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Comparative study of agar gel immunodiffusion (AGID) protocols for the diagnosis of equine infectious anemia in Brazil

Estudo comparativo de protocolos de imunodifusão em gel de ágar para o diagnóstico da anemia infecciosa equina no Brasil

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Abstract

To evaluate the Equine Infectious Anemia (EIA) agar gel immunodiffusion (AGID) protocols, two different kits commercially available in Brazil were used: an imported kit (kit A) and a domestically produced kit (kit B). Kit A was submitted to the protocols recommended by the World Organization for Animal Health (OIE) and the protocol recommended by the *Ministério da Agricultura Pecuária e Abastecimento* (MAPA). Kit B, the Brazilian kit, was submitted only to the MAPA-recommended protocol and was used as a reference in this study. A total of 345 equid serum samples, including field samples, serum sets from official laboratories and a weak positive serum control from National Veterinary Services Laboratories (NVSL, USA), were used. Parameters such as the sensitivity of kit A in the two protocols, the detection limit of kits and the occurrence of nonspecific reactions or non-identity were evaluated. When Kit A was used for an AGID procedure performed according to the OIE-recommended protocol, the kit demonstrated good agreement with kit B and 99 % relative sensitivity. However, when kit A was processed according to the MAPA-recommended protocol, it failed to detect 1.16 % of weak positive samples and its relative sensitivity decreased to 96 %. The detection limit of kit A was lower than the detection limit of kit B for weak positive samples in both protocols. The occurrence of non-identity reactions was higher with kit B than with kit A. The training of veterinarians to ensure the correct execution of the AGID test protocol should be intensified in Brazil.

Key words: Equine infectious anemia, diagnosis, sensitivity, agar gel immunodiffusion

Resumo

Para avaliar diferentes protocolos da imunodifusão em gel de ágar (IDGA) para diagnóstico da anemia infecciosa equina (AIE), foram utilizados dois kits comerciais de IDGA: kit A importado e kit B fabricado no Brasil. O kit A foi submetido aos protocolos recomendados pela Organização Mundial de Saúde Animal (OIE) e Ministério da Agricultura Pecuária e Abastecimento (MAPA). O kit B, nacional, foi submetido somente ao protocolo recomendado pelo MAPA e foi utilizado como referência nesse estudo. Foi utilizado um total de 345 amostras de soro que incluiu amostras de campo, amostras de laboratórios oficiais e controle fraco positivo proveniente do *National Veterinary Services Laboratories* (NVSL, EUA). Foram avaliados parâmetros tais como a sensibilidade do kit A nos dois protocolos, o

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limite de detecção dos kits e a ocorrência de reações não específicas ou não-identidade. O teste IDGA com o kit A, quando realizado de acordo com o protocolo recomendado pela OIE, demonstrou boa concordância com o kit B e 99% de sensibilidade relativa. No entanto, quando o kit A foi executado com o protocolo recomendado pelo MAPA, houve falha na detecção de 1,16% de amostras fracas positivas, e sua sensibilidade relativa diminuiu para 96%. O limite de detecção do kit A foi menor do que o limite de detecção do kit B para amostras fracas positivas em ambos os protocolos. A ocorrência de reações inespecíficas foi maior com kit B do que com o kit A. Deve ser intensificado o treinamento de médicos veterinários, para garantir a correta execução do protocolo do teste IDGA para diagnóstico da AIE no Brasil.

Palavras-chave: Anemia infecciosa equina, diagnóstico, sensibilidade, imunodifusão em gel de ágar

Equine Infectious Anemia (EIA) represents an obstacle to the development of the equine industry. The disease is caused by a *lentivirus*, a member of *Retroviridae* family that exclusively infects equids (COOK, 2009). This disease is under a regulatory control program in many countries, including Brazil.

