

Reproductive efficiency of asymptomatic *Theileria equi* carriers mares submitted to an embryo transfer program¹

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ABSTRACT. Bezerra L.L., Jacob J.C.F., Santos H.A., Massard C.L., Silva P.C.A., Gaudêncio F.N. & Sá M.A.F. 2015. **Reproductive efficiency of asymptomatic *Theileria equi* carriers mares submitted to an embryo transfer program.** *Pesquisa Veterinária Brasileira* 35(3): 265-269. Departamento de Reprodução e Avaliação Animal, Departamento de Parasitologia Animal, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ 23890-000, Brazil. E-mail: luluzootec@uol.com.br

This study aimed to assess and evaluate the effects of *Theileria equi* infection on embryonic recovery, gestation and early embryonic loss. Thirteen Mangalarga Marchador *Theileria equi* positive donors (diagnosed through nested-PCR) and 40 embryos receptors were used. Donors were submitted to two embryo collections in two consecutive estrous cycles (GId); after, the same mares were treated with imidocarb dipropionate (1.2mg/kg IM.) in order to collect more embryos in two more estrous cycles (GIId). Receptors were divided into two groups (control and with treated) with 20 animals each, where one group was the control (GIr) and the other one (GIIr) treated with 1.2mg/kg IM of imidocarb dipropionate assessing the gestation rate at 15, 30, 45 and 60 days. After 52 embryo collections, the embryonic recovery rates were 53.84% (14/26) and 65.38% (17/26) ($p > 0.05$) for GId and GIId, respectively. The gestation rate was 70% (14/20) ($p > 0.05$) at 15, 30, 45 and 60 days in group GIr and for GIIr was 85% (17/20) ($p > 0.05$) at 15 days, 80% (16/20) ($p > 0.05$) at 30, 45 and 60 days. The treatment with imidocarb dipropionate did not cause significant improvement in the reproductive efficiency at an ET program.

INDEX TERMS: *Theileria equi*, babesiosis, biotechnology, nPCR, mares.

RESUMO. [Eficiência reprodutiva de éguas portadoras assintomáticas de *Theileria equi* submetidas a um programa de transferência de embriões.] Este estudo teve por objetivo avaliar a influência da infecção por *Theileria equi* nas taxas de recuperação embrionária, gestação e perda embrionária precoce. Foram utilizadas 13 doadoras e 40 receptoras de embrião da raça Mangalarga Marchador, positivas para *Theileria equi* através da técnica de nested-PCR. Nas doadoras foram realizados duas coletas de embriões em dois ciclos estrais consecutivos (GId), em sequência, esses mesmos animais foram tratados com dipropionato de imidocarb (1,2mg/kg IM.) para realização de mais duas coletas de

embriões em dois ciclos estrais (GIId). As receptoras foram divididas em dois grupos de 20 animais cada, onde um grupo foi o controle (GIr) e, o outro grupo, foi tratado (GIIr) com 1,2mg/ Kg IM de dipropionato de imidocarb, com intuito de avaliar a taxa de gestação aos 15, 30, 45 e 60 dias. Após a realização de 52 coletas de embrião, as taxas de recuperação embrionária foram de 53,84% (14/26) e 65,38% (17/26) ($p > 0,05$) para GId e GIId, respectivamente. A taxa de gestação foi de 70% (14/20) ($p > 0,05$) aos 15, 30, 45 e 60 dias no grupo GIr e para o GIIr foi 85% (17/20) ($p > 0,05$) aos 15 dias, 80% (16/20) ($p > 0,05$) aos 30, 45 e 60 dias. O tratamento com dipropionato de imidocarb não promoveu melhora significativa na eficiência reprodutiva em um programa de TE.

TERMOS DE INDEXAÇÃO: *Theileria equi*, babesiose, biotecnologia, nPCR, éguas.

INTRODUCTION

The success of embryo transfer programs (ET) in equines can be affected by several known factors such as animal

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body score, uterine status, day of embryo collection, quality and handling of the embryo, besides requirement of highly specialized workers. However, there are also some unknown factors that could interfere in the ET. Animals free of diseases, ectoparasites and endoparasites are necessary for an ideal embryo transfer program; however, due to Brazilian tropical climate and the lack of sanitary control, it is difficult obtaining animals free of some parasitosis, such as babesiosis.

The demand for assisted reproduction techniques development in equideoculture has considerably increased. Brazil is one of the leading countries in the use of Embryo transfer (ET), along with the USA and Argentina. Although ET use has increased over the last decades, its numbers have fluctuated along with the financial health of the equine industry. Due to the high cost of the technique it has only been used in genetically superior animals as donors (Squires et al. 1999).

