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Comparison of therapeutic efficacy of different drugs against *Trypanosoma vivax* on experimentally infected cattle

Thiago Souza Azeredo Bastos[a], Adriana Marques Faria[a], Alliny Souza de Assis Cavalcante[a], Darling Mélany de Carvalho Madrid[a], Dina Maria Beltrán Zapa[a], João Eduardo Nicaretta[a], Leonardo Bueno Cruvinel[a], Luciana Maffini Heller[a], Luiz Fellipe Monteiro Couto[a], Vando Edésio Soares[b], Fabiano Antônio Cadioli[c], Welber Daniel Zanetti Lopes[d,*]

[a] Centro de Parasitologia Veterinária, Escola de Veterinária e Zootecnia, Universidade Federal de Goiás – UFG, Goiânia, GO, Brazil
[b] Universidade Brasil, Campus de Descalvado, São Paulo, SP, Brazil
[c] Departamento de Clínica, Cirurgia e Reprodução Animal, Faculdade de Medicina Veterinária, Universidade Estadual Paulista - Unesp, Araçatuba, SP, Brazil
[d] Departamento de Biociências e Tecnologia, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás - UFG, Goiânia, GO, Brazil

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ABSTRACT

In this study, we evaluated the therapeutic efficacy of diminazene diaceturate at a dose of 7 mg/kg (DA), imidocarb dipropionate at 4.8 mg/kg (IMD), isometamidium chloride at 0.5 and 1.0 mg/kg (ISM 0.5 and ISM 1.0) and combinations applied through different methods to treat *Trypanosoma vivax* in experimentally infected calves. Thirty male Girolando calves were kept indoors and infected intravenously with *T. vivax* trypomastigotes (approximately 1×10^6). On D-1, the calves were randomized based on the quantity of infecting parasites per animal, yielding six groups of five animals each: G1: positive control group without treatment; G2 animals treated with DA on Day 0 intramuscularly (IM); G3 animals treated with IMD on Day 0 and D + 14 subcutaneously; G4 animals treated with ISM 0.5 on Day 0 IM; G5 animals treated with ISM 1.0 on Day 0 IM; G6 animals received DA on Day 0 and ISM 1.0 on D + 14, both IM. Throughout 180 days, blood samples were collected for the evaluation of *T. vivax* using the Woo, Brener and PCR methods. The results indicated that the treatment protocols with DA and/or ISM 0.5 and ISM 1.0 had high efficacy (100 %) against *T. vivax*. Interestingly, cattle that received ISM remained free of parasites until D + 180. In contrast, animals treated with IMD had relapsed *T. vivax* detected on the 10th and 14th days post-treatment (DPT). Cattle that received ISM 1.0 did not exhibit relapsed *T. vivax* in the blood, even after reinfection performed on the 50th DPT. However, treatment with DA on Day 0 failed to prevent a new infection of *T. vivax* on the 50th DPT. The animals that received ISM 1.0 had a transient decrease in packed cell volume similar to that found in the control group. The reappearance of *T. vivax* in herds in Brazil treated with DA likely occurred due to the short half-life of the drug and not necessarily due to *T. vivax* resistance to DA.

1. Introduction

Bovine trypanosomosis is a disease caused by the hemoparasite *Trypanosoma vivax*, which leads to considerable financial losses in cattle herds (Gutiérrez et al., 2013). The losses occur due to animal mortality, reduced fertility and feed conversion in the herd or the direct loss of daily income due to lower milk production (Abrão et al., 2009). In bovines, the transmission of *T. vivax* may occur through iatrogenic means (Bastos et al., 2017) as well as biting flies, such as *Tabanidae*, *Stomoxys calcitrans* (Dagnachew and Bezie, 2015) and *Haematobia irritans* (Salas et al., 2017). Clinical signs in affected animals include fever, anemia, loss of appetite, progressive weakening, decubitus, weight loss, subcutaneous edema, ascites, reproductive problems, abortion, neurologic disturbances (blindness and muscular tremors) and, ultimately, death (Silva et al., 1996).

The use of trypanocidal drugs, such as diminazene diaceturate and isometamidium chloride, is currently the most effective way to combat the disease (Dagnachew and Bezie, 2015; Cossic et al., 2017). However, field reports commonly describe the inefficacy of diminazene against *T. vivax* in Brazil (Paiva et al., 2000; Van den Bossche et al., 2000; Gall et al., 2004; Cadioli et al., 2012; Bastos et al., 2017; Mulandane et al., 2018) and the use of isometamidium chloride for treatment and

* Corresponding author.
E-mail address: wdzlopes@hotmail.com (W.D.Z. Lopes).

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prevention is reported in other regions of the world (Mulandane et al., 2018; Degneha et al., 2019). This is possibly related to development of strains of the protozoan that are resistant to these compounds. Therefore, research is being conducted to find new diamines with action against *T. congolense* and *T. vivax* (Gillingwater et al., 2017). Another issue related to treating cattle affected by *T. vivax* is the fact that producers often use imidocarb dipropionate to combat outbreaks, although the manufacturers do not indicate the drug for this purpose (Costa et al., 2016).

These formulations are relatively old and, to the best of our knowledge, there are no new studies that have evaluated the effectiveness of different formulations administered with different treatment protocols in the same experimental design using cattle experimentally infected with *T. vivax*. Moreover, the reference drug for treatment of this protozoan has become isometamidium, which was only launched on the market in Brazil in 2016 (Bastos et al., 2017) and there are no studies performed in this country involving this active ingredient, which motivated the execution of the present investigation.

The aim of this study was to evaluate the therapeutic efficacy of diminazene diaceturate, imidocarb dipropionate and isometamidium chloride administered with different methods for the treatment of *T. vivax* in experimentally infected cattle.

2. Materials and methods

This project received approval from the Animal Experimentation Ethics Committee of the Federal University of Goiás, Brazil, (certificate number: 032/16) and was conducted in accordance with the guidelines of the National Animal Research Ethics Committee (CONCEA).

2.1. Animals, housing and experimental design

The study involved 30 male Girolando calves raised in tie stalls in a commercial herd free of *T. vivax* located in the municipality of Santo Antônio de Goiás in the state of Goiás, Brazil. At six months of age, the animals (average body weight 120 ± 34 kg) were transported to the university to be used as experimental units in the present trial.

