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EVALUATION OF A MICROIMMUNODIFUSION ASSAY FOR THE DIAGNOSIS OF ENZOOTIC BOVINE LEUKOSIS

(Avaliação de um ensaio de microimunodifusão para diagnóstico da leucose enzoótica em bovinos)

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ABSTRACT

This work was developed to evaluate how a micro-AGID using simple protocol for obtaining the antigen compared to a macro-AGID. A total of 450 serum samples from 92 herds in 23 counties that make up the dairy herd of Maranhão were used. The antigen used in micro-AGID was obtained by desalting of supernatant of FLK cells infected with BLV against the polyethylenoglicol. In micro-AGID 10µL of antigen and positive serum control was used and 30 µl of test serum, in the macro-AGID 25 µl of all reagents were used. Of the compared sera, 57.56% (n=259) and 54.44% (n=245) showed positive animals reagents results in micro-AGID and macro-AGID, respectively. There was a very good agreement between both techniques (K=0.91), with sensitivity and specificity of the macro-AGID for micro-AGID of 93.43% and 98.43% with an accuracy of 95.96%. Micro-AGID showed clearer lines than those observed in the macro-AGID and reading can be made 24 hours before the macro-AGID. It is concluded that micro-AGID can be used successfully in the serological diagnosis of EBL, with the advantage of greater speed in issuing the results and obtaining the antigen with a simple technique.

Keywords: Bovine, macro-AGID, EBL, antigen.

RESUMO

O presente trabalho foi realizado com o objetivo de avaliar uma micro-IDGA usando protocolo simples para obtenção do antígeno comparativamente a uma macro-IDGA. Foram utilizadas 450 amostras de soro bovino provenientes de 92 rebanhos de 23 municípios que compõem a bacia leiteira do estado do Maranhão. O antígeno usado na micro-IDGA foi obtido por diálise frente ao polietilenoglicol de sobrenadante de células FLK infectadas pelo VLEB. Na micro-IDGA utilizou-se 10µL de antígeno e soro controle positivo e 30 µl do soro teste; na macro-IDGA 25 µl de todos os reagentes. Dos soros comparados, 57.56% (n=259) e 54.44% (n=245) apresentaram animais reagentes na micro-IDGA e macro-IDGA, respectivamente. Houve ótima concordância entre as duas técnicas (K=0,91), com sensibilidade e especificidade da macro-IDGA em relação à micro-IDGA de 93.43% e 98.43%, respectivamente. A micro-IDGA apresentou linhas mais claras que as observadas na macro-IDGA e a leitura pode ser feita 24 horas antes da macro-IDGA. Conclui-se que a micro-IDGA pode substituir à macro-IDGA no diagnóstico sorológico da LEB, com a vantagem de maior rapidez na emissão dos resultados e da obtenção do antígeno com técnica simples.

Palavras-chave: Bovino, macro-IDGA, EBL, antígeno.

INTRODUCTION

Bovine Enzootic Leukosis (EBL) is a disease of adult cattle caused by the EBL virus (Bovine Leukemia vírus - BLV), isolated by (MILLER *et al*., 1969), member of the family *Retroviridae,* subfamily *Orthoretrovirinae* and genus *Deltaretrovirus* (ICTV, 2022). Most animals infected by the vírus show no clinical signs, however 30 to 70% of them may develop

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persistent limphocytosis and a small proportion (0,1 – 10%) develop lymphosarcomas in several organs, which induce diggestive disturbances, inappetence, weight loss, weakness, general debility and neurological manifestations (BRAGA *et al*., 1998; KORNIIENKO *et al*., 2020). The superficial lymph nodes may be enlarged e palpable. Some reports had revealed the possibility of the EBL be imunossupressive, which could predispose to secondary infeccions (FETROW e FERRE, 1982).

The BLV can be transmitted mainly by direct exposure of animals to biological fluids contaminated with infected lymphocytes, specifically blood, milk, semen, spittle, urine, nasal and tracheal secretions (JOHNSON e KANEENE, 1992). Natural transmission occurs, for example, during parturition, however it depends on the transfer of infected cells and it only occurs in cattle, water buffaloes and capybaras. Also, lateral transmission can occurs in the presense of blood-contaminated, needles and surgical equipment (OIE, 2018). Some studies suggested the elimination and separation of soropositive animals as an alternative to control the infeccion of the BLV and mixed management of animals in order to prevent transmission of the virus as well (BRAGA *et al*., 1997).

