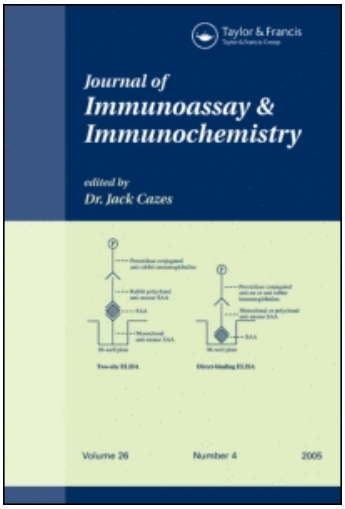

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Validation of a Recombinant Based Antibody ELISA for Diagnosis of Human and Canine Leishmaniasis

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Abstract: In this study, a recombinant chimeric antigen (CA) ELISA was validated as a single test for both human and dog leishmaniasis. Serum panels included 327 human and 339 canine IFAT-positive and 1113 human and 1078 canine IFAT-negative samples. CA-ELISA was carried out using the same serum dilution, and labelled protein A as secondary reagent. Test performances were calculated using ROC analysis. For the human panel, the test showed diagnostic accuracy (DA) 0.974, specificity (Sp) 97.12%, sensitivity (Se) 91.44%, and concordance (K) 0.88. The dog panel showed DA 0.998, Sp 99.54%, Se 98.54%, and K 0.98. The proposed method is the best recombinant antigen-based ELISA, and can be used as IFAT substitute for mass screening.

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Keywords: Canine sera; Chimeric antigen; ELISA; Human sera; IFAT; Leishmaniasis

INTRODUCTION

Leishmaniases are human and animal parasitic infections caused by several members of the protozoan hemoflagellate *Leishmania*. The diseases have a worldwide distribution in both the Old (Mediterranean basin, Middle East and Indian sub-continent) and New Worlds (Latin America).[1–3] Phlebotomine sand flies (*Phlebotomus* and *Lutzomyia* spp.) transmit *Leishmania* promastigotes when biting on a mammalian host,[4] where they differentiate into amastigotes and replicate as intracellular parasites of the mononuclear phagocyte system.

Wild canids and hounds are the major reservoirs for Zoonotic Visceral Leishmaniasis (ZVL); above all, domestic dogs play a strategic role for infection transmission and maintenance.[5] In the past decades, human factors and environmental changes promoted the diffusion of the disease in the areas which were not traditionally considered suitable for the spreading of leishmaniasis.[1,6–8] Furthermore, leishmaniasis is an opportunistic infection in immunocompromised patients, especially HIV-positive individuals, in endemic areas.[9–12] Human Visceral Leishmaniasis (VL) represents a severe form of the disease which is fatal without suitable therapy.[1,13] The severity of the disease and the role of dogs as reservoir hosts both make monitoring and surveillance of leishmaniasis infections important to prevent the spread of disease.[9,14–16]

The immunofluorescent antibody test (IFAT) is a widely utilized test for leishmaniasis diagnosis, being the most sensitive and specific method. Although being considered a standard reference test,[17] the main disadvantage of IFAT consists in the subjective interpretation of results, often non-repeatable from different laboratories.[18] Furthermore, it is laborious and time consuming to screen large numbers of sera.

Rapid and unfailing indirect diagnosis are necessary tools for ZVL detection and surveillance, both in human and in veterinary practice. The large variability of clinical symptoms and the presence of asymptomatic infectious dogs[19] makes antibody detection the most suitable diagnostic tool and many efforts have been carried out in the last decades for the development of reliable, cheap, and easy to perform diagnostic methods. Enzyme Linked Immunosorbent Assay (ELISA) is the candidate of choice for the development of a rapid and reliable leishmaniasis diagnosis. ELISA is practical, easy to standardize and suitable for a massive screening of sera rather than the IFAT method. Whole cell lysate is usually sensitive, but several *Leishmania* antigens have been genetically and antigenically characterized with the aim of increasing specificity.

Some of them have been shown to be expressed in the amastigote stage, thus representing a pool of potential markers during mammal infection. The antigenicity of K9, K39, and the repetitive region of K26 showed independent and complementary immunoreactivity and reached a good agreement with IFAT when used in parallel.[20] Recombinant technology can improve both specificity and sensitivity of relevant antigens by affinity purification of recombinant antigens and by joining immunodominant linear epitopes in a single fusion protein. A K9-K39-K26 recombinant chimeric antigen (CA) has been proposed and evaluated in a previous study.[21]

In this study, the same chimeric construct was employed and evaluated as a single ELISA protocol for serological diagnosis of both human and canine leishmaniasis infections. A large panel of sera, previously characterised by IFAT, was tested by CA-ELISA. Modifications of the previously proposed protocol were carried out by using the same serum dilution for human and canine samples, incubation time, and peroxidase conjugate protein-A as secondary antibody. Results indicate that the modified CA-ELISA performance had improved and showed excellent agreement in comparison with IFAT and may be proposed as a single diagnostic tool for both human and canine leishmaniasis diagnosis.