The babesiosis has been described as the main equine parasitosis, due to direct damages such as reduced performance and mortality, besides the indirect damages as commercialization restraint and specially exports (Friedhoff 1990). The mortality in infections by *Theileria equi* is low; in general the animals recover from the disease acute phase and of remaining parasite asymptomatic carriers. During the infection chronic phase, unspecified clinical signs, as impatience, weight loss and reduced physical and reproductive performance are common (Schein 1988).

Cases of congenital babesiosis have been observed, suggesting transplacental transmission (Santos et al. 2008), at the first trimester of gestation (Allsopp et al. 2007), besides the occurrence of aborted fetuses with jaundice and anemia, premature or born sick foals, showing symptoms as anemia, prostration, jaundice and reluctance to feed (Du Plessis & Basson 1966). Thus, considering the small number of embryos obtained per mare a year, a better pregnancy rate and lower embryonic loss are required. Accordingly, it is important assessing the influence of *T. equi* infection on embryonic recovery rates, gestation and early embryonic loss in a Commercial Program of Equine Embryo Transfer.

MATERIALS AND METHODS

The present study took place in a Commercial Center for Embryo Transfer, located in the city of Itaguaí, and on two stud farms in the city of Seropédica/RJ, Brazil. Thirteen donors and 40 receptors of embryos from the "Mangalarga Marchador" breed were used. The animals were between 3 and 10 years old, presented body score between four and five (NRC 2007) and were previously examined for *T. equi* by the nested-PCR method (Nicolaiewsky et al. 2001) at the Laboratory of Hematoparasites and Vectors of the Federal Rural University of Rio de Janeiro.

During the whole study period, the control of endoparasites was performed through the association of 1,2g ivermectin and 15,0 g praziquantel, and the ectoparasites control were accessed by a weekly cypermethrin 15% pulverization. The embryo donor mares were kept in individual stalls, fed with four quilograms of commercial feed per day with 13% of protein, alfalfa hay, commercial mineral salt and water ad libitum. The embryo receptor mares were kept in pickets, being fed in troughs with two kilos of commercial feed per day with 13% protein, alfalfa hay, mineral salt and water ad libitum.

The blood samples were collected from a puncture in the jugular vein, with 40x12 needles, placed on flasks with ethylenediaminetetraacetic acid 11% (EDTA) anticoagulant and sent in isothermal boxes to the Laboratory of Experimental Chemotherapy in Veterinary Therapy of the Federal Rural University of Rio de Janeiro. To access the hematimetry and leucometry a Poch 100 IV Roche electronic equipment was used. The differential leukocytes count was performed by stained blood smears and the total solids determination was accessed through refractometry.

For the reproductive test, the mares were every two days examined by transrectal palpation and ultrasonography until they presented about 30mm diameter follicle, when they began to be daily examined. When the largest follicle reached ≥ 35 mm diameter and uterine echogenicity suitable with estrus, ovulation was induced with 1000 UI of intravenous human chorionic gonadotropin (hCG). The Artificial Insemination (AI) was performed with fresh semen of assured quality 24 hours after the hCG administration. Whether there was no ovulation 48 hours after the AI, mares would be submitted to the same procedure. The dose of 500x106 progressively motile spermatozoa was used to inseminate the mares. Seven and nine days post-ovulation (D7 and D9), embryo recovery attempt was performed in the donor mares (Fleury et al. 2001) at two consecutive estrous cycles (GI_d). After, these same animals were treated with imidocarb dipropionate (1.2mg/kg IM monthly) for two more embryos in two estrous cycles (GI_{ld}). Embryo receptors were divided into two groups of 20 animals each, where one was the control (GI_r) and, the other one treated with (GI_{lr}) imidocarb dipropionate. The receptors were then inoovulated with a synchronicity degree from -1 to +5 (ovulation one day before, up to five days after the donors), and then the gestation rates were evaluated at 15, 30, 45 and 60 days.

The hematological parameters were transformed (natural log) and submitted to the t test ($p>0.05$) and the embryonic recovery rates, gestation and animals embryo quality were assessed by chi-square test with 5% significance level and 95% interval according to Sampaio, 2002.

RESULTS

In the present study, as the 13 embryo donors as the 40 receptors presented positive result for *T. equi* based on the nested-PCR diagnostic method at the beginning of the procedures. At the end of the experiments, after imidocarb dipropionate treatment, 69% (9/13) of embryo donors and 50% (10/20) of embryo receptors presented negative results for *T. equi* in the nPCR (Table 1 and 2). The receptors untreated group of receptors presented 20% (4/20) of negative animals, using the same diagnostic method (Table 2).

