



Original Article

Non-specific reactions caused by vaccination in agar gel immunodiffusion for diagnosis of equine infectious anemia

Reações inespecíficas provocadas por vacinação na imunodifusão em gel de ágar para diagnóstico da anemia infecciosa equina

Clidilene Nogueira de Alencar¹ , Analy Castro Lustosa Cavalcante² , Luciano Santos da Fonseca³ , Erlin Cely Cotrim Cavalcante² , Tiago da Silva Teófilo² , Daniel Praseres Chaves^{4*} 

¹ Mestranda do Programa de Pós-graduação Profissional em Defesa Sanitária Animal

² Médico(a) Veterinária do Laboratório de Bioprodutos Ltda

³ Professor Adjunto I, curso de Medicina Veterinária da Universidade da Região Sul Tocantina/UEMASUL – Programa de Pós-graduação Profissional em Defesa Sanitária Animal/UEMA

⁴ Docente departamento de Patologia – CCA/UEMA (autor para correspondência)

ARTICLE INFO

Article history

Received 18 June 2019

Accepted 22 January 2020

Keywords:

Diseases

Tests

Seroconversion

Palavras-chave:

Enfermidades

Testes

Soroconversão

ABSTRACT

Equine infectious anemia (EIA) is one of the most important diseases from the health and economic point of view for equidae breeding, as it does not have treatment and vaccines. The Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) instituted mandatory sanitary measures that include the official diagnosis and sacrifice of seropositive animals to control this disease. Seventy-two seronegative equines, challenged with different vaccines, were used to verify the occurrence of non-specific reactions in the AGID techniques. Five serological controls were performed one week after vaccination, at seven-day intervals. The results indicated that the use of vaccines in equines in a period that precedes the performance of laboratory tests for the diagnosis of EIA does not induce seroconversion. However, 11.11% of the equines vaccinated against influenza, encephalomyelitis, equine rhinopneumonitis, and tetanus, and 15.38% of those vaccinated against leptospirosis had non-specific negative reactions to AGID. In this study, there was a non-specific line in the AGID for EIA.

RESUMO

Anemia Infecciosa Equina é uma das enfermidades mais importantes sob o ponto de vista sanitário e econômico para a equideocultura, por não possuir tratamento e vacinas. Para controle desta doença, o Ministério da Agricultura, Pecuária e Abastecimento (MAPA) instituiu medidas sanitárias obrigatórias em todo território nacional que incluem o diagnóstico e sacrifício dos animais soropositivos. Para verificar a ocorrência de reações inespecíficas na técnica de IDGA utilizou-se 72 equinos soronegativos, desafiados com diferentes vacinas. Uma semana após a vacinação, realizou-se cinco controles sorológicos, em intervalos de sete dias. Os resultados indicaram que o uso de vacinas em equinos em período que antecede a realização de exames laboratoriais para diagnóstico de AIE, não induz a soroconversão. Entretanto, 11,11% dos equinos vacinados contra influenza, encefalomielite, rinopneumonia equina e tétano, e 15,38% dos que foram vacinados contra leptospirose apresentaram reações negativas inespecíficas ao IDGA. Neste estudo, verificou-se uma linha inespecífica no IDGA para AIE.

* Corresponding author: daniel@cernitas.com.br

INTRODUCTION

Equidae are of great economic and social importance in Brazil due to the various activities they have in sport, traction, transport, and treatment of diseases in people through equine therapy. However, some diseases cause irreparable economic damage, including the equine infectious anemia (EIA), for which the Brazilian National Equine Health Program (PNSE) obligates the sacrifice of infected animals because there is no treatment (BRASIL, 2004).

Considering that more than 95% of infected animals are asymptomatic for EIA, as well as the fact that there is no available cure, vaccine, or effective treatment, laboratory diagnosis plays an important role in the control and prevention of this disease. Thus, the official control program determines the euthanasia of infected animals (BRASIL, 2004).

Several techniques have been developed for EIA diagnosis. Agar gel immunodiffusion (AGID) is one of the official methods, according to the OIE, applied in several countries, including Brazil (BRASIL, 2004b, 2004c). AGID is used on a large scale, and despite its sensitivity, it does not detect specific antibodies in the early EIA stages. These limitations compromise the strategies for disease control and eradication, as serology has a determining value in a disease with no treatment and ineffective vaccines (VERMA et al., 1990; REIS, 1997; NEUBAUER et al., 2005; NAUREEN et al., 2007; TELES et al., 2012; OIE, 2013a, 2013b).

