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Development of Recombinant Chimeric Antigen Expressing Immunodominant B Epitopes of *Leishmania infantum* for Serodiagnosis of Visceral Leishmaniasis

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Wild canids and domestic dogs are the main reservoir of zoonotic visceral leishmaniasis (VL) caused by *Leishmania infantum* (syn.: *Leishmania chagasi*). Serological diagnosis of VL is therefore important in both human and dog leishmaniasis from a clinical and epidemiological point of view. Routine diagnosis of VL is traditionally carried out by immunofluorescent antibody test (IFAT), which is laborious and difficult to standardize and to interpret. In the last decade, however, several specific antigens of *Leishmania infantum* have been characterized, allowing the development of a recombinant-based immunoassay. Among them, the whole open reading frame encoding K9 antigen, the gene fragment encoding the repetitive sequence of K26, and the 3′-terminal gene fragment of the kinesin-related protein (K39sub) were previously evaluated as diagnostic markers for canine leishmaniasis and proved to be independent in their antibody reactivity. Since sensitivity of serological test is usually higher in multiple-epitope format, in this study the relevant epitopes of K9, K26, and K39 antigens were joined by PCR strategy to produce the chimeric recombinant protein. The resulting mosaic antigen was found highly expressed in *Escherichia coli* and efficiently purified by affinity chromatography. Antigenic properties of this recombinant antigen were evaluated by indirect enzyme-linked immunosorbent assay (ELISA) using a panel of human and dog sera previously characterized by parasitological and/or serological techniques. Chimeric ELISA showed 99% specificity in both human ($n = 180$) and canine ($n = 343$) control groups, while sensitivity was higher in canine VL (96%, $n = 213$) than in human VL (82%, $n = 185$). Accordingly, concordance between IFAT and canine chimeric ELISA ($k = 0.95$, 95% confidence interval = 0.93 to 0.98) was higher than between IFAT and human chimeric ELISA ($k = 0.81$, 95% confidence interval = 0.76 to 0.87). Results suggest the potential use of this new antigen for routine serodiagnosis of VL in both human and canine hosts.

Animal and human leishmaniases are parasitic infections caused by protozoan hemoflagellates belonging to the genus *Leishmania*.

Parasites are transmitted by the bite of phlebotomine sand flies to the mononuclear phagocyte system of the vertebrate host, where the infecting promastigotes differentiate into and replicate as amastigotes. The geographical distribution and the spreading of the infection depend on the presence of sand fly vectors and of animal reservoirs (3).

Wild canids and domestic dogs represent the main reservoir hosts of zoonotic visceral leishmaniasis (VL), playing a strategic role for diffusion and maintenance of the infection (25). Zoonotic VL is caused by *Leishmania infantum* (syn. *Leishmania chagasi*) (24) and spread in the Mediterranean basin, in the Middle East, and in Latin America.

In the past decades, human factors and environmental changes have promoted the diffusion of the disease in areas originally not considered suitable for the spreading of leishmaniasis (9, 11, 32, 14). Leishmaniasis is also an opportunistic infection in immunocompromised patients, especially human immunodeficiency virus (HIV)-positive subjects, in areas of endemicity (2, 10, 15, 17). Human VL represents the most severe form of the disease, and, left untreated, it is nearly always fatal (9, 38). The severity of the disease and the role of dogs as reservoir hosts make important monitoring and surveying *L. infantum* infections to prevent spreading of the disease (12, 26, 39). Rapid and unfailing indirect diagnoses are necessary tools for zoonotic VL detection because of the large variability of clinical symptoms and the presence of asymptomatic but infective dogs (13). Serology methods are frequently used for mass screening of infected dogs, and immunofluorescent antibody test (IFAT) is widely diffused for diagnosis, being the most sensitive and specific test. Although IFAT represents the reference test, it is limited by the subjective interpretation of results often nonrepeatable from different laboratories (31).