This trial was conducted from April to September 2017 in the large animal barn of the School of Veterinary Science and Animal Husbandry of the Federal University of Goiás. The animals were lodged in 12 stalls measuring 9 m^2 protected with nylon screen (mesh: 3 mm) to prevent attacks from hematophagous flies. Each stall had two to three animals.

Upon entering the experimental pens on D-12, all calves received preventive treatment against intestinal parasites (albendazole 5 mg/kg, Valbazen®, Zoetis Animal Health), ticks (Potenty®, MSD Animal Health), *Eimeria* spp. (toltrazuril 15 mg/kg, Baycox®, Bayer Animal Health) and *Anaplasma marginale* (enrofloxacine 7.5 mg/kg, Kinetomax®, Bayer Animal Health).

The animals remained indoors in their assigned pens throughout the entire experimental period with free access to water and grass hay and were supplemented daily with concentrate at 1.5 % of their body weight. The adaptation period lasted seven days (D-12 to D-5). Blood was collected from all animals at the beginning and end of the trial to confirm the absence of *Babesia* sp. and *A. marginale*, as described elsewhere (Dagnachew and Bezie, 2015). Moreover, the absence of *T. vivax* just prior to experimental infection was confirmed using the Woo method (Woo, 1970), blood smears (Dagnachew and Bezie, 2015) and cPCR (Cortez et al., 2009).

2.2. Animal infection procedures

The infective dose was prepared with the *T. vivax*-Ipameri strain (Bastos et al., 2017) (Genbank number MK392089) cryopreserved in 8% glycerol and kept in liquid nitrogen at the Center for Veterinary Parasitology of the Federal University of Goiás. On D-9, samples this specific strain of *T. vivax* were thawed and injected intravenously into a six-month-old Girolando calf, which maintained isolated as a parasite donor within the trial site. On D-5, a high quantity of circulating trypomastigotes (approximately 3×10^6) was detected in the blood of the donor calf using the Brener method (1961). After the quantification of the parasites, aliquots were prepared with blood and EDTA containing approximately 1×10^6 viable trypanosomes, which were injected into all 30 animals used in this study on D-5.

On D-1, after confirmation of the *T. vivax* infection in all 30 animals, the calves were divided into six blocks of five animals each based on trypanosome count and location in the stall. Calves in each block were then randomly allocated to the different treatment groups. Sets of two and three subsequent blocks were designated to six nearby stalls each and the animals within each of these blocks were randomly allocated to the stalls within each set.

2.3. Experimental groups and treatment criteria

Day zero of the study (D0) was established as the day on which animals received the first treatment for *T. vivax*. No other activity was performed on this day, such as blood collection, rectal temperature reading, etc. The drugs chosen to perform the treatment for *T. vivax* infection were diminazene diaceturate at a dose of 7 mg/kg (DA), imidocarb dipropionate at a dose of 4.8 mg/kg (IMD), isometamidium chloride at doses of 0.5 and 1.0 mg/kg (ISM 0.5 and ISM 1.0) and combinations using different administration routes.

The following were the experimental groups: G1 - positive control group (PCG) without treatment; G2 – animals treated on D0 intramuscularly with DA (Ganaseg®, Elanco Animal Health); G3 – animals treated subcutaneously on D0 and D + 14 with IMD (Imizol®, MSD Animal Health); G4 – animals treated on D0 intramuscularly with ISM 0.5 (Vivedium®, Ceva Animal Health); G5 – animals treated on D0 intramuscularly with ISM 1.0 (Vivedium®, Ceva Animal Health); and G6 – animals treated on D0 with DA (Ganaseg®, Elanco Saúde Animal) and on D + 14 with ISM 1.0 (Vivedium®, Ceva Animal Health), both intramuscularly (Table 1).

It was also pre-planned in the experimental design that animals could receive another treatment on the 14th day post-treatment (DPT) if *T. vivax* reappeared in the blood stream (detected either by the Woo and/or Brener methods) before this time. Animals diagnosed as free of *T. vivax* through to the 50th DPT received a second injection of 1×10^6 trypomastigotes of *T. vivax* on D + 50 (Table 1).

Table 1
Experimental groups, products used, dose, route, treatment regimen and post-treatment challenge of calves experimentally infected with 1×10^6 trypomastigotes of *Trypanosoma vivax*.

Treatment	Animlas per group	Groups	Dose	Route	Treatment regime	Post-treatment re-infection day
PCG	5	Control	10 mL	Deep intramuscular	D 0	—
DA	5	Diminazene	7.0 mg/kg	Deep intramuscular	D 0	D + 50
IMD	5	Imidocarb	4.8 mg/kg	Subcutaneous	D 0 and D + 14	—
ISM 0.5	5	Isometamidium	0.5 mg/kg	Deep intramuscular	D 0	—
ISM 1.0	5	Isometamidium	1.0 mg/kg	Deep intramuscular	D 0	D + 50
DA + ISM 1.0	5	Diminazene + Isometamidium	7.0 mg/kg +1.0 mg/kg	Deep intramuscular	D 0 and D + 14	D + 50

2.4. Clinical signs, rectal temperature and packed cell volume

Each animal had an identification card and all animals were evaluated visually on a daily basis from D-1 and D + 30 to determine alterations in the eyes, body, skin, hair, digestive system, central nervous system, lymphatic and urinary systems. A reduction in appetite and feed intake, apathy and signs of pale mucosa were also observed.

Rectal temperature and packed cell volume (PCV) were also monitored in all animals daily. Rectal temperature was read between 06:00 and 07:00 AM. Temperature values between 37.5 and 39.2 °C were considered normal for the animal category (Almeida et al., 2010a). PCV was measured using the micro-tube technique. For such, 4 mL of blood was collected from the jugular vein of each animal in vials with anticoagulant (EDTA). At the university lab, the samples were homogenized and the blood was transferred to micro-tubes. The samples were then centrifuged at 13,000 RCF for 5 min just before PCV reading, as described elsewhere (Gomes et al., 2007). Values equal to or above 24 % were considered normal. From D + 31 to D + 90, all the above procedures were performed every other day. After D + 90, one last sample was collected at D + 180 only from the animals in ISM 0.5, ISM1.0 and DA + ISM1.0 groups.

