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Optimization of canine Anti-IGG conjugate in Canine Visceral Leishmaniosis (CVL) diagnostic kit

Otimização do conjugado Anti-IGG canino no kit diagnóstico da Leishmaniose Visceral Canina (LVC)

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ABSTRACT

Introduction: The diagnosis of canine visceral leishmaniasis (CVL) is of great importance in the control of visceral leishmaniasis, a disease that is neglected worldwide and represents a significant public health problem. Enzyme-linked immunosorbent assay (ELISA) test is widely used because of the possibility of large-scale diagnosis and low cost. **Objective:** The objective of this study was to optimize the stoichiometry of the conjugate used in the EIE/CVL kit; for this purpose, some conjugation and monitoring parameters were modified through molecular exclusion chromatography to improve the final product. **Method:** The conjugate produced by Bio-Manguinhos was optimized by purification using SEC gel filtration chromatography and evaluated using the EIE-leishmaniasis-visceral-canine-Bio-Manguinhos kit (EIE-LVC). The conjugates were produced with different stoichiometries (IgG X peroxidase): current conjugate, optimized conjugate and optimized and purified conjugate. **Results:** The results obtained were validated using the manual Canine Visceral Leishmaniasis test. The homogeneity of the conjugate means in the ELISA test was evaluated using the chi-square test, thereby demonstrating the normality and reliability of the data. Based on statistical data, positive samples obtained by the ELISA test showed homogeneity at a significance level of 0.05, i.e. a data reliability level of 95%. The optimization of the production of the canine anti-IgG conjugate, an extremely important component used in the composition of the Bio-Manguinhos EIE LVC KIT, contributed to ensuring the quality of the kit and improving the diagnosis of canine visceral leishmaniasis disease, making it more sensitive and inexpensive for public health. **Conclusions:** This work demonstrates that it is possible to optimize the anti-IgG canine peroxidase (HRP) conjugate used in the EIE CVL kit to make it more specific and ultimately more economical.

KEYWORDS: Peroxidase; Canine Visceral Leishmaniasis; Diagnostic Kit

RESUMO

Introdução: O diagnóstico da leishmaniose visceral canina (LVC) é de grande importância no controle da leishmaniose visceral, uma das doenças mais negligenciadas no mundo e de grande importância no campo da saúde pública. O teste *Enzyme-linked immunosorbent assay* (ELISA) é amplamente utilizado pela possibilidade de diagnóstico em larga escala e baixo custo. **Objetivo:** Para otimizar a estequiometria do conjugado utilizado no kit EIE/CVL, foram modificados alguns parâmetros de conjugação e monitorização através de cromatografia de exclusão molecular no melhoramento do produto final. **Método:** O conjugado produzido por Bio-Manguinhos por purificação foi otimizado utilizando a cromatografia de gel de filtração SEC e foi avaliado através do desempenho do kit EIE-leishmaniose-visceral-canina-Bio-Manguinhos (EIE-LVC). Os conjugados foram produzidos com diferentes estequiometrias (IgG X peroxidase): conjugado atual, conjugado otimizado e conjugado otimizado e purificado. **Resultados:** A homogeneidade das médias dos conjugados no teste ELISA foi avaliada através do teste qui-quadrado, evidenciando assim a normalidade e confiabilidade dos dados. Com base nos dados estatísticos, amostras positivas obtidas pelo teste ELISA apresentaram homogeneidade ao nível de significância

de 0,05, ou seja, grau de confiabilidade dos dados de 95%. A otimização da produção do conjugado anti-IgG canino, componente de suma importância utilizado na composição do Kit Bio-Manguinhos EIE LVC, contribuiu para assegurar a qualidade do kit e melhor diagnóstico da doença leishmaniose visceral canina, tornando-os mais sensíveis e de baixo custo para a saúde pública. **Conclusões:** Este trabalho demonstra que é possível otimizar o conjugado Anti-IgG Peroxidase Canina (HRP) utilizado no Kit EIE CVL, tornando-o mais específico e, em última análise, mais viável economicamente.

