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Evaluation of cross-reaction test positivity in horses: a comparison between the use of plasma and serum

Avaliação da positividade do teste de reação cruzada em equinos: comparação entre o uso do plasma e soro

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Highlights

Cross-reaction technique in horses.
Plasma and serum for cross-reaction in horses.
Positive cross-reaction test in horses that had never received blood transfusions.

Abstract

Horses have more than 400,000 possible blood type combinations, making it impossible to perform a blood transfusion with complete compatibility between the animals. The compatibility test or cross-reaction test verifies *in vitro* the blood compatibility between the recipient and donor. The likelihood of incompatibility during the first transfusion is low because the presence of natural antibodies in horses is rare. Therefore, cross-reactivity tests are not commonly performed during a horse's first blood transfusion. However, studies indicate that the positivity of the cross-reaction is associated with a reduction in the lifespan of blood cells; therefore, it is recommended that this test be performed. Few studies have evaluated the need for a compatibility test during the first blood transfusion in horses. This study aimed to verify the occurrence of positive cross-reactions among horses that had never received blood transfusions and to compare the use of plasma and serum for the cross-reaction technique. This study evaluated 20 equines, including males and nulliparous mares. Blood samples from these animals were tested, resulting in 380 cross-reactions. The results were presented by verifying the occurrence of positive reactions in the plasma and serum. In the serum group, 14.5% of the samples were positive, whereas in the plasma group, 37.9% were positive. A statistically significant difference was observed

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between the two groups, in which the serum presented a lower percentage of positive results than the plasma, and there were positive results even in animals that had never received a blood transfusion. This study demonstrated the occurrence of positive cross-reactions in horses that had never received a blood transfusion and showed that using serum for cross-reaction tests was more reliable than using plasma.
Key words: Agglutination. Blood compatibility. Blood transfusion. Equine.

Resumo

Os equinos possuem mais de 400.000 possíveis combinações de tipos sanguíneos, dessa forma é praticamente impossível realizar uma transfusão sanguínea na qual exista completa compatibilidade entre os animais. O teste de compatibilidade ou reação cruzada, verifica *in vitro* a compatibilidade sanguínea entre receptor e doador. A chance de incompatibilidade na primeira transfusão é muito baixa, já que a presença de anticorpos naturais em equinos é rara, dessa forma, o teste de reação cruzada não é comumente realizado na primeira transfusão sanguínea em equinos. Porém, estudos indicam que a positividade da reação cruzada está associada a diminuição do tempo de vida das hemácias, e, portanto, recomenda-se que este teste seja sempre realizado. Atualmente, são poucos os estudos que avaliam a real necessidade do teste de compatibilidade na primeira transfusão sanguínea em equinos. O objetivo desse trabalho foi verificar a ocorrência de reações cruzadas positivas entre equinos que nunca receberam transfusões sanguíneas, além de comparar a utilização do plasma e do soro para a técnica de reação cruzada. O estudo foi realizado avaliando-se 20 cavalos, machos e/ou fêmeas nulíparas. As amostras de sangue destes animais foram testadas entre si resultando em 380 reações cruzadas. O resultado foi apresentado verificando a ocorrência de reações positivas entre eles em dois grupos: plasma e soro. No grupo soro, 14,5% das amostras foram positivas, enquanto no grupo plasma, 37,9% foram positivas. Houve diferença estatística entre os dois grupos, na qual o soro, apresentou menor porcentagem de resultados positivos quando comparado com o plasma, além de indicar que, mesmo em animais que nunca receberam transfusão sanguínea, houve resultados positivos. Este trabalho demonstrou a ocorrência de reações cruzadas positivas em equinos que nunca receberam transfusão sanguínea e que a utilização de soro para a realização da prova de reação cruzada foi a mais confiável do que o plasma.
Palavras-chave: compatibilidade sanguínea, cavalos, transfusão sanguínea, aglutinação.

Introduction

Blood transfusions are uncommon in horses; however, they are an emergency therapy for cases of acute hemorrhage and severe anemia. In such cases, the patient typically presents with low hematocrit and hemoglobin values and clinical signs of low perfusion and oxygen delivery, such as pale mucous membranes, tachycardia, tachypnea, prolonged capillary refill time, and hypotension. Therefore, equine veterinarians should perform this procedure. Ideally, the transfused red blood cells should have a normal half-life (on average 20 days) in the recipient (patient); however, for this to occur, compatibility between the donor's blood and the recipient's blood is required (Mudge, 2014; Radcliffe et al., 2022).