Donor and receptor mares presented normal erythrogram before and after treatment with imidocarb dipropionate. Regarding the leukogram, the embryo donors presented a decrease in leucocytes and total neutrophil, and a slight increase of lymphocytes and monocytes after treatment. The embryo receptors presented a blood count within normal parameters.

Before donor mares have been treated with imidocarb dipropionate, the embryonic recovery rate was 53.8% (14/26). After the treatment, the recovery rate increased to 65.9% (17/26). Even with this numerical superiority observed after treatment, the chi-square test did not show significative difference ($p>0.05$).

The gestation rate at 15, 30, 45 and 60 days in the un-

Table 1. DNA detection of *Theileria equi* by the nested PCR technique in embryo donor mares before and after treatment with imidocarb dipropionate

DNA detection of <i>Theileria equi</i> by Nested-PCR in embryo donors		
Donors	Positives	Negatives
Before treatment	13 (100%)	0
After treatment	4 (31%)	9 (69%)

Table 2. DNA detection of *Theileria equi* by the nested PCR technique in embryo receptor mares not treated and treated with imidocarb dipropionate

DNA detection of <i>Theileria equi</i> by Nested PCR in embryo receptors				
Receptors	Not treated		Treated	
	Positives	Negatives	Positives	Negatives
Beginning of experiment	20 (100%)	0	20 (100%)	0
End of experiment	16 (80%)	4 (20%)	10 (50%)	10 (50%)

treated receptor group was 70% (14/20) with no embryonic loss. In the treated group the gestation rate at 15 days was 85% (17/20) and at 30, 45 and 60 days it was 80% (16/20), considering there was embryonic loss at 30 days of gestation in one of the treated mares. No significative difference ($P>0.05$) was observed among the gestation rates.

The treatment with imidocarb dipropionate caused improvement on the quality of the recovered blastocysts. Before treatment, it was obtained 71.43% of embryos (10/14) classified as grade I. After treatment the number of grade I embryos was 94.12% (16/17). An increase of 22.69% in the quality of grade I embryos was observed. The percentage of embryos classified as grade II before treatment was 28.57% (4/14) and after treatment was 5.88% (1/17). No significative difference was observed ($p>0.05$) among the results after the chi-square test.

DISCUSSION

Aiming to characterize the occurrence of *Theileria equi*, several researches have been performed in Brazil. Serological tests have been in epidemiological studies, showing the presence of antibodies and indicating, therefore, that these animals were in contact with the parasite in some moment of their lives. The greatest obstacle of these serological techniques lies in the fact that the antibodies can remain circulating for months or even for the entire life of the animals, characterizing positivity in serological tests. For asymptomatic carriers, the molecular tests are more indicated, since they are able to detect the presence of blood circulating protozoa at low rates. The nPCR has been cited as the best technique due to its amplification reactions, which ensures greater sensitivity to the technique (Baldani 2004). The highest percentage of negative animals in the treated donors and receptors groups is probably due to the prophylactic treatment with imidocarb dipropionate. It is believed that, once animals did not present any symptomatology, the parasitemia was already low, even when still detectable; and after treatment the parasitemia level became lower and probably undetectable by the method. The nPCR method based on the gene sequence *ema-1* of *T. equi*,

is able to detect parasitemias up to 0.000006%, the equivalent of 5 infected cells from a total of 10^8 erythrocytes (Nicolaiewsky et al. 2001). In another evaluation of this PCR technique for *T. equi* routine detection (Rampersad et al. 2003) through blood samples analyses from healthy and clinically sick equines, the nPCR method was able to detect the parasite 3.6 times over than stained blood smears and 2.2 times over than the detection at the first PCR amplification, showing higher sensitivity when compared to the conventional parasitological methods. The negative results obtained with no treatment receptors at the end of the experiment have possibly been associated to the reduction in the parasitemia levels due to a better handling of these animals during mating season, reducing the contact with the vector tick and possible reinfections.

The treatment with imidocarb dipropionate in infected equines has demonstrated that this compound causes parasite eradication from blood; however, according to Bruning (1996) the horses remained always infected. Clinically infected animals receiving imidocarb dipropionate (5mg/kg) treatment presented parasitemia presented decrease after two days, from 20% to 10% of infected erythrocytes and untreated ponies presented an increase from 20 to 60% in three days (Simpson & Neal 1980).