Considering the difficulties of isolation and viral identification, the sorological techniques are widely used, such as agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA) (ALTANER *et al*., 1982; TÎRZIU *et al*., 2014). Those tests are currently recommended for international certification by the World Organisation for Animal Health and European Union (OIE, 2018). The AGID is the most widely used sorological test due its low cost, high specificity and easy to handle. However, the interpretation of the results is relatively subjective and may request some experience from the executor.

Several AGID protocols have been developed from the original description of (VAN DER MAATEN *et al*., 1974; BRAGA *et al*., 1997) using the main viral antigens (p24 or gp51), obtained from fetal lamb kidney cell (FLK) infected with BVL.FLK

Many procedures are used to obtain virus, such as polyethylene glycol followed by desalting, precipitation with ammonium sulfate followed by ultrafiltration, precipitation with polyethylene glycol followed by desalting and separation on a polyacrylamide bead column (OIE, 2018). There are also many formulations of gels, size, shape and distance between the wells in the mold, as well as the arragement and drilling of the gel in the mold. Nowadays, the antigen recommended for the diagnosis of EBL is gp51 (OIE, 2018).

Based on the amount of reagents used, the gel drilling mold model and the reagent volumes, all tests previously developed for the diagnosis of EBL refers to micro-AGID[1]. A comparative study between macro-AGID and micro-AGID for the diagnosis of Small Ruminant Lentiviruses – SRLV demonstrated that micro-AGID is more sensitive and present earlier reaction reading with clearer precipitation lines (ARRUDA *et al*., 2011). Therefore, this study was carried out with the objective of evaluating a micro-AGID using a simple protocol for obtaining a antigen comparatively to a macro-AGID, aiming its use in the sorological diagnosis of Bovine Enzootic Leukosis.

MATERIAL AND METHODS

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Micro-AGID – Production of antigen

The antigen was produced from supernatants of fetal lamb kidney cell (FLK), chronically infeccted by the BLV as described by Van Der Maaten *et al.* (1974), cultivated in growth medium (essential minimum medium – MEM, plus 10% fetal bovine serum - FBS) and incubated at 37 ºC. After the confluence of the monolayer, the growth medium was replaced with maintenance medium (MEM with 2% FBS). From 7th day culture, the supernatant was collected from each bottle that was submitted to the new passage. The supernatants were storaged at -20 ºC until purification.

After three freeze and thaw cycles, the supernatants were centrifuged at 3.300g for 30 minutes and concentrated 50-100 fold by polyethylene glycol (PEG 8.000) followed by desalting at 40% in PBS (pH 7.6) at 4 ºC, for 48 to 72 hours, and then collected and storaged at –20 ºC. After thawing, the antigen was tested againt TECPAR kits (Parana, Brazil) and *Behringwerke AG* (Margburg, Germany), according to manufacturers, and showed identity lines with stardard sera from both kits. The antigen was titrated according to micro-AGID in twofold dilution, against the standard sera and two precipitating units[2] of the antigen were used in the test, as recommended by the World Organisation for Animal Health (OIE, 2018).

Production of the control serum

The control serum was prepared from the serum of a bovine naturally infected by BLV, which material was provided by the Garanhus Cattle Clinic of the Federal University of Pernambuco (UFRPE). The serum was precipitated with caprylic acid followed by fractionation with saturated ammonium sulfate solution (PAGE e THORPE, 1998). After incubation and centrifugation at 5.000g for 30 minutes at 4 ºC, the supernadant was discarded and the precipitate was ressuspended up to ¼ of the initial volume in PBS pH 7.4 and submitted to desalting for 48 hours at 4 ºC (SANTOS *et al*., 2011). Then, the serum was tested against TECPAR kits (Parana, Brazil) and *Behringwerke AG* (Margburg, Germany), showing identity lines with stardard sera from both kits. Finally, the serum was titrated at the micro-AGID in twofold dilution and two precipitating units of the antigen were used.

Procedure of micro-AGID and macro-AGID

The macro-AGID was performed as recommended by the Technological Institute of Parana (TECPAR, Parana, Brazil). The micro-AGID was performed in disposable petri dish, of 90mm of diameter, which contained 16mL of 1% (w/v) agarose in sodium borate buffer solution (108mM, pH 8.6). During the test, the gel was punctured with a hexagonal shape mold, in order to form seven wells, one in the center, in which the antigen (Ag) was added, and six equidistante peripherals, in which were alternately added the control serum (CS) and the serum to be tested. The wells intended to SS and the antigen had three mm of diameter, with capacity to 10µL of each reagente, while the wells intended to serum to be tested had five mm (30µL), all equidistants to two millimeters. When finished adding the reagents, the plates were incubated at 25 ºC and the reading was made after 24 to 48 hours of incubation.

