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Efficacy of oxyclozanide against adult *Paramphistomum leydeni* in naturally infected sheep

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ABSTRACT

The aim of the current study was to assess oxyclozanide (OCZ) efficacy against *Paramphistomum leydeni* in naturally infected adult sheep. OCZ concentrations in blood stream and gastrointestinal fluids collected from treated animals were also measured. Fifteen *P. leydeni* naturally infected sheep were randomly divided into two groups: untreated control ($n = 5$) and treated ($n = 10$). The treated group was orally drenched with OCZ (20 mg/kg, day 0). A second dose was administered 72 h later. Faecal samples were taken at days 0, +3 and +5. Five sheep from both groups were slaughtered at day +5. At necropsies, rumen, abomasum and small intestine were examined for adult and immature flukes. All recovered flukes were counted and the treatment efficacy was estimated. Additionally, serum and gastrointestinal fluid content (ruminal, abomasal and small intestine) samples, obtained from five treated animals at day +5, were analyzed by HPLC to measure OCZ concentrations. OCZ showed high efficacy (99%) against mature *P. leydeni*. The post-treatment egg reduction was also high after the first dose with values ranging from 98.4% (day +3) to 99.5% (day +5). The highest OCZ concentrations were measured in serum (20.7 ± 11.5 μg/mL) followed by the small intestinal fluid (6.00 ± 4.50 μg/mL). Very low OCZ concentrations (ranging between 0.05 and 0.02 μg/mL) were measured in ruminal and abomasal fluids. OCZ administered to sheep twice (20 mg/kg) by the oral route was highly efficacious against mature stages of *P. leydeni* in naturally infected sheep. Despite a high drug concentration at the intestinal fluid, OCZ efficacy against immature stages could not be assessed. OCZ efficacy and assessment of its concentration profiles in different tissues are considered a contribution to the scarce information available on this ruminant fluke.

1. Introduction

Paramphistomosis is a parasitosis caused worldwide by several amphistome species, mainly belonging to the families *Paramphistomidae* and *Gastrothylacidae* (Sey, 1991), of which *Paramphistomum* is the mostly widely spread genus (Over et al., 1992). The concern about paramphistomes has usually been associated with the tropics and subtropics, with little or insignificant impact on temperate regions. However, numerous recent cases of clinical paramphistomosis with considerable morbidity and mortality have been mainly reported in Britain and Ireland (Zintl et al., 2014). In Argentina, its spread has increased

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over the last years, in some regions being an emerging parasitosis (Sanabria et al., 2009, 2011).

Paramphistomes have a heteroxenous life cycle that involves snails from Lymnaeidae, Bulinidae and Planorbidae families (intermediate hosts). The parasite larvae develop until reaching the cercaria stage, when this emerges from the mollusk, typically encysting on vegetation and then developing into a metacercaria, which is ingested by grazing ruminants (final host). Juvenile parasites first reach the small intestine and feed on the intestinal mucosa. As they grow, they migrate upwards to the reticulum and rumen where they live as adults, shedding eggs into the environment (González-Warleta et al., 2013). Regarding the paramphistomosis pathology, it has been revealed that the immature worms migrating in the small intestine cause more severe damage (including death) than the adult worms (Dorchies et al., 2002; Mavenyengwa et al., 2005). Nevertheless, ruminal lesions have also been associated with heavy infections by adult worms, affecting digestion and absorption and resulting in diarrhea, anorexia, anaemia and weakness (Mavenyengwa et al., 2010; Dorny et al., 2011).

Most of the fasciolicide drugs have no or scarce effect against both immature and adult stages of amphistomes. For instance, closantel (10 mg/kg) was not efficacious, and triclabendazole showed only 50% efficacy at 100 mg/kg (Rolfe and Boray, 1987). Praziquantel, fenbendazole and albendazole were not effective in sheep. In the same experiments, niclosamide had 94–99% efficacy against immature amphistomes, administered at doses of 50 and 100 mg/kg (Rolfe and Boray, 1987).