2.5. Blood sampling for Trypanosoma vivax count using Woo, Brener and cPCR methods

Between D-1 to D + 30, 4 mL of blood was collected from the jugular vein of each animal in vials with EDTA and the presence of *T. vivax* was determined using the Woo (Woo, 1970) and Brener (Brener, 1961) methods. Between D + 31 and D + 90, these procedures were performed at every other day. From D + 90 to D + 180 evaluations were performed only for animals in the ISM 0.5, ISM 1.0 and DA + ISM1.0 groups. cPCR (Cortez et al., 2009) was performed on samples taken on D-8 and D-1. On D + 1, D + 2, D + 3, D + 7, D + 14, D + 21, D + 28, D + 35, D + 45, D + 55, D + 65, D + 75, D + 85, D + 90 and D + 180, these analyses were performed only for samples taken from treated groups.

PCR assays were performed for the amplification of the CatL-like gene in *T. vivax*. For such, DNA was extracted from 200 µl of blood from each sample using the Mini Spin® DNA extraction kit (Kasvi®) following the manufacturer's directions. DNA was amplified by PCR using the primers TviCatL [5´GCCATCGCCAAGTACCTCGCCGA3´] and DT0155 [5´TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA3´] (Invitrogen®), which amplify a specific fragment of 177 base pairs (bp) from the CatL-like region responsible for the cathepsin L-like (CatL) enzyme of *T. vivax*. Amplifications were conducted in 50 µl of reaction mixture containing 5 µl of buffer 10 X, 2 µl (1.5 mM) of $MgCl_2$, 1 µl of the four dNTPs (200 mM each), 1 µl (100 pmol) of each primer, 3.75 µl (7.5 % of the reaction) of dimethyl sulfoxide (DMSO), 1 µl (0.1 mg/mL) of bovine serum albumin (BSA), 0.25 µl (2.5 units) of Taq DNA polymerase and 1 µl of DNA (approximately 25 ng/µl). All reactions were performed in a Mastercycler DNA Engine (Eppendorf) for 35 cycles. Each cycle consisted of a denaturation step (94 °C for 1 min), an annealing step (65 °C for 1 min) and an extension step (72 °C for 1 min), followed by 10 min of final extension at 72 °C. To ensure the absence of contamination of the PCR process, water instead of DNA was used as the quality control, following the same procedure (Cortez et al., 2009).

The positive control for the PCR was performed with DNA extracted from cattle experimentally infected with *T. vivax* (confirmed by DNA sequencing) with a parasite count in blood of approximately 6×10^6 trypanosomes/mL. The negative control consisted of DNA extracted from cattle free of *T. vivax* housed at the School of Veterinary Science and Animal Husbandry of the Federal University of Goiás in a region free of *T. vivax* infection. The PCR products were submitted to gel electrophoresis with 1.5 % agarose gel stained with ethidium bromide in TBE buffer, pH 8.0 (44.58 M of Tris-base, 0.44 M of boric acid and 12.49 mM of EDTA) and examined with ultraviolet light to determine the size of the amplified fragments compared to controls with a molecular weight of 100-bp DNA ladder (Invitrogen).

2.6. Evaluation of efficacy of different formulations against Trypanosoma vivax and calculation of percentage reduction in parasites in blood

Efficacy was determined with arithmetic means for the calculation of the percentage reduction in *T. vivax* trypomastigote counts (Brener, 1961). The same procedure was performed in all groups daily using the following formula:

2.6.1. Efficacy percentage

$$\mathbf{EP} = \frac{\mathbf{MC} - \mathbf{MT}}{\mathbf{MC}} \times \mathbf{100}$$

EP: efficacy percentage

MC: mean number of *T. vivax* trypomastigotes in control group on Day X

MT: mean number of *T. vivax* trypomastigotes in treated group on Day X

2.6.2. Reduction percentage

$$\mathbf{RP} = \frac{\mathbf{MTD0} - \mathbf{MT}}{\mathbf{MTD0}} \times \mathbf{100}$$

RP: reduction percentage

MTD0: mean number of *T. vivax* trypomastigotes in treated group on Day 0 (before treatment)

MT: mean number of *T. vivax* trypomastigotes in treated group on Day X

The efficacy of each formulation was also evaluated considering the presence or absence of *T. vivax* diagnosed by cPCR.

2.7. Data analysis

The experimental records of rectal temperature, PCV and *T. vivax* trypomastigote counts (Brener, 1961) were grouped in seven-day intervals for the statistical analysis. The data were checked for normality and variance through residual evaluation. As a result, rectal temperature and PCV were analyzed in a factorial design in a repeated-measures structure and means were compared using the Tukey-Kramer test. The data from the Brener exams of the different treatments were analyzed using the non-parametric Kruskal-Wallis test with the Simes-Hochberg method. All the statistical analyses were performed with the aid of Statistica version 12 (StatSoft, Inc., 2014), considering a 95 % confidence level ($P \leq 0.05$).

3. Results

The cattle that received DA and IMD did not exhibit any clinical signs of the *T. vivax* disease throughout the study. However, a reaction at the injection site was observed in the animals that received ISM 0.5 and ISM 1.0, as some animals presented leg swelling and ambulation problems. Three animals in the control group were excluded from the study on Days 10, 16, and 31 and were immediately treated intramuscularly with DA 7.0 mg/kg (Ganaseg®, Elanco Animal Health). However, these animals did not recover and were euthanized for welfare reasons on Days 12, 20 and 35. The main post-mortem findings in these three carcasses were anemia, petechiae in the heart, dehydration, cachexia and anasarca.

The therapeutic efficacy and parasite reduction rates achieved by the different drugs and treatment schemes are shown in Table 2. Twenty-four hours after treating the animals, 100 % therapeutic efficacy was attained by DA, ISM 1.0 and DA + ISM 1.0. In the animals treated with IMD and ISM 0.5, viable trypomastigotes were identified in the blood on D + 1 (99.9 % efficacy). From the 2nd DPT until the end of

Table 2
Percentages of efficacy and reduction in *Trypanosoma vivax* trypomastigote counts in experimentally infected animals with this protozoan, and subsequently treated with different formulations or maintained as controls.