Statisctical Analysis

The micro-AGID was compared to the macro-AGID through kappa adjusted agreement indicator (k), sensitivity and specificity trought the statistic program OPENEPI

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versão 3.01/2013, calculated based on the test of 450 serum samples of dairy cattle collected during a soroepidemiological survey, with 92 herds, located in dairy basin of the state of Maranhao (MEIRELLES *et al*., 2009).

The readings of the micro-AGID and macro-AGID reactions were made 24 to 48 hours after performing the tests. The serums were considered positive when a precipitation line is formed between the midway well (Ag) and the serum tested, showing identity line between the standard serum and the Ag. The results were registered as follow: negative (-), weakly positive (+), positive (++), strongly positive (+++) and unspecific. During the reading the sharpness of precipitation lines of the tests were compared.

RESULTS AND DISCUSSION

Up to 450 serums tested, 57.56% (n = 259) and 54.44% (245) were reactives to the micro-AGID and macro-AGID, respectively. When comparing the results, it was observed great accordance between the two tests (k = 0,91), with sensitivity, specificity and accuracy of 93.43%, 98.43% e 95.56%, respectively (Tab. 01).

Table 01: Test results of 450 serum samples of catte submitted to the techniques macro and micro agar gel immunodiffusion for detection of antibodies in Bovine Leukemia vírus.

Macro-IDGA	**Micro-IDGA**		**Total**
	Positive	**Negative**	
Positive	242	3	**245**
Negative	17	188	**205**
Total	**259**	**191**	**450**

Sensitivity = 93,43%. Specificity = 98,43%. Accuracy in diagnosis = 95,56%.

Considering only positive results, regarding the precipitation line intensity for the results of macro and micro-AGID, it was observed that up to 204 negative samples in macro-AGID, 16 were positive in micro-AGID and 48 positive samples in macro-AGID improved line intensity in micro-AGID. On the other hand, a sample weakly positive in macro-AGID was negative in micro-AGID and two positive samples in macro-AGID had less intense lines in micro-AGID (Tab. 02).

These results from micro-AGID and macro-AGID differ from those found by Meirelles *et al*. (ASTUDILLO e KANTOR, 1981) in a study carried out with dairy herds in the years of 2000, 2003 and 2006 with prevalence of 63,33%, 81,08% e 55,56%, respectively.

The validity of a diagnostic test can be estimated based on its intrinsic values (sensitivity and specificity), which are proper to the test and are not influenced by prevalence or disease (GONZÁLEZ *et al*., 2007). Classicaly, the sensitivity is defined as the percentage of true positive identified in the test and the specificity of the true negatives, when compared to a gold standart test or another test usually used. González et al. (FILHO *et al*., 2019), using indirect ELISA for the detection of antibodies for BLV with immunodifusion test as a reference, found values of 98.93% of sensitivity, 79.74% of specificity and correspondence

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between the two tests was 83.90%, classified as good. On the other hand, Filho *et al*. (WINWARD *et al*., 1979), found sensitivy of 98.6%, 72% of specificity and kappa coefficient of 0.55. According to the authors, the lack of accordance between diagnostics methods was problably due to the high sensitivity to ELISA, which makes it possible to detect antibodies even in situations with low serum levels.

Table 02: Distribution of the 450 serum samples reading results of catte submitted to the techniques macro and micro agar gel immunodiffusion for detection of antibodies in Bovine Leukemia vírus.

Macro-AGID	N	Micro-AGID	N
(-)	204	(-)	187
		(+)	16
		(++)	1
(+)	183	(-)	1
		(+)	134
		(++)	44
		(+++)	4
(++)	49	(+)	1
		(++)	48
(+++)	14	(+)	1
		(+++)	13

(-) = negative, (+) = weakly positive, (++) = positive, (+++) = strongly positive.

According to the results of Tab. 02, it was found that micro-AGID, in addition to detecting a greater number of positive animals, presents a better reading of the reactions. This is important because it minimizes the effects of subjectivity of the reading, contributing to more consistent results. Similar findings were described by Winward et al. and Arruda (ONUMA *et al*., 1975; ARRUDA *et al*., 2011) using micro-AGID for the diagnosis of Small Ruminant Lentiviruses – SRLV (CAEV e Maedi-Visna).

