Evaluation of the Fasciolicidal/Nematicidal Efficacy of an Intramuscular Combination of Clorsulon/Levamisole in Naturally Infected Dairy Cattle

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Abstract
The aim of the present study was to assess the fasciolicidal and nematicidal efficacy of a new combination of clorsulon/levamisole, which was formulated for intramuscular administration, in dairy cattle. For the study, sixteen Holstein-Freisian dairy cows were selected for inclusion in the trial based on finding Fasciola hepatica eggs or gastrointestinal nematodes (GIN) in the feces. Ten of these cows were naturally infected with F. hepatica, and 6 were naturally infected with GIN. On Day 0 (zero), all animals were treated with a combination of clorsulon (2.5 mg/kg/IM and levamisole (2.5 mg/kg/IM) administered at the dose recommended by the manufacturer (1 ml/20 kg/bw). Subsequently, their feces were analyzed on days 0, 7, 14 and 21 post treatments by the Flukefinder sedimentation method and McMaster technique to determine the percentages of egg reduction in the animals, with reference to the egg load count on day 0. The results obtained in the current work indicated an efficacy of 100% that was determined by the percentage reduction of eggs of F. hepatica and/or GIN. This new fasciolicide/nematicide formulation exerted a remarkable effect and can be considered a new alternative to control F. hepatica and gastrointestinal worms in dairy cattle.

Keywords
Efficacy, Fasciola Hepatica, Gastrointestinal Nematodes, Clorsulon, Levamisole, Cattle

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1. Introduction

_Fasciola hepatica_ and gastrointestinal nematodes (GIN) are common worldwide parasite infections in ruminants. They are relevant because of the large economic losses that occur in livestock worldwide [1]. On the other hand, for decades, chemical control has been the main strategy used to address these diseases. Several options depend on a wide number of products, such as clorsulon, nitroxinil, closantel, albendazole, triclabendazole or oxyclozanide [2]. Triclabendazole (TCBZ) has been the drug of choice to treat liver fluke infections in ruminants for over 38 years. Resistance against TCBZ was reported in the mid-1990s in Australia [3], and there have been several reports worldwide [4]. In cattle, reduced efficacy of TCBZ to _F. hepatica_ has been reported in the Netherlands [5] and in Scotland [6]. Therefore, new alternatives should be sought to control fasciolosis or GIN.

Two compounds to be highlighted are clorsulon and levamisole, which are highly effective against adult liver flukes and GIN, respectively [5] [7] [8]. Clorsulon belongs to the benzenesulfonamide family that is recommended for the treatment and control of adult liver flukes (e.g., _Fasciola hepatica_ and _Fasciola gigantica_) in cattle in the form of suspensions for oral use or injectable formulations. It causes severe damage to the tegument of _F. hepatica_ [9] [10] [11]. Levamisole is the hydrochloride salt of the synthetic imidazothiazole derivative, levamisole, with anthelminthic and immunomodulating activities. It is an anthelmintic drug that is commonly used to treat parasitic, viral, and bacterial infections. It was manufactured by Janssen and was first used in 1969 as an agent to treat worm infestations [12]. This drug has long been used to effectively control these important parasitic diseases.

Likewise, the administration of combined oral compounds (fasciolicide/nematicide) has been used to obtain a broad spectrum of efficacy, and more recently, some anthelmintic compounds have been solubilized and formulated for parenteral application [13].

The aim of the present study was to assess the anthelmintic efficacy of a combined formulation of clorsulon/levamisole, which was prepared for intramuscular application, in dairy cows naturally infected with _F. hepatica_ and/or GIN.

2. Material and Methods

2.1. Study Location

The study was carried out on a farm located at Tulancingo, Hidalgo, central Mexico.

2.2. Animals

Sixteen Holstein-Freisian dairy cows naturally infected with _Fasciola hepatica_ and/or gastrointestinal nematodes (GIN) were used. All of them were individually ear tagged for further monitoring. This selection was made based on the
highest load of eggs/animal, as determined by the Flukefinder sedimentation technique and/or McMaster technique.

Likewise, to reduce stress, the selected animals were housed at their specific premises for adaptation to food and mutual group coexistence.

2.3. Compounds

Clorsulon and levamisole were formulated for intramuscular application by Laboratorio Salud Animal, S.A. de C.V., México, containing 2.5 mg/ml clorsulon and 6 mg/ml levamisole chlorhydrate.

2.4. Experimental Design

On Day 0 (zero), all animals were treated with an intramuscularly administered combination of clorsulon/levamisole at the dose recommended by the manufacturer (1 ml/20kg/bw). Subsequently, their feces were collected and analyzed on days 0, 7, 14 and 21 post treatment by the Flukefinder fecal sedimentation method to determine the *F. hepatica* egg counts [14] and by the McMaster technique to obtain the percentages of egg reduction in the animals.