EXPERIMENTAL

Serum Samples

According to the Office Internationale des Epizooties (OIE) guidelines for validation of diagnostic tests, which suggest that a minimum of 300 positive samples and 1,000 negative samples should be used to make a preliminary estimate of sensitivity and specificity,[22] 1423 human and 1417 canine sera were collected. Among the former, a first group of 326 IFAT-positive (different titres $\geq 1/160$) and 331 IFAT-negative (titre $< 1/40$) sera were from the serum collection maintained at Istituto Superiore di Sanità (Rome, Italy) and including samples from all regions of Italy; a second group of 1 IFAT-positive and 389 IFAT-negative were collected from an ipoendemic area of North-West Italy (Asti province); a third group of 393 IFAT-negative samples were kindly provided by a blood-donor bank (AVIS, Torino) and were collected from Italian healthy donors. Thus, a total amount of 327 positive and 1113 negative human sera were tested. As far as hound samples are concerned, a first group was from an endemic zone (Liguria, North-West Italy), the second group was from an ipoendemic zone (Valle d'Aosta, North-West Italy), and a third group was collected from Torino (Italy) at the Faculty of

Veterinary Medicine, a small animal hospital. A total amount of 339 positive and 1078 negative canine sera were tested.

All samples were tested with the IFAT method, at the Istituto Superiore di Sanità (human sera) and at the Istituto Zooprofilattico Sperimentale of Imperia (canine sera), according to the standard protocol.[16] Both institutes utilize a titre of 1/160 as cut-off for the IFAT positive response. This IFAT threshold value is recognized as suitable to reduce the number of uncertain sera.[23] All samples giving discordant results between the IFAT and CA-ELISA tests were re-tested using the same techniques.

ELISA Procedure

The CA-ELISA procedure was essentially as described in the previous study, with some modifications according to a single test strategy for human and canine sera.

Briefly, microplates (TPP immunomaxi) were coated, in odd columns, with 25 ng/well of purified CA diluted in water (50 μL/well) and, in even columns, with water as a negative antigen. Plates were dried overnight at 37°C, blocked with 150 μL/well of 2.5% bovine milk casein pH 7.0 for 1 h at 37°C and rinsed three times with 300 μL/well of 138 mM NaCl, 0.05% Triton-X100 (washing solution WS).

Both human and canine serum samples were diluted 1/40 with dilution buffer consisting of PBS, 1.25% bovine milk casein pH 7.0 and dispensed 100 μL/well (both odd and even wells) and incubated at 37°C for 1 h. Plates were rinsed four times with 150 μL/well of WS, and incubated as above with 100 μL/well of peroxidase-labeled A-protein anti-immunoglobulin (Pierce, Rockford, IL) diluted 125 ng/mL in DBS. After the final rinsing step the reaction was developed using 100 μL/well of ABTS and plates read at 405 nm after 10–15 minutes.

To take into account a plate effect, one positive and one negative control serum, available in large quantity for each species, were tested in each plate. The net absorbance (NA) was obtained for each sample by subtracting the absorbance against the negative antigen from the absorbance against the positive antigen and expressed as percentage of reactivity (PR) compared to NA of the relative positive control serum.

Calculation and Statistical Analyses

Sensitivity of the ELSA test was estimated by the fraction of IFAT-positive sera that tested positive, whereas specificity was estimated as the fraction of IFAT-negative sera that tested negative. The receiver operating characteristic analyses (ROC) were used to find optimal cut-off

values, to consider all possible combinations of diagnostic sensitivity (Se) and diagnostic specificity (Sp) that can be achieved by changing the CO value.

Diagnostic accuracy of the test was expressed as the area under the ROC curve (AUC). AUC was calculated following the equation of Greiner et al.:[24]

$\mathrm{AUC} = \Phi\left(A/\sqrt{1+B^2}\right)$ developed in the formula $\mathrm{AUC} = 1/2\ \Sigma[(\mathrm{Se}^n + \mathrm{Se}^n + 1) \cdot ((1 - \mathrm{Sp}^n) - (1 - \mathrm{Sp}^{n+1}))]$.