PALAVRAS-CHAVE: Peroxidase; Leishmaniose Visceral Canina; Kit Diagnóstico

INTRODUCTION

Leishmaniasis is a zoonotic infectious protozoan disease transmitted by more than 70 species of female phlebotomine worms and is one of the most neglected diseases in the world. It is the second most prevalent parasitic disease in the world, after malaria[1]. The natural history of the manifestation of this disease shows that it is possible to find parasites in different tissues that present immunopathological alterations according to the progression of the clinical forms[2]. In terms of diversity, the disease consists of four main clinical forms: cutaneous leishmaniasis, mucosal or mucocutaneous leishmaniasis, visceral leishmaniasis, also known as Kalazar, and post-Kalazar dermal leishmaniasis[3]. Visceral leishmaniasis (VL) is the most serious form of the disease according to the World Health Organization (WHO) and can cause the death of approximately 20,000 people a year worldwide[4]. Generally, cases of leishmaniasis affect poor communities, whether in rural or urban areas. For reasons that are still unknown, but which are thought to be associated with the parasite and the host's effective immune responses, the most severe form of the disease is often not observed[5]. Most seropositive dogs are apparently healthy, but with a high infectious load they are easily infected by vectors, so dogs are considered a better reservoir than humans, even in the early stages of infection, as they infect phlebotomines in both the asymptomatic and symptomatic stages[6]. To date, serological tests are the best option for detecting leishmaniasis, as they are less invasive methods and represent a good cost-benefit ratio compared to other alternatives[7]. The test considered confirmatory for canine visceral leishmaniasis (CVL) is the enzyme-linked immunosorbent assay (ELISA), which is easy to read and used in control and epidemiological programs[8]. Diagnostic reagents play an important role in public health and epidemiological surveillance, identifying and monitoring diseases, as well as assessing the quality of blood in Hemotherapy Services[9]. The ELISA kit, used in all public laboratories in the country, is produced by the Immunobiological Technology Institute (Bio-Manguinhos), controlled by the Ezequiel Dias Foundation (FUNED) and regulated by the Ministry of Agriculture, Livestock and Supply (MAPA). The canine anti-IgG conjugate is an essential component of the CVL enzyme-linked immunosorbent assay (EIA) kit and is an extremely important input for the reaction response of this kit. The study aimed to optimize the conjugation process using the monomeric forms of immunoglobulin, modifying some conjugation parameters and monitoring by molecular exclusion chromatography. Better specificity of IgG binding to peroxidase was obtained, which contributed to better stability of the canine anti-IgG/peroxidase conjugate and optimization of conjugate quality before reaching the final kit.

METHOD

Nine conjugates were initially produced at the Conjugates and Support Supplies Section of the Bio-Manguinhos Institute of the Oswaldo Cruz Foundation (Fiocruz). Different stoichiometries were used for the method of conjugating goat anti-rabbit IgG with the enzyme Horseradish Peroxidase (HRP): Group 1: concentration (w/v) conjugate A: with stoichiometry currently used in the diagnostic kit (current stoichiometry) 45 mg/mL of IgG conjugated with 22.5 mg/mL of HRP; test group B: 45 mg/mL of IgG conjugated with 45 mg/mL; test group C: 45 mg/mL of IgG conjugated with 11.3 mg/mL. Using the ELISA assay, we chose the best conjugate from group 1 to start further tests. In group 2, conjugation was based on the molar ratio - Conjugate D: current stoichiometry - 0.3 µM IgG: 0.5 µM HRP; E: conjugate C produced from group 1 - 0.3 µM IgG: 1 µM HRP and F: test stoichiometry, decreasing the ratio between IgG and HRP - 0.2 µM IgG: 1 µM HRP. After the ELISA test, new conjugates were produced, group 3: G: conjugate with current stoichiometry - 0.3 µM IgG: 0.5 µM HRP; H: conjugate F produced in group 2 - 0.2 µM IgG: 1 µM, and I: conjugate with stoichiometry group F, using only the immunoglobulin monomer after purification - 0.2 µM purified IgG: 1 µM HRP, highlighted in Table 1.

The best conjugates from groups 2 and 3 were selected for the production of the following groups, which we called "mini", as we used fewer inputs, group 4: - J: H conjugate produced in group 3 with the following stoichiometry 0.2µM IgG: 1µM HRP; K: conjugate I, produced in group 3, using a purified IgG (only the monomeric form), with the following stoichiometry - 0.2µM IgG:1µM, and conjugate L: conjugate I, with a change in reaction time (shorter incubation in the borohydride step), shown in Table 2. All the conjugates were analyzed for homogeneity and molecular weight using size exclusion chromatography (SEC) and the data obtained was validated using the CVL EIE manual test. The homogeneity of the means of the conjugates in the ELISA test was assessed by the chi-square test, thus demonstrating the normality and credibility of the data with a data reliability level of 95%.