Several transfusion reactions can occur in the recipient, with the acute hemolytic immune-mediated reaction considered one of the most serious. This can lead to the animal's death, resulting in rapid destruction of the transfused red blood cells due to blood incompatibility between the donor and recipient. Delayed hemolytic immune-mediated reactions occur more than 24 h after transfusion and decrease the lifespan of transfused red blood cells, which is considered an ineffective blood transfusion (Mudge & Willians, 2016).

In horses, blood groups are classified into eight systems with 30 different factors, resulting in more than 400,000 combinations (Reichmann & Dearo, 2001; Mudge, 2014). Therefore, it is difficult to perform a transfusion in which the donor and recipient blood are completely compatible (Casenave et al., 2019). Donor horses should ideally be negative for Aa and Qa alloantigens because these are most commonly associated with hemolytic reactions. However, horse blood typing in Brazil is not easily accessible because of a lack of awareness regarding the laboratories that perform this test.

The cross-reaction test is an *in vitro* test that evaluates the blood compatibility between two individuals. This test aimed to detect the presence of antibodies that could lead to the hemolysis of transfused red blood cells. The test is divided into primary (antibodies in the recipient's plasma) and secondary (antibodies in the donor's plasma) tests and is considered positive or incompatible in the presence of hemolysis and/or macroscopic and/or microscopic agglutination (Brown & Vap, 2006; Kretsch et al., 2023). The blood is considered compatible, and blood transfusion is permitted when there is no hemolysis and/or agglutination at either test stage (Lacerda, 2008).

Physiologically, equine blood cells tend to form rouleaux, a microscopic characteristic in which the cells appear as stacked coins. This was due to the low ionic charge of the equine red blood cell membrane. Conditions in which the animal presents with hyperglobulinemia and/or hyperfibrinogenemia cause this stacking to occur in an exacerbated form, increasing the erythrocyte membrane's electronegativity (Grondin & Dewitt, 2010).

Therefore, cross-reactions in equine species can be difficult to interpret, as rouleaux are often confused with agglutination (Mudge & Willians, 2016). As Lacerda (2008) and Mudge and Willians (2016) described, cross-reactions can be performed using plasma and red blood cell concentrates. However, Tiwari et al. (2009), Kumar (2017), Fenn et al. (2020), and Jamieson et al. (2022) showed that this technique should be performed using serum instead of plasma. These authors stated that, with the use of serum, there was a decrease in the formation of rouleaux, facilitating their interpretation.

According to Tomlinson et al. (2015), the low rate of natural antibodies in horses indicates that the first transfusion is well tolerated. However, clinicians lack the information to predict transfused cells' lifespan, often approximately 20 days. In a prospective study that aimed to analyze the relationship between cross-reaction compatibility and incompatibility and the lifespan of red blood cells, the results indicated that cross-reaction incompatibility was associated with a decreased lifespan of red blood cells; therefore, cross-

reactions should be performed before blood transfusion (Mudge, 2014; Tomlinson et al., 2015).

In addition to comparing the use of plasma and serum in the cross-reactivity technique, this study aimed to verify the occurrence of positive cross-reactions in horses that had never received blood transfusions.

Material and Methods

This study was approved by the Ethics Committee on Animal Use of the Universidade Estadual de Londrina (protocol number CEUA-UEL 030.2022) and conducted at the Animal Transfusion Medicine Laboratory of the same institution. Twenty healthy adult horses with good body condition (score 5/9) (Henneke et al., 1983), including 12 males and eight females, were selected for the study. The selected animals had never received blood transfusions, and the females were nulliparous. Blood was collected via jugular vein puncture using 21 G needles (25 × 0.8 mm) in vacuum flasks containing EDTA and in flasks containing a clot activator, totaling 20 mL of blood from each horse.

For the cross-reaction technique, blood samples were divided into two groups: cross-reaction of red blood cell suspension (RCS) with plasma (RCSP) and cross-reaction of red blood cell suspension with serum (RCSS). All blood samples from the 20 horses were tested against each other; thus, 380 cross-reactivity tests were performed.

Preparation of blood samples

All blood samples were centrifuged (1000 rpm for 5 min) to separate the plasma, red blood cell concentrate, and serum into labeled flasks.