2.5. Efficacy

Flukicidal or nematicidal activities were assessed based on the percentage reductions of *F. hepatica* or GIN eggs on the days analyzed post treatment with respect to the number of eggs present on Day 0.

Efficacy was calculated using the following formula: [15].

\[
\text{Efficacy} = \frac{\text{FEC}_0 - \text{FEC}_{21}}{\text{FEC}_0} \times 100
\]

FEC0 and FEC21 represent the mean fecal egg counts at 21 days post treatment or at the start of treatment, respectively.

3. Results and Discussion

The information obtained is shown in Tables 1-4.

The efficacy conferred by the compounds consisted of a 100% reduction in eggs either for *F. hepatica* and/or gastrointestinal worms.

**Table 1** shows the coprological analysis of the fluky cows, where it is clearly seen that from day 7 post treatment, all animals were negative for the presence of *Fasciola* eggs, and maintained this high fasciolicidal activity in the analyses on days 14 and 21.

The egg counts on day 0 (beginning of treatment) showed a minimum of 4 eggs/animal and maximum of 131 eggs/animal (\( \bar{x} = 24.9 \) eggs/animal).

**Table 2** shows the outstanding efficiency of the combined compounds, which, when applied intramuscularly, provided 100% efficacy in all treated animals.

**Table 3** shows the coprological analysis of the cows infected with GIN. Here, the percentage of egg reduction was 100%, observed from the sampling on Day 7, and this reduction remained constant until day 21, the end of the study.
Table 1. Coprological analyses of cows naturally infected with fasciola hepatica before and after treatment with a combined formulation of clorsulon/levamisole*.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Weight (kg)</th>
<th>Dose (ml)</th>
<th>Number of eggs (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 (Tx.)**</td>
</tr>
<tr>
<td>1</td>
<td>220</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>380</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>400</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>280</td>
<td>14</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>270</td>
<td>13.5</td>
<td>31</td>
</tr>
<tr>
<td>6</td>
<td>330</td>
<td>16.5</td>
<td>13</td>
</tr>
<tr>
<td>7</td>
<td>380</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>160</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>260</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>290</td>
<td>14.5</td>
<td>131</td>
</tr>
</tbody>
</table>

* = Clorsulon 2.5 mg/kg/IM/Levamisole 6 mg/kg/IM., ** = Day of treatment. Tx = Treatment.

Table 2. Efficacy of intramuscular combined clorsulon/levamisole against fasciola hepatica in naturally infected dairy cows.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Efficacy (%) (days)</th>
<th>Global Efficacy %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0*</td>
<td>7</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>100</td>
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<tr>
<td>5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
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</tr>
<tr>
<td>7</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* = Day of treatment.

For GIN, the minimum and maximum egg counts were 50 and 150 (\(\bar{x} = 83.3\) eggs/animal).

Therefore, in terms of efficacy, it can be seen that the combined administration of clorsulon/levamisole generated remarkable results, where from day 7 post treatment, all animals remained negative for GIN eggs until the end of the study (Table 4).

Wood et al. (1995) [16] reported that this clinical field test for egg reduction
Table 3. Coprological analyses of cows naturally infected with gastrointestinal nematodes before and after treatment with a combined formulation of clorsulon/levamisole*.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Weight (Kg)</th>
<th>Dose (ml)</th>
<th>No. of eggs (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 (Tx.)**</td>
</tr>
<tr>
<td>11</td>
<td>360</td>
<td>18</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>340</td>
<td>17</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>320</td>
<td>16</td>
<td>150</td>
</tr>
<tr>
<td>14</td>
<td>420</td>
<td>22</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>260</td>
<td>13</td>
<td>50</td>
</tr>
<tr>
<td>16</td>
<td>390</td>
<td>19.5</td>
<td>50</td>
</tr>
</tbody>
</table>

* = Clorsulon 2.5 mg/kg/IM/Levamisole 6 mg/kg/IM., ** = Day of treatment. Tx = Treatment.

Table 4. Efficacy of an intramuscular combined formulation of clorsulon/levamisole against gastrointestinal nematodes in naturally infected dairy cows.

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Efficacy % (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0*</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

* = Day of treatment.

provides valuable information, even though the number of flukes removed is unknown. In addition, these authors mentioned that a suitable time to evaluate the efficacy of a compound is at days 14 or 21 post-treatment. Similar data regarding the high efficacy of clorsulon administered to cattle were obtained by others: 100% adult fluke reduction [5] and 91.2% adult fluke reduction [7].

Coles et al. (2006) [17] considered a compound efficacious if it reduced the EPG by 95%. Our findings showed that this combination of drugs represents a remarkable alternative for treating fasciolosis and GIN.