Table 1. Ratio of IgG/HRP used to obtain the conjugate subgroups.

	IgG	HRP
Group 1 conjugates		
A (Current stoichiometry)	45 mg/mL	22.5 mg/mL
B (Stoichiometry test)	45 mg/mL	45 mg/mL
C (test stoichiometry)	45 mg/mL	11.3 mg/mL
Group 2 conjugates		
D (Current stoichiometry)	0.3 µM	0.5 µM
E (Conjugate C group 1)	0.3 µM	1 µM
F (Stoichiometry test)	0.2 µM	1 µM
Group 3 conjugates		
G (current stoichiometry)	0.3 µM	0.5 µM
H (F-conjugate from group 2)	0.2 µM	1 µM
I (Stoichiometry of the F conjugate of group 2 - IgG monomer	0.2 µM	1 µM

Source: Prepared by the authors, 2020.

Table 2. Ratio of IgG/HRP used to obtain the "mini" conjugates.

Mini conjugates group 4	IgG	HRP
J (H conjugate of group 3)	0.2 µM	1 µM
K (conjugate I of group 3)	0.2 µM	1 µM
L (conjugate K change in conjugation time)	0.2 µM	1 µM

Source: Prepared by the authors, 2020.

Functionality was analyzed by indirect ELISA test, positive and negative controls of canine serum samples were used at 1:100 dilution and secondary antibody labeled with horseradish peroxidase (HRP) enzyme at 1/60000, which is based on soluble antigens of promastigote forms of *Leishmania major*-like. The kit is produced by Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil, and all procedures were carried out according to the manufacturer's instructions.

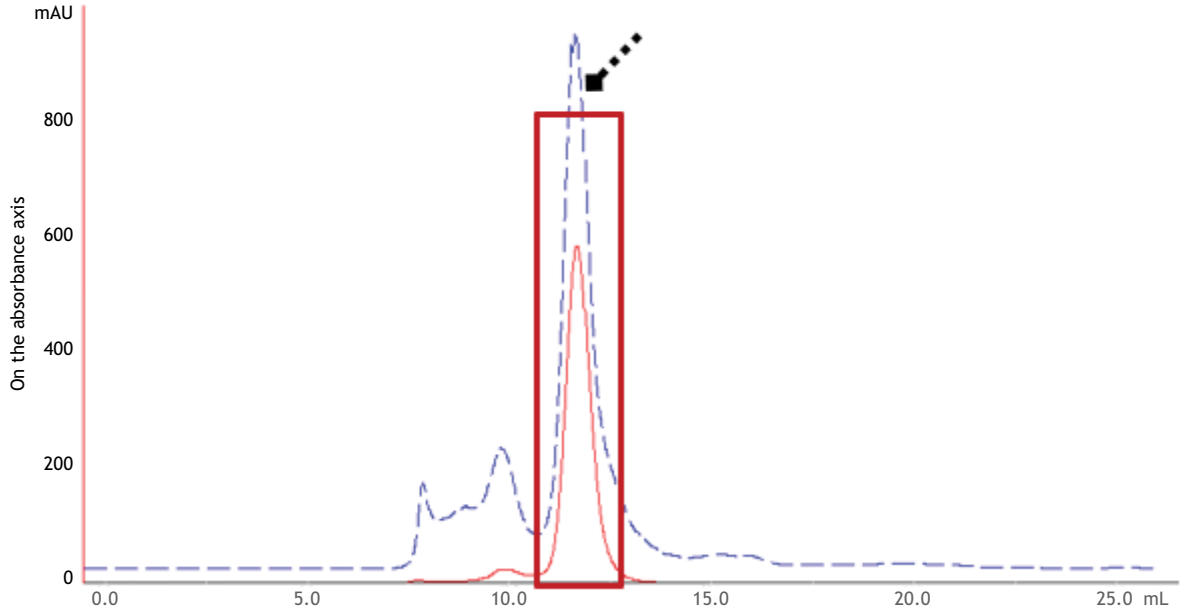
The immunoglobulin used was obtained after purification using ion exchange chromatography and subjected to preparative molecular exclusion chromatography on a Superdex 200 HR 30/10 column, carried out on a G&E Heathcare workstation. The column was equilibrated with 0.01 M phosphate buffer solution (PBS), pH 7.4, at a flow rate of 0.5 mL/min with the system pressure limit set at 1.5 MPa. A volume of 100 µL of the sample was applied. The protein fractions were detected by absorbance readings at 220 nm and 280 nm on the equipment (GE Healthcare).