The red blood cell concentrate was washed three times with a buffered saline solution (0.9% NaCl), centrifuged (1000 rpm for 5 min), and the supernatant was discarded. After washing, a 5% red blood cell suspension (RCS) was prepared by mixing five drops of red blood cell concentrate with 0.9% NaCl (0.5 mL).

Cross-reaction technique

The test was initiated after preparing the samples (plasma, serum, and red blood cell suspensions). The flasks from each horse were identified as the major crossmatch (Flask A) and control (Flask C). For the RCSP group, 100 µl of plasma from one horse and 50 µl of the RCS from another horse were added to Flask A, and 100 µl of plasma and 50 µl of the RCS from the same horse were added to Flask C. For the RCSS group, the same RCS was used but was added serum instead of plasma (Figure 1).

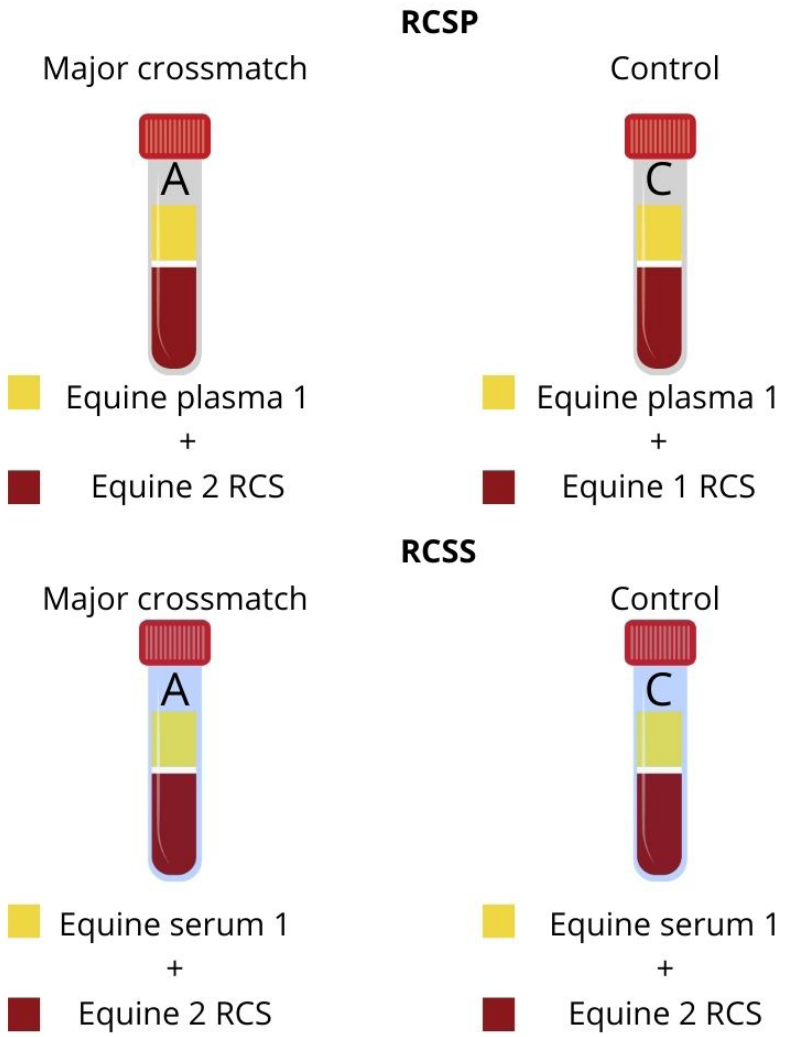

Figure 1. Schematic model of red blood cell solution, plasma, and serum distribution during horse cross-reactions. RCS refers to the red blood cell solution, RCSP refers to the group with red blood cell solution and plasma, and RCSS refers to the group with red blood cell solution and serum.

The flasks were homogenized and incubated for 15 minutes in a water bath at 37°C. After incubation, samples were centrifuged for 30 s. The supernatant was checked for the presence or absence of hemolysis. Red blood cells were resuspended to check for the presence or absence of macroscopic agglutination. All samples were examined for microscopic agglutination.

Statistical results were generated using Jamovi software, analyzing the data obtained from the cross-reactions with serum and plasma. For the analysis, the chi-square test was performed due to the presence of categorical and independent variables, considering $p < 0.05$ as statistically significant.