Day of	Experimental groups / Mean number of trypomastigotes of *T. vivax*						% EFFICACY					% REDUCTION				
study	T01:Positive control gourp	T02: Diminazene 7 mg/kg day 0	T03: Imidocarb (4.8 mg/kg) days 0 and 14	T04: isometamidium (0.5 mg/kg) day 0	T05: isometamidium (1.0 mg/kg) day 0	T 03: diminazene (7 mg/kg) day 0 and isometamidium (1.0 mg/kg) day 14	DA	IMD	ISM 0.5	ISM 1.0	DA + ISM 1.0	DA	IMD	ISM 0.5	ISM 1.0	DA + ISM 1.0
−1	555600.0	555600.0	551600.0	553600.0	553600.0	553600.0										
1	792000.0	0.0	4.0	4.0	0.0	0.0	**100.0**	**99.9**	**99.9**	**100.0**	**100.0**	**100.0**	**99.9**	**99.9**	**100.0**	**100.0**
2	897600.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
3	340004.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
4	695204.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
5	1846008.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
6	1115208.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
7	1127600.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
8	1210004.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
9	4054120.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
10	3755200.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
11	3755200.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
12	2025000.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
13	1585015.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
14	5250030.0	0.0	1735200.0	0.0	0.0	0.0	**100.0**	**66.9**	**100.0**	**100.0**	**100.0**	**100.0**	**0.0**	**100.0**	**100.0**	**100.0**
15	3250020.0	0.0	7604.0	0.0	0.0	0.0	**100.0**	**99.8**	**100.0**	**100.0**	**100.0**	**100.0**	**98.6**	**100.0**	**100.0**	**100.0**
16	1050015.0	0.0	72.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
17	4834500.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
18	5237500.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
19	1934515.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
20	1766833.3	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
21	390000.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
22	530000.0	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
23	366746.7	0.0	0.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
24	63366.7	0.0	4.0	0.0	0.0	0.0	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**
25	392006.7	0.0	320000.0	0.0	0.0	0.0	**100.0**	**18.4**	**100.0**	**100.0**	**100.0**	**100.0**	**42.0**	**100.0**	**100.0**	**100.0**
26	50673.3	0.0	NR	0.0	0.0	0.0	**100.0**	NR	**100.0**	**100.0**	**100.0**	**100.0**	NR	**100.0**	**100.0**	**100.0**
27	93.3	0.0	NR	0.0	0.0	0.0	**100.0**	NR	**100.0**	**100.0**	**100.0**	**100.0**	NR	**100.0**	**100.0**	**100.0**
28	300266.7	0.0	NR	0.0	0.0	0.0	**100.0**	NR	**100.0**	**100.0**	**100.0**	**100.0**	NR	**100.0**	**100.0**	**100.0**
29	290053.3	0.0	NR	0.0	0.0	0.0	**100.0**	NR	**100.0**	**100.0**	**100.0**	**100.0**	NR	**100.0**	**100.0**	**100.0**
30	213353.3	0.0	NR	0.0	0.0	0.0	**100.0**	NR	**100.0**	**100.0**	**100.0**	**100.0**	NR	**100.0**	**100.0**	**100.0**
35	1765000.0	0.0	NR	0.0	0.0	0.0	**100.0**	NR	**100.0**	**100.0**	**100.0**	**100.0**	NR	**100.0**	**100.0**	**100.0**
41	280000.0	0.0	NR	0.0	0.0	0.0	**100.0**	NR	**100.0**	**100.0**	**100.0**	**100.0**	NR	**100.0**	**100.0**	**100.0**
45	10.0	0.0	NR	0.0	0.0	0.0	**100.0**	NR	**100.0**	**100.0**	**100.0**	**100.0**	NR	**100.0**	**100.0**	**100.0**
51	32030.0	0.0	NR	0.0	0.0	0.0	**100.0**	NR	**100.0**	**100.0**	**100.0**	**100.0**	NR	**100.0**	**100.0**	**100.0**
55	60.0	479010.0	NR	0.0	0.0	0.0	**0.0**	NR	**100.0**	**100.0**	**100.0**	**13.8**	NR	**100.0**	**100.0**	**100.0**
61	0.0	NR	NR	0.0	0.0	0.0	NR	NR	**NA**	**NA**	**NA**	NR	NR	**100.0**	**100.0**	**100.0**
65	0.0	NR	NR	0.0	0.0	0.0	NR	NR	**NA**	**NA**	**NA**	NR	NR	**100.0**	**100.0**	**100.0**
71	0.0	NR	NR	0.0	0.0	0.0	NR	NR	**NA**	**NA**	**NA**	NR	NR	**100.0**	**100.0**	**100.0**
75	0.0	NR	NR	0.0	0.0	0.0	NR	NR	**NA**	**NA**	**NA**	NR	NR	**100.0**	**100.0**	**100.0**
81	0.0	NR	NR	0.0	0.0	0.0	NR	NR	**NA**	**NA**	**NA**	NR	NR	**100.0**	**100.0**	**100.0**
85	0.0	NR	NR	0.0	0.0	0.0	NR	NR	**NA**	**NA**	**NA**	NR	NR	**100.0**	**100.0**	**100.0**
90	0.0	NR	NR	0.0	0.0	0.0	NR	NR	**NA**	**NA**	**NA**	NR	NR	**100.0**	**100.0**	**100.0**
97 to 180	NR	NR	NR	0.0	0.0	0.0	NR	NR	**NA**	**NA**	**NA**	NR	NR	**100.0**	**100.0**	**100.0**

NR = Unrealized. Group was excluded from the study after this time.
NA = Not applied as a function of T. vivax trypomastigote counts present in the control group.

Table 3
Parasitemia by *Trypanosoma vivax* trypomastigotes, diagnosed by the Brener technique, in experimentally infected cattle with 1 × 106 trypomastigotes of the protozoan in question, and subsequently treated with different formulations, or maintained as control.