The affinity binding measurement by microscale thermophoresis (MST) was carried out on a NanoTemper Monolith NT.115 device (Nano Temper Technologies). Briefly, the *Leishmania major*-like antigen was tagged using a Monolith RED-NHS 2nd Generation protein labeling kit. The data was obtained with MO.Control V2.1.1 software (NanoTemper Technologies, Munich, Germany) and the recorded data was analyzed with MO.Affinity Analysis V3.0.4 software (NanoTemper Technologies, Munich, Germany).

The data was compiled using the Microsoft Excel 2013 program. The statistics for conjugate J ("mini" without purification), conjugate K ("mini" monomer), and conjugate L ("mini" monomer in a shorter time) were evaluated using the mean, standard deviation (SD), and coefficient of variation of the positive results. A statistical study of each conjugate group was analyzed, using the chi-square test with a 95% data reliability level.

RESULTS

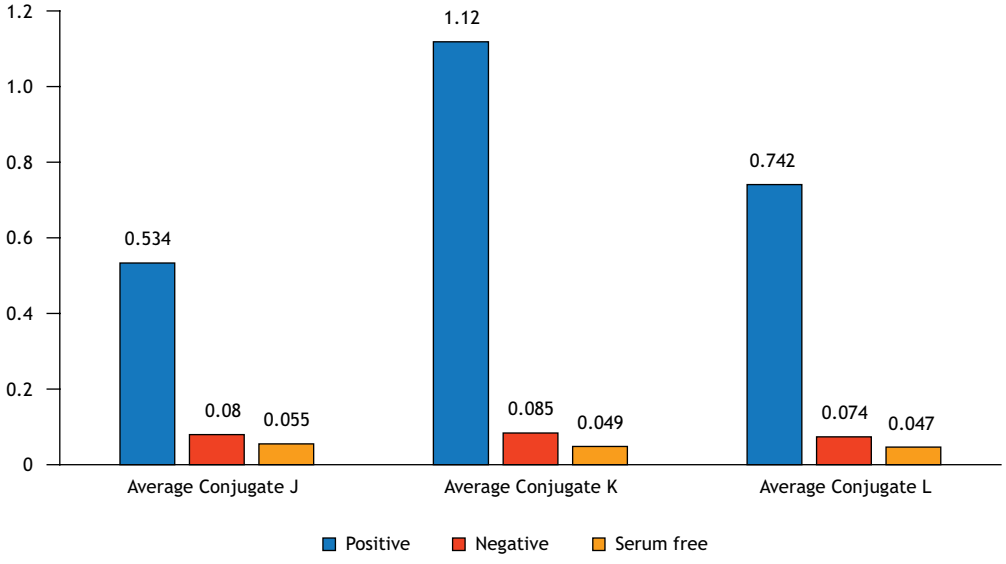
The best result obtained among the conjugates produced with different stoichiometries was the IgG:HRP µM molar ratio, which showed less IgG and free HRP visualized by SEC, a result corroborated by the ELISA test; however, the protein peak referring to the conjugate had a high molecular weight (over 300 kDa). Conjugates produced strategically considering the molecular weight of IgG and the HRP molar ratio (µM) showed a single protein peak by size exclusion chromatography, despite the high molecular weight (over 300 kDa). The IgG:HRP µM molar ratio conjugate produced using purified IgG (only the monomeric form) showed a single protein peak by size exclusion chromatography with 190 kDa (Figure 1), which showed the best ratio according to the ELISA procedure, and also proved to be 8% more sensitive than the approved kit standard. After producing the "mini" conjugates, due to their small volume, they were only analyzed in the indirect ELISA assays n = 9 for positive, negative, and serum-free (SF). The K conjugate showed the best result according to the highest O.D. curve for the positive control, the lowest O.D. for the negative control and (SF), as shown in Figure 2. The means of the "minis" conjugates (Figure 3) were shown by the chi-squared test at a 95% degree of data reliability. The results of these values showed a 95% probability of occurrence (number of repetitions in the experiment). The evaluation of the functionality of the conjugates produced against the *Leishmania major-like* antigen was compared by microscale thermophoresis analysis which verified that the variation in the concentration of the *Leishmania major*-like antigen caused an increase in the response at higher concentrations, indicating the presence of intermolecular interaction between all the conjugates tested, it was observed that the current conjugate had an EC50 between 235.1-333.3 pM, the purified conjugate showed 182.1-664.1 pM, and finally, the optimized conjugate, 77.45-2,258.1 pM. It can therefore be inferred that the samples showed similar results in terms of the effective dose for which half of the target molecule is in the bound state (Figure 4).