The antigen-antibody reaction detected by AGID is characterized by precipitation and formation of visible line in the gel. In order to improve AGID method, many varibles can be studied, such as: reagent concentration, gel and buffer components and mold used to drill the gel (shape, diameter and distance between the wells). Depending on the variation of the antigen and antibody concentration, there must be a displacement of these lines closer to one of the reagentes or even inhibit their formation. Therefore, the reagente titration was made, adjusted to 2 UP, as recommended by OIE (2018).

The line precipitation may be influenced by physical-chemical conditions, such as eletrolytic concentration of the gel, buffer solution used in the procedure, pH, temperature and humidity. In both tests, high purity agarose was used, allowing better migration of reagents and visualization of precipitation lines. In macro-AGID, the buffer solution used was PBS with EDTA, pH 7.3; in micro-AGID was used borato buffer solution, which has been shown as the most suitable for the diagnosis of SRLV (ARRUDA *et al*., 2011).

In micro-AGID an hexagonal shape mold with diferent well diameters was used, that enable the use of different reagent volumes, which is not yet used in the diagnosis of EBL. For the antigen and control serum, wells of 10µL were used while for the serums to be tested, 30µL wells were used. This may explain the better lines observed in micro-AGID compared

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to macro-AGID, as well as the greater capacity of detecting positive animals through micro-AGID, possibly those with lower antibody titers, insufficient to form visible lines in macro-AGID. Similar results were described by Winward et al. and e Arruda (ONUMA *et al*., 1975; ARRUDA *et al*., 2011) when studying micro-AGID for the diagnosis of SRLV.

Recently, an European Union regulation about the diagnosis of EBL recommended that the reagents be distributed in a central cavity with 4 mm in diameter (32µL of antigen) and around which six peripheral cavities with 6 mm in diameter (73µL of serum control or test) are arranged in a circle. This arrangement may has the same purpose of this study, however, any research developed for this purpose was mentioned in the regulation (OIE (2018).

The main antigen of BLV are the protein p24 and glycoprotein gp51. This is the envelope protein that has important biological functions by interacting more directly with the host through cell receptors. After several studies, it was concluded that the antigen most recommended for the diagnosis of EBL is gp51, because the antibodies to glycoprotein gp51 appear earlier in infected animals than to p24 as well as they persist for longer periods than those against p24 (ONUMA *et al*., 1975).

The antigens obtained from the FLK cells infected with BLV are made up of several proteins, mainly p24 and pg51, however, according to its titration for diagnostic use, AGID will be able to detect antibodies against one and/or other antigen. (VAN DER MAATEN *et al*., 1974; MILLER *et al*., 1976). The antigen prepared for the micro-AGID was titrated against two commercial standard serums reagents for gp51 in parallel to the antigens supplied by the manufacturers and presented a single line of perfect identity. Thus, the micro-AGID is considered a test able to detect antibodies to gp51, which meets the requirements for certification of animals for international transit (OIE 2018).

CONCLUSIONS

According to the conditions described in this experiment, it is suggested that micro-AGID can be used as a alternative for the diagnosis of Enzootic Bovine Leukosis and will henceforth replace macro-AGID with higher sensitivity, specificity and speed in obtaining the results.

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Publicado: set./2023.

REFERENCES

ALTANER, C.; ZAJAC, V.; BÁN, J. A simple and inexpensive metod for detection of BLB infected cattle based on modified ELISA principal. **Zentralblatt für Vetemärmedizin**, v.29, n.8, p.583-590, 1982.

ARRUDA, E.; OLIVEIRA, M.; NASCIMETO, S.; CASTRO, R. Avaliação de uma microimunodifusão em gel de ágar para diagnóstico de Lentivírus de pequenos ruminantes (LVPR) em caprinos. **Ciência Animal Brasileira**, v.1, n.3, p.560-565, 2011.

ASTUDILLO, V.M.; KANTOR, I.N. El problema de la validez de una prueba diagnostica para uso masivo como procedimento estatístico de clasificación. **Boletin Centro Panamericano Fiebre Aftosa**, v.43/44, n.1, p.37-43, 1981.

BRAGA, F.M.; VAN DER LAAN, C.W.; HALFEN, D.C.; VIDOR, T. Avaliação de métodos de controle da infecção pelo vírus da leucose enzoótica bovina. **Ciência Rural**, v.27, n.4, p.635-640, 1997.

BRAGA, F.M.; VAN DER LAAN, C.W.; SCHUCH, L.F.; HALFEN, D.C. Infecção pelo vírus da Leucose Enzoótica Bovina. **Ciência Rural**, v.28, n.1, p.163-72, 1998.