The enzyme-linked immunosorbent assay (ELISA) is the

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A

Primer	Sequence (5′-3′)
K9s:	5′-TTGGATCCATGGGAGCCTACTGCACGAAGGAC-3′
K9-K39a:	5′-**GCGAAGCTGCTGCTCTAG**GTTGCCGGCAACCTGCTC-3′
K9-K39s:	5′-**GAGCAGGTTGCCGGCAAC**CTAGAGCAGCAGCTTCGC-3′
K39a:	5′-TTGAATTCCGTGGCCCTCGTGCTC-3′
K39-K26a:	5′-**TTGAATTCGCCATCGCCGTCATTTTTCTGTGTACGGCCGTCCT** **CCTTAGGGCCATCGCCGTCATT**CGTGGCCCTCGTGCTCTCGCG-3′

B

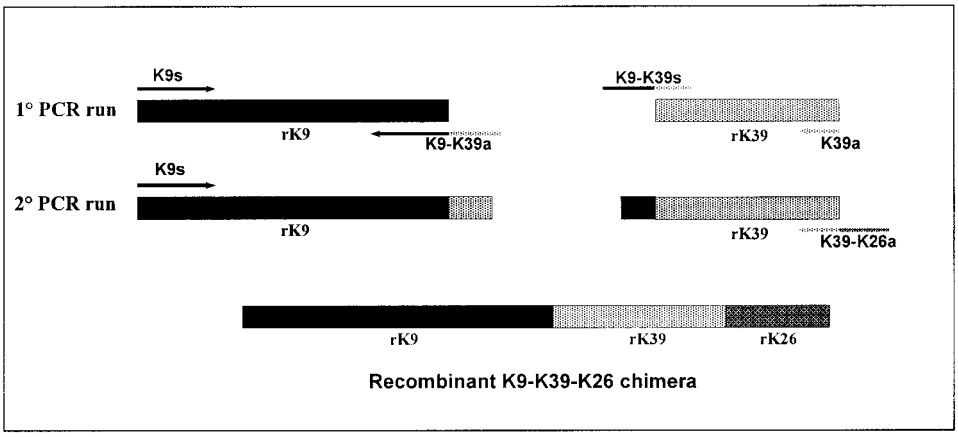

FIG. 1. A: primer sequences used for amplification and joining of the *K9-K39-K26* synthetic gene. Primer K9-K39a contains at the 5′ end an 18-bp sequence overlapping to the 5′ end of K39 sequence (bold). Primer K9-K39s contains at the 5′ end an 18-bp sequence overlapping to the 3′ end of K9 sequence (bold). The antisense primer K39-K26a contains a 5′ extension complementary to the sequence coding for 19 aa identified from K26 epitope mapping. (bold). Restriction sites BamHI (sense) and EcoRI (antisense) are underlined. B: PCR strategy used for joining of K9, K39sub, and K26 sequences. The primer set K9s/K9-K39a and K9-K39s/K39a were used in the first PCR step to produce two overlapping fragments. Purified amplicons were joined together in the second step using external primers K9s and K39-K26a.

candidate of choice for the development of a rapid and reliable *Leishmania* diagnostic method, because it is more practical, standardizable, and suitable for mass screening than IFAT. Specificity and sensitivity of the ELISA-based immunoassay strictly depends on antigen quality and can be improved by the use of recombinant technology, which drives the expression, and purification of diagnostically relevant proteins in large amounts (31, 38).

In the last decade, several *Leishmania* antigens have been genetically and antigenically characterized. Some of them have been shown to be expressed in the amastigote stage, thus representing a pool of potential markers during vertebrate infection. Recombinant K39 antigen (rK39) is a 39-amino-acid-repetitive immunodominant B-cell epitope of the 230-kDa kinesin-related protein of *L. chagasi* (4, 8, 41). The rK39 ELISA has been demonstrated suitable for detection of human VL (1, 4, 7, 8, 17, 18, 21, 27, 28, 36) and of both clinical and asymptomatic canine VL (4, 27, 33, 35, 40). K9 and K26 are two related hydrophilic antigens of *L. chagasi* that differ for the presence of 11 copies of a 14-amino-acid repeat in the open reading frame of K26 (5).