There is no effective treatment for EIA or vaccine; thus, its control depends on laboratory diagnosis with identification, segregation and euthanasia of seropositive animals (ISSEL; COOK, 2004). Laboratory diagnosis of animals is generally required for movement, congregations, interstate travel, change of ownership, international trade and control programs (PARÉ; SIMARD, 2004; ALVAREZ et al., 2007). The official diagnostic test for EIA in Brazil and in most countries around the world is the AGID (Agar Gel Immunodiffusion) test, also known as the Coggins test (MAPA, 2004; OIE, 2008). This test is simple and highly specific for identifying animals infected with the EIAV, as a positive AGID test correlates with EIAV presence (COGGINS; NORCROSS; NUSBAUM, 1972; ISSEL; COOK, 1993). The basis for the AGID test is the concurrent migration of antigen and antibodies through an agar gel. A visible precipitation line is produced when an insoluble antigen-antibody complex becomes visible between the serum and EIAV antigen in the agar gel (COGGINS; NORCROSS; NUSBAUM, 1972). One of the limitations of AGID is that it is considered less sensitive and more subjective in reading than other tests such as ELISA or immunoblotting (ISSEL; COOK, 1993; CULLINANE et al., 2007).

The AGID protocol for EIA diagnosis recommended by the World Organization for Animal Health (OIE) is different from the protocol implemented in Brazil by the national animal health authorities (Ministério da Agricultura, Pecuária e Abastecimento - MAPA). The AGID OIE based-test is performed in a Petri dish using larger volumes of antigen, control sera and samples (50 µL) (OIE, 2008) than the AGID protocol that is recommended by MAPA in Brazil (MAPA, 1992). The second protocol recommends performing the AGID on a microscope slide and utilizes wells that are 4 mm in diameter. The well size promotes the use of a lower volume of reagents per well (25 µL).

The imported commercially available EIA-AGID kits in Brazil, which were standardized according to the OIE instructions, were processed according to the MAPA-recommended protocol until the authorities updated the AGID procedures in 2010 (MAPA, 2010). However, this document only recommends that the AGID test be performed in accordance with the manufacturer's instructions in the case of imported kits, and it is necessary to discourage laboratories use small volumes of reagents to allow more samples per kit, as the volume of reagents indicated in the MAPA protocol is half that used in the OIE protocol. The evaluation of the loss of analytical sensitivity in AGID diagnosis has become an important objective for this study due to the use of imported AGID kits being employed using a protocol that does not match the one for which they were designed (SILVA et al., 2009). This fact may have impaired the efficiency of imported kits and compromised EIA control in Brazil, as the non-identification of EIA-positive animals is the main factor contributing to the maintenance of the EIAV in herds (MCCONNICO et al., 2000).

The aim of this study was to show the influence of changes in the procedures of AGID test that Brazilian authorities have recommended in 2010 (MAPA, 2010) on the performance of the two official AGID protocols for EIA diagnosis performed in Brazil using two commercial kits: one imported (kit A) and one national (kit B). The loss of analytical sensitivity of the imported kit was evaluated when it was performed according to the MAPA-recommended protocol (MAPA, 1992). Other testing aspect such as the occurrence of non-identity lines in the AGID test was evaluated as well. At the first time in Brazil, this work was performed as a partnership with MAPA to emphasize the importance of the use of the correct procedures under the new official protocol for the EIA AGID test when using imported kits (MAPA, 2010).

A total of 345 serum samples obtained from field horses, mules and donkeys, including a positive and negative serum control from official laboratories and a weak positive serum control from National Veterinary Services Laboratories (Ames, USA), and two different AGID commercial kits were used in this analysis. The first kit was an imported kit (kit A) standardized according to OIE instructions (OIE, 2008). The second kit was a Brazilian kit (kit B) standardized according to MAPA instructions (MAPA, 1992). AGID testing with kit B was used as a reference in our study. All kits from the same manufacturer belonged to the same lot number. Both kits were stored following the manufacturer's recommendations. All of the AGID tests were performed blinded and were read and interpreted independently by at least two trained EIA-certified analysts who were familiar with running the assay at 48 h of incubation. In the case of a disagreement in the interpretation of results between the analysts, the sample was retested.

