Identification of new antigens for the diagnosis of visceral leishmaniasis

6. Abstract

Visceral leishmaniasis (VL), also known as kala-azar, is a systemic infectious disease with a chronic course, characterized by long feverish periods, weight loss, anemia and changes in internal organs such as the spleen and liver. Such changes lead to a progressive weakness of the individual that can progress to death when not treated early. VL is endemic in several countries and represents a serious public health problem with a high incidence.

In the Americas and southern Europe, VL is zoonotic, with dogs being its main reservoir in urban areas. In these regions, VL is considered a disease of great human and