Results and Discussion

There was a statistically significant difference in the use of serum and plasma, with a P-value <0.001 (Table 1).

Plasma and serum are differentiated by the presence or absence of fibrinogen in the sample (Thrall et al., 2016), indicating the presence of immunoglobulin in both blood products. Consequently, positive reactions are those in which agglutination of red blood cells occurs because of cross-incompatibility between antigens and antibodies. Of all the evaluated samples, 57.4% were negative for both serum and plasma, whereas 28.2% were negative for serum but positive for plasma. When cross-reactions performed with serum were analyzed, 85.5% were negative, and 14.5% were positive. In contrast, cross-reactions performed with plasma showed that 62.1% of the samples were negative, and 37.9% were positive.

Table 1
Chemical composition of the silage and experimental diets

	Primary Reaction with Plasma		
	Negative result	Positive result	Total of reactions
Primary Reaction with Serum			
Negative result	57.4% (n = 218)	28.2% (n = 107)	85.5% (n = 325)
Positive result	4.7% (n = 18)	9.7% (n = 37)	14.5% (n = 55)
Total of reactions	62.1% (n = 236)	37.9% (n = 144)	100% (n = 380)

n = amount of cross-reactions.

Vap et al. (2012) reported that one of the most common causes of false-positive results in the compatibility test is the presence of rouleaux in the sample, a physiological characteristic of equine blood that can be exacerbated in the presence of proteins such as fibrinogen.

In this study, it was observed that by removing the intrinsic factor that favors reading errors (false positives), as in the case of fibrinogen, the formation of rouleaux was reduced, and consequently, the visualization of supposedly positive results, which has already been reported by Tiwari et al. (2009), Kumar (2017) and Jamieson et al. (2022) (Figure 2). The control test graph further supports this conclusion; five of the 20 control tests using plasma were positive (25%), whereas none of the 20 control tests with serum showed a positive result (Figure 3).

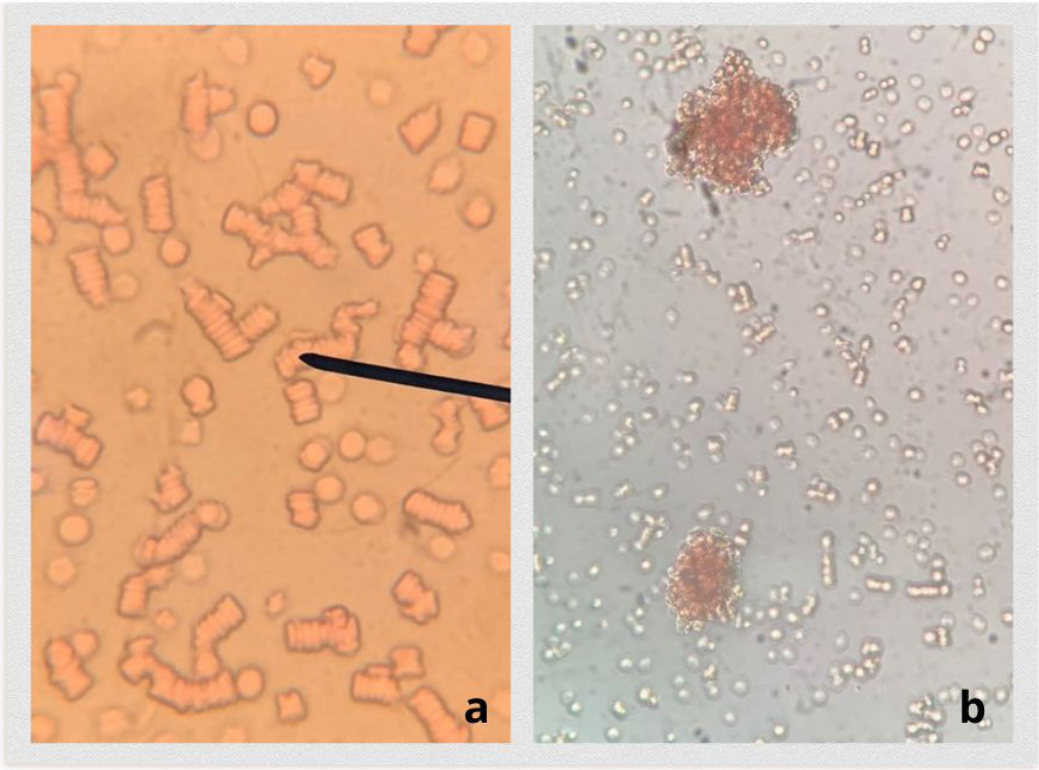

Figure 2. Photographic image showing the microscopic difference between Rouleaux (image a, 40x objective) and agglutination (image b, 10x objective) in primary cross-reaction in horses using plasma.