According to Oliveira et al. (2013), the training of veterinarians to ensure the correct execution of the AGID test protocol for diagnosing EIA in Brazil should be intensified. The volumes of antigen, standard serum, and test serum used in the diagnosis of EIA can be used according to the quantity established by MAPA (25 µL) or as recommended by OIE (50 µL). The use of reduced volumes can represent an advantage, especially in terms of cost reduction, since the number of tests can be doubled without additional costs (BELTRÃO et al., 2015).

The AGID test for EIA detection has as a limitation the lower sensitivity and more subjective reading than tests such as ELISA and immunoblot (ISSEL; COOK, 1993; CULLINANE et al., 2007).

Therefore, investigating the inoculation of these substances in animals could compromise the quality and effectiveness of laboratory tests because the serological diagnosis recommended by the legislation is essential to establish the frequency and assist in the strategic EIA control plan. The use of this test associated with the need to use vaccines in the health management of equidae is one of the determining factors for the sanitary slaughter of healthy animals and the calculation of economic losses in a herd. Thus, we sought to verify the occurrence of non-specific reactions in EIA tests after the application of vaccines against influenza, encephalomyelitis, equine rhinopneumonitis, tetanus, and leptospirosis.

MATERIAL AND METHODS

Seventy-two adult male and female equines belonging to three Training Centers (TCs) for barrel racing and pole bending, located in the municipalities of São Luís, Paço do Lumiar, and Raposa, in the State of Maranhão, Brazil, were used in this study. TCs were chosen because they have animals with breed standardization, good nutritional status, and, especially, uniform food and sanitary management. For research purposes, the properties were identified by letters A, B, and C. This research was approved by the Animal Ethics and Experimentation Commission (CEEA) under the protocol No. 026/2014.

A pre-vaccination period was established, in which animals were subjected to two consecutive serological controls at 60-day intervals using the AGID technique to ensure the absence of specific antibodies for EIA. Animals were divided into three groups, one in each TC, which were challenged with different vaccination schedules (Table 1). Five tests were performed at seven-day intervals one week after vaccination.

Table 1. Vaccination before the AGID serological test for EIA diagnosis.

Group	Property	No. of animals	Treatment
1	A	10	Triple vaccine ¹
2	B	36	Eightfold vaccine ²
3	C	26	Anti-leptospirosis vaccine ³
Total		72	

¹Triple inactivated vaccine, 1 mL (IM), composed of equine encephalomyelitis virus, east and west strains, equine influenza virus, types A1 and A2 (including Kentucky 92), and tetanus toxoid. ²Eightfold inactivated vaccine, 3 mL (IM), composed of east and west equine encephalomyelitis virus; equine influenza strains: A/equine1/Prague/1/56, A/equine2/Kentucky/94, and A/equine2/South Africa 4/03; equine herpesvirus types 1 and 4 and tetanus toxoid. ³Inactivated vaccine, 2 mL (IM), composed of cultures of *Leptospira icterohaemorrhagiae*, *L. canicola*, *L. bratislava*, *L. copenhageni*, *L. pomona*, *L. grippotyphosa*, *L. tarassovi*, *L. hardjo prajitno*, *L. andamana*, *L. ballum*, *L. wolffii*, and *L. pyrogenes*.

The criterion for choosing vaccines was based on the frequency they are used in the sanitary management of the herd, while the dose followed the manufacturers' rules and was registered in MAPA.

A disposable needle was used to collect 10 mL by jugular venipuncture. Samples were taken under refrigeration to the Cernitas Laboratory, accredited by MAPA. After

centrifugation, the serum was aliquoted in microtube tubes with a capacity of 1.5 mL, identified, and stored in a freezer at -20°C until processing.

Antibodies against the EIA virus were detected by the 1% agar gel immunodiffusion (AGID) test, using a commercial antigen and standardized positive control serum stored at -20°C , following the recommendations of Ordinance No. 84/1992 by MAPA (BRASIL, 1992).

The non-specificity of reactions by the AGID test was evaluated by counting all reactions in the samples with no continuous line formation compared to the positive control. The shapes of precipitation lines that corresponded to non-specific reactions were characterized as double or simple, strong or smooth, and shiny or opaque.

Statistical analysis: The experimental design was completely randomized by applying Fisher's exact non-parametric test to verify the occurrence of non-specific reactions in the diagnostic test for AGID in five replications at seven-day intervals after the previous vaccination. The procedure PROC MIXED of the statistical program SAS[®] version 9 was used considering a 5% significance level ($P < 0.05$).

RESULTS

The 72 equines distributed into the three experimental

groups and submitted to five serological controls (7th, 14th, 21st, 28th, and 35th days) were negative for EIA in the AGID test and did not present specific negative reaction at any time during the pre-vaccination period (Table 2).

