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Dynamics of humoral immune response in pregnant mares and foals vaccinated with *Theileria equi* recombinant EMA-2[1]

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ABSTRACT.- Santos A.C., Leite F.P.L., Vianna A.M., Weege G.B., Finger I.S., Müller V., Curcio B.R. & Nogueira C.E.W. 2018. **Dynamics of humoral immune response in pregnant mares and foals vaccinated with *Theileria equi* recombinant EMA-2.** *Pesquisa Veterinária Brasileira 38(6):1105-1109*. Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Capão do Leão, Cx. Postal 354, Pelotas, RS 96010-900, Brazil. E-mail: alice.cs@live.com

Theileria equi is an infectious hemoprotozoan agent of equine piroplasmosis, a disease that has severe economic and sanitary impact internationally. In addition to its common clinical features, piroplasmosis can cause gestational losses and neonatal damage, which makes neonates susceptible to this disease. The aim of this study was to evaluate the dynamics of humoral immune response to recombinant EMA-2 of *T. equi* in pregnant mares and foals, as well as the transfer of vaccine antibodies through the colostrum ingested by sucking foals. For vaccine production, the EMA-2 expression gene was cloned and expressed in the yeast species, *Pichia pastoris*. Thirty-six horses were used, of which 18 were pregnant mares and 18 were foals. The mares were divided into control and vaccinated groups, and the vaccinated group received three doses of rEMA-2 every 21 days starting at 300 days of gestation. Foals from vaccinated and control groups were evaluated until the sixth month of life. The production of antibodies by foals on the rEMA-2 vaccination schedule was also evaluated from the second month of life. Foals in the vaccinated group had received three doses of the vaccine every 21 days. The method used to evaluate serum and colostrum samples was indirect ELISA, and plates were sensitized with the rEMA-2 protein. At the end of the vaccination schedule, vaccinated mares showed a 2.3-fold increase in antibody levels when compared to baseline values. The colostrum of vaccinated mares presented antibody levels of 1.0432±0.33. Foals delivered by vaccinated mares presented levels of antibodies greater than those of foals delivered by control mares after their first time sucking (at about twelve hours after birth). Foals vaccinated in the second month of life showed an 8.3-fold increase in antibody levels when compared to baseline values. The vaccination schedule with rEMA-2 was able to stimulate humoral immunity in pregnant mares. Vaccine immunoglobins were concentrated in the colostrum of vaccinated mares and foals delivered by these mares showed an increase in serum levels of vaccine antibodies after the first-time sucking.

INDEX TERMS: *Theileria equi*, equine piroplasmosis, hemoprotozoan, humoral immunity, rEMA-2, horses, parasitoses.

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RESUMO.- [**Dinâmica da resposta imune humoral em éguas gestantes e potros vacinados com EMA-2 recombinante de *Theileria equi*.**] *Theileria equi* é um hemoprotozoário, agente da piroplasmose equina, doença de impacto sanitário e econômico internacional. Em éguas gestantes além da doença clínica, podem ocorrer abortos e danos ao neonato, caracterizando grande susceptibilidade à doença no período neonatal. O objetivo deste estudo foi avaliar a dinâmica da

resposta imune humoral à EMA-2 recombinante de *T. equi* em éguas gestantes e potros, bem como a transferência de anticorpos vacinais no colostro. Foram utilizados 36 equinos, sendo 18 éguas gestantes e 18 potros. As éguas foram divididas em grupo controle e vacinado, que receberam rEMA-2 a partir dos 300 dias de gestação em três doses com intervalos de 21 dias. Para produção da vacina, o gene de expressão de EMA-2 foi clonado e a proteína expressa em *Pichia pastoris*. Os potros provenientes de éguas dos grupos vacinado e controle foram avaliados até o 6º mês de vida. Avaliou-se também a produção de anticorpos em potros submetidos ao esquema vacinal com rEMA-2 a partir do 2º mês de vida, que receberam três doses da vacina em intervalos de 21 dias. O método escolhido para a avaliação das amostras de soro e colostro foi ELISA indireto, com sensibilização pela proteína rEMA-2. Nas éguas gestantes vacinadas com rEMA-2 ocorreu o incremento de 2,3 vezes o valor basal ao final do esquema vacinal. O colostro de éguas vacinadas apresentou título médio de anticorpos de 1,0432±0,33, e potros provenientes de éguas vacinadas apresentaram média maior que os provenientes de éguas controle após a primeira mamada (12 horas). Os potros que passaram por esquema vacinal a partir do 2º mês de vida obtiveram incremento de 8,3 vezes o valor basal de anticorpos. O esquema vacinal com rEMA-2 foi capaz de estimular a imunidade humoral em éguas gestantes. Éguas gestantes vacinadas concentraram imunoglobulinas vacinais no colostro, e os potros provenientes destas obtiveram incremento nos níveis séricos de anticorpos vacinais após a primeira mamada.

