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Clinical Efficacy of Enrofloxacin HCl-2H_2O (ENRO-C) in a Sheep Leptospirosis Outbreak

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Abstract

The favorable pharmacokinetics of enrofloxacin HCl-2H_2O (enro-C) prompted an experimental/clinical trial in a field outbreak of reproductive problems due to leptospirosis in a 92 unvaccinated flock in México. The selected treatments were either 5 daily intramuscular injections of 15 mg/kg of enro-C ($n = 14$ sheep) or 25 mg/kg of streptomycin combined with 15,000 IU/kg of procaine-penicillin G ($n = 13$). Polymerase chain reaction and microscopic agglutination tests were performed for the initial diagnosis and after the treatments to assess their efficacy. Bratislava, Canicola, Grippotyphosa, Hardjo, Pyrogenes, Pomona, and Wolffi were the most frequently serovars found. Lower antibody titers after treatment in both groups were observed but were significantly lower in the enro-C group (118.18) as compared to the group treated with streptomycin/penicillin G (192.59) ($p < .05$). Five days after the enro-C treatment, 10 out of 14 samples were PCR negative, and 28 days later, all PCR tests were negative. For streptomycin/penicillin, 8 out of 11 samples were PCR negative on days 5 and 28 ($p < .05$). Adverse effects were not observed in any of the enro-C-treated animals. This is the first report of successful treatment of leptospirosis in sheep with a fluoroquinolone and serological and PCR data suggest that a high rate of bacteriological cure was achieved.

Keywords: Clinical trial, enrofloxacin HCl-2H2O, Leptospira, sheep

Introduction

Leptospirosis is a zoonosis caused by pathogenic spirochetes of the genus *Leptospira*. It affects wild and domestic animals causing significant losses in small ruminant production (Ellis, 2015). It is distributed worldwide with an emphasis in tropical and subtropical regions, where environmental conditions allow the survival of the bacteria and its dissemination among various species, including humans (Costa et al., 2015). It has been described as one of the most widely distributed zoonoses in the world (Abela-Ridder et al., 2010; Haake & Levett, 2015). Sheep are an important reservoir for *Leptospira* spp. (Fang et al., 2015; Martins & Lilenbaum, 2014), but it is often rarely diagnosed. Yet, it has been estimated that it causes significant losses in production (Sanhueza et al., 2020). The infection in sheep occurs after having contact with contaminated food or water, through wounds or abrasions on the skin, and of mucous membranes. The clinical manifestations become apparent within the following 2 weeks after contamination. Usually, there is the liver, spleen, kidney, and genital involvement and on rare occasions, some signs involving the nervous system are observed. Unfortunately, initial signs may go unnoticed, as they are manifested subclinically (Adler & de la Peña, 2010). The treatments of choice described in texts and the literature indicate that single to triple high doses of streptomycin and beta-lactamic drugs are good therapeutic options (Ellis, 2015). In a field outbreak, some deaths as well as last-third of the gestation period abortions occur. Often some animals remain as carriers despite their treatment (Gerritsen et al., 1993). Treated or not, sheep show a slight recovery after some days, and the infection often becomes chronic. Affected animals who have become healthy carriers show intermittent elimination of leptospires in the urine. This condition may last months and even years (Almeida et al., 2019; Silva et al., 2019). Hematuria, abortions, infertility, embryonic and fetal death, mummification of offspring, premature deliveries, and the birth of weak lambs become frequent in the flock (Martins et al., 2012), although abortions can be sporadic and become endemic (da Silva, et al., 2019).