Oxyclozanide (OCZ) is a salicilanylide compound introduced over 40 years ago. It is routinely used as fasciolicide, showing high efficacy against adult fluke infections in ruminants (Mooney et al., 2009). Unlike other salicilanylides (closantel or rafoxanide), OCZ shows a broad security range (Sinclair, 1969), except for some peripartum restrictions (Spence et al., 1996). Sey (1989) reported less than 90% efficacy against adult paramphistomes after a single OCZ dose (20 mg/kg). In addition, Rolfe and Boray (1987) demonstrated 99–100% reduction against adult and juvenile flukes for OCZ given twice (18.7 mg/kg) in cattle. Spence et al. (1996) reported a significant faecal egg count reduction (FECR) in amphistomes' from dairy cattle treated with of OCZ (16.5 mg/kg). In more recent works, OCZ was found to be highly effective against adult stages of *Calicophoron daubneyi* in goats (Paraud et al., 2009), at a 15 mg/kg dose schedule, since Arias et al. (2013), found a high anthelmintic performance of oxyclozanide in dairy cattle (99% FECR), at a single dose of 15 mg/kg. Conversely, a lower amphistome FECR (83.53%) was observed after a single dose of 15 mg/kg in Indian sheep (Jeyathilakan et al., 2005)

Scarce information concerning the OCZ plasma pharmacokinetic behavior is available. A mean plasma half-life of 6.4 days was reported in sheep (Ali and Bogan, 1987). Plasma and milk concentrations were detectable up to 7.4 days, and 30–47 h, respectively, after a single administration of OCZ in dairy cattle (Fujinuma et al., 2006).

The aim of the current study was to assess OCZ efficacy against *Paramphistomum leydeni* in naturally infected adult sheep. Additionally, OCZ concentrations in serum and gastrointestinal fluids obtained from the same treated animals were measured.

2. Materials and methods

2.1. Animals and efficacy assessment

The study was carried out on a farm located in Entre Ríos, Argentina (32°52′S, 59°25′W), with a history of high prevalence of *P. leydeni* in both sheep and cattle. This species was previously identified throughout Argentina, including the involved farm, by morphological (Sanabria et al., 2009) and molecular (Sanabria et al., 2011) methods.

Preliminary faecal samples were randomly taken from a flock of adult crossbreed Romney Marsh and Corriedale sheep. This flock remained grazing during the year in the same paddock, which is crossed by a stream that contributes to flooding. Antiparasitic treatments are regularly administrated against *Fasciola hepatica* and/or *Haemonchus contortus* infections, which mainly involves the use of benzimidazoles, macrocyclic lactones and salicilanylides such as closantel. Although recently reported as effective against amphistomes (Arias et al., 2013), closantel have had no effect in amphistome's eggs or flukes reduction in our experience (data not published), or in previous studies (Rolfe and Boray, 1987, 1993). Faeces were processed according to Ueno and Gonçalves (1988), with a sensitivity of 1 egg per 2 g of faeces. As advised by Wood et al. (1995), a controlled test was performed by selecting 15 sheep with at least 20 eggs per 2 g of faeces. Sheep were identified by ear tags, weighed and randomly divided into two groups: untreated control (*C*) group ($n = 5$) and treated (*T*) group ($n = 10$). All experimental animals were separated from the flock and relocated into a high paddock to avoid parasite reinfestation.

Since Rolfe and Boray (1987) found more consistent results giving a two-dose oxyclozanide schedule than a single one, the treated group was orally drenched with OCZ (EcoMilk 10%®, Cibeles, Uruguay) at 20 mg/kg dose (day 0), and a second dose was administered 72 h later (day +3). The five animals of *C* group and five randomly selected from *T* group were slaughtered 48 h after the second dose (day +5), following the regulations mentioned in the Animal Terrestrial Health Code (OIE, 2010). Faecal samples were taken from sheep on days 0, +3 and +5 to assess *P. leydeni* eggs. Clinical examination was performed after treatment looking for signs of toxicity and/or adverse reactions, such as mouth rash or hypersensitivity.

At necropsies, rumen, reticulum, omasum, abomasum and the first 5 m of small intestine were removed from carcasses and examined for adult and immature flukes. The pre-stomach content was carefully revised, whereas the abomasum content was completely removed and filtered through a 250 μm sieve. The small intestine was longitudinally opened and the mucosa was scraped with an enterotome. All this content was filtered in the same way. The recovered contents were kept in individual plastic containers properly labeled. The organs were refrigerated and taken to the laboratory. Contents were studied under stereomicroscope (Nikon SMZ-2 T, 1.5 X), and all recovered

flukes counted. According to the fluke counts, the efficacy was calculated by Abbot's formula as follows:

Efficacy (%) = 100 x (C-T/C), where C is the geometric mean (x + 1) of adult worm counts for the untreated control group, and T is the geometric mean (x + 1) of adult worm counts for OCZ treated group. For the coprological efficacy study, only T group was taken into account and initial egg counts (day 0) were contrasted with those found on days +3 and +5. In this case, the efficacy was as follows:

$$\text{Efficacy}(\%) = 100(T(\text{day } 0) - T(\text{day } X)/T(\text{day } 0)).$$

This analysis was preferred in order to improve the reliability of results since group T included more animals than group C. Geometric means (x + 1) were also calculated here.