TERMOS DE INDEXAÇÃO: *Theileria equi*, piroplasmose equina, hemoprotozoário, imunidade humoral, rEMA-2, equinos, parasitoses.

INTRODUCTION

The hemoparasites *Theileria equi* and *Babesia caballi* are the causative agents of equine piroplasmosis. While *B. caballi* parasites only infect erythrocytes, *T. equi* has an intra-erythrocyte and a pre-erythrocyte phase in peripheral blood mononuclear cells (PBMCs) (Wise et al. 2014).

Piroplasmosis has international epidemiological impact, especially in areas such as tropical, subtropical, and some temperate areas where there are vectors of this hemoparasite (Friedhoff & Soule 1996). In addition, horses frequently become a chronic carrier of this hemoparasite (Wise et al. 2014). Besides affecting the sanitary aspect, this disease has significant economic impact due the costs of the treatment, and abortions, neonatal damage, reduced performance of athlete horses, as well as restrictions on transportation and participation in equestrian events in countries that are free of this disease (Hussain et al. 2014). In the state of Rio Grande do Sul, Brazil, *T equi.* is endemic in the equine population. Therefore, its control and prophylaxis are of great importance, although monitoring of the infectious agent is not very effective (Nizoli et al. 2008).

The classical routes of transmission are getting bitten by ixodidae ticks and the iatrogenic pathway. Transplacental transmission of *T. equi* has additionally been described in recent years (Allsopp et al. 2007, Santos et al. 2009, Chhabra et al. 2012); however, the specific mechanism of transmission has not been elucidated (Wise et al. 2014). Transplacental transmission can lead to abortion in the final trimester of gestation and to the birth of either stillbirths or foals that show clinical signs of piroplasmosis in the first few days after birth. Effective methods to prevent transplacental infection have not been described yet (Rothschild 2013).

The recognition of the *T. equi* by the immunological system occurs mainly through the antigens on the surface of the merozoite (EMAs - equi merozoite antigen) (Kumar et al. 2015). These antigens are expressed at different stages of the protozoan cycle. EMA-1 and EMA-2 are expressed at several stages; however, EMA-2 is one of the first antigens to be recognized by the immune system (Kumar et al. 2013), which makes it potentially useful for the development of prophylactic tools such as vaccines (Kumar et al. 2013, Vianna et al. 2014).

T. equi is important during the gestational and perinatal periods since it can lead to abortion and represents a risk for neonates. In this context, the immunization of pregnant mares is an approach to enhance maternal immunity and promote the transfer of antibodies to the foal via passive immunity. The aim of this study was to evaluate the dynamics of humoral immune response to recombinant EMA-2 of *T. equi* in pregnant mares and foals, as well as the transfer of vaccine antibodies through the colostrum.

MATERIALS AND METHODS

Animals. The present study was performed with 36 horses, including 18 mares and their 18 foals, from the Centro de Ensino e Experimentação em Equinocultura da Palma (CEEEP), Universidade Federal de Pelotas/RS, Brazil. All mares recruited in this experiment were crossbreed, multiparous, and aged between ten and sixteen years old. Mares were monitored monthly through the entire gestation period by ultrasonography. In addition, physical exams and serologic evaluation by indirect ELISA with rEMA-2 were performed prior to the commencement of the study.

Mares were kept on cultivated pasture (*Lolium multiflorum*) with water *ad libitum* until foaling and were dewormed every three months, alternating the active agent each time (ivermectin, febendazole, and ivermectin with praziquantel). During the second stage of labor, mares were kept in clean stalls and all foalings were observed and assisted when needed. Within 24 hours of delivery, mares and their foals returned to the pasture, where they were kept until the end of the study.