Experimental period (days)	Experimental groups / Means and Standard deviation*																						Value of P
	PCG			DA			IMD			ISM 0.5				ISM 1.0				DA + ISM 1.0						
0	555,600	±	592,998	Aab	555,600	±	390,956	Aa	551,600	±	613,989	Aa	553,600	±	612,531	Aa	553,600	±	355,591	Aa	553,600	±	923,334	Aa	0.8645
1–7	973,375	±	1,287,947	Aab	0	±	0	Bb	1	±	1	Bb	1	±	1	Bb	0	±	0	Bb	0	±	0	Bb	0.0002
8 to 14	3,090,653	±	2,006,711	Aa	0	±	0	Bb	247,886	±	548,240	Bb	0	±	0	Bb	0	±	0	Bb	0	±	0	Bb	< 0.0001
15 to 21	2,637,626	±	2,700,325	Aab	0	±	0	Bb	1097	±	2450	ABb	0	±	0	Bb	0	±	0	Bb	0	±	0	Bb	< 0.0001
22 to 28	243,308	±	194,064	Aabc	0	±	0	Bb	53,155	±	118,858	ABb	0	±	0	Bb	0	±	0	Bb	0	±	0	Bb	< 0.0001
29 to 35	583,901	±	192,509	Aa	0	±	0	Bb					0	±	0	Bb	0	±	0	Bb	0	±	0	Bb	< 0.0001
37 to 41	301,683	±	53,031	Aab	0	±	0	Bb					0	±	0	Bb	0	±	0	Bb	0	±	0	Bb	< 0.0001
43 to 47	36,693	±	25,923	Abcd	0	±	0	Bb					0	±	0	Bb	0	±	0	Bb	0	±	0	Bb	< 0.0001
49 to 53	282,010	±	163,113	Aab	0	±	0	Bb					0	±	0	Bb	0	±	0	Bb	0	±	0	Bb	0.0028
55 to 59	20	±	14	Bcd	479,010	±	696,078	Aa					0	±	0	Bb	0	±	0	Bb	0	±	0	Ab	0.9956
61 to 65	0	±	0	Ad									0	±	0	Ab	0	±	0	Ab	0	±	0	Ab	0.9854
67 to 71	0	±	0	Ad									0	±	0	Ab	0	±	0	Ab	0	±	0	Ab	0.9948
73 to 77													0	±	0	Ab	0	±	0	Ab	0	±	0	Ab	0.9713
79 to 90													0	±	0	Ab	0	±	0	Ab	0	±	0	Ab	–
Value of P	0.0020				0.0001				0.00001				0.00001				0.00001								

*: Values followed by the same letter, uppercase in the line and lowercase in the column, do not differ from one another by the Kruskal-Wallis test (p > 0.05).
PCG = Positive control group DA = Diminazene 7 mg/kg (day 0) – IM IMD = Imidocarb (4.8 mg/kg) Days 0 and 14 – SC ISM 0.5 = Isometamidium 0.5 mg/kg – IM.
ISM 1.0: Isometamidium 1 mg/kg – IM DA + ISM 1.0 = Diminazene 7 mg/kg D0 and Isometamidium 1 mg/kg D + 14.

the study (180th DPT), the formulations containing ISM 0.5, ISM 1.0 and DA + ISM 1.0 achieved maximum therapeutic efficacy (100 %). In contrast, the animals that received IMD had relapsed circulating *T. vivax* on the 14th DPT (66.9 % efficacy) and, in accordance with the design of the present study, received a new treatment with IMD on the same day. Even after this second treatment, however, the animals had another *T. vivax* relapse on the 25th DPT (18.4 % efficacy). Therefore, the group was excluded from the study after this time. For DA, therapeutic efficacy and parasite reduction were 100 % from the 1st to the 50th DPT. On the 55th DPT (five days after the experimental re-infection of the animals), the efficacy and parasite reduction rates dropped to 0.0 % and 13.8 %, respectively, and the DA group was also excluded from the trial. In contrast, the therapeutic efficacy and parasite reduction rates in the animals treated with ISM 1.0 and DA + ISM 1.0 were 100 % throughout the entire study, even after re-infection on the 50th DPT with 1 × 10^6 trypomastigotes of *T. vivax* (Table 2).

The statistical analysis performed based on the *T. vivax* count method proposed by Brener confirmed the therapeutic results described in the paragraph above (Table 3). *T. vivax* counts in the blood of the animals treated with IMD did not differ (P > 0.05) from the mean parasite counts in the blood of the animals in the control group from the 15th to 28th DPT. Moreover, the quantity of trypomastigotes of *T. vivax* in the blood of the animals treated with DA was higher from the 55th to 59th DPT (P = 0.0028) compared to the number in the other experimental groups (Table 3 and Fig. 1). The mean *T. vivax* counts were always lower in the animals treated with ISM 0.5, ISM 1.0 and DA + ISM 1.0 (P ≤ 0.05) than the counts in the control group, as shown in Table 3 and Fig. 1.

During the acute phase of *T. vivax* infection (1st to 28th DPT), only the PCG control groups showed fever between 1st to 7st DPT, differing statistically (p = 0.0001) from the other groups (Table 4). No significant differences in rectal temperature within the same experimental group were found in the treated animals (P > 0.05). In the control group, a significant (p = 0.0023) variation in body temperature was found from the 55th to 65th DPT compared to the previous periods (Table 4).

The statistical analysis revealed no significant differences (P > 0.05) in mean PCV among the cattle treated with DA, ISM 0.5, ISM 1.0 and DA + ISM 1.0. At some time points, however, the animals treated with ISM 1.0 and DA + ISM 1.0 had lower PCV values (P ≤ 0.05), which were similar to those found in the positive control group (Table 5 and Fig. 2). These lower PCV values were also found in the animals that received IMD. The mean PCV in the animals treated with IMD did not differ from that of the animals in the positive control group from the 8th to 14th DPT (p = 0.0003) and from the 22nd to 28th DPT (p = 0.0010) (Table 5).

The cPCR results clearly show that even after treatment with IMD and ISM 0.5, it was possible to detect *T. vivax* in one animal in each group on D + 1, in one animal treated with IMD on Day 2 as well as two other animals on the 14th DPT and another one on the 24th DPT in the group treated with IMD. Moreover, the four animals treated with DA on D0 and re-infected on the 50th DPT had viable *T. vivax* parasites according to cPCR on the 55th DPT. The other samples were free of *T. vivax* following the treatment assignments.

4. Discussion

To the best of our knowledge, this is the first study to test the therapeutic efficacy of different drugs for the control of *T. vivax,* such as diminazene diaceturate, imidocarb dipropionate and isometamidium chloride, in experimentally infected animals using the same experimental design. The treatment protocols with DA and/or ISM 0.5 and ISM 1.0 had high efficacy (100 %) against *T. vivax* in experimentally infected cattle. However, treatment with DA on Day 0 failed to prevent a new infection. IMD exhibited 66.9 % of efficacy and the animals that received this treatment had relapsed circulating *T. vivax* on 14th and