The antigenicity of K9, of a single 39-amino-acid unit of K39 (K39sub hereafter), and of the repetitive region of K26 was determined in multiple-well ELISA using infected dog sera. The three recombinant antigens showed independent and complementary immunoreactivities and reached an excellent agreement with IFAT when used in parallel (34). An ideal test would therefore employ a combination of relevant epitopes in a single recombinant antigen, more specific than crude antigen preparation and more sensitive than single epitope-based ELISA.

The aim of the present study was to produce a recombinant chimera resulting from the fusion of K9, K39sub, and K26 antigens and to develop an indirect ELISA for the diagnosis of VL in human and dog. Epitope mapping of the repetitive region of K26 was previously carried out to define immunodominant epitope(s). A PCR strategy was developed to generate a synthetic construct, which was cloned into prokaryotic expression vector. The recombinant chimera was expressed in *Escherichia coli* and purified by double affinity steps. A panel of well-characterized human and dog sera was then used to validate this novel chimeric ELISA.

MATERIALS AND METHODS

Plasmid and bacterial host. Plasmids pGex-2T (Amersham Biosciences) and pGex-6H (modified in our laboratories) were used as prokaryotic expression vectors.

In both plasmids the gene of interest is expressed in fusion with glutathione *S*-transferase (GST) under the control of the inducible *tac* promoter. Fusion protein can be purified by affinity chromatography using glutathione Sepharose.

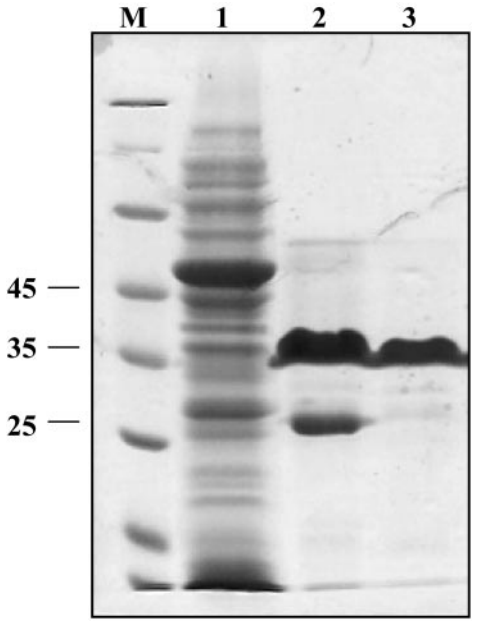

FIG. 2. Expression and purification of K9-K39-K26 chimera. Lane M: molecular weight marker; lane 1: total cell lysate of *E. coli* expressing GST-K9-K39-K26 fusion protein; lane 2: K9-K39-K26 chimera after Sepharose 4 B purification and cleavage of the GST carrier moiety; lane 3: K9-K39-K26 chimeric antigen after IMAC purification.

PGex-6H vector comes from pGex-6P (Amersham Biosciences) that has been modified by adding an in-frame six-histidine-encoding sequence between EcoRI and SalI restriction sites. Recombinant protein expressed in pGex-6H contains a carboxy-terminal six-histidine tag (F-H-H-H-H-H-H-V), which allows a further affinity purification step by immobilized metal chelate affinity chromatography (IMAC).

Two recombinant plasmids containing, respectively, the whole open reading frame of K9 antigen (GenBank accession number AF131227) and the K39sub (GenBank accession number L07879) have been developed previously (34) and served as PCR templates in this study.

E. coli strain BL21star (Invitrogen) was used as the bacterial host.

K26 epitope mapping. Five overlapping oligonucleotide pairs, 48 bp long, were synthesized encompassing two adjacent K26 repetitive sequences (single repetition; PKEDGRTQKNDGDG; GenBank accession number AF131228), to express five peptides 14 amino acids (aa) long offset two to three residues. Annealing of each oligonucleotide pair was performed in a 10-μl reaction mixture containing 500 pmol of each primer at 60°C for 5 min, with a first denaturation step at 95°C for 2 min. Annealing of each set generated five double-strand sequences with a 5′ single-strand overhang which allowed directional cloning into BamHI/EcoRI sites of pGex-2T expression vector. *E. coli* BL21star was transformed with recombinant plasmids, plated on selective agar, and screened by colony PCR using vector primers pGex5′/pGex3′.