All procedures carried out in this study were approved by the Ethical Committee on Animal Experimentation at the Universidade Federal de Pelotas (CEEA-UFPel) under protocol #8247.

Vaccination schedule and sampling. The EMA-2 was obtained as per the protocol described by Vianna et al. (2014). The vaccine was prepared by adding 200µg of the recombinant protein in 10% aluminum hydroxide adjuvant. The final dose of the vaccine was 2ml. The 18 pregnant mares recruited for this study were assigned to two groups: vaccinated group (n=9) and control group (n=9). Local antiseptic treatment was performed on the pectoral muscle region, and mares from the vaccinated group received three doses of the rEMA-2 vaccine intramuscularly with 21-day intervals between doses. The vaccination schedule started at 300 days of gestation. At this point, mares from both groups were monitored, underwent physical exams, and had blood collected weekly. The clinical parameters evaluated were the heart rate,

respiratory rate, capillary refill time, color of mucous membranes, and rectal temperature. Blood samples were collected by jugular venipuncture using tubes without anticoagulant.

At the second stage of labor, 2ml of amniotic fluid was collected in sterile syringes from all mares. After birth, foals received assistance and were assigned to two groups: foals delivered by vaccinated mares (n=9) and foals delivered by control mares (n=9). Prior to first sucking by foals, 2ml of colostrum was collected from each mare. In addition, blood was collected from foals within twelve hours of birth (foals had already sucked), seven days of birth, and monthly from the first to the sixth month of life.

In the second part of this study, 18 foals (two months of age) were assigned to two groups: vaccinated foals (n=4) and control foals (n=14). Vaccinated foals were put on the vaccination schedule with the rEMA-2 vaccine. The first set of vaccinations were performed in the second month of life and the second set of vaccinations were performed in the fifth month of life. Both groups underwent physical examinations weekly and had blood collected monthly until the sixth month of life.

Blood samples were allowed to clot and then centrifuged for five minutes at 400 *g* to obtain serum. Samples were aliquoted in 2-ml microcentrifuge tubes and stored at -20°C before conducting further analyses. Amniotic fluid and colostrum samples were aliquoted in 1.5-ml cryotubes and stored at -20°C before conducting further analyses.

Laboratorial analysis. Samples (serum, amniotic fluid, and colostrum) were analyzed by enzyme linked immunonosorbent assay (ELISA), as described previously by Vianna et al. (2014). The serum of an experimentally infected horse was used as positive control, while the serum of a newborn foal delivered by a mare negative for *T. equi* infection was used as negative control. The resulting plates were read in a micro-plate reader (TP-READER - Thermo Plate) at 492nm.

Statistical analysis. The commercial software Statistix 8.0® was used for statistical analysis. The Shapiro-Wilk test was used to verify normality, followed by frequency distribution analysis. The normally distributed data was compared among groups by ANOVA and Tukey's test, and the abnormally distributed data was compared among groups by Kruskal-Wallis test. Significance was set at $p<0.05$ for all tests. Graphs were prepared using the commercial software GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, EUA).

RESULTS

Results of the weekly physical examinations were normal for vaccinated mares and vaccinated foals. They did not show either reactions or side effects after vaccination with rEMA-2, and were healthy during the study period. Tick infestations were not observed in the animals used in this study. Figure 1 shows the mean absorbance in optical reading (492nm) of samples obtained from vaccinated mares (n=9) and control mares (n=9). The rEMA-2 vaccine induced humoral immunity in vaccinated mares. At the end of the vaccination schedule, vaccinated mares showed an increase in absorbance that was 2.3 times the basal value.

The absorbance of the amniotic fluid of vaccinated mares was 0.029±0.02, and no statistical difference was found when compared to the control group (0.027±0.07). When evaluating the absorbance of the colostrum, vaccinated mares had values of 1.043±0.33, while control mares had values of 0.468±0; these results were statistically different ($p<0.05$).

The mean absorbance (492nm) for foals delivered by vaccinated mares (n=9) and for foals delivered by control mares (n=9) is shown in Figure 2. Foals delivered by vaccinated mares presented absorbance values of 0.719±0,220 twelve hours after birth, which represents 69% of that observed for the colostrum of vaccinated mares (1.043±0.33). In the group of foals delivered by control mares, the absorbance of immunoglobulins was 0.243±0.104, which represents 51% of that observed in the colostrum of control mares (0.468±0.345). In the second month of life, foals from both groups had similar absorbance values. It is worth mentioning that the foals vaccinated later, at two months of life (n=4), were not included in the statistical analysis when evaluating foals delivered by vaccinated or control mares until six months of life to avoid confusion in the results.