After vaccination, non-specific negative reactions were observed in four animals (11%) from Group 2 by the AGID method. Group 3 had four equines (15.38%) with non-specific negative reactions to AGID (Table 2).

In Group 2, four equines vaccinated with the eightfold vaccine showed non-specific negative reactions to the AGID test in samples from the fifth collection (35th day). The non-specific reaction was observed from the 21st day only in one animal. Non-specificity started in two animals in samples from the fourth collection (28th day) and on the 35th day after vaccination.

Among four animals vaccinated against leptospirosis in Group 3, the first animal showed non-specific lines in five serological controls to which it was submitted. In the second animal, the reactions started from the 14th day and extended until the 35th day. In the third animal, this reaction was limited to the 7th day, while in the fourth animal, the reaction was restricted to the 21st day after vaccine inoculation (Table 3). The sera that showed non-specific negative reactions were subjected to counterproof and confirmed the first results.

Table 2. Non-specific negative reactions using the AGID technique after different vaccination schedules.

Group	Treatment	No. of animals	Non-specific reactions	negative Occurrences (%)
1	Triple vaccine	10	0	0
2	Eightfold vaccine	36	4	11.11
3	Anti-leptospirosis vaccine	26	4	15.38
Total		72	8	11.11

Table 3. Equines Xc, Yc, Wc, and Zc inoculated with anti-leptospirosis vaccine that showed non-specific reactions by the AGID method at seven-day intervals (2014).

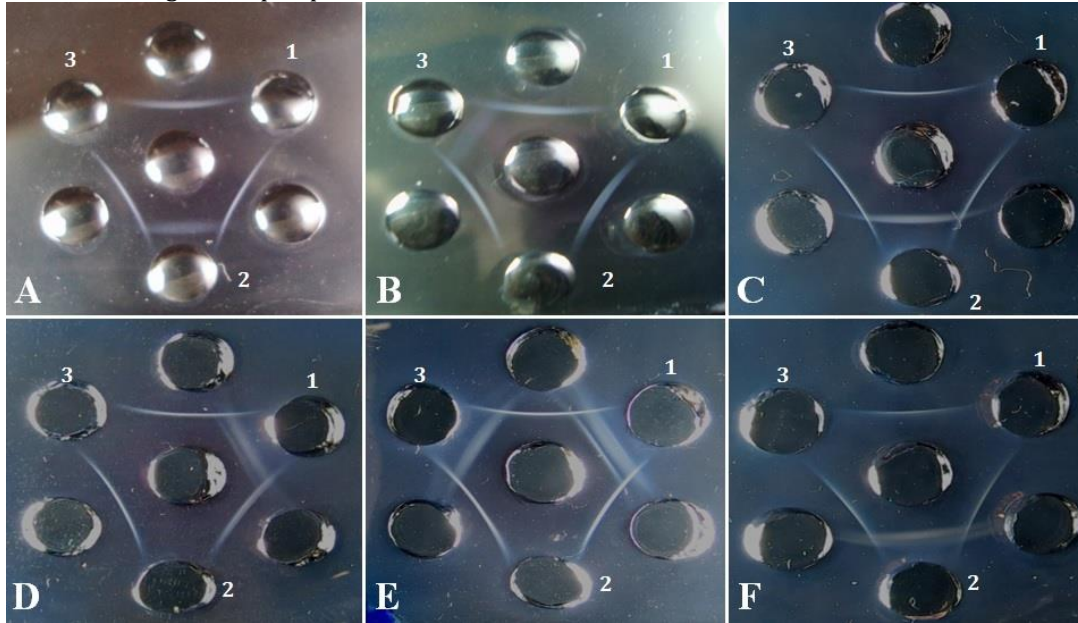
Animal	AGID tests				
	7th day	14th day	21st day	28th day	35th day
Xc	N (R.I) ^a	N (R.I)	N (R.I)	N (R.I)	N (R.I)
Yc	N ^b	N (R.I)	N (R.I)	N (R.I)	N (R.I)
Wc	N (R.I)	N	N	N	N
Zc	N	N	N (R.I)	N	N

^aNegative with non-specific reaction, ^bN – negative.

Non-specific reactions verified by the AGID method were characterized by the presentation of simple, smooth, and low-shine straight lines in Group 2 (Figure 1A and B). In Group 3, specific lines were the most varied, ranging from simple and composed, strong and shiny, to straight

and slightly curved (Figure 1C–F). However, despite reactions presenting marked and peculiar characteristics, no statistical significance was observed in both tested groups.