Early log phase cultures of positive clones were induced for 2 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) under agitation to express GST fusion peptides. The five recombinant peptides were recovered in the soluble fraction and purified in batch using glutathione Sepharose 4B (Amersham Biosciences). Purity and yield of each recombinant peptide were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Bradford method (6).

Microplates (Immunomaxi; TPP) were coated with 100 ng/well of the fusion partner GST as negative control and with equimolar amounts of each of the five peptides (104 ng/well). A panel of eight sera from IFAT-positive and four sera from IFAT-negative dogs, both living in endemic areas, were used for ELISA tests. Dog sera were diluted 1/40 in phosphate-buffered saline–0.05% Tween 20–1% yeast extract and incubated 1 h at 37°C. After four washes, 20 ng/well of peroxidase-labeled protein A, diluted in the same buffer, was added and the plates were incubated as described above. After four final washes, 100 μl/well of substrate solution 2,2′azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) was added and absorbance was measured at 405 nm after 10 min. Net absorbance was determined for each serum and each recombinant K26 subunit by subtracting the absorbance value against GST as the negative antigen.

Primer design, PCR strategy, cloning, and sequencing. The list of primers used for amplification of synthetic gene and the PCR strategy is schematically presented in Fig. 1. Briefly, in the first run of amplification, primers K9/K9-K39a and K9-K39s/K39a were used to generate overlapping K9 and K39sub fragments (offset by 36 bp), which were joined in a second run using the most 5′ and 3′ primers. The latter (K39-K26a) contained a 5′ extension complementary to the sequence coding for 19 amino acids (aa), identified from K26 epitope mapping.

The first PCR was carried out in 50 μl of a volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates, 20 pmol each primer, 1 ng of template, and 1 U of *Taq* DNA polymerase (QIAGEN). PCR was performed in 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, after a single denaturation step of 94°C for 5 min. Amplified products were resolved and visualized after agarose gel electrophoresis and ethidium bromide staining, gel purified, and combined to form the template for the second PCR run.

The second reaction mixture (50-μl volume) contained the same buffer as described above, 20 pmol each primer (K9s/K39-K26a), and 1 U of *Taq* DNA polymerase (QIAGEN). The latter primer set contained, respectively, at the 5′ terminus, restriction sites for BamHI (sense) and EcoRI (antisense) to facilitate cloning. PCR was performed in 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, after a single denaturation step of 94°C for 5 min. During the annealing step, overlapping fragments joined each other, accomplishing the full-length synthesis of the gene encoding the chimeric protein. Amplified product of the expected length was purified and digested with EcoRI and BamHI restriction enzymes and cloned into pGEX-6H expression vector, in frame with GST. Transformed *E. coli* BL21star bacteria were plated on selective agar and screened by colony PCR using vector primers. The plasmid preparation from one positive clone was sequenced to confirm the in-frame fusion of the three subunits with ABI 310 (Applied Biosystems) by a cycle sequencing method using fluorescently labeled chain terminating dideoxynucleotide triphosphates.

K9-K39-K26 chimera expression and purification. To express K9-K39-K26 chimera, early log phase cultures of positive clone were induced for 2 h with 0.5 mM IPTG under agitation. Bacterial cells were recovered by centrifugation and lysed by conventional physicochemical methods.

In pGex-6H plasmid, foreign gene is expressed in fusion with a 26-kDa GST carrier in N terminal and with a six-histidine tag in C terminal. Upstream to the cloning site, the Prescission Protease (Amersham Biosciences) recognition sequence allows subsequent separation of recombinant protein from the GST carrier moiety.

Recombinant K9-K39-K26 protein was recovered in the soluble fraction and purified using two different affinity principles. The first step of purification was performed in batch using glutathione Sepharose 4B (Amersham Biosciences). GST cleavage was achieved in situ using 80 U of Prescission Protease per ml of glutathione Sepharose bed volume. Eluted fraction was dialyzed for 24 h to remove reducing and chelating agents and loaded into a Hi-trap chelating HP column (Amersham Biosciences), positively charged with nickel ions.

Purity and yield of purified recombinant protein were estimated by SDS-PAGE, conducted under denaturing conditions, Western blotting, and DC protein assay (Bio-Rad).