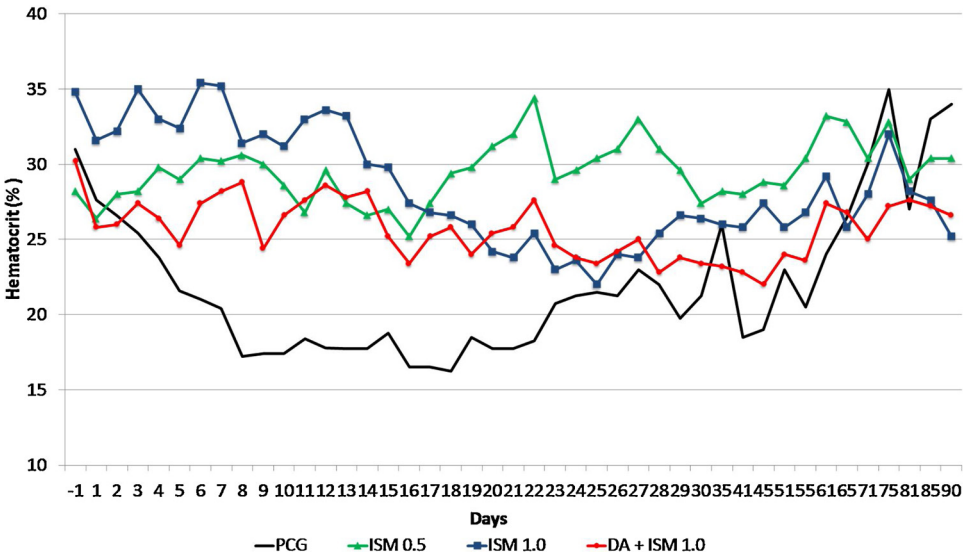

Fig. 1. Mean globular volume in calves experimentally infected with *Trypanosoma vivax* and subsequently treated with different formulations or maintained as control. PCG = Positive control group; DA = diminazene aceturate (7 mg/kg) on day 0; IMD = imidocarb dipropionate (4.8 mg/kg) on days 0 and 14; ISM 0.5 = isometamidium (0.5 mg/kg) at day 0; ISM 1.0 = Isometamidium (1 mg/kg) at day 0; DA + ISM 1.0 = Diminazene (7 mg/kg) on day 0 and Isometamidium 1.0 (1.0 mg/kg) on day 14.

28th DPT (7604 and 320,000 trypomastigotes/mL respectively). These results are discussed below.

Several comparisons of efficacy between diminazene diaceturate and isometamidium chloride have been performed in the past using different protocols. For example, Paiva et al. (2000) tested two commercial formulations of diminazene diaceturate (3.5 mg/kg) for *T. vivax* in cows, reporting good efficacy, although one animal had a relapsed *T. vivax* infection at the end of the study. In contrast, Desquesnes et al. (1995) concluded that the efficacy of diminazene diaceturate at 7.0 mg/kg was nearly null and not acceptable for bovine trypanosomosis after treating zebu cattle infected with strains of *T. vivax* from a natural outbreak in French Guiana. In the same study, the authors reported that treatment with isometamidium chloride was effective at a dose of 0.5 mg/kg, with no circulating parasite found for up to 113 days after treatment. Schönefeld et al. (1987) evaluated the sensitivity of African strains of *T. vivax* to isometamidium chloride and found no curative effect of the drug. According to the authors, the results suggest parasite resistance to the drug due its indiscriminate use in the region. A possible explanation for these contrasting results may reside in the fact that isometamidium chloride was not used for decades in Brazil and has only recently been approved for use on cattle (Bastos et al., 2017). As a result, the Brazilian *T. vivax* strains were not exposed to this compound and did not have time to acquire resistance.

Relapsed *T. vivax* infections after treatment with diminazene diaceturate, imidocarb dipropionate and isometamidium chloride has been reported previously both for natural and experimental infections (Bassi et al., 2018; Bastos et al., 2017; Batista et al., 2007; Cadioli et al., 2012; Dagnachew and Bezie, 2015; Paiva et al., 2000). The main hypothesis to explain this event is the acquirement of resistance by the parasite to the drugs (Mulandane et al., 2018). It is also possible that the parasite is able to escape from trypanocide drugs by invading areas of the body in which the drug does not reach levels high enough to eliminate it, such as the central nervous system (Ardelli and Woo, 2001; Batista et al., 2011), eye globe (Whitelaw et al., 1988), skin (Capewell et al., 2016) or even adipose tissue (Trindade et al., 2016).

In the present study, diminazene diaceturate at a dose of 7 mg/kg was able to eliminate *T. vivax* trypomastigotes. It should be pointed out that the *T. vivax* strain used in this study was the same as that reported to be resistant to diminazene by Bastos et al. (2017) as well as other research groups (Batista et al., 2007; Bassi et al., 2018; Cadioli et al., 2012). Diminazene diaceturate has a half-life of 107.5 ± 8.5 h (≈ 4.5 days) in calves and 65 % of the administered dose is excreted within 24 h after treatment (Kaur et al., 2000). Thus, this drug has a very short residual effect on *T. vivax*. Indeed, the relapsed cases described by Bastos et al. (2017) and other researchers were likely due to the short residual effect of diminazene diaceturate rather than parasite resistance. Thus, caution is recommended when interpreting the resistance of *T. vivax* to this drug in Brazil, as described by Bastos et al. (2017), and these findings should be reviewed to avoid erroneous conclusions, as the relapses may have been due to natural re-infection and not necessarily parasite resistance.

The preventive protection effect of ISM 1.0 in the present study, even after re-infection on the 50th DPT with 1×10^6 trypomastigotes of *T. vivax* may be explained by findings described by Kinabo and Bogan (1988) and Eisler (1996). These researchers report that isometamidium chloride (1.0 mg/kg) has a half-life of 286 h (approximately 12 days), in some cases reaching 463 h and being detected in the serum of treated animals for as long as 64 days.

For animals with an active *T. vivax* infection treated with imidocarb dipropionate, there is only one report mentioning its use to combat *T. vivax* during an epidemic outbreak in a dairy herd in Brazil, when a dose of 2.4 mg/kg of the drug failed to cure the infected cattle (Costa et al., 2016). Experiments performed in mice using imidocarb dipropionate also demonstrated poor therapeutic results against *Trypanosoma* (Schafer da Silva et al., 2008), which is in agreement with the findings of the present study for the dose of 4.8 mg/kg. However, this is the first report in a controlled design showing the poor efficacy of imidocarb in experimentally infected cattle.

In the present study, the animals treated with imidocarb exhibited no adverse events due to parasitism by *T. vivax*, as these animals did not have persistently high parasitemia (7604 and 320,000 trypomastigotes/mL on Days 14 and 28, respectively) compared to the control animals (2.03×10^6 from Days 1–14 and 1.44×10^6 from Days 15–28). Therefore, the globular volume and dehydration of these animals were less severe. The animals treated with imidocarb had high parasitemia for a short period (one or two days) before they were treated again on Day 14 or excluded from the study on Day 28. In this case, the clinical complications were likely due to the persistence of high parasitism during the acute phase of the infection. Imidocarb can currently be used to keep reservoir bovines for *T. vivax* or when the aim is to decrease the degree of infection by this protozoan temporarily during the acute stage of infection in an experimental infection study with primo-infected animals so that these animals do not die and, at the same time, do not eliminate *T. vivax* from the body (personal communication).