Additionally, serum and gastrointestinal contents (ruminal, abomasal and small intestine) samples were obtained at 48 h post the last treatment (day +5), for OCZ quantification by HPLC.

Statistical differences between C and T groups were compared by t test for unequal variances, whereas the coprological differences between day 0 and days +3 and +5, only for T, were estimated by paired t test.

2.2. OCZ HPLC analysis

Sample clean-up: Spiked and experimental serum, ruminal, abomasal and intestinal content samples were extracted to quantify OCZ. An aliquot (0.5 mL) of serum or gastrointestinal content samples (previously centrifuged for 15 min at 4000 g) were combined with 1 mL of acetonitrile. After mixing for 10 min (Multi-tube Vortexer; VWR Scientific Products, West Chester, PA, USA) and 10 min of sonication (Transsonic 570/H, Lab-line Instruments Inc., Melrose Park, Illinois, USA) the solvent-sample mixture was centrifuged (4 °C) at 2000 g for 15 min (Legend Micro 17R, Thermo Fisher Scientific, Osterode, Germany). The supernatant was manually transferred into a tube and concentrated to dryness in a vacuum concentrator (Speed-Vac®; Savant, Los Angeles, CA, USA) and then reconstituted with 200 μL of mobile phase. An aliquot (50 μL) of this solution was injected directly into the chromatograph system.

HPLC analysis: Experimental and spiked serum/gastrointestinal content samples (used for validation) were analysed by HPLC (Shimadzu 10 A-HPLC System, Kyoto, Japan) with a UV detector set at 254 nm. Fifty microliter of each previously extracted sample were injected and the analyte eluted (flow 1.2 mL/min) from the analytical column (5 μm, 250 mm × 4.6 mm, C_{18} column, Kromasil®; Eka Chemicals AB, NY, USA) using an acetonitrile/0.025 M ammonium acetate (pH = 8.5) mobile phase (40/60, isocratic) at 30 °C. The compound was identified by the retention times of pure reference standards. Retention time for OCZ was 4.70 min. There was no interference of endogenous compounds in the chromatographic determinations. Calibration curves for OCZ for each matrix were constructed by least squares linear regression analysis, giving correlation coefficients (r) ≥0.995. Mean absolute recovery percentage for concentrations ranging between 0.1 and 40 μg/mL (n = 6) were between 75% and 86%. The precision of the method (intra- and inter-assay) was determined by analysing serum or gastrointestinal fluid samples (n = 6) fortified with OCZ at three different concentrations (0.25, 2 and 10 μg/mL). The CVs for the intra- and inter-assay precision were between 3% and 11%. The limit of quantification (LOQ) was defined as the lowest measured concentration with a CV < 20%, an accuracy of ±20%, and an absolute recovery ≥70%. The LOQ obtained for OCZ was 0.1 μg/mL.

3. Results and discussion

Previous OCZ animal safety data in cattle and sheep showed that even relatively low single doses (15 mg/kg) could have adverse effects on the central nervous system and intestinal function (behavioral depression, diarrhea and anorexia) (EMEA, 2004). Furthermore, severe signs of toxicity and mortality have been reported to occur at doses ≥50 mg/kg (EMEA, 2004). However, in the current experimental work, no adverse effects were observed at the 20 mg/kg dose administered twice to sheep.

The mature and immature worm counts and anthelmintic OCZ efficacy obtained against *P. leydeni* is shown in Table 1. The faecal egg counts and egg count reduction in T group are presented in Table 2. In agreement with previous findings in other amphistome genus (Rolfe and Boray, 1987), OCZ showed high efficacy (99%) against mature *P. leydeni*, when administered to sheep twice at 20 mg/kg. The post-treatment egg reduction was also high after the first dose with values of 98.4% (day +3) and 99.5% (day +5). When the efficacy of OCZ was evaluated against *Calicophoron daubneyi* in goats, worm burdens were reduced by nearly 95% at both 15 and 22.5 mg/kg (single doses) (Paraud et al., 2009). However, the efficacy against immature stages (10 days post-infection) was moderate (82%) after a single dose of 22.5 mg/kg (Paraud et al., 2009).

Table 1
Efficacy of oxyclozanide against mature *Paramphistomum leydeni* in naturally infected sheep. Post-mortem mature and immature worm mean counts (range) at different parasite location sites in control and treated groups are also shown.

Experimental Group	Rumen	Abomasum	Small intestine
Control	195.1 (59–498)	18[a]	7[a]
Treated	0	0	0
Efficacy (%)	99	–	–
p Value	0.015	–	–

[a] All immature flukes belonged to only one sheep.