Mass spectrometry and N-terminal sequencing. The recombinant protein solution was desalted in $ZipTip_{C4}$ devices (Millipore, Bedford, MA). After elution with 9 μl of 60% methanol plus 1% formic acid, 4 μl of the protein solution was applied into a gold-coated borosilicate capillary (New Objective, Cambridge, MA) and analyzed in an LCQ Thermo (San Jose, CA) ion trap mass spectrometer fitted with a nano-electrospray ionization source. The capillary voltage was set at 46 V and spray voltage at 1.9 kV. The data were managed by the Xcalibur software (Thermo, San Jose, CA).

The amino acid NH_2-terminal amino acid sequence was then determined using a Procise 492 protein sequencer (Applied Biosystems, Foster City, CA). All chemicals used were from Applied Biosystems.

Serum samples and chimeric ELISA procedure. A panel of 384 human and 609 canine sera were used in the study. Among human sera, 185 were from immunocompetent patients with confirmed VL; 19 from HIV-positive patients with confirmed VL but having a negative IFAT result; 41 from healthy blood donors; and 139 from *Leishmania*-negative immunocompetent patients affected by other pathologies including undetermined fever and/or splenomegaly (n = 122), Chagas' disease (n = 13), African trypanosomiasis (n = 2), falciparum malaria (n = 1), and autoimmune disease (n = 1).

Among dog sera, 232 were from animals in which a leishmanial infection was detected by parasitologic examination (n = 19) or having IFAT titer ≥ 80 (n = 213); 362 were from animals proven to be negative by parasitologic examination

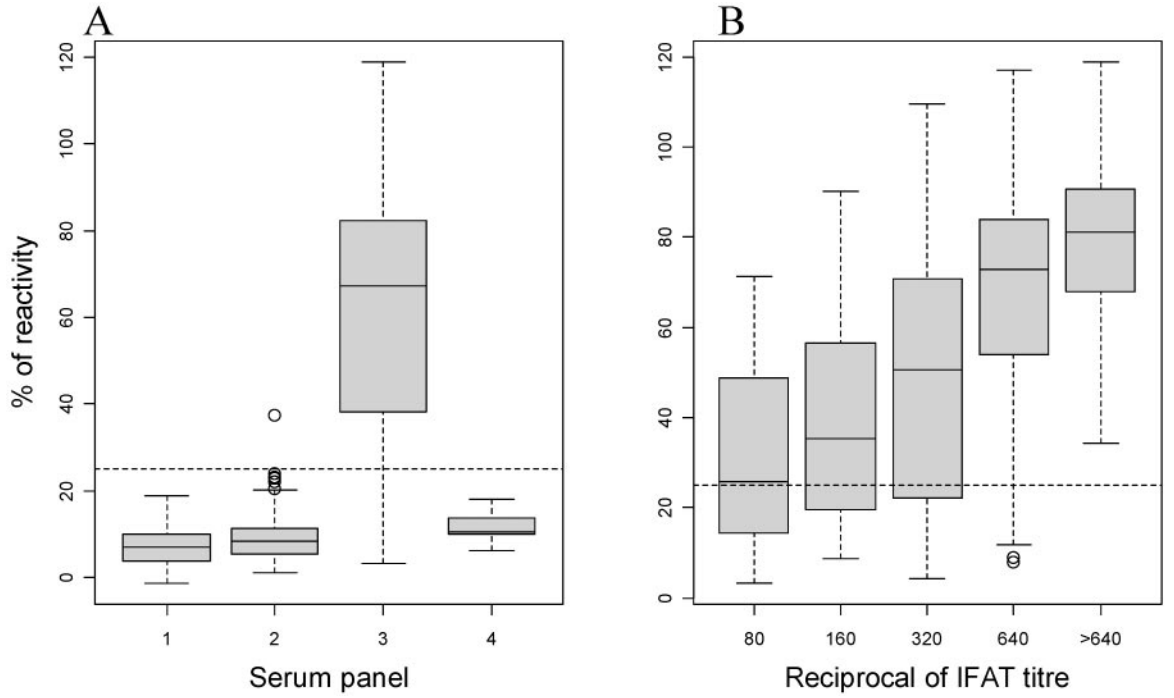

FIG. 3. Human chimeric ELISA. Panel A: boxplots of net absorbance (expressed as percentage of reactivity of the positive reference serum) of healthy blood donors (group 1), immunocompetent patients affected by other pathologies (group 2), immunocompetent patients with confirmed VL (group 3), and HIV-VL-positive/IFAT-negative patients (group 4). Panel B: boxplots of net absorbance of 185 positive patients grouped by their reciprocal IFAT titer.