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There are no reports on the use of fluoroquinolones to treat neither acute nor chronic leptospirosis in sheep (Sykes et al., 2011). In experimental models in hamsters, ciprofloxacin and gatifloxacin have been used at high doses, achieving superior survival rates compared to untreated animals, but with many side effects, including severe diarrhea (Griffith et al., 2007). In contrast, a high success rate of enrofloxacin in its recrystallized form as HCl-$2H_2O$ (enro-C) has been documented in experimental leptospirosis in hamsters (Carrascosa et al., 2017) and in clinical cases of acute canine leptospirosis (Gutierrez et al., 2019). Furthermore, the pharmacokinetics/pharmacodynamics (PK/PD) analysis and the Monte Carlo simulations in cattle indicate that the use of enro-C is viable (Mendoza et al., 2019). These data and the existence of an outbreak of leptospirosis in sheep prompted this clinical trial of ovine leptospirosis treated with enro-C.

Methods

Study Design and Animals

All study procedures and animal care activities were carried out following the Institutional Committee for Research, Care, and Use of Experimental Animals of the National Autonomous University of Mexico (UNAM), per Official Mexican Regulation NOM-062-ZOO-1999 (Mexican NOM-062, 1999). The outbreak occurred in the Municipality of Villahermosa, Tabasco, México, located between 17°59′ N 92°55′ W at 32 ft elevation. This location has a humid tropical climate with rainfall all year round (1800 mm^3), a mean temperature of 27°C, and atmospheric relative humidity is greater than 90%. These conditions are ideal for the survival and dissemination of *Leptospira* spp. The study was carried out in a flock with 92 Pellibuey sheep (3 males and 89 females). The flock was raised under continuous grazing mixed with beef cattle and there was no story of vaccination. A history of recent miscarriages, weak or dead lambs, premature deliveries, and generally decreased fertility prompted veterinary assistance. However, all sheep included in this study were clinically healthy upon physical examination. This study separated 27 sheep from the flock, which were housed in raised-floor pens with 2.2 m^2 per sheep with shade, food, and water *ad libitum* throughout the day. Animals had a mean weight of 39.06 ± 6.74 kg and were 1.5–4 years old.

Microscopic Agglutination Test

Seven days before the test, blood samples were taken by jugular venipuncture, centrifuged at 2000 g for 10 minutes, the serum was recovered, and it was sent to the Microbiology Laboratory of the Faculty of Veterinary Medicine and Zootechnics for the titration of antibodies through microscopic agglutination (MAT) according to Goris and Hartskeerl (2014), using a panel of 12 serovars of *Leptospira:* Autumnalis (Akiyami A), Bataviae (Van Tienen), Bratislava (Jez Bratislava), Canicola (Hond Utrch IV), Celledoni (Celledoni), Grippotyphosa (Moska V), Hardjo (Hardjopradjitno), Icterohaemorrhagiae (RGA), Pomona (Pomona), Pyrogenes (Salinem), Tarassovi (Perepelicin), and Wolffi (3705). This procedure was also repeated 28 days after ending the treatment.

Real-Time Polymerase Chain Reaction Test

Urine samples (10–20 mL) were collected in a 40 mL sterile container 7 days before the test and 5 and 28 days after the end of the treatment. Urination was stimulated by placing the hand over the nostrils until the sample was obtained. The collected urine was processed for DNA amplification of pathogenic leptospires using polymerase chain reaction (PCR). Urine samples were centrifuged at 12,000 g for 20 minutes, the supernatant was discarded, and the pellet was resuspended in 200 µL of phosphate-capped saline. The PCR analysis was conducted according to the method described by Stoddard (2013): LipL32-45F (5´-AAG CAT TAC CGC TTG TGG TG-3´) and LipL32-286R (5´-GAA CTC CCA TTT CAG CGA tt-3´). Real-time PCR was performed using TaqMan PCR Master Mix (Thermo Scientific, Mexico City, Mexico) in a volume of 20 µL containing 400 nM of forwarding primer, 400 nM reverse primer, 12.5 µL of Master Mix, and 5 µL of DNA clinical extract. The amplification protocol consisted of 5 minutes at 94°C followed by 40 cycles (30 seconds at 94°C, 30 seconds at 68°C, and 30 seconds at 72°C). After the reaction, samples were cooled to 40°C for 120 seconds.

