 200–203.
Martínez-Ibeas, A.M., González-Warleta, M., Martínez-Valladares, M., Castro-Martínez, J.A., González-Landa, T., Núñez, C., Merlo, M., Martínez González, M.Y., 2012. Development and validation of a mtDNA multiplex PCR for identification and discrimination of Calicophoron daubneyi and Fasciola hepatica in the Galá truncate snail. Vet. Parasitol. 195, 57–64.
Martin, A., Deplazes, P., Eckert, J., 1996. An improved test system for PCR-based specific detection of Echinococcus multilocularis eggs. J. Helminthol. 70, 219–222.
Michiels, M., Meulders, W., Heykants, J., 1987. The metabolism and fate of closantel in cattle (Flukovir) in situ in cattle. Drug. Dev. Res. 14, 235–231.
Toboli, G., Koudelá, R., Marinucil, A., Rajský, D., Pybus, M., 2008. Sequence analysis of ribosomal and mitochondrial genes of the giant liver fluke Fascio
coides magna (Trematoda: Fasciolidae): intraspecific variation and differentiation from Fasciola hepatica. J. Parasitol. 94, 58–67.
Martin, P.J., Chambers, M., Hennessey, D.R., 2009. Efficacy against Fasciola hepatica and the pharmacokinetics of triclabendazole administered by oral and topical routes. Aust. Vet. J. 87, 200–203.
Martínez-Ibeas, A.M., González-Warleta, M., Martínez-Valladares, M., Castro-Martínez, J.A., González-Landa, T., Núñez, C., Merlo, M., Martínez González, M.Y., 2012. Development and validation of a mtDNA multiplex PCR for identification and discrimination of Calicophoron daubneyi and Fasciola hepatica in the Galá truncate snail. Vet. Parasitol. 195, 57–64.
Martin, A., Deplazes, P., Eckert, J., 1996. An improved test system for PCR-based specific detection of Echinococcus multilocularis eggs. J. Helminthol. 70, 219–222.
Michiels, M., Meulders, W., Heykants, J., 1987. The metabolism and fate of closantel in cattle (Flukovir) in situ in cattle. Drug. Dev. Res. 14, 235–231.
Toboli, G., Koudelá, R., Marinucil, A., Rajský, D., Pybus, M., 2008. Sequence analysis of ribosomal and mitochondrial genes of the giant liver fluke Fascio
coides magna (Trematoda: Fasciolidae): intraspecific variation and differentiation from Fasciola hepatica. J. Parasitol. 94, 58–67.
and triclabendazole efficacy against _Fasciola hepatica_ by coproantigen ELISA in naturally infected sheep. Vet. Parasitol. 190, 272–276.
Novobilský, A., Engström, A., Sollenberg, S., Gustafsson, K., Morrison, D.A., Höglund, J., 2014. Transmission patterns of _Fasciola hepatica_ to ruminants in Sweden. Vet. Parasitol. 203, 276–286.
Novobilský, A., Katrný, M., Beran, L., Rondelaud, D., Höglund, J., 2013. _Lymnaea palustris_ and _Lymnaea fuscus_ are potential but uncommon intermediate hosts of _Fasciola hepatica_ in Sweden. Parasit. Vect. 6, 251.
Novobilský, A., Sollenberg, S., Höglund, J., 2015. Distribution of _Fasciola hepatica_ in Swedish dairy cattle and associations with pasture management factors. Geospat. Health 9, 293–300.
Oberhauserová, K., Bazsalovicsová, E., Krállová-Hromadová, I., Major, P., Reblanová, M., 2010. Molecular discrimination of eggs of cervid trematodes using the teflon (PTFE) technique for eggshell disruption. Helminthologia 47, 147–151.
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., Guzman, 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.
Robles-Pérez, D., Martínez-Pérez, J.M., Rojo-Vazquez, 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.
Rolfe, P.F., Boray, J.C., Fitzgibbon, C., Parsons, G., Kemsley, P., Sangster, N., 1990. Closantel resistance in _Haemonchus contortus_ from sheep. Aust. Vet. J. 67, 29–31.
Skuce, P.J., Fairweather, I., 1990. The effect of the hydrogen ionophore closantel upon the pharmacology and ultrastructure of the adult liver fluke _Fasciola hepatica_. Parasitol. Res. 76, 241–250.
Skuce, P.J., Zadok, R.N., 2013. Liver fluke – a growing threat to UK livestock production. Cattle Pract. 21, 138–149.
Thienpont, D., Rochete, F., Vanpris, O.F., 1979. Diagnosing Helminthiasis Through Coprological Examination. Janssen Research Foundation, Beerse, Belgium.
Titi, A., Rondelaud, D., Mekroud, A., Vignoles, P., Dreyfuss, G., 2014. Natural light influences cercarial emergence of _Calicophoron daubneyi_ but not that of _Haploometra cylindracea_ from temperature-challenged _Galba truncatula_. Parasitol. Res. 113, 3557–3563.
Torgerson, P.R., Paul, M., Furrer, R., 2014. Evaluating faecal egg count reduction using a specifically designed package “eggCounts” in R and a user friendly web interface. Int. J. Parasitol. 44, 299–303.