(n = 19) or having IFAT titer < 40 (n = 343); and 15 were previously classified as IFAT indeterminate (titer = 40).

A subset of human and canine well-characterized sera were preliminary tested at different dilutions to define, for each species, the best signal-to-noise ratio and to evaluate the cutoff value for both human and canine chimeric ELISA. Optimal dilution and cutoff are reported thereafter.

ELISA microplates (Immunomaxi; TPP) were coated, in odd columns, with 25 ng/well of chimeric antigen diluted in water (50 μl/well) and, in even columns, with water as negative antigen. Plates were allowed to dry overnight al 37°C and then blocked with 2.5% casein for 1 h at 37°C. Serum samples were diluted 1/100 (human sera) or 1/40 (dog sera) in phosphate-buffered saline containing 1.25% casein and incubated 1 h at 37°C. After four washes, peroxidase-labeled anti-human or anti-dog immunoglobulin G (Sigma), diluted in the same buffer, was added and the plates were incubated as described above. After a final wash step, the reaction was developed with 100 μl/well ABTS and plates were read at 405 nm after 15 to 25 min. For each serum sample, ELISA net absorbance was obtained by subtracting the absorbance against negative antigen from the absorbance against chimeric antigen and expressed as the percentage of reactivity of the positive reference serum. Cutoff was defined as a reactivity of ≥25% (human chimeric ELISA) or ≥40% (canine chimeric ELISA) of that of the respective positive reference serum enclosed in each plate.

Concordance between chimeric ELISA and IFAT was evaluated using R software and Kappa (k) statistic (19).

Nucleotide sequence accession number. The nucleotide sequence data corresponding to the synthetic construct have been submitted to GenBank database and given the accession number AY944410.

RESULTS

Epitope mapping. Epitope mapping of K26 was based on ELISA evaluation of the most immunoreactive subunit of the K26 repetitive sequence. Five purified peptides and GST were immobilized on the same ELISA plate and probed against dog sera that were found highly reactive to K26 repetitive sequence. A sequence of 19 residues (amino acid sequence ND GDGPKEDGRTQKNDGDG) shared by the most immunoreactive peptides was obtained, and the respective coding sequence was reverse complemented and added at the 5′ terminus in the antisense primer K39-K26a.

K9-K39-K26 chimera production and purification. After the first PCR run, two single bands of the correct length (K9s + K9K39a = 266 bp; K9K39s + K39a = 143 bp) were detected (not shown). In the second PCR step, a 430-bp amplicon was obtained, according to the expected length of synthetic gene *K9-K39-K26* (not shown).

Cloning of the K9-K39-K26 fragment in the pGex-6H expression vector, transformation of the bacterial host, and expression of the fusion protein were successfully achieved. Sequence analysis of the cloned fragment confirmed the correct fusion and orientation of the insert. Bacterial cells, analyzed by SDS-PAGE after induction, showed a new highly expressed band of about 45 kDa, close to the expected size of GST/K9-K39-K26 fusion protein (Fig. 2 lane 1). After glutathione affinity chromatography and protease cleavage (Fig. 2, lane 2), chimeric antigen was further purified by IMAC (Fig. 2, lane 3). Chimeric antigen showed an aberrant migration in SDS-PAGE, with an apparent molecular mass of ~35 kDa instead of the predicted 17 kDa, which has been already reported for the recombinant K9 and K26 antigens (5) and is consistent with dimerization of the protein via disulfide bond. Mass spectrometry analysis showed the presence of two molecular weights (17,416 and 34,827, respectively) corresponding to the monomeric and dimeric form of the chimeric antigen, although the latter form was predominant. The N-terminal sequence of the purified product provided a single sequence corresponding to the chimeric antigen. Yield of chimeric antigen was estimated at ~10 mg/liter bacterial culture. Contamination of