Enro-C Production and Treatment

Overall, 27 sheep were included in this study and they were assigned to either of 2 treatments aided by a random number generator. The treatments were either a daily intramuscular (IM) injection for 5 days of 15 mg/kg of enro-C ($n = 14$ sheep) (Mendoza et al., 2019) or a daily IM injection for 5 days of 25 mg/kg of streptomycin combined with 15,000 IU/kg of procaine-penicillin G, Strepto Bio-Benzipen (Biozoo, Jalisco, Mexico) ($n = 13$) (Alt et al., 2001; Ellis, 2015). The chosen injection sites were the semitendinosus and semimembranosus muscles, and the volume injected ranged from 4 to 7 mL. The batch of enro-C was synthesized as described in Mexican patent number MX/a/2013/014605 (Instituto Mexicano de Protección Industrial, Mexico City), based on good manufacturing practices and following Mexican regulation as laid out in regulation known as NOM-012 (NOM-012-ZOO-1993). The enro-C powder was suspended in water to 10% and shaken just before injection (pH 6.7 and non-irritant). Sheep with initial antibody titers >100 (OIE World Organization for Animal Health, 2018) and positive real-time PCR of urine samples 7 days before treatment were included in the study. Lactating animals were not considered for this trial.

Statistical Analysis

The average antibody titers of the serovars observed 5 days before and 28 days after each treatment were analyzed using *t*-tests for paired samples (Sheskin, 2011). The statistical analysis between treatments considering the average of antibody titers was carried out using a *t*-test for independent samples, and non-parametric analysis of Kruskal–Wallis was carried out to determine the bacteriological cure rate. The Statistical Package for the Social Sciences software package was used (IBM SPSS Inc., Chicago, Ill, USA). A significance level of $p < .05$ was established.

Results

Seven days before treatment, the 92 serum samples of the herd were MAT-tested and 57.60% resulted positive. Twenty-eight (30.43%) sheep were PCR positive to the urine samples at the beginning of the study, but one sheep was lactating and was not included in the trial. Hence, 27 were considered to be treated with either enro-C or streptomycin/penicillin. Eight sheep had a record of having previously been treated with procaine-penicillin G and 4 were assigned to each group to block this feature. Of the 27 initial serum samples, 5 were reactive to 1 serovar, 11 to 2, 7 to 3, and 5 to more than 3 serovars. Bratislava, Canicola, Grippotyphosa, Hardjo, Pyrogenes, Pomona, and Wolffi were the most frequent 12 serovars found. Table 1 shows the total number of seropositive samples before and

Table 1

The Number of Seropositive Samples to Leptospira *spp. in Sheep Using Microscopic Agglutination Test (Titers ≥100) 7 Days Before and 28 Days After Receiving 15 mg/kg of Enrofloxacin HCl-2H$_2$O (Enro-C) or 25 mg/kg of Streptomycin Plus 15,000 IU of Penicillin G.*

	Enro-C		Strepto plus penicillin G	
Serovar	**Before**	**After**	**Before**	**After**
Bratislava	8	5	7	6
Canicola	4	2	3	3
Griphotyphosa	4	3	3	3
Hardjo	7	6	4	3
Pirogenes	3	2	5	4
Pomona	3	2	3	3
Wolffi	4	3	2	1

28 days after either treatment scheme. The mean significantly lower antibody titers after treatment in both groups are shown in Table 2 ($p < .05$). The average antibody titers after treatment were significantly lower in sera collected from the enro-C group as compared to those in the group treated with streptomycin/penicillin G ($p < .05$) (Table 2).

Five days after the end of the enro-C treatment, 10 of 14 samples were PCR negative and 28 days later all the cases were negative. Of the cases treated with streptomycin/penicillin, 8 and 11 were negative 5 and 28 days after treatment, respectively (Table 2). The number of PCR negative urine samples 28 days after treatment with enro-C is statistically higher than those observed in the streptomycin/penicillin G group ($p < .05$).