Source: Prepared by the authors, 2020.

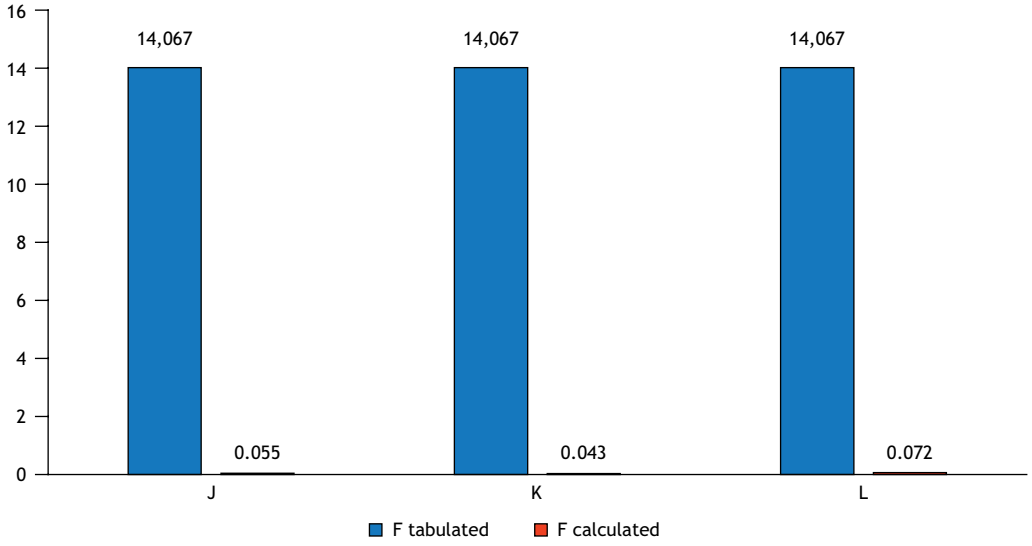
Figure 1. Chromatographic profile of immunoglobulin purification by Superdex 200 (24 mL) at 280 nm in 0.01 M PBS solution pH 7.2. The rectangle indicates the overlap of the IgG monomer after purification (full line) under the chromatographic profile of the original immunoglobulin (dashed line). Application volume 100 µl.

CONTROLS	CONJUGATE J (O.D.)	STANDARD DEVIATION	K-CONJUGATE (O.D.)	STANDARD DEVIATION	CONJUGATE L (O.D.)	STANDARD DEVIATION
POSITIVE	0.534	0.06	1.12	0.077	0.742	0.081
NEGATIVE	0.08	0.01	0.085	0.007	0.074	0.002
SS	0.055	0.018	0.049	0.009	0.047	0.006
UA	5.339	N/A	11.267	N/A	9.399	N/A