Despite the fluctuations in body temperatures, the parasitemia of 93,737 and 243,308 from Days 1–7 and 22–28, respectively, coincided with hyperthermia in the animals in the control group, as previously described by several authors (Almeida et al., 2010b; Dagnachew and

Table 4
Mean values of rectal temperature measured in experimentally infected animals with 1 × 10^6 *Trypanosoma vivax* trypomastigotes and subsequently treated with different formulations, or maintained as controls.

Experimental period (days)	Experimental groups / Means and Standard deviation*																				Value of P
	PCG				DA				IMD				ISM 0.5				ISM 1.0				DA + ISM 1.0				
0	38.60	±	0.90	Aabc	38.16	±	0.61	Aa	38.14	±	0.46	Aa	38.50	±	0.33	Aa	38.22	±	0.69	Aa	37.86	±	0.47	Aabc	0.9561
1–7	39.56	±	0.79	Aa	37.59	±	0.37	Ba	37.83	±	0.33	Ba	37.86	±	0.14	Ba	37.99	±	0.65	Ba	37.93	±	0.53	Babc	0.0001
8 to 14	38.88	±	0.42	Aab	37.90	±	0.21	Ba	38.10	±	0.66	ABa	37.86	±	0.39	Ba	38.44	±	0.42	ABa	38.16	±	0.50	Bab	< 0.0001
15 to 21	38.66	±	0.19	Aabc	37.81	±	0.22	BCa	38.34	±	0.33	ABa	37.63	±	0.53	Ca	38.39	±	0.39	ABa	38.23	±	0.21	ABCab	< 0.0001
22 to 28	39.09	±	0.54	Aab	38.28	±	0.23	Ba	38.33	±	0.27	ABa	37.71	±	0.41	Ba	38.42	±	0.59	ABa	38.25	±	0.28	ABab	< 0.0001
29 to 35	38.23	±	0.34	Abc	37.67	±	0.30	Aa					37.84	±	0.22	Aa	37.97	±	0.35	Aa	37.68	±	0.33	Aabc	0.6421
37 to 41	38.64	±	0.12	Aabc	37.75	±	0.55	Ba					37.91	±	0.24	ABa	38.31	±	0.70	ABa	37.89	±	0.28	ABabc	0.0123
43 to 47	38.24	±	0.31	Abc	37.68	±	0.11	Ba					37.82	±	0.21	ABa	37.78	±	0.30	Ba	37.71	±	0.14	Babc	0.0013
49 to 53	38.49	±	0.22	Abc	37.68	±	0.29	Ba					37.92	±	0.37	ABa	37.91	±	0.45	AB	37.61	±	0.37	Bbc	0.0014
55 to 59	36.99	±	0.22	Ad									37.69	±	0.55	Aa	37.54	±	0.59	Aa	37.33	±	0.13	Ac	0.3452
61 to 65	36.97	±	0.24	Ad									37.71	±	0.54	Aa	37.69	±	0.54	Aa	37.66	±	0.26	Aabc	0.4160
67 to 71	37.87	±	0.42	Acd									38.08	±	0.71	Aa	37.96	±	0.58	Aa	37.84	±	0.18	Aabc	0.6741
73 to 77													37.75	±	0.69	Aa	37.81	±	0.74	Aa	37.89	±	0.35	Aabc	0.4621
79 to 90													38.17	±	0.35	Aa	38.61	±	0.65	Aa	38.42	±	0.46	Aa	0.8741
Value of P	0.0023				0.7563				0.8745				0.8621				0.8962				0.0116				–

*: Values followed by the same letter, uppercase in the line and lowercase in the column, do not differ from one another by the Kruskal-Wallis test ($p > 0.05$).
PCG = Positive control group DA = Diminazene 7 mg/kg (day 0) – IM IMD = Imidocarb (4.8 mg/kg) Days 0 and 14 – SC ISM 0.5= Isometamidium 0.5 mg/kg – IM.
ISM 1.0: Isometamidium 1 mg/kg – IM DA + ISM 1.0= Diminazene 7 mg/kg D0 and Isometamidium 1 mg/kg D + 14.

Table 5
Mean hematocrit values of experimentally infected animals with 1 × 10^6 *Trypanosoma vivax* trypomastigotes and subsequently treated with different formulations, or kept as control.

Experimental period (days)	Experimental groups / Means and Standard deviation*																				Value of P
	PCG				DA				IMD				ISM 0.5				ISM 1.0				DA + ISM 1.0				
0	31.00	±	9.06	Aa	33.00	±	4.36	Aa	26.40	±	9.48	Aa	28.20	±	3.42	Aa	34.80	±	8.93	Aa	30.20	±	6.65	Aa	0.8796
1–7	23.77	±	90.1	Aa	30.11	±	4.70	Aab	24.94	±	7.32	Aa	28.86	±	3.32	Aa	33.54	±	8.49	Aa	26.54	±	3.87	Aa	0.9541
8 to 14	17.67	±	4.97	Ba	27.61	±	2.22	Aabc	27.14	±	4.90	ABa	28.51	±	3.69	Aa	32.06	±	8.47	Aa	27.43	±	2.48	Aa	0.0003
15 to 21	17.43	±	5.01	Ba	26.46	±	2.51	Abc	25.86	±	4.58	Aa	28.86	±	6.03	Aa	26.37	±	3.63	Aa	24.97	±	3.06	ABa	0.0016
22 to 28	21.14	±	5.21	Ba	25.57	±	1.27	Abc	27.06	±	3.35	ABa	31.20	±	4.02	Aa	23.89	±	8.31	ABa	24.49	±	3.08	ABa	0.0010
29 to 35	22.32	±	4.21	Aa	23.50	±	0.92	Ac					27.37	±	1.59	Aa	26.03	±	5.55	Aa	23.00	±	3.88	Aa	0.6874
37 to 41	21.91	±	0.76	Aa	25.83	±	2.88	Abc					27.47	±	2.02	Aa	26.80	±	9.08	Aa	24.87	±	4.46	Aa	0.6474
43 to 47	19.00	±	6.59	Ba	25.33	±	3.05	Abc					28.13	±	2.59	Aa	27.60	±	6.43	ABa	24.60	±	3.00	ABa	0.0014
49 to 53	20.80	±	7.16	Aa	26.67	±	2.20	Abc					28.07	±	2.64	Aa	26.73	±	8.79	Aa	24.60	±	4.06	Aa	0.6521
55 to 59	19.77	±	6.11	Ba									30.73	±	4.37	Aa	28.00	±	7.34	ABa	25.53	±	4.78	ABa	0.0038
61 to 65	22.60	±	6.89	Aa									32.53	±	4.37	Aa	28.00	±	7.86	Aa	27.00	±	4.73	Aa	0.6741
67 to 71	20.75	±	5.59	Aa									31.53	±	6.93	Aa	30.00	±	9.29	Aa	26.60	±	3.48	Aa	0.4624
73 to 77													31.53	±	6.70	Aa	30.60	±	8.12	Aa	27.60	±	2.84	Aa	0.6251
79 to 90													29.93	±	4.09	Aa	27.00	±	6.58	Aa	27.13	±	1.82	Aa	0.3457
Value of P	0.9420				0.0001				0.7652				0.8765				0.7564				0.8743				–