Table 2
Faecal egg reduction (%) obtained in sheep treated twice with oxyclozanide (20 mg/kg) by the oral route. Counts at day 0 (before treatment), 72 h after the first dose (day +3) and, 48 h after the second dose (day +5) are shown.

	Day 0	Day + 3	Day + 5
Geometric mean	53.2	1.84	1.26
Range	20–293	0–8	0–1
Eggs count reduction (%)		98.4	99.5
UL 95% CI		100	100
LL 95% CI		90.9	95.4
p Value		<0.0001	<0.0001

References: UL: Upper limit; LL: Lower limit; CI: confidence interval.

Table 3
Oxyclozanide concentrations measured in serum and ruminal, abomasal and intestinal fluid samples at 48 h after its administration (20 mg/kg, twice) to sheep naturally infected with *Paramphistomun leydeni*.

Animal number	Concentration (μg/mL)			
	Serum	Ruminal fluid	Abomasal fluid	Intestinal fluid
168	24.5	0.13	ND	2.6
091	13.2	ND	ND	6.2
050	12.6	ND	ND	4.0
006	39.3	0.12	0.10	13.6
053	14.0	ND	ND	3.4
Mean	20.7	0.05	0.02	6.0
SD	11.5			4.5

ND: Not detected. SD: standard deviation.

Unfortunately, in the current work the efficacy against immature worms could not be assessed properly, since this stage was only found in one sheep from the untreated control group (Table 1).

OCZ concentrations measured in serum and ruminal, abomasal or intestinal fluid samples at 48 h after the last OCZ administration are shown in Table 3. The highest drug concentrations were quantified in the blood stream followed by the intestinal fluid. The high OCZ concentrations quantified in serum indicate that the drug was well absorbed after its oral administration. This finding was consistent with that previously obtained in sheep after OCZ oral administration (12.5 mg/kg), where a peak plasma concentration (C_{max}) of 25–29 μg/mL was reached at 22 h (t_{max}) post-treatment (EMEA, 2004).

Although a high efficacy was obtained against mature worms, very low concentrations were quantified in abomasal and ruminal fluid contents (Table 3). This could be the consequence of several factors, such as the dilution of the drug in the large volume of rumen after its oral administration and/or its adsorption to the digesta particulate material. As described before for low water soluble molecules (Hennessy, 1993), a physical adsorption of the drug to ruminal particulate digesta may have taken place. The association of the drug with rumen digesta cellulose limited the amount of "free" drug in the ruminal fluid. In fact, the extensive association of oxfendazole (Hennessy et al., 1994), closantel (Hennessy and Ali, 1997) and ivermectin (Lifschitz et al., 2005) with rumen digesta cellulose have been reported. Closantel, another salicylanilide compound, was almost completely (99%) associated with rumen particulate material with minimal amount in rumen digesta fluid (Hennessy and Ali, 1997). Although OCZ association to particulate digesta has not been described, it may explain the low OCZ concentrations measured at ruminal fluid level. As only free molecules may passively diffuse across the external trematode surface, drug action would be related to the very low OCZ concentrations measured at the ruminal fluid.

On the other hand, OCZ concentrations quantified in the intestinal fluid were higher than those measured both in ruminal and abomasal fluids. It has been described that the proportion of particulate digesta associated to xenobiotics appears to decrease on entry to the duodenum (Lees et al., 1988). In addition, it has been reported that following absorption, OCZ reaches the highest concentrations in liver, kidney, and intestines, being excreted into the bile (Roberson and Courtney, 1995), which would contribute to the highest concentrations measured at the intestinal fluid at 48 h post-dosing. These high OCZ concentrations at the intestine are an interesting feature for a paramphistomicide effect, since as mentioned above, the immature worms migrating in the small intestine cause the most severe damage.

In conclusions, OCZ orally administered to sheep twice was safe, without any adverse reactions detected in treated animals. This two-dose schedule is preferred not only because of its effectiveness, but also because of the variable results that single treatments showed in some reports (Rolfe and Boray, 1987; Jeyathilakan et al., 2005; Paraud et al., 2009). In addition, previous data reported very low residue levels, thus allowing a short withdrawal period for animals intended for slaughter and zero withdrawal for milk (Roberson and Courtney, 1995). The efficacy against mature stages of *P. leydeni* in naturally infected sheep was high. All these properties are desirable and make OCZ a good paramphistomicide drug. Despite a high drug concentration at the intestinal fluid, OCZ efficacy against immature stages could not be assessed. Further controlled infection studies would be necessary to confirm the complete OCZ efficacy against *P. leydeni* in sheep, even involving larger groups. OCZ efficacy and assessment of its concentration profiles in different tissues are considered a contribution to the scarce information available in the literature on this ruminant fluke.

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