Figure 1. AGID test of vaccinated equines showing non-specific line(s) (NSL). **A** – NSL (well 2) with a negative result of an equine vaccinated against influenza, encephalomyelitis, equine rhinopneumonitis, and tetanus. **B** – Simple, smooth, and opaque-type NSL (well 3) in a negative result of an animal vaccinated against influenza, encephalomyelitis, equine rhinopneumonitis, and tetanus. **C** – Simple, slightly curved, smooth, and shiny NSL (well 2) in a negative result of an equine vaccinated against leptospirosis. **D** – Non-specific negative reaction (well 1) with compound (double), straight, and opaque NSL in a negative result of an animal vaccinated against leptospirosis. **E** – Double and shiny NSL (wells 1 and 3) in negative results observed in an equine vaccinated against leptospirosis. **F** – Double and opaque NSL (well 2) with a negative result from an equine vaccinated against leptospirosis.



DISCUSSION

The negative results obtained by the AGID method demonstrated that the use of vaccines in the period before the performance of laboratory tests for EIA diagnosis in animals does not induce seroconversion since the animals were subjected to the same serological controls during the pre-vaccination phase of the study and showed the same result.

Our results differ from those found in the literature that used 82 seronegative equines subjected to treatments with vaccines and mineral complex with organic compounds and demonstrated that 16.7% of the group of animals vaccinated against equine influenza and 16.7% of those inoculated with mineral complex with plant extracts were false positive by the AGID method. Another percentage of equines (25%) that received two doses of the last product also presented non-specific results, which confirms the absence of seroconversion in the treatments (Jacobo et al., 2004). However, the similarity between the methodologies of both studies was based on the use of products with biological components (vaccines and plant extracts) as treatments that preceded serology, with the hypothesis that these components would be responsible for non-specific reactions.

Ordinance No. 84/1992 by MAPA (BRASIL, 1992) indicates as the cause for the occurrence of non-specific reactions to AGID the formation of other antigen-antibody interactions (Ag-Ab) not specific to the EIA virus p26, but with antigens molecularly very similar. In

the present study, no statistical significance was observed for the correlation of occurrences of negative reactions associated with non-specific precipitation lines and the vaccine protocol used in this research.

The presence of non-specific lines associated with negative reactions provides data that can be added to the legislation, as Ordinance No. 84/1992 by MAPA (BRASIL, 1992) reports only the simultaneous occurrence of specific positive reaction to EIA associated with the non-specific precipitation line, in contrast to the reading protocol for the AGID test, recommended by the United States Department of Agriculture, in which non-specific negative reactions are also demonstrated (USAHA, 2008). More recently, the Normative Instruction No. 52/2018 included information on the reactions of non-specific lines, including non-specific reactions, whether positive or negative, for EIA.

Moreover, the most prominent non-specific negative reactions characterized by the presentation of double (compound) and shiny non-specific lines were observed in the experimental group exposed to the leptospirosis vaccine. That one challenged with the eightfold vaccine (against influenza, encephalomyelitis, equine rhinotracheitis, and tetanus) showed non-specific lines but smoother and with dashes or unitary curves (simple). The scarcity of studies aimed at the characterization of non-specific lines by the AGID technique after using vaccines limited the discussion of the obtained results.

Regarding the interpretation of results found by the

AGID method, Oliveira (2011) observed that the non-specific reactions verified when comparing diagnostic kits did not interfere with test readings after the 48-hour incubation period. The results found in this study corroborate the data of Oliveira (2011) because the occurrence of these reactions did not harm the reading and interpretation of this research. Despite the non-interference, Silva (2007) highlighted during the evaluation of a commercial antigen and control serum used in the AGID technique that non-specific reactions are undesirable phenomena that can be used as a quality parameter to analyze commercial kits.

Another experiment correlated the use of vaccines to the AGID method and reported the occurrence of suspicious results in EIA diagnosis in seronegative animals previously vaccinated against equine influenza. According to Jacobo et al. (2004), it allows us to infer that immunogen components would be responsible for the presentation of non-specific reactions in serology.

CONCLUSIONS

Vaccination before performing the AGID test does not interfere with EIA diagnosis. The occurrence of negative reactions associated with non-specific lines in AGID for EIA diagnosis can serve to support changes in the current legislation on the diagnosis of this disease.