Discussion, Conclusion and Recommendations

This study was conducted under controlled conditions, that is, the sheep included in the study were isolated from the rest of the herd. They were kept on raised-floor pens restricting contact with potential carriers such as rodents and other sheep from the herd. The water and food source were free of possible contaminants expected after filtering it and using chlorine levels up to 4 ppm. Hence the possibility of reinfections during the study was minimized. MAT and PCR results were available 7–10 days after the initial sample collection. Therefore, all the sheep clinically diagnosed at the beginning with leptospirosis were isolated. However, only seropositive and PCR-positive sheep were included in this study. As recommended by the World Organization for Animal Health (OIE) (OIE, 2018), the use of MAT as a diagnostic tool allowed to elucidate the circulating serotypes in the herd and together with PCR to confirm healthy carriers with urinary elimination of *Leptospira* spp. Infections with the serovars detailed in the serological study (Table 1) can be explained by the coexistence of the sheep herd with other species in the area, including horses during grazing and constant contact with dogs in the herd (Pinto et al., 2017), as well as wildlife mammals in the area (Vieira et al., 2018) and rodents (Boey et al., 2019).

None of the sheep included in the study was previously vaccinated against leptospirosis. Therefore, following OIE (2018) recommendations, titers ≥100 were considered seropositive. In this study, seropositivity of 57.60% was found in the herd, which suggests a natural exposure to *Leptospira* spp. (Vallée et al., 2015). Additionally, 30.43% of the sheep were healthy carriers as identified by PCR, and therefore they were eliminating leptospire organisms through their urine. The serotypes identified here have also been identified in other species (Campos et al., 2017). Leptospires of the Sejroe group are known to be the main cause of leptospirosis in sheep. However, leptospires of other species and serovars can infect and cause disease (Martins & Lilenbaum, 2014). This is one of the reasons why vaccination is not completely effective as protection is limited to the serovars included in the particular bacterin (Fernandes et al., 2016) and the infection can remain in the herd through carrier animals, apparently healthy (Martins & Lilenbaum, 2014). From this outbreak, it is then concluded that, as in other parts of the world, vaccination is not a common practice in sheep herds (Wilson et al., 2021) and identification of circulating serovars in the herd is essential to manufacture appropriate bacterins for a given region.

The chosen dose of 15 mg/kg/day of enro-C was based on the probability of achieving the target ratio of Cmax/MIC = 10, which was established for enrofloxacin through Monte Carlo simulations (Mendoza et al., 2019) and extrapolating the successful rate of treating bovine leptospirosis (Mendoza, 2021). Although the dose can be regarded as high, neither toxicity nor side effects were seen in this and other studies (Carrascosa et al., 2017; Gutierrez et al., 2019). The commercial preparation of streptomycin plus procaine-penicillin G was selected as the reference treatment based on the recommendations in the literature (Alt et al., 2001; Ellis, 2015). Streptomycin has been adopted as the drug of choice in the treatment of leptospirosis in ruminants, despite having a low volume of distribution <0.2 L/kg (Riviere & Papich, 2018), which impedes it from achieving the urinary elimination of *Leptospira* spp., especially in mixed infections

Table 2

Mean ± SE of Serum Antibody Titers Determined by Microscopic Agglutination Test (MAT) and Proportion of Negative Urine Samples by Polymerase Chain Reaction (PCR) in Sheep Affected by Leptospira *spp. Treated with Enrofloxacin HCl-2H$_2$O (Enro-C) or Streptomycin Plus Penicillin G.*

	Microscopic Aglutination Test		Real-Time PCR Negative/Positive (%)		
Treatment	**Before**	**28 Days After Tx**	**Before**	**5 Days After Tx**	**28 Days After Tx**
Enro-C	396.69 ± 52.12^{a}	$118.18 \pm 14.88^{b,A}$	$14/14\ (100)^{a}$	$10/4\ (71.42)^{b}$	$14/0\ (100)^{b,A}$
Streptomycin plus penicillin G	392.59 ± 58.93^{a}	$192.59 \pm 22.28^{b,B}$	$13/13\ (100)^{a}$	$8/5\ (61.53)^{b}$	$11/2\ (84.61)^{b,B}$

^{a,b}Different letters in a row indicate a statistically significant difference between values ($p < .05$).
^{A,B}Different letters in a column indicate a statistically significant difference between values ($p < .05$).
PCR, polymerase chain reaction.