AUC may usually range from 0.5 and 1. Values between 0.5 and 0.7 indicate low accuracy, between 0.7 and 0.9 indicate moderate accuracy; values between 0.9 and 1 indicate high accuracy.[24,25]

Agreement behind chance between IFAT (regarded as golden standard) and CA-ELISA was evaluated by K statistic.

Positive and negative predictive values (PV+, PV−) were obtained for human and canine serum panels following the equations suggested by Tijssen;[26] these values describe what fraction of the obtained results, positive or negative, are a correct results. The CA-ELISA test efficiency (Ef), which describes what fraction of all results are correct (true positive and negative), was calculated following the indication of Zweig and Campbell.[25]

All analyses and calculations, plots and graphic images, were obtained using R© (''The R Foundation for Statistical Computing, Version 2.1.1'' (2005-06-20), ISBN 3-900051-07-0).

RESULTS

Human Sera Response

The human control sera showed a NA of 1.752 ± 0.129 (mean $\pm$ SD, $n = 82$) and -0.014 ± 0.031, with a range from 1.538 to 2.012 and −0.152 to 0.049 for positive and negative sera, respectively.

The frequencies of assessment of negative and positive sera for IFAT are shown in Figure 1a. Table 1 shows the number of true negative (TN), true positive (TP), false negative (FN), false positive (FP), PV+, PV− and Ef for a selected range of CO values.

The box-plots of PR for different groups of IFAT titres (negative and positive) are shown in Figure 2a; the NA gap between the IFAT negative sera group and the positive group was slight. The mean PR of positive human sera groups showed a rising trend which follows the IFAT titres. The group with IFAT titres 1/160 showed a PR average of 33.50% (±28.52 SD) versus 86.32% (±25.87 SD) of the group with IFAT titre over 1/10240.

Table 1. Result of IFAT and CA-ELISA serum panels and calculation

	IFAT Positive		IFAT Negative							
Human		327		1113						
ELISA	TP	FN	TN	FP	PV+	PV−	Ef (P = 13%)	Ef (P = 20%)	Ef (P = 35%)	Ef (P = 50%)
CO 2%	310	17	1066	47	0.8683	0.9843	0.9565	0.9558	0.9544	0.9529
CO 3%	299	28	1081	32	0.9033	0.9748	0.9639	0.9599	0.9513	0.9428
CO 6%	290	37	1100	13	0.9571	0.9675	0.9751	0.9680	0.9528	0.9376
CO 11%	276	51	1106	7	0.9753	0.9559	0.9743	0.9638	0.9413	0.9189
CANINE		339		1078						
ELISA	TP	FN	TN	FP	PV+	PV−	Ef (P = 13%)	Ef (P = 20%)	Ef (P = 35%)	Ef (P = 50%)
CO 23%	335	4	1068	10	0.971	0.996	0.990	0.990	0.990	0.989
CO 27%	334	5	1073	5	0.985	0.995	0.994	0.993	0.992	0.990
CO 30%	333	6	1075	3	0.991	0.994	0.995	0.994	0.992	0.990

Source: TP: true positive, positive response vs both IFAT and ELISA. FN: false negative, positive to IFAT and negative to ELISA. TN: true negative, negative response vs both IFAT and ELISA. FP: false positive, negative to IFAT and positive to ELISA. PV+: positive predictive value calculated as $VP+ = TP/(TP+FP)$. PV−: negative predictive value calculated as $VP- = TN/(FN+TN)$. Ef test efficiency calculated as $Ef = P \cdot Se + (1 - P)\ Sp$, where P is the Prevalence of disease in target population.

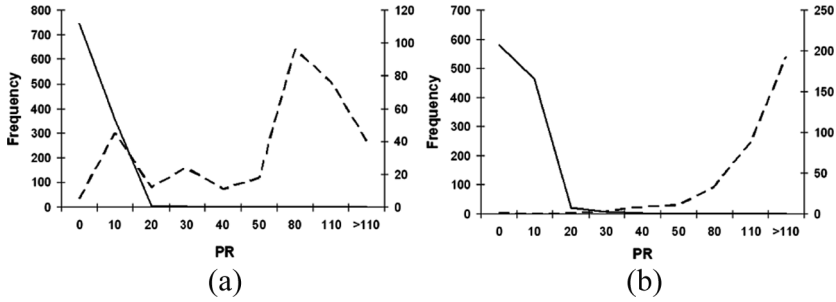

Figure 1. CA-ELISA frequencies. The frequencies of IFAT negative (continues line) and IFAT positive (dashed line) serum panel expressed as percentage of reactivity (PR). (a) frequencies of human serum panel; (b) frequencies of canine serum panel.