FETROW, J.; FERRE, J.F. Bovine leukemia virus infection and mastitis. **Journal Dairy Science**, v.65, n. 05, p.881-882,1982.

FILHO, L.C.F.B.; FERNANDES, A.C.C.; SILVA, T.I.B.; RAMOS, T.R.R.; MELO, L.E.H. Performance assessment of imported ELISA in the serodiagnosis of the enzootic bovine leukosis in herds of Pernambuco state, Brazil. **Arquivo do Instituto Biológico**, v.86, n.1, p.1-4, 2019.

GONZÁLEZ, T.; LICURSI, M.; BONZO, E. Leucose Bovina Enzoótica: Desempenho de um ELISA indireto empregado no diagnóstico sorológico. **Brazilian Journal of Microbiology**, v.38, n.1, p.1-5, 2007.

JOHNSON, R.; KANEENE, J. Bovine leukemia virus and enzootic bovine leukosis. **Veterinary Bull**, v.62, n.3, p.287-314, 1992.

KORNIIENKO, L.Y.; PYSKUN, A.V.; TSARENKO, T.M.; UKHOVSKYI, V.V.; KYIVSKA, G.V.; MOROZ, O.A.; KOVALENKO, V.L.; ALIEKSEIEVA, G.B. Retrospective analysis of the epizootic situation of enzootic bovine leukosis in Ukraine in 1994–2019. **Regulatory Mechanisms in Biosystems**, v.11, n.3, p.372-377, 2020.

MEIRELLES, C.; DITTRICH, T.; CIPRIANO, F.; OLLHOFF, R.D. Evolution of Enzootic Bovine Leukosis prevalence in a university dairy herd. **Semina: Ciências Agrárias**, v.30, n.1, p.671-678, 2009.

MILLER, J.; VAN DER MAATEN, M. Sorologic detection of Bovine Leukemia Virus infection. **Veterinary Microbiololy**, v.31, n.6, p.47-55, 1976.

MILLER, J.M.; MILLER, L.D.; OLSON, C.; GILLETE, K.G. Virus-like particles in phytohemaggIutinin-stimulate lymphocyte cultures with reference to bovine lymphosarcoma.

Recebido: out./2022.
Publicado: set./2023.

Journal of the National Cancer Institute, v.43, p.1297-1305, 1969. Disponível em: http://ictvonline.org/virusTaxonomy.asp. Acesso em: 01 jan. 2022

OIE. **"Enzootic Bovine Leukosis" in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals**, 2018. Disponível em: https://www.woah.org/Terrestrial Manual. Acesso em: 01 jan. 2022.

ONUMA, M.; OLSON, C.; BAUMGARTENER, L.E.; PEARSON, L.D. An ether sensitive antigen associated with bovine leukemia virus infection. **Journal of the National Cancer Institute**, v.55, n.5, p.1155-1158, 1975.

PAGE, M.; THORPE, R. **Methods in Molecular Biology: Immunochemical Protocols**. 2. ed. Pound JD (Totowa, NJ: Humana Press), 1998.

SANTOS, H.P.; PEREIRA, H.M.; NASCIMENTO, S.A.; COUTINHO, L.C.A.; TEIXEIRA, W.C.; ARRUDA, R.C.N.; BEZERRA, N.P.C.; BEZERRA, D.C.; CASTRO, R.S. Frequência de anticorpos e fatores de risco associados à leucose enzoótica bovina em rebanhos da bacia leiteira do estado do Maranhão. **Arquivos do Instituto Biológico**, v.78, n.3, p.351-358, 2011.

TÎRZIU, E.; CUMPĂNĂȘOIU, C.; NICHITA, I.; REMAN, G.D.; SONEA, C.; SERES, M. Performance assessment of three tests applied in enzootic bovine leukosis diagnosis. **Romanian Biotechnological Letters**, v.19, n.5, p.9666-9677, 2014.

VAN DER MAATEN, M.; MILLER, J.M.; BOOTHE, A.D. Replicating type-C virus particles in monolayer cell cultures of tissues from cattle with lymphosarcoma. **Journal of the National Cancer Institute**, v.52, n.2, p.491-497., 1974.

WINWARD, L.D.; LEENDERTSEN, L.; SHEN, D.T. Microimmunodiffusion test for diagnosis of ovine progressive pneumonia. **American Journal of Veterinary Research**, v.40, n.7, p.564-6, 1979.

Recebido: out./2022.
Publicado: set./2023.