Kit A testing was performed according to the AGID OIE-recommended protocol and AGID MAPA-recommended protocol. Tests with kit B, used as a reference in this study, were performed only according to the MAPA-recommended protocol. Briefly, for the OIE-recommended protocol-based AGID test, procedure was performed in a 100-mm diameter Petri dish with 17 mL of 1 % Noble agar. After the agar had hardened sufficiently, it was cut using a template that contained a central hole and six peripheral holes. These holes measured 5.3 mm in diameter and had a 2.4 mm distance between them. The serum samples (50 µL) were distributed and interspersed with the positive control sera in peripheral wells (50 µL), and the antigen (50 µL) was placed in the center well. For the MAPA-recommended protocol-based AGID test, the procedure was performed on a microscope slide (25x75 mm) with 4.5 mL of 1 % Noble agar in borate buffer (0.15 M, pH 8.6). The agar was cut after it had hardened sufficiently using a template that contained a central hole and six peripheral holes measuring 4 mm in diameter and with a 3 mm distance between them. The serum samples were distributed (25 µL) and interspersed with the positive control sera in peripheral wells (25 µL), and the antigen was placed in the center well. In both protocols, the results were read after 48 h of incubation and the testes were performed at room temperature (20-25°C).

The AGID test results were designed to be interpreted either as positive or negative by visual reading of precipitation line curvature, but positive-sample reactions may vary in intensity based on the level of anti-p26 antibody present. In this study, we adopted a semi-quantitative numerical AGID test-scoring system based on United States Department of Agriculture (USDA) standards (ISSEL; COOK, 2004).

The detection limit of kit A in the two different protocols was compared with the reference EIA-AGID (kit B) by testing two-fold serial dilutions of positive samples with different levels of positivity from weak to strong: "+1" = weakest positive, "+2" = weak positive, "+3" = positive and "+4" = strongest positive. The samples were tested undiluted and diluted from 1:2 to 1:256. The endpoint dilutions of the sera in each kit were tabulated. We included the weak positive control both undiluted and diluted.

To evaluate the occurrence of nonspecific reactions in the AGID test, after 48 h of incubation, we recorded all nonspecific reactions or non-identity precipitation lines that occurred with the positive control during the AGID testing of all 345 serum samples with kit A or reference kit B.

The performances of kit A and kit B were quantified by estimating the *kappa* score and relative sensitivity with a 95 % confidence interval using STATA software version 10.0 (STATA corp., College Station, TX, USA). Data interpretation was conducted according to the procedures suggested by Dohoo, Martin e Stryhn (2003).

The results of the EIA-AGID test performed with kit A according to the OIE-recommended protocol was highly correlated with the results of the reference EIA-AGID test (kit B) after 48 h of incubation (Table 1). The majority of results were identical, except for one sample previously known to be positive for EIA that yielded a negative result with kit A and a positive result with kit B. The relative sensitivity of kit A was 99 %.

Table 1. Relative sensitivity and kappa (95 % confidence interval) of the AGID with kit A according to the OIE-recommended protocol, Kit A according to the MAPA recommended protocol relative to the reference AGID (kit B).

	Relative Sensitivity [a]	Kappa [a]
Kit A (OIE)	99 % (93.8 % to 99.9 %)	0.993 (0.979 to 1.0)
Kit A (MAPA)	96 % (89.5 % to 98.7 %)	0.972 (0.944 to 0.999)

[a] Results presented were obtained after 48 h of incubation.
Source: Elaboration of the authors.

Even though kit A presented a high correlation with kit B (*kappa* = 0.972), when the MAPA-recommended protocol was utilized (testing performed in microscope slide with half volume of reagents), kit A failed to detect 1.16 % of weak positive samples (4/345), with a relative sensitivity of 96 % (Table 1). According to the study presented here, the imported EIA-AGID kit (kit A) is equivalent in performance to the domestically produced reference kit (kit B) when the kit procedures are conducted according to the OIE-recommended protocol in a Petri dish with 50 μL of reagents.

In contrast, when the MAPA-recommended protocol is used with kit A (on a microscope slide with 25 μL of reagents), the testing failed to detect the weak positive samples, including the weak positive control sera from the NVSL. In this case, the AGID readers reported that the precipitation lines formed between the antigen, the positive control and the samples were very thin and light, making reading and interpretation extremely difficult. The same observation was reported previously that examined the differences in the visibility of precipitation lines obtained with different commercial AGID systems employed for EIA diagnosis, however this study used a few number of samples in their evaluations (SILVA et al., 2009).

