Serological Diagnosis of Canine Visceral Leishmaniasis

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Abstract

Canine Visceral Leishmaniasis (CVL) is an infection of veterinary importance, involving a complex interaction between trypanosomatid protozoa of the *Leishmania donovani* complex, sand fly vectors, environmental conditions that influence the distribution of the vector, dogs that are the major urban reservoirs and humans that are hosts that harbor these parasites. Dogs are a key piece in the epidemiological chain of VL because it has been shown that the high prevalence of canine infection is related to a high risk of disease in humans. Detection of the infection in dogs is essential to proceed to the chemotherapeutic treatment in infected individuals, in epidemiological studies and control programs, for this reason, the implementation of rapid, sensitive and specific diagnostic tests is essential. The diagnosis of CVL commonly uses extracts from the promastigote stage of *Leishmania*, which are complex and heterogeneous mixtures that may have values of low specificity (false positives) and percentages of minimal sensitivity (false negatives). In order to achieve the improvement of diagnostic tests, numerous investigations based on Molecular Biology techniques have been carried out over several years, which have allowed the obtaining of recombinant antigens from *Leishmania donovani* complex cDNA libraries. In this way excellent antigens have been obtained, such as rk39 and rk28, which are very specific, sensitive, obtained in large quantities at a relatively low cost through an easy and fast purification process. The recombinant protein rK39 is a 39 amino acid repetitive immunodominant B cell epitope that is part of a 230 kDa kinesin-related protein expressed predominantly in the amastigotes of viscerotropic *L. chagasi*, has proved to be an exceptionally strong marker of disease, allows the detection of canine VL in asymptomatic animals, as well as in those that present clinical symptoms. The levels of anti-rK39 antibodies are highly correlated with active disease can be used to monitor the effectiveness of chemotherapeutic compounds and clinical monitoring of patients. The incorporation of this antigen in a dipstick format allows the production of a Rapid Diagnostic Test (RDT), easy to use, which provides reliable results, without the need for equipment or specialized personnel, all these advantages allow its implementation in the field; this is very important for handling in rural areas where humans and dogs of low socioeconomic conditions live. Subsequent studios have allowed to express rK9 and rK26, 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. Some evaluations have shown that the sensitivity of the rK26 antigen is only 20% to 40% while rK9 yields 78% sensitivity. The antigenicity of K9, K39, and K26 was determined in multiple-well ELISA using infected dog sera, these antigens showed independent and complementary immunoreactivities, which led to the idea of producing an antigen formed by the fusion of said proteins. Thus to solve these drawbacks, the technological advances of the last few years have been used to develop new generation of recombinant chimeric protein, resulting from fusion of *L. infantum* genes: k9, k39 and k26, denominated rK28 chimeric protein with multiple tandem repeat sequences, increasing antigen epitope density, with a sensitivity of 92% to 100% in Sudan. Due to the multiple advantages of rK28 has been used in the development of RDT, for example, DPP CVL a screening method established by the Brazilian government. this test shows many benefits: i) high levels of sensitivity and specificity for canine VL, ii) shows results in 15 minutes, which allows to evaluate a large number of samples, iii) in association with the confirmatory test EIE CVL give results within 15 days. Their incorporation in the current protocol accelerates the implementation of the control measures in endemic areas endemics. In conclusion, this assay constitute an approximation to the ideal test because it employs a combination of relevant epitopes in a single recombinant antigen in the form of quimera, more specific than crude antigen preparation and more sensitive than single epitope-based ELISA.