During the chronic phase there is no significative alteration between uninfected equines hematocrit *T. equi* carries (Hailat et al. 1997). According to that author, the infected equines and those ones previously treated with imidocarb dipropionate did not present any clinical sign of the disease. The same authors still reported equines highly infected presenting a reduction in their hematocrit from around 20 to 30% (Hailat et al. 1997).

According to Schein (1988), equines infected by *T. equi* develop a solid immunity protecting them against clinical diseases in cases of re-exposure to the parasite. This protection has been attributed to a continuous stimulation of the immune system by parasites persisting in the organism during the chronic phase of the disease, even with low parasitemia rates. According to Bruning (1996) imidocarb dipropionate treatment to *T. equi* has proven removing the parasite from blood circulation, although, the infected equines have remained carriers through out their lives, being responsible for the propagation and maintenance of infection.

According to the study performed by Squires et al. (2003) the embryonic recovery rate was between 50 and 75%. Some studies have shown variations in the embryonic recovery rate of: 50.9 and 59.5% (Fleury et al. 2001), 63.4% in 658 collections (Jacob et al. 2002), and 60% (Gusmão et al. 2010). The stress caused in *T. equi* carriers animals with disease clinical signs and treated with imidocarb dipropionate at therapeutic dose (4mg/kg, q72h, in four applications) has caused great reduction in the embryonic recovery rate. Besides, this parasiticide is related to episodes of cramps, salivation, irritability, gastrointestinal hyper-motility, renal and liver failure, etc. However, using prophylactic doses (1.2mg/kg) in chronic carrier mares, no undesirable side effects were observed, as well as, there was no decrease in the embryonic recovery rate and ges-

tation rate according to what was reported in the present study.

Several factors directly influence the gestation rate in a ET, among the most important are the ones related to the embryo receptor (handling and selection), the quality of the embryo, day of ovulation, and the technical factor. Commercial ET programs in Brazil have present critical and experienced professionals for the "Mangalarga Marchador" breed (Jacob et al. 2002, 2010, Gomes et al. 2004); these programs have demonstrated the transcervical transfer method presenting gestation rates around 70% according to the reported data, Rocha et al. (2007) assessing six reproductive seasons obtained a general average for 15 days of gestation of 73.4%. Evaluating the pregnancy rate and early embryonic loss of "Mangalarga Marchador" mares in a non-surgical commercial ET program during six reproductive seasons, the same authors obtained rates of 69.9 and 4.8%; 66.7 and 9.0%; and 64.5 and 12.2% at 30, 45 and 60 days of gestation, respectively. The gestation rates of the present study corroborate to the findings previously reported, although none of these earlier studies accessed the infection by *T. equi* in the animals correlating it with gestation rates. In the present study, the gestation rates obtained were better after imidocarb dipropionate treatment, however, there was no significative difference ($p>0.05$).

During the mating season, most of the obtained receptors have presented the lowest cost at the moment of acquisition due to their low body score and high infestation by ectoparasites, nevertheless, feeding improvement, vermifugation and anti-tick baths have been enough to solve the animals physical conditions problems, however, it has been neglected that animals may be *T. equi* chronic carriers and the treatment with prophylactic dose (1.2mg/kg) might improve the results obtained at the end of mating season.

In literature no similar work has been described in the relation between embryonic quality and imidocarb dipropionate treated animals. It is known that after animals prophylactic treatment, there is a reduction on the parasitism in the blood stream, thus, avoiding the occurrence of anemia and feverish peaks in carrier animals, which can directly influence the embryo recovery rates, embryonic absorption, gestation rates and embryonic death in several ET programs. In a study, the increase of internal temperature in 2°C, produced by 30 minutes of induced exercise, caused a decrease in embryonic recovery rate (from 63 to 34%), in the embryos quality (from 73 to 36% grade I), in the size of ovulatory follicles and induced a delay in the return of the oestrus period for approximately seven days after prostaglandin administration (Mortensen 2009), demonstrating that the stress and elevation of temperatures in donor mares, also observed in theileriosis, can affect the dynamic of the estrous cycle.

CONCLUSIONS

It can be concluded that treatment with imidocarb dipropionate did not cause a significant improvement ($p>0.05$) in the embryonic recovery rates and gestation rates at 15, 30, 45 and 60 days.

However, there were important increases of 11.54% in the embryonic recovery rate, 15% in the gestation rate at 15 days and 10% in the gestation rate at 30, 45 and 60 days in the group treated with imidocarb dipropionate in relation to the untreated group.

The treatment of *Theileria equi* infection with imidocarb dipropionate raised the quantity of collected grade I embryos.

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