The detection limit for the +2, +3 and +4 samples were similar for kit A compared to reference kit B when testing was performed according to the OIE-recommended protocol. However, testing with reference kit B resulted in the formation of visible precipitation lines at dilutions higher than kit A for the EIA weak positive control and weak positive sample (+1) when the OIE-protocol was followed

(Table 2). These results emphasize the loss of analytical sensitivity of the imported AGID kit when it is used with a lower reagent volume-based procedure. As we mentioned previously, the main limitation of AGID testing is the subjective reading of results (ISSEL; COOK, 1993; CULLINANE et al., 2007). Many published studies present equivocal results with weak positive samples due to the difficulty in visualizing the curvature of the precipitation lines in samples with low levels of EIAV antibody (ISSEL; ADAMS, 1982; MCCONNICO et al., 2000). The use of imported kits with the MAPA-recommended protocol may have only served to strengthen this limitation of the AGID test. The major importance of the loss of sensitivity of the imported EIA-AGID kits relates to the increased chance of reporting false negative results when the kit testing is performed according to the MAPA-recommended protocol, as the control of EIA depends on the detection of EIA-positive animals with this test (ISSEL; ADAMS, 1982; MAPA, 2004; OIE, 2008). The failure of laboratory testing to detect EIA-positive animals could have important consequences, especially if the animals in question move freely and put other equids at risk (MCCONNICO et al., 2000). Thus, any failure to detect EIA-positive animals enhances the difficulty of controlling EIA in Brazil.

Table 2. Comparison of results obtained for two fold serial dilutions of serum samples tested in AGID with kit A according to the OIE-recommended protocol, Kit A according to the MAPA-recommended protocol and reference AGID (kit B).

	End point of positive reactions [a]		
	Kit A(MAPA)	Kit A(OIE)	Kit B(MAPA)
Weak positive control	Undiluted	1:2	1:4
Pos +1	Undiluted	1:2	1:4
Pos +2	1:2	1:8	1:8
Pos +3	1:4	1:8	1:8
Pos +4	1:128	1:256	1:256

[a] Results presented were obtained after 48 h of incubation.
Source: Elaboration of the authors.

Precipitation lines not associated with EIA were observed during routine AGID testing for infection with serum obtained from horses that had been given multiple vaccinations (GASKIN; NEAL; RUBIN, 1977). However, these lines of identity can be distinguished from lines of non-identity with a positive control in the AGID test. According to the OIE recommendations, the antigen used for the AGID test can be prepared from the spleens of acute EIAV-infected horses, from persistently infected cell cultures or from a recombinant expression system (ALVAREZ et al., 2007). The occurrence of non-identity reactions was higher with kit B than with kit A. Kit A, when used according to the MAPA-recommended protocol, demonstrated 0.87 % nonspecific reactions. When using kit A and employing the OIE-recommended protocol, the nonspecific reaction occurrence was 1.45 %. Kit B demonstrated a 3.48 % nonspecific reaction occurrence rate. The nonspecific reactions observed in this study were readily distinguished from the true EIA-associated reactions and did not result in false positive interpretations of the test such as those observed in a prior study conducted by Gaskin, Neal e Rubin (1977). The differences in the occurrence of nonspecific reactions in the EIA-AGID observed with both kits used in this study can be attributed to differences in the process of antigen purification by each kit manufacturer. Both kits used native viral proteins produced from EIAV-infected lineage cells as antigen. From the obtained results we can deduce that kit A has more highly purified antigen than kit B. Furthermore, with the rise of newer reagent purification processes, as well as the use of technology for the production of recombinant antigens, the occurrence of nonspecific reactions has decreased (ISSEL; COOK, 2004; ALVAREZ et al., 2007).

According to the results obtained in this study, the kit A showed reduced sensitivity when performed following AGID MAPA-recommended protocol. The choice of EIA-AGID kits and the protocol used must strictly follow the manufacturer's recommendations, as recommended by a new statement of sanitary authorities in Brazil (MAPA, 2010). This is to ensure a lower incidence of false results, as EIA control is dependent exclusively on the results of AGID testing. This is because of the risk of reporting false negative results when the manufacturer's recommendations are not followed. Through detection limit testing, we could verify the lower analytical sensitivity of the imported kit A when AGID was performed with the MAPA-recommended protocol. The AGID test is internationally recognized and is the gold standard for EIA diagnosis. Thus, the training of veterinarians to ensure the correct execution of the AGID test protocol should be intensified in Brazil. If an accredited laboratory for the diagnosis of EIA chooses to use imported EIA-AGID kits, all testing should be performed according to the OIE-recommended protocol.

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