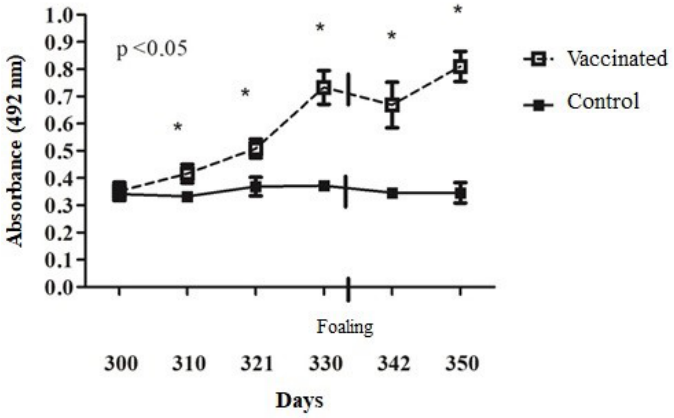

Fig.1. Indirect ELISA for equine serum. Absorbance in optical density (492nm) for vaccinated mares (n=9) and control mares (n=9) are expressed as mean ±SE. The doses of the rEMA-2 of *Theileria equi* vaccine were administered on days 300, 321, and 342 of gestation in the vaccinated group. The asterisks (*) indicate statistical difference between groups ($p<0.05$).

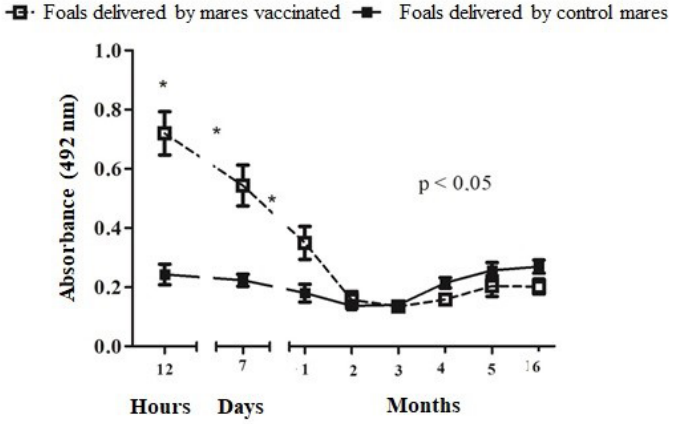

Fig.2. Indirect ELISA for equine serum. Absorbance in optical density (492 nm) for foals delivered by mares vaccinated with rEMA-2 of *Theileria equi* vaccine (n=9) and for foals delivered by control mares (n=9) is expressed as mean ±SE, and determined from twelve hours after birth to the sixth month of life. The asterisks (*) indicate statistical difference between groups ($p<0.05$).

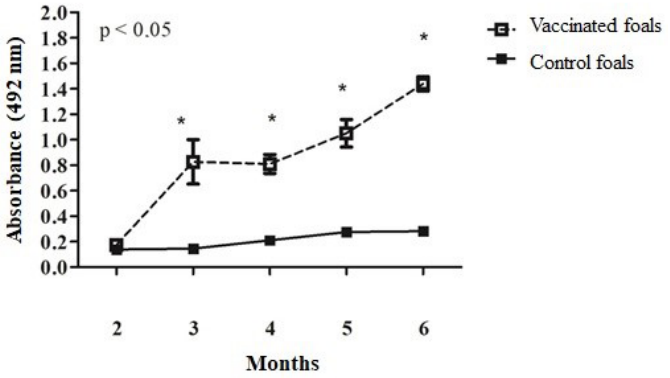

Fig.3. Indirect ELISA for equine serum. Absorbance in optical density (492 nm) for foals vaccinated with rEMA-2 of *Theileria equi* vaccine (n=4) and for control foals (n=14) is expressed as mean ±SE. Vaccination schedules were started at the second and fifth months of life for the vaccinated group. The asterisks (*) indicate statistical difference between groups (p<0.05).