*: Values followed by the same letter, uppercase in the line and lowercase in the column, do not differ from one another by the Kruskal-Wallis test ($p > 0.05$).
PCG = Positive control group DA = Diminazene 7 mg/kg (day 0) – IM IMD = Imidocarb (4.8 mg/kg) Days 0 and 14 – SC ISM 0.5= Isometamidium 0.5 mg/kg – IM.
ISM 1.0: Isometamidium 1 mg/kg – IM DA + ISM 1.0= Diminazene 7 mg/kg D0 and Isometamidium 1 mg/kg D + 14.

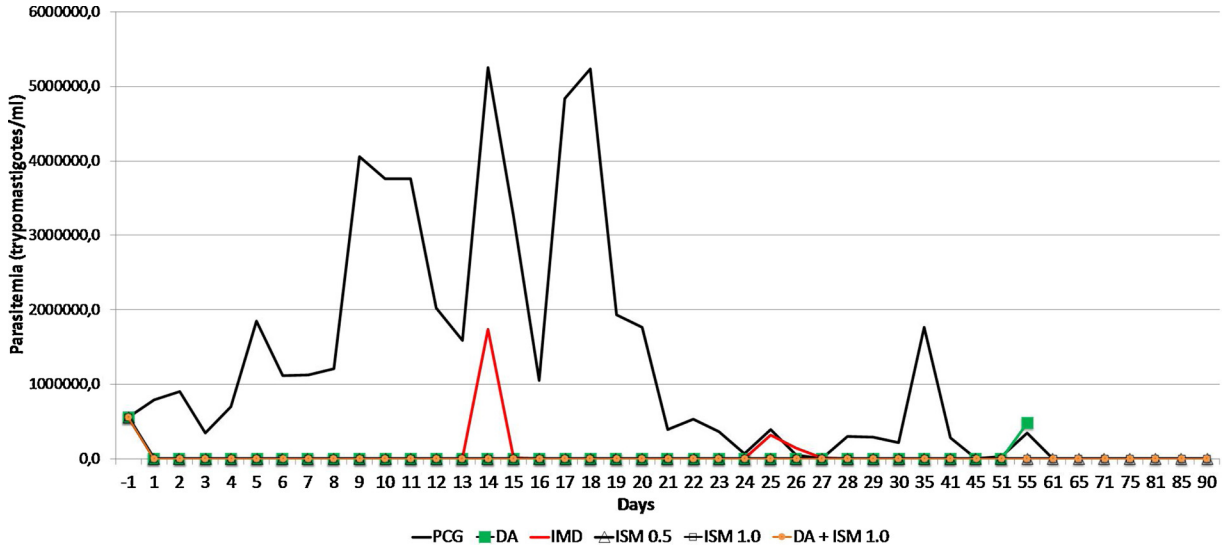

Fig. 2. Mean values of parasitemia in calves experimentally infected with *Trypanosoma vivax* and later treated with different formulations or maintained as controls. PCG = Positive control group; DA = diminazene aceturate (7 mg/kg) on day 0; IMD = imidocarb dipropionate (4.8 mg/kg) on days 0 and 14; ISM 0.5 = isometamidium (0.5 mg/kg) at day 0; ISM 1.0 = Isometamidium (1 mg/kg) at day 0; DA + ISM 1.0 = Diminazene (7 mg/kg) on day 0 and Isometamidium 1.0 (1.0 mg/kg) on day 14.

Bezie, 2015; Desquesnes, 2004; Schenk et al., 2001).

The PCV results were interesting. The animals treated with ISM 1.0, which was effective against *T. vivax*, experienced a drop in average PCV at some time points, approaching the values found in the negative controls. Schillinger et al. (1985) described a drop in PCV and hemoglobin to about 1/3 of initial values in cattle treated intravenously with isometamidium chloride (1 mg/kg). These results are very important for practitioners in the field and highlight the need to avoid the use of isometamidium chloride at a dose of 1 mg/kg in animals that are extremely ill.

Regarding the methods for diagnosing *T. vivax*, animals that received DA or ISM 1.0 did not exhibit circulating *T. vivax* within 24 h post-treatment with any of the diagnostic methods employed. This clearance effect has also been seen in dogs, ewes and cattle infected by *T. evansi*, *T. congolense* and *T. vivax* following treatment with isometamidium chloride (Schillinger et al., 1985) or diminazene diaceturate (Dagnachew and Bezie, 2015). Other researchers state that cPCR provides considerable sensitivity and specificity (Maganga et al., 2016; Gall et al., 2004; Cossic et al., 2017; Rabelo et al., 2018) even in the presence of low parasite levels. However, one cannot discard the occurrence of false negatives with cPCR when compared to other molecular techniques, such as LAMP (Cadioli et al., 2015). Moreover, hemoglobin, heparin or even DNAases cause the destruction of DNA and the specific sequence investigated (Cuglovici et al., 2010). However, the number of repetitions performed on the same animal over time in the present study decreases this possibility.

5. Conclusion

The treatment protocols with diminazene (7 mg/kg) and/or isometamidium (0.5 and 1.0 mg/kg) achieved 100 % therapeutic efficacy against *Trypanosoma vivax* in experimentally infected cattle. In contrast, imidocarb (4.8 mg/kg) was not effective against *T. vivax*.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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