(Alt et al., 2001). Undoubtedly, streptomycin is very effective to treat the phase of leptospiremia or during the early stages of *Leptospira* renal accumulation but it is highly unlikely that this or other aminoglycosides reach therapeutic concentrations in other tissues such as the reproductive tract (Cabral et al., 2018; Di Azevedo et al., 2020). Other antibacterial drugs have shown some degree of efficacy in the treatment of leptospirosis in ruminants, for example, amoxicillin (Cortese et al., 2007; Smith et al., 1997), oxytetracycline, ceftiofur, tylosin, and tilmicosin (Alt et al., 2001). In contrast, fluoroquinolones have not been effective against *Leptospira* spp. microorganisms, except enro-C that has shown high efficacy in the hamster model (Carrascosa et al., 2017), in dogs (Gutierrez et al., 2019), and cows (Mendoza, 2021).

Excepting the information presented in the patent document to the World Intellectual Property Organization (World Intellectual Property Organization, 2015), neither pharmacokinetic nor PK/PD studies of enro-C have been published in sheep. However, if data published for enrofloxacin and its derivative enro-C are considered, a large volume of distribution in sheep is expected (2.97 kg/L) (Rahal et al., 2006). It is well established that enrofloxacin has a notable penetration into various tissues (Elsheikh et al., 2002) and in other species, the pharmacokinetic behavior of enro-C Cmax and relative bioavailability values (Carrascosa et al., 2017; Gutierrez et al., 2019; Mendoza et al., 2019; Sumano et al., 2018). It is therefore tempting to correlate the significantly lower values in antibody titers observed after administration of enro-C as compared to the group treated with streptomycin-penicillin G. A similar serological profile was observed in dogs (Gutierrez et al., 2019) after administering 10 mg/kg of enro-C and in cows after administering 25 mg/kg of streptomycin (Gerritsen et al., 1993). This decrease in antibody titers is associated with the decline and perhaps leptospires eradication (Gerritsen et al., 1993), as the sharp decrease in urine PCR positive cases on day 5 implies, and after a total PCR negativity was obtained on day 28 after initiation of the treatment with enro-C. This is likely to indicate that a bacteriological cure was achieved.

This clinical study in treating a field outbreak of sheep leptospirosis was prompted by the background information on the ability of enro-C to achieve bacteriological cures in hamsters, dogs, and cows. However, it is necessary to define the pharmacokinetics of enro-C in sheep and derive its PK/PD ratios. With this information, further clinical work to establish the ideal dosing scheme should be done. Nonetheless, it is worth stating that the administration of enro-C did not induce any observable adverse effect, and in contrast with the reference solution of enrofloxacin (pH 10.4), the injection of a 10% water suspension did not induce irritation or discomfort at the injection site (pH 6.5–6.7). Also, it is important to state that the administration of streptomycin in combination with procaine-penicillin G was due to the lack of a commercial preparation of streptomycin alone in Mexico. Finally, the use of antibacterials reduces the risk of transmission and presentation of leptospirosis. However, it is very important to consider vaccination and the implementation of sanitary measures to control this disease.

This is the first report of successful treatment of leptospirosis in sheep with a fluoroquinolone, that is, enrofloxacin HCl-2H_2O, and serological and PCR data suggest that a total bacteriological cure was achieved. Together with sanitary measures and vaccination, it is possible to control better this disease in endemic areas, a premise that must be subjected to experimental tests to be able to recommend the scheme used in this trial.

Ethics Committee Approval: This study was approved by an ethics committee from the Facultad de Medicina Veterinaria y Zootecnia at UNAM: SICUAE-Subcomité Institucional para el Cuidado y Uso de Animales Experimentales. Approval No: DC2018/2-7.

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