The mean absorbance values for vaccinated foals (n=4) and control foals (n=14) are shown in Figure 3. At the end of the vaccination schedule (at six months), vaccinated foals showed an increase in absorbance that was 8.3 times the basal value (at two months of age)

DISCUSSION

According to the findings, there was an increase in specific antibodies in vaccinated mares when compared to control mares. Concentration of anti-*Theileria equi* immunoglobulins in the colostrum, as well as in the serum of the newborn foals, demonstrated the passive transfer of maternal antibodies. In addition, foals that were a part of the vaccination schedule in the second month of life had higher levels of antibodies than the levels in control foals.

The method chosen to perform the serological evaluation of animals in this study was indirect ELISA. The protocol used had been already tested in a previous study and had showed 83.3% specificity and 90.9% sensitivity (Vianna et al. 2014). Several variations of ELISA with different antigens have been successfully used in the diagnosis of and in studies with *T. equi* (Kumar et al. 2013, El-Sayed et al. 2015, Kumar et al. 2015). The use of the protein rEMA-2 in the vaccine formulation and as an antigen for the ELISA test is justifiable by the fact that EMA-2 is expressed in several stages of the protozoan life cycle. In addition, it is one of the first antigens to be recognized by the immune system through its expression on the membrane and cytoplasm of erythrocytes (Vianna et al. 2014).

The placenta of the mare is classified as "diffuse epitheliochorial, which prevents the passage of immunoglobulins to the fetus. However, if the equine fetus is challenged, it can produce neutralizing antibodies at around 200 days of gestation" (Perkins & Wagner 2015). Kumar et al. (2008) did not observe anti-*T. equi* antibodies in the serum of neonatal foals delivered by mares positive for theileriosis. Results of the analysis of the amniotic fluid of vaccinated mares in this study indicated that there is no transfer of antibodies through the placenta.

The production of good quality colostrum is essential to supply antibodies to the foal and to allow it to respond to pathogens present in the environment. Therefore, several vaccines are administered to broodmares in the final trimester of gestation. The foal should ingest colostrum as soon as possible after birth, specifically within a maximum time period of twelve hours (Curcio & Nogueira 2012). After that, the absorption of immunoglobulins by the foal is reduced and the mare begins to produce milk instead of colostrum (Jeffcott 1971). In this study, the concentration of anti-*T. equi* antibodies in the colostrum of vaccinated mares was higher than in the colostrum of control mares. It was also observed that foals delivered by vaccinated mares showed increased levels of serum immunoglobulins when compared to the levels in foals delivered by control mares after the first sucking. These findings demonstrate that the transfer of vaccine antibodies via passive immunity was effective.

Although foals delivered by vaccinated mares showed adequate absorption of antibodies (0.7197±0.220), levels decreased over time reaching minimal values in the second month of life (0.1566±0.086). Kumar et al. (2008) obtained similar results when evaluating the passive immunity of neonates delivered by mares infected by *T. equi* and observed a decrease in antibody levels within 63-77 days of birth. IgG3 is the most abundant class of immunoglobulins when discussing *T. equi*. Perkins & Wagner (2015) reported that IgG3 decreases to low levels when the foal reaches around ten weeks of life; this report correlates with the results obtained in this study.

Foals have an immunological window between the second month of life, when passive immunity decreases, and the fourth month of life, when adequate levels of immunoglobulins are established by the adaptive immunity (Perkins & Wagner 2015). The ideal period for the first vaccination in young individuals of several species is still undecided since the interactions among antigens, antibodies, the complement system, and cytokines in an organism in development are still not completely understood (Hodgins & Shewen 2012). It was observed in this study that vaccination in the second month of life induced the production of vaccine antibodies. This shows the ability of the vaccine to stimulate humoral immunity in a period of great vulnerability for foals.

CONCLUSIONS

The vaccination schedule with rEMA-2 stimulated humoral immunity in the pregnant mares and vaccinated foals. Vaccine immunoglobulins were concentrated in the colostrum of the pregnant mares, and their foals showed an increase in serum antibodies that decreased until the second month of life.

It was observed that rEMA-2 of *Theileria equi* stimulated humoral immunity in vaccinated animals; however, as a next step, it is necessary to expose these animals to the pathogen to verify the efficacy of this vaccine *in vivo*.

Conflict of interest statement.- The authors have no competing interests.

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