Introduction

Canine Visceral Leishmaniasis (CVL) is among the most important vector-borne parasitic diseases of dogs, occurring on all continents except Oceania [1]. It is constitute a neglected disease

worldwide of great importance in public health, what has emerged within non endemic areas, mostly because of transportation of dogs from endemic areas and climatic changes with the expansion of the geographical range of the sand fly vector. It is endemic in over 70 countries, mainly in the Mediterranean region and South America [2]. Many authors have determined prevalence rates ranging from 3.4% to 67% in several regions of Brazil [3,4], the ecological niche it´s shared by reservoirs, humans and vectors in some of these areas, what contributes to the persistence of the infection. Moreno and Alvar concluded that at least 2.5 million dogs are infected in only four countries in southeastern portion of Western Europe, representing 16.7% of the canine population [5]. This infection is caused by species of the complex *Leishmania (Kinetoplastida:Trypanosomatidae)*, *Leishmania infantum* in the Mediterranean area, Middle-East and Asian countries and some regions of Latin America and *Leishmania donovani* is restricted to the (sub) tropics of Asia and Africa, transmitted by the bite of female phlebotomine sand flies and affects domestic, wild animals and humans [6]. Diagnosis is usually carried out for to confirm disease, to investigate the presence of infection for epidemiological studies, for the prevention of transmission from asymptomatic carriers by blood transfusion, to avoid the importation of infected dogs to non-endemic countries, to monitor response to treatment [7] or reduce transmission of infection from asymptomatic dogs to sand flies and humans [8]. The molecular biology techniques have allowed the development of recombinant antigens of high quality, expressed using the metabolic machinery of prokaryotic host, produced in large amounts making standardization processes easier, and eliminating the cross-reactivity due to posttranslational modifications [9]. These proteins show high degree of sensitivity, specificity, the variations between lots is minimal (guaranteeing high reproducibility), the method of purification can be easily optimized, favors large-scale production by adding affinity tags to the proteins [10], allow better standardization of the tests and reduce the costs of elaboration. rK39 and rK26 proteins of *L. chagasi* are among the recombinant antigens with higher values of sensitivity and specificity in CVL serodiagnosis [11,12]. Using rK9, rK39 and rK26 it has developed a recombinant chimera protein called rK28, that takes the advantages of those three antigens to improve the quality of diagnostic tests [13].

Role Epidemiologic of the Canines in the Transmission of CVL

The domestic dogs (*Canis familiaris*) are highly susceptible to VL infection, present a intense cutaneous parasitism, serves as a food source, attracting the vector, which is *Lutzomyia longipalpis* in the Americas, and are considered to be the most important reservoirs for zoonotic VL transmission because of their strong relationship with humans [14]. Routinely, CVL was considered circumscribed to rural areas, but with migration to urban areas the disease has moved to these regions. An example is Brazil, where CVL has been detected in all regions. In fact, in the period from 2006 to 2012, 5010 cases of dogs positive for VL were detected in Mossoro [15]. In Teresina (Brazil) a strong association was found between canine and human infection that is closely related to poverty [16]. These conditions of misery are a difficulty to raise dogs properly and create a problem in the surroundings of large urban centers. In addition, coupled with all this there is the difficulty of early diagnosis, use of inaccurate serological tests [17]. The seroprevalence of CVL in endemics areas in the Mediterranean basin, Latin America and Middle East, including Iran, varies from 10% to 37% [18,19]. The measure of control of the dogs

infected with *Leishmania* is euthanasia, but it has been demonstrated that the elimination of the animals no eradicated canine leishmaniasis [16]. A round of the world numerous large-scale epidemiological studies have been carried out to obtain seroprevalence data, these studies use as a methodological tool the serological evaluation of a large number of samples, this allows to detect the presence of dogs asymptomatic infected [20,21]. Using these type of studies it has been estimated that exist around 2.5 million dogs were infected in southwestern Europe (5 Moreno 2002). South America have millions of dogs infected and it has been demonstrated high percentages of canine infection is observed in some regions of Venezuela and Brazil, which is considered a risk factor of human transmission [22,23]. The dogs play an important role in the transmission cycle of *Leishmania* to humans because constitute the main domestic reservoir of this parasite [5,24], according to the following reasons: a) high susceptibility of the dogs to the parasite, b) may act as sources of infection of the phlebotomine vector, c) in areas where the zoonotic visceral leishmaniasis (ZVL) is endemic, the prevalence of canine infection is high, with a high proportion of asymptomatic animals, d) due to its permanence in the dwelling and in the peridomicilio, favors the maintenance of the transmission cycle; e) they can remain in a carrier condition, without showing clinical signs of LVC for many years, or throughout their life, f) canine cases appear first than human cases [25], g) the high amount of parasites on the skin of dogs which is a very important source of infection for vectors [26,27], h) CVL is crucial from an epidemiological point of view, as it is more prevalent than human VL. For all these reasons it is extremely important to determine the prevalence of visceral leishmaniasis in canines.