REFERENCES

- BELTRÃO, M. R. O. C.; ALENCAR, C. A. S.; LEITE, A. S.; FREITAS, L. T.; GONZALEZ, J. C.; SANTANA, V. L. A.; MANSO-FILHO, H. C. Comparison of Two Protocols of Agar Gel Immunodiffusion (AGID) Used to Diagnose of Equine Infectious Anemia (EIA). *Open Journal of Veterinary Medicine*, v. 5, n. 07, p. 169, 2015.
- BRASIL 1992. Portaria nº 84, de 19 de outubro. Normas de credenciamento e monitoramento de laboratórios de anemia infecciosa equina. Ministério da Agricultura Pecuária e Abastecimento (MAPA). Disponível em www.agricultura.gov.br Acesso em 10 jul. 2014.
- BRASIL, 2004b. IN nº 24, de 05 de abril. Normas para o controle e a erradicação do mormo. Ministério da Agricultura Pecuária e Abastecimento (MAPA). < www.agricultura.gov.br > Acesso em 11 jul. 2014.
- BRASIL, 2004c. IN nº45, de 15 de junho. Normas para prevenção e o controle da Anemia Infecciosa Equina – A.I.E.
- CULLINANE, A. et al. Diagnosis of equine infectious anaemia during the 2006 outbreak in Ireland. *The Veterinary Record*, v. 161, n. 19, p. 647-652, 2007.
- ISSEL, C. J.; COOK, R. F. A review of techniques for the serologic diagnosis of equine infectious anemia. *Journal of Veterinary Diagnostic Investigation*, v. 5, n. 1, p. 137-141, 1993.
- JACOBO, R.A. et al. Reacciones inespecíficas en el diagnóstico de anemia infecciosa equina. *Revista Veterinaria*, v. 15, n. 1, p. 17-20, 2004.
- Ministério da Agricultura Pecuária e Abastecimento (MAPA). < www.agricultura.gov.br > Acesso em 20 maio 2012.
- NAUREEN, A. et al. Comparative evaluation of Rose Bengal plate agglutination test, mallein test, and some conventional serological tests for diagnosis of equine glanders. *Journal of veterinary diagnostic investigation*, n. 19, p. 362-367, 2007.
- NEUBAUER H. et al. Serodiagnosis of Burkholderia mallei infections in horses. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health*, n. 52, p. 201-205, 2005.
- OIE, 2013a. Equine Infectious Anaemia. OIE Terrestrial Manual. <<http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/>> Acesso em 25 maio 2014.
- OIE, 2013b. Glanders. OIE Terrestrial Manual. <<http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/>> Acesso em 09 jul. 2014.
- OLIVEIRA F. G. 2011. Validação da imunodifusão em gel de ágar para o diagnóstico da anemia infecciosa equina em equídeos e comparação com o elisa rgp90 e imunoblot. Dissertação de Mestrado em Ciência Animal, Universidade Federal de Minas Gerais, Belo Horizonte, 2011.
- OLIVEIRA, F. G.; DINIZ, R. S.; CAMARGOS, M. F.; OLIVEIRA, A. M.; RAJÃO, D. S.; BRAZ, G. F.; LEITE, R. C.; REIS, J. K. P. Comparative study of agar gel immunodiffusion (AGID) protocols for the diagnosis of equine infectious anemia in Brazil. *Semina: Ciências Agrárias*, v. 34, n. 6. Suplemento 12, p. 3909-3916, 2013.
- REIS, J. K. P.; MELO, L.M.; REZENDE, M.R.; LEITE R.C. Use of an ELISA test in the eradication of an equine infectious anaemia focus. *Tropical Animal Health and Production*, n. 26, p. 65-68, 1994.
- SAS 2004. Statistical Analysis System: user's guide statistics. Versão 9.1.3. Cary: SAS Institute, 1 CD-ROM.
- SILVA, A.R.S. 2007. Diagnóstico da Anemia Infecciosa Equina: análise comparativa de sistemas comerciais de diagnóstico por imunodifusão. Dissertação de Mestrado em Ciências em Microbiologia Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ. 66p.
- TELES, J.A.A.; CAMPOS, A.C.; SILVA, K.P.C.; SANTOS, A.S.; SANTANA, V.L.A.; CASTRO, R.S.; MOTA, R.A. 2012. Desenvolvimento e avaliação de um teste ELISA indireto para o diagnóstico sorológico do mormo em equídeos. *Pesquisa Veterinária Brasileira*, v. 31, n. 9, p. 838-842, 2012.
- USAHA. 2008. United States Animal Health Association. Foreign Animal Diseases. 7.ed. Canada: Boca Publications Group. p. 281-286.
- VERMA, R.D.; SHARMA, J.K.; VENKATESWARAN, K.S.; BATRA, H.V. Development of an avidin-biotin dot enzyme-linked immunosorbent assay and its comparison with other serological tests for diagnosis of glanders in equines. *Veterinary Microbiology*, n. 25, p. 77-85, 1990.