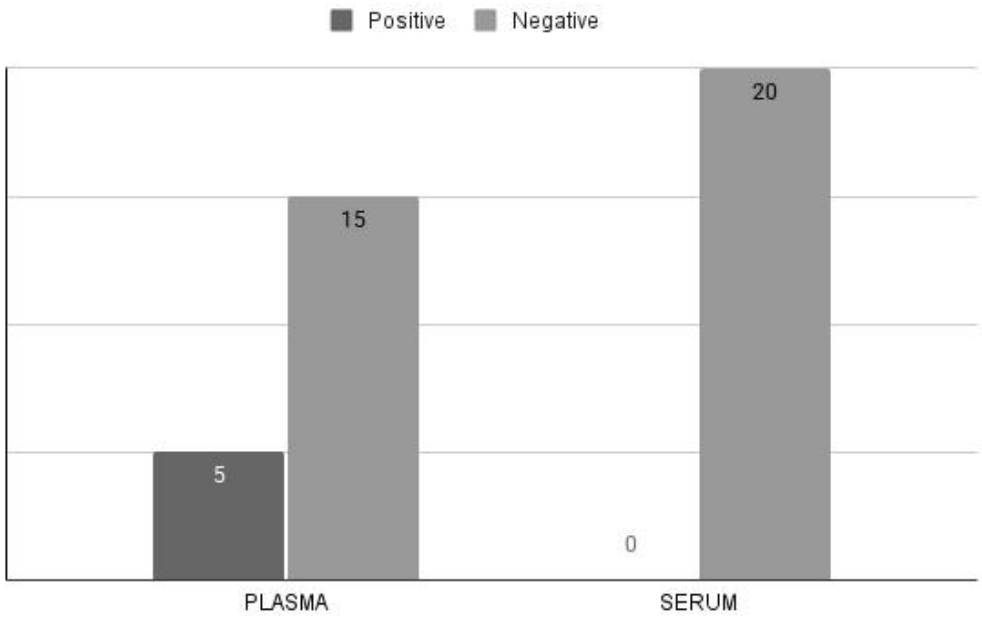

Figure 3. Results of control tests for cross-reactions in horses, comparing the outcomes with the use of plasma and serum.

Mudge (2014) stated that compatibility tests may not be necessary in emergencies, especially when the recipient has no history of receiving blood components. However, the same author reinforced that cross-reactivity is necessary at the time of the second transfusion, as alloantibodies are already present one week after the first transfusion.

Tomlinson et al. (2015) reported that the first blood transfusion in horses is generally well tolerated owing to the low occurrence of natural antibodies; however, there may be a reduction in the lifespan of red blood cells in the presence of natural antibodies. This study demonstrated the presence of positive cross-reactions *in vitro* in healthy horses that had never received a blood transfusion. Thus, in agreement with the results of Tomlinson et al. (2015), the presence of positive reactions *in vitro* may be associated with the presence of natural antibodies, leading to the early destruction of transfused red blood cells (delayed hemolytic immune-mediated transfusion reaction).

In a study by Hurcombe et al. (2007), adverse reactions were observed in seven of 44 blood transfusions (16%), including urticaria, worsening hemolysis, and anaphylactic shock. The adverse reactions persisted even when blood type and cross-reactivity testing were performed before transfusion. However, their incidence was significantly lower when compatibility testing was conducted, allowing for a more appropriate treatment approach for patients.

Conclusion

This study demonstrated positive results in cross-reactivity tests, even in horses that have never received blood or other blood component transfusions, indicating possible blood incompatibility. This finding reinforces the recommendation to perform the test before transfusion. Additionally, it showed that using serum for the cross-reaction test in horses is more reliable than the plasma technique.

Further studies are needed to determine whether these *in vitro* positive reactions are clinically significant in these animals and whether they have the potential to cause severe acute transfusion reactions or delayed hemolytic immune-mediated reactions.

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