Clinical Manifestations of CVL

The dogs that suffer VL can be classified according to its clinical manifestations as follows: asymptomatic, oligosymptomatic, or symptomatic [28]. The asymptomatic are animals serologically and parasitologically positive but they do not present symptoms suggestive of the disease, the oligosymptomatic, positives through methods serologics and parasitologics, with three clinical signs of the disease and symptomatic, what present tests serologic and parasitologic positives together with more than three clinical symptoms of CVL, this dogs usually die, and the clinical manifestations are varied, such as lymphadenopathy, onychogryphosis, cutaneous lesions, alopecia, apathy, vomiting, fever, diarrhoea, polyuria, polydipsia, splenomegaly, and pale mucous membranes [29,30]. Infected dogs can also remain asymptomatic, and even be classified as false-negative in both clinical evaluations and serological trials performed [28]. This is an important problem, since infected dogs (even asymptomatic ones) are important domestic reservoirs of parasites, and can further contribute to transmission between sand flies and humans [20]. This entire scenario indicates that the diagnosis must be made during the initial stages of the disease, through a procedure accurate and effective.

Serodiagnosis of CVL

The diagnosis of CVL constitutes a problem for the following reasons:

a) This infection has a wide range of clinical symptoms, similar to those of other pathologies, which is why it tends to be confused with other diseases (for example, ehrlichiosis).

b) Histopathological studies are costly tests, difficult to implement and need a large investment of money.

c) In endemic regions of low economic resources, detection is particularly difficult because the methods serologicals or moleculars are inaccessible.

d) The existence of asymptomatic animals.

e) The lack of diagnostic kits with high levels of specificity and sensitivity [31].

Serological tests are based on the presence of specific humoral immune responses against proteins of the pathogen, through the detection of immunoglobulin G (IgG). The most commonly employed serological tests for the diagnosis of CVL, including ELISA, indirect Immunofluorescence Test (IFAT), and Direct Agglutination Test (DAT), which using as antigen source whole promastigotes or crude lysate of *Leishmania* [32].

These antigens constitute complex mixtures that present several disadvantages:

a) The results are obtained in large lapses of time, because constitute a laborious technique and therefore, there is a delay in the delivery of results and for this reason, in the administration of treatment or removal of infected dogs of the affected zones.

b) Originate the appearance of cross-reactions (positives false results) with sera from individuals infected with other *Leishmania* species or with other pathogens such as *Toxoplasma gondii*, *Ehrlichia canis* [33,34] and parasites of the Trypanosomatidae family such as *Trypanosoma cruzi* [35,36] or *Trypanosoma caninum* [37,38].

c) There are reports of low reproducibility between batches of ELISA based on whole parasites or crude antigens, since different isolates of *Leishmania* sp. were used and depending on the culturing batch, distinct antigenic compositions can be detected, what results in problems of standardization of the tests [39,40].

d) Origins problems of sensitivity (negatives false) due to the use of specific antigens of strain or morphological stage of *Leishmania* [41].

e) Problems to producing large quantities of antigen in a reproducible manner [42].

The use of tests with low levels of specificity and sensitivity (not very precise) has serious epidemiological consequences: lack of detection of positive dogs (false negatives) which allows to maintain the life cycle of the parasite in endemic areas, and the high rate of detection of false positive dogs causes excessive sacrifice of healthy dogs. The lack of a reliable gold control test for CVL makes it difficult to evaluate the performance of the diagnostic protocol. In fact, although the commonly used gold standard, the culture of *L. infantum*, is highly specific, its low sensitivity makes it difficult to evaluate other diagnostic techniques (13 Fraga 2016). The employment of diagnostic efficient tests is fundamental because to the epidemiological importance of canines in the control of the transmission of visceral leishmaniasis and the need to determine the real impact of the infection in the endemic areas [43,44].