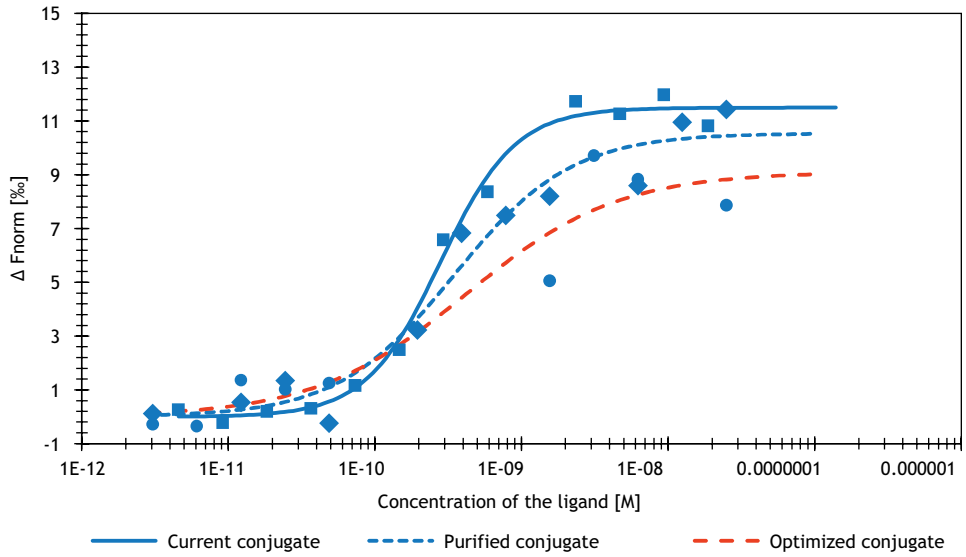
Source: Prepared by the authors, 2020.

Figure 2: Results of the mean O.D., standard deviation and AU for the "mini" conjugates. Conjugate J (best conjugate produced in group 3, without purifying the IgG): O.D.: optical density of the control media (positive, negative, SF), standard deviation and arbitrary unit (AU); Conjugate K (best conjugate produced in group 3): controls (positive, negative, SF), standard deviation and AU: arbitrary unit and conjugate L, best conjugate from group 3 (shortest conjugation time): (positive, negative, SS), standard deviation and arbitrary unit.



Source: Prepared by the authors, 2020.

Figure 3. Analysis of the positivity of the "mini" J, K, and L conjugates using the chi-square test. Chi-square test (X^2) of the J, K, and L conjugates where calculated X^2 = 0.055, 0.043, and 0.072 - tabulated X^2 = 14.067 for the three "mini" conjugates. The strikethrough bars represent F tabulated and the undivided bars represent F calculated.



Source: Prepared by the authors, 2020.

Figure 4. Evaluation of binding affinity using the microscale thermophoresis (MST) technique: graph of the ΔFnorm dose-response curve as a function of the different conjugate concentrations.

As the curve was not completely saturated due to insufficient titers, the dissociation constant (Kd) could not be calculated.

DISCUSSION

CVL has become an urban disease that needs to be continuously investigated, as it is fatal in untreated patients. Diagnostic reagents help in the monitoring of various diseases, as they generate fast, accurate and effective results, which are essential for early detection. In 2011, the Ministry of Health notified the replacement of the CVL diagnostic protocol, which used the ELISA test as a screening method and the indirect immunofluorescence test (RIFI) as a confirmatory test (Technical Note No. 01/2011).

Conventional serological tests for diagnosing CVL (indirect immunofluorescence, direct agglutination test, and ELISA) are used both for individual diagnosis and in epidemiological studies.

In general, many serological tests can be used as markers of infectious diseases, but none of them have 100% sensitivity and specificity.[11]

The ELISA test is based on the binding of an antibody to a single antigen detectable through enzymatic reactions. This test is characterized by its speed and therefore the possibility of performing a greater number of samples, obtaining automated results, which exclude the subjectivity of the reading and its high specificity[12,13,14]. Other advantages of ELISA include its stability in relation to microscopic agglutination serum, the small volume of reagents required, and the quantitative nature of the results obtained, which allows rapid diagnosis and presents a low level of biological risk. This technique is also used for epidemiological studies and offers simplicity of execution and low cost.[15,16]