The ROC curve (Figure 3a) shows that the optimal CO value, nearest the upper left corner, was 3% of PR, giving a Sp and Se of 97.12% and 91.44%, respectively. Concordance between CA-ELISA and IFAT was K = 0.88 (CI 95%, 0.85 to 0.91) for the same CO value. Diagnostic accuracy of CA-ELISA, expressed as AUC, was 0.974.

The CO that gave the lowest number of discordant sera, when compared with IFAT, was 6% of PR, giving a Sp and Se of 98.83% and 88.69% and a K value of 0.90 (CI 95%, 0.87 to 0.93).

To improve the Sp of the CA-ELISA, a CO of 11% of PR, gave an acceptable concordance value of 0.88 (CI 95%, 0.85 to 0.91), comparable to optimal CO and Sp and Se of 99.37% and 84.40% respectively.

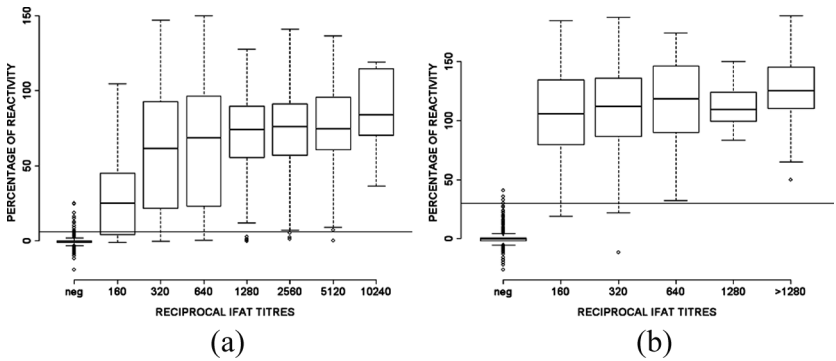

Figure 2. Boxplot of tested sera related to reciprocal IFAT titres. (a) boxplots of human serum panel; (b) boxplots of canine serum panel. Horizontal lines are cut-off values chosen for each species.

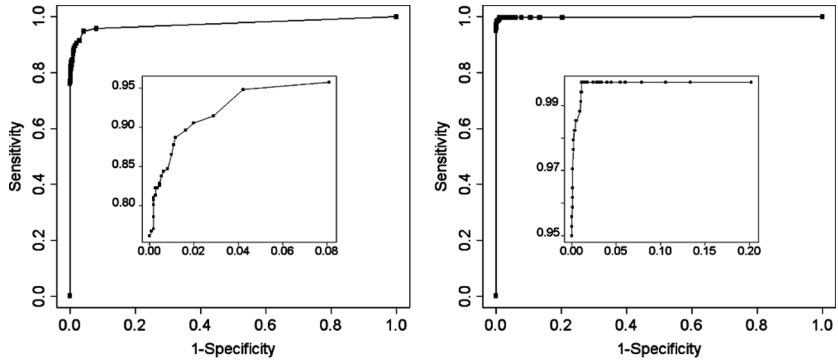

Figure 3. ROC curves of human and canine serum panel. (a) ROC curve of human sera and its detail of upper left corner; the nearest point to the corner indicate the optimal cut-off; (b) ROC curve of canine sera and its detail of upper left corner; the nearest point to the corner indicate the optimal cut-off.

Dog Sera Response

The dog control sera showed a NA of 1.654 ± 0.162 (mean $\pm$ SD, n = 82) and 0.020 ± 0.078, with a range from 1.177 to 2.013 and -0.209 to 0.210 for positive and negative sera, respectively.

The frequencies of assessment of negative and positive sera for IFAT are shown in Figure 1b.

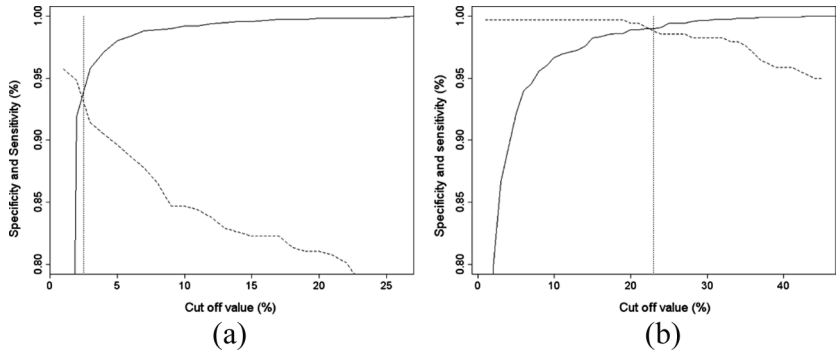

Figure 4. Plot of specificity and sensitivity related to cut-off value, graphic view of optimal cut-off definition. ROC analyse indicate that the point where specificity (continues line) cross the sensitivity (dashed line) indicates the optimal cut-off value (vertical dotted line). (a) human serum panel; (b) canine serum panel.