Despite the practicality and simplicity of serological tests, they do not have 100% sensitivity because some dogs, especially those that are resistant or in the early stages of the disease, have negative results. Thus, the results of such tests should be evaluated carefully, always associating test results with epidemiological history, clinical state of the animal, and the result of a more specific diagnostic test

[45]. In addition, since titers of anti-*Leishmania* antibodies remain detectable for long periods, serological tests are not a good alternative for assessing healing or monitoring dogs after treatment [46].

Diagnosis using Recombinants Antigens

The advances in Molecular Biology of the last times have allowed solving many problems in the diagnosis by obtaining quality recombinant antigens in order to achieve the approximation to the ideal antigen. In the diagnosis of CVL, numerous studies with recombinant antigens have been carried out over many years, trying to improve the detection of this disease that constitutes a scourge for canines around the world. Burns et al. [40] identified a kinesin-related gene product, LcKin, as a candidate antigen by screening a Brazilian *L. infantum (L. chagasi)* genomic library with serum of an *L. donovani* patient. It is expressed predominantly by amastigotes and shared by members of the *L. donovani* complex (*L. donovani, L. infantum*). A part of the coding sequence, comprising a 46aa region followed by 6.5aax39aa repeats, forms the recombinant diagnostic protein rK39. Has been observed a high prevalence of antibodies to rK39 in VL patients from different geographically regions, whereas patients with CL or mucocutaneous leishmaniasis remained negative [37]. rK39 provides high sensitivity to clinical cases in ELISA. . In recent multicentre evaluations, the use of the rK39 in a lateral-flow immunochromatographic, rapid test format reported less success in East Africa than in the Indian subcontinent for point-of-care diagnosis of VL [47]. Underlying explanatory factors may reside in molecular divergence between East African *L. donovani* kinesin gene homologues and the Brazilian *L. infantum* (L. chagasi)-derived rK39 sequence, and/or may be due to differential immunocompetence and antibody levels produced among African and Asian human populations [47]. This antigen has been widely applied in a strip test or in an ELISA format and validated for field use in areas endemic for VL [48] or for immigrants from endemic areas with VL [49] but there is little experience on its performance in people from non-endemic areas who got infected abroad. Badaró et al. [50] demonstrated that rK39 seroreactivity correlated with active disease. A serological follow-up of patients with VL after specific treatment revealed a decline of K39 and IFA antibodies in all successfully treated individuals within the first 12 months [51]. In an operational setting, a Rapid Diagnostic Test (RDT) with high sensitivity that would allow for timely in situ diagnosis of infection would thus be invaluable for large scale control of infected dogs [52]. The RDT most widely used are immunochromatographic dipstick tests based on the *Leishmania* rK39 antigen, are ready to use, provide results in few minutes, without needing to use sophisticated equipment, like an Elisometer and require low maintenance in field settings. The overall specificity of rK39 RDTs in dogs was indeed very high (99%), slightly higher than that reported in humans [53]. However, 3 of 11 studies reported much lower specificities of 65% to 85% [54]. Specificity might be expected to be highest in healthy non-endemic controls, compared to healthy endemic controls (a proportion of which may have unapparent infection) or controls infected with other diseases (which may have cross-reacting antibodies). A few false positive rK39 RDT results were reported in dogs with *Ehrlichia canis*, *Trypanosoma cruzi* or *Neospora caninum* infection [55], though few studies tested dogs with potentially cross-reacting infections. rK39 is restricted to species of the visceralizing *L. donovani* complex [37], but cross-reactions have been reported using rK39 ELISA in 3/9 dogs infected with *L. braziliensis* [56], and using the rk39 RDT in two Iranian dogs infected with *L. tropica* [57]. In the current study, the sensitivity of rK39 ELISA was somewhat higher than rK39 RDTs, but much

lower than that of CLA (Crude Leishmanial Antigen) ELISA. These results suggest that low sensitivity to detect asymptomatic infection is largely due to the use of a single defined antigen. The sensitivity of rK39 RDTs to detect infected dogs is too low for their effective use in epidemiological studies or operational control programmes. Further research is needed to develop more sensitive RDTs, using combinations of antigens. rK39 Immunochromatographic Tests (ICT) assay is used as a reference standard for the diagnosis of VL but its inability to discriminate between clinical and subclinical infection in endemic population drew our attention towards novel antigens more specific and sensitive. The high variability in the humoral responses to the different parasite antigens of infected dogs required efficient diagnosis based on purified antigens might require a mixture of recombinants antigens or the use of chimera antigens containing several leishmanial proteins [58].