In our study we used the indirect ELISA test, which is widely used to detect and/or quantify antibodies in serum samples. Several indirect ELISA methods use whole viruses or recombinant Maedi-Visna virus (MVV)[17,18]. As the conjugate is important because it is produced from the binding of the antibody and the antigen in question, using the k-conjugate ("mini" monomer) with a purified IgG (only the monomeric form), it is necessary to optimize the conjugation processes to obtain more specific results. Despite this[19], it mentions the possibility of binding more than one HRP molecule (5-6) to an IgG molecule, which causes the formation of a very large conjugate, reducing the rate of tissue penetration, in the case of immunohistochemical assays, which is not verified if the conjugate is used only for antigen detection, as in microscopy, the size of the conjugate does not affect the assay[19]. In the case of the ELISA assay, the presence of non-specific fluorescence can generate adsorption of antibody molecules to the cell surface without an antigen-antibody reaction, which can show a false event. To avoid false positivity interfering with the results, it is necessary to quantify and eliminate the non-specific fluorescence[20]. In addition, the presence of immunoglobulin aggregates in the conjugate could affect biological activity, causing a decrease in the specificity as well as the reproducibility of the assay.[21]

The functionality of the conjugates produced was evaluated against the Lsh antigen using MST analysis[22]. It should be noted that the measurement check showed low signal-to-noise for both conjugates, indicating the presence of detectable intermolecular interaction at the concentration evaluated. The binding affinity assay provided the dose-response curve data shown in Figure 4, where varying the concentration of the Lsh conjugates resulted in an increase in the ΔFnorm at the highest concentrations. The confidence interval (95% CI) for the EC50 determined by the software indicated no statistically significant difference between the samples. For example, the current conjugate was found to have an EC50 between 235.1-333.3 pM, the purified conjugate showed 182.1-664.1 pM, and finally, the optimized conjugate, 77.45-2.258.1 pM. It can therefore be inferred that the samples showed similar results in terms of the effective dose for which half of the target molecule is in the bound state.

According to current data, the stoichiometry recommends the production of immunoglobulin conjugates with peroxidase ranges of 1:1-1:4 (IgG:HRP), always considering the possibility of post-conjugation purification to separate free forms (Protocols *Roche Diagnostics GmbH, Interchim, Synapse, Sigma-Aldrich, GE Life Science, Thermofisher*). Based on this information about the production of conjugates and the need to optimize the CVL detection kit, we strategically designed our experiments by producing four groups of conjugates, in which we studied the ideal stoichiometry and improvement conditions. Based on the literature, we obtained the following data showing the best average readings of the reactivity of the conjugates, in arbitrary units (AU), in which the normal time of the conjugate with monomer resulted in a value of 11.267, conjugate with monomer in reduced time L = 9.399 and the conjugate produced with unpurified IgG and the same stoichiometry used for the monomers = 5.339. It can be seen that the highest UA value obtained was related to the conjugate, which was produced with the monomeric form of immunoglobulin, in the established stoichiometry. It was also possible to see that the conjugates with monomers performed better than the other conjugate, indicating that despite having the same stoichiometry, the fact of using the monomeric form of immunoglobulin instead of the protein without purification optimized the conjugation process, even using a shorter reaction reduction time with NaBH4. The best titration chosen was the one that showed the highest UA results, with low readings in the blank and negative samples and high readings in the positive samples, as shown previously[23]. The variation parameter expresses the precision and repeatability of an assay, and it was found that the conjugate produced with purified antibody (monomeric form) has a lower coefficient of variation when compared to the conjugate produced with unpurified antibody, indicating greater precision of the data and ensuring greater reproducibility and homogeneity of the data from the tests carried out. The conjugate produced using the monomeric form of IgG at a shorter incubation time also showed a lower coefficient of variation compared to the conjugate produced without antibody purification.

These data suggest that the optimization of the stoichiometry is important in the production of the conjugate, but the purification of the antibody used in this reaction is essential to guarantee the reproducibility and accuracy of the data obtained. Based on our statistical study, we have shown that the positive samples obtained by the ELISA test are homogeneous at a significance level of 0.05, i.e. a 95% data reliability level.[24]

CONCLUSIONS

The present study reported on the optimization of the production of canine anti-IgG conjugate, as an essential input used in the

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Authors' Contribution

Chermont SA - Conception, planning (study design), acquisition, analysis, data interpretation, and writing of the paper. Nascimento HJ† - Data interpretation. Andrade MAL - Conception. Pinto CT - Writing of the paper. Bastos RC - Data interpretation. Pereira KAB - Analysis and writing of the paper. Jurgilas PB - Data interpretation. Delgado I - Data interpretation, analysis, and revision of the paper. All the authors approved the final version of the paper.

Conflict of Interest

The authors inform that there is no potential conflict of interest with peers and institutions, political or financial, in this study.