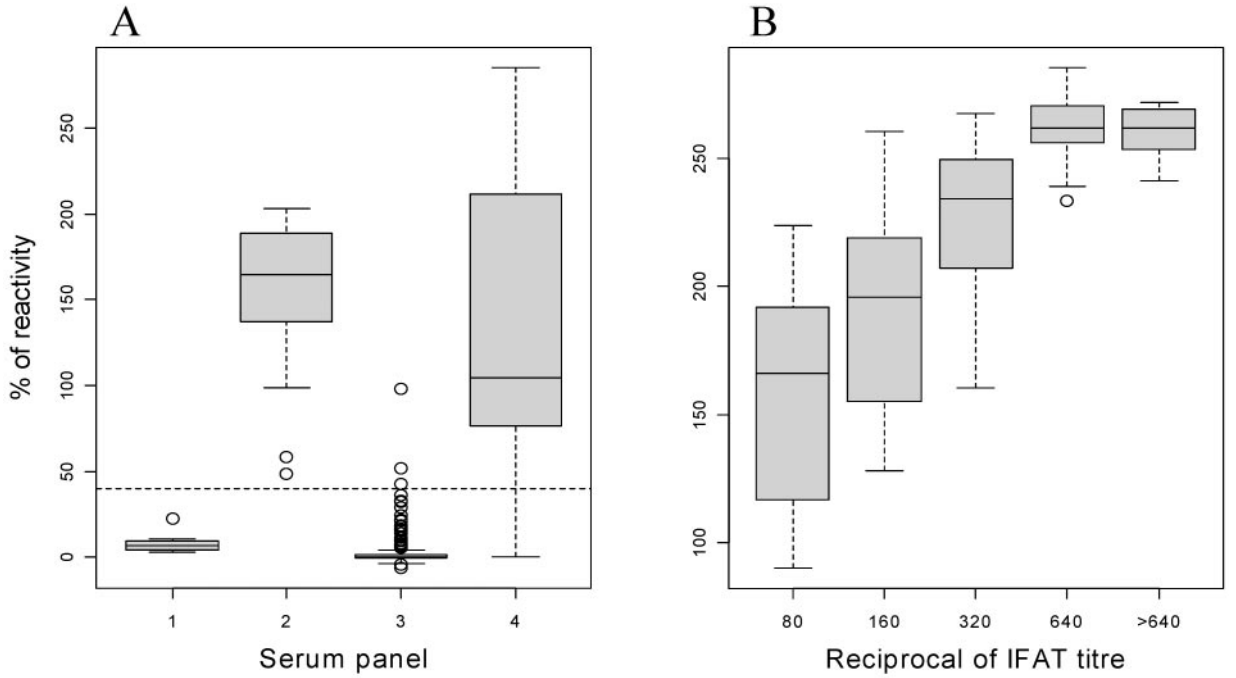

FIG. 4. Canine chimeric ELISA. Panel A: Boxplots of net absorbance (expressed as percentage of reactivity of the positive reference serum) of parasitologically negative (group 1) and positive (group 2) dogs and of IFAT-negative (group 3) and IFAT-positive (group 4) dogs. Panel B: Boxplots of net absorbance of 103 positive dogs grouped by their reciprocal IFAT titers.

copurified bacterial proteins and/or GST was slightly detected after the first step of purification by SDS-PAGE or Western blotting analysis, using a pool of true negative sera, but not after the IMAC step (not shown).

Chimeric ELISA. (i) Human chimeric ELISA. Boxplots of ELISA net absorbances, expressed as percentages of reactivity of the positive reference serum, in negative and positive human sera are shown in Fig. 3A. Among 180 *Leishmania*-negative patients (41 healthy blood donors and 139 patients affected by other pathologies), 179 were negative with chimeric ELISA, including all patients affected by Chagas' disease (specificity 99.4%), while among 185 immunocompetent patients with confirmed VL, 152 were reactive against chimeric ELISA (sensitivity 82.2%). All 19 HIV-positive patients with confirmed VL and having a negative IFAT titer were also negative to chimeric ELISA. Concordance between chimeric ELISA and IFAT was 90.7% ($k = 0.81$, 95% confidence interval = 0.76 to 0.87). Distribution of reactivity in the 185 VL patients with reciprocal IFAT titer is shown in Fig. 3B.