In general terms, the intramuscular combination of clorsulon/levamisole administered at the dose recommended by the manufacturer exerted a high fasciolicidal/nematicidal efficacy in all dairy cows studied.

The fasciolicidal efficacy obtained here agrees with that obtained by [14], where dairy cows from Australia were treated with clorsulon at a dose of 2 mg/kg/bw, which achieved 100% egg removal.

Perhaps a point to mention is why a control group without treatment was not included and why the efficacy was evaluated with respect to the egg counts on
day 0. The reason is because the cattle owner feared that the animals would continue to be parasitized; because of this, the effectiveness was calculated with the formula reported by [15].

Salgado et al. (2019) [18] mentioned that control of *F. hepatica* and GIN infections requires a deep understanding of parasite epidemiology and of the production system. It is also of paramount importance to preserve the effectiveness of compounds [19].

The pharmaceutical industry has not brought a new fasciolicide to market in more than 38 years due to extremely high production costs. However, a viable alternative approach is to combine the chemical structures of existing compounds with the goal of obtaining higher and/or wider anthelmintic efficacy.

In the present study, due to the small number of animals that were positive for *F. hepatica* and the GIN egg counts, it was not possible to incorporate additional groups to determine if there was an additive, synergistic and/or enhancing effect for the efficacy obtained. However, the results obtained clearly indicate that there was no effect that could interfere with or reduce the efficacy exerted by each of the compounds, since the activity was 100% for both *F. hepatica* and GIN.

Given the difficulty of launching new drugs, there is growing interest in the combined use of drugs [20]. Therefore, combined anti-*Fasciola* and anti-GIN therapy using the parenteral route seems to be a promising alternative. Apparently, this route of administration is preferred for veterinary drugs, as injections enable direct distribution to the circulatory system without the need to pass through the digestive system, where drugs can be inactivated or decomposed [21].

Miller and Craig (1996) [22] demonstrated that a combination of fenbendazole and levamisole produced a synergistic effect by reducing the EPG by 62% in a sheep flock where the efficacy of each individual drug was 1% and 23%, respectively. Other combinations, such as ABZ and IVM to control resistant GIN [23] as well as mebendazole and levamisole for resistant *H. contortus* [24], have been shown to have synergic and additive effects in sheep, respectively.

Due to the aforementioned factors, combined intramuscular formulations can provide convenience and ease of use when parasitic diseases must be controlled.

In addition, combined products have been used to control the immature stages of trematodes. High effectiveness of moxidectin + triclabendazole has been indicated, unlike the mixtures, ivermectin + closantel or ivermectin + clorsulon [25].

Triclabendazole is one of the most widely used anthelmintic drugs to control fasciolosis and began to be used from the 1980s onward [3]. Rolfe and Boray (1987) [26] reported for the first time the emergence of resistance to triclabendazole and since then, there have been numerous reports of this issue [27] and [28]. Therefore, new alternatives for treating *F. hepatica* should be sought.

Ico-Gómes et al. (2021) [29] evaluated the efficacy of a combination of iver-
mectin + clorsulon against *F. hepatica*. Their results indicated an efficacy of 89.8%. The present study has shown that the clorsulon/levamisole combination is an interesting alternative for controlling fasciolosis and GIN.

This study is the first report showing the remarkable efficacy of an intramuscular combination of clorsulon and levamisole in a dairy cow flock with fasciolosis and GIN.

However, it is intended to conduct more field evaluations and controlled tests to establish an efficiency interval with greater precision, as occurred with the introduction of TCBZ, where studies reported varying efficiencies between 93 and 100% when using the same dose orally and with the same stages of flukes [30].

Unfortunately, the aim of the study focused only on comparing the efficacy of the tested compounds for 21 days. Further confirmatory studies aimed at elucidating the actual anthelmintic potential as well as the emergence of resistance to these and other compounds should be encouraged.

4. Conclusion

The new clorsulon/levamisole intramuscular formulation demonstrated remarkable fasciolicidal/nematicidal efficacy in dairy cows.

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Availability of Data and Material

All datasets are included in this manuscript.

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Contributors

FIV contributed to the study design, carried out the field study, wrote the paper, manuscript revision and discussion. YVM and IOL collaborated with the field study, fecal sampling and results interpretation. AFC, ICM and JAM provided assistance and performed laboratory analysis. All authors supervised the experimental procedures, read and approved the final version of the manuscript.

Animal Research

The work was carried out adhering to the guidelines of the Institutional Committee for Use and Care of Experimental Animals of the institution, according to the Mexican Official Regulation NOM-062-ZOO-1999 and Animal Research: Reporting of in Vivo Experiments guidelines was followed.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.
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