The detection of asymptomatic *L. infantum* - infected dogs is considered crucial in epidemiological studies, for laboratory diagnosis of the disease [59]. Many other *L. donovani*-specific antigens have been characterized, demonstrating variable specificity and sensitivity and evaluated on symptomatic VL of endemic populations but the diagnostic accuracy of the rK39 antigen is the strongest worldwide. The sensitivity of the rK26 antigen developed from *Leishmania chagasi* is only 20% to 40% in [60]. The rK9 antigen, which possesses 11 copies of a 14 - amino-acid repeat in the open reading frame of K26, yields only 78% sensitivity. Important drawbacks of rK39 in any format are its low sensitivity in Africa, which to some extent has improved with the rapid tests manufactured by DiaMed Cressier sur Morat (Switzerland), and a large proportion (up to 32%) of asymptomatic healthy individuals from an endemic area testing positive. The antigenicity of rK9, of a single 39-amino-acid unit of rK39 (K39sub hereafter), and of the repetitive region of rK26 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 set. 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. Although rK39 is one of the best recombinant antigens to detect visceral leishmaniasis (sensitivity: 67% to 100%; specificity: 93% to 100%), shows a low sensitivity in Africa [61]. Thus to resolve these drawbacks, the technological advances of the last few years have been used to develop new generation of recombinant chimera protein, resulted from fusion of *L. infantum* genes: k9, single repeat units of k39 and k26 [62], what esresinglti-epitope denominated rK28 chimeric protein (rK9 + rK26 + rK39), with multiple tandem repeat sequences, increasing antigen epitope density, this results in an increment of the sensitivity of the test rK28 with improved sensitivity (92% to 100% in Sudan) without any changes of its sensitivity in the Indian subcontinent. rK28 ELISA is also useful in diagnosis of cutaneous VL in Brazil. Another limitation of ELISA is that it can be only done in research labs or well-equipped hospitals and thus cannot be practical at field setting in endemic areas. For this reason, it has been decided to use the rK28 antigen in the development of RDT has shown improved performance in Brazil [13].

DPP CVL is a rapid test based on rK28 that has been adopted as the screening method in a new protocol established by the Brazilian government.

This kit offers several advantages:

a) rK28 provide very high levels of sensitivity and specificity for canine VL.

b) DPP CVL constitutes a rapid test that shows results in 15 minutes, a very important feature when evaluating a large number of samples.

c) DPP CVL in association with the confirmatory test EIE CVL give results within 15 days, in comparison to previous protocol (EIE CVL + IFI CVL) that results were only released after a lengthy time interval that varied from one to two months. Thus, the incorporation of this rapid test into the current protocol accelerates the implementation of the control measures in endemic areas. In addition, this procedure uses only small blood samples and does not require specialized equipment and supplies [63].

In conclusion, the rK28 protein affords higher sensitivity in detecting active VL cases compared to rK39 both on ELISA and RDT format. The rK28 protein is a fusion protein that was designed to improve the diagnosis of CVL. This antigen is more sensitive than rK39 in the determination of the presence of active LV infection in ELISA and RDT. rK28 has become a very powerful tool for diagnosing CVL [64].

Conclusions

The DNA recombinante technology has allowed the development of antigens that are approximated to the ideal, that presented multiple advantages: great sensitivity and specificity, high degree of purity, can be purified in large amounts, obtained easily due to the addition of affinity tags what it favors its purification using columns of affinity and this decreases the costs of production, there is good reproducibility between lots. rK28 is one of the recombinantes proteins that shows favorable and promising results in the diagnosis of CVL.

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