(ii) Canine chimeric ELISA. Boxplots of ELISA net absorbances, expressed as percentages of reactivity of the positive reference serum, in negative and positive dog sera are shown in Fig. 4A, while distribution of reactivity in positive sera with known IFAT titers is shown in Fig. 4B. Among 343 IFAT-negative sera, 340 were negative in recombinant ELISA (specificity 99.1%) while 204 of 213 IFAT-positive sera were also positive by chimeric ELISA (sensitivity 95.8%). Among 15 IFAT-indeterminate sera (titer = 1/40) 4 were ELISA negative (27%) and 11 ELISA positive (73%). The observed concordance between chimeric ELISA and IFAT was 97.8% ($k = 0.95$, 95% confidence interval = 0.93 to 0.98).

DISCUSSION

To date several *Leishmania* antigens have been genetically and antigenically characterized, and recombinant technology has been used for the development of novel immunoassays based on recombinant antigens for serological diagnosis of infections (20, 22, 23, 29, 30). Most of these diagnostic formats employed a single recombinant protein or protein subunit. While specificity of recombinant-based immunoassays is in general high, due to efficient purification by affinity chromatography, sensitivity is usually lower than with crude antigen preparations, such as IFAT, which is based on multiple epitope reactivity. In a recent study (34), we showed that a single 39-aa subunit of K39, the recombinant K9, and the repetitive sequence of K26 carry immunodominant epitopes which can be useful for serological diagnosis of canine leishmaniasis. All three antigens were independent in their antibody reactivity, and higher sensitivities were obtained when they were used in parallel, suggesting that their combination in a single-well test could further improve the performance of the assay. To our knowledge, a single study report was published on the validation of a recombinant multicomponent antigen for serodiagnosis of canine VL (37). The authors described a fusion of recombinant ribosomal proteins LP2a, LP2b, LiP0, and histone H2A, and the resulting ELISA had sensitivity ranging from 79% to 93% and specificity ranging from 96% to 100% depending on the negative control panel used. In our study, the chimeric ELISA had a very good specificity both in human (99%) and in canine (99%) control groups. This may be due to the nature of the *Leishmania* immunodominant epitopes selected; notably, none of 13 sera from Chagas' disease patients

reacted with the antigen, while they did so in the IFAT assay. The double-step purification described in our methods may also explain the low occurrence of false-positive reactions. This procedure was necessary, since we experienced some aspecific background after protease cleavage of fusion protein in immunoblotting, using negative dog sera, which recognized contaminating *E. coli* proteins or eluted GST. Sensitivity of chimeric ELISA was greater in canine VL (96%) than in human VL (82%). Accordingly, concordance between chimeric ELISA and IFAT was greater for canine sera than for human sera. Interestingly, this excellent concordance for canine sera was higher than that obtained in a previous study using the repetitive region of K26 ($k = 0.92$) or K9 and K39sub used in parallel ($k = 0.87$) as ELISA antigens (34).

Concerning human sera, we tested samples from 19 HIV-positive patients (including both HIV-asymptomatic and AIDS individuals) with confirmed VL. None of these sera was positive by chimeric ELISA. As indicated in Materials and Methods, they were selected among those which proved to be nonreactive to IFAT. Our results are not surprising, as about 40% of all HIV-VL patients have no detectable anti-leishmanial antibodies to several serological tests (2, 10, 16), suggesting that during the immunodeficiency stage of HIV infection, only direct methods of diagnosis can be reliable.

In conclusion, a recombinant chimeric antigen expressing three immunoreactive epitopes of *L. infantum* was generated to develop an indirect ELISA for serological diagnosis of canine and human VL infection. The simple strategy employed for epitope fusion will allow us to incorporate any additional sequences which could be available in the future to further increase the test sensitivity.

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