Table 1 shows the number of true negative (TN), true positive (TP), false negative (FN), false positive (FP), PV+, PV− and Ef for a selected range of CO values.

The box-plots of PR for different groups of IFAT titres (negative and positive) are shown in Figure 2b and the NA gap between the group of IFAT negative sera and the group of positive was high. The mean PR of the groups of positive human sera showed a rising trend which follows the IFAT titres, the group of sera with IFAT titres 1/160 was a mean PR of the 103.57% (±38.88 SD) against the mean PR of the 124.94% (±26.22 SD) for the group with IFAT over 1/1280.

The ROC curve (Figure 3b) shows that the optimal CO value, nearest the upper left corner was the 27% of PR, giving a Sp and Se of 99.54% and 98.54%, respectively. Concordance between CA-ELISA and IFAT was K = 0.98 (CI 95%, 0.97 to 0.99) for the same CO value. Diagnostic accuracy of CA-ELISA, expressed as AUC, was 0.998.

The CO that gave the lowest number of discordant sera, when compared with IFAT, was 30% of PR, giving a Sp and Se of 99.72% and 98.23% and a K value of 0.98 (CI 95%, 0.97 to 0.99).

The Sp of the CA-ELISA cross the Sp at CO value of 23% of PR, gave a K value of 0.97 (CI 95%, 0.96 to 0.99) and Sp and Se of 99.07% and 98.82%, respectively.

DISCUSSION

During the last decades, many rapid diagnostic tests have been developed in order to follow rapid diagnosis and interventions both in humans and dogs.[27–34]

Until now, many researchers focused their efforts to adapt either serological markers or methods to a single host species and validation of the new assay has been frequently carried out using an insufficient number of well-characterised sera. A new contribution of this work is the possibility to perform a single ELISA test working equally well in human and canine species. Validation of the assay was carried out following the principles of validation of diagnostic assays for infectious diseases suggested by OIE. Particularly, the number of negative and positive sera, as well as the method of ROC analyses for evaluation of test accuracy, were taken into account. The CA-ELISA has been preliminarily evaluated, in a previous work, on a restricted panel of human and canine sera in a separate format, with good performance.[21] In the present study, Sp, Se, and K values were improved for both species. This could be attributable to changes in the ELISA method (human sera dilution, secondary antibody) and the number and conservation of sera.

Several methods have been proposed to define the CO value, based on absorbance values of a minimum number of negative control sera and 2 or 3 times the standard deviation.[35,36] Such methods would require a greater number of controls in each plate to be consistent. Indeed, a defined absorbance value as CO is no longer accepted for high variation between plates. In this work the CO was expressed as PR of samples versus the positive reference serum (regarded as 100% reactivity) enclosed in each plate. To our knowledge, this method is widely used in commercial ELISA formats, and minimized the inter-plate variation, allowing comparison of a large panel of sera tested in different batches of reagents and environmental conditions. On the basis of PR of samples, optimal CO was calculated using ROC analyses, which can provide additional information, such as AUC value and Ef, allowing comparison of different test methods.[24,25]

In the proposed format, a positive and negative control for each species analysed was enclosed in each plate. However, since protein A, as secondary antibody, binds with the same efficiency both human and canine IgG, a single control species (e.g., canine sera) might be considered. Simulation of such situation did not affect the correct classification of human sera analysed (not shown).

Several *Leishmania* antigens have been characterised to date at the genetic level and most of them have been successfully used to develop a single recombinant based immunoassay. A few examples employed a recombinant multicomponent antigen which is expected to mimic the performance of the golden standard IFAT.[21,37] The epitopes selected in our recombinant chimera derived from *Leishmania* proteins that are mainly or exclusively expressed in the amastigote (vertebrate) stage of infection. Conversely, the crude whole promastigote, used as antigen in IFAT, may lead to false positive reactions due to cross reactivity with other protozoa. Nevertheless, to our knowledge, the performance of the proposed method is the best recombinant based ELISA, in terms of K value, with the IFAT and may be proposed as an alternative method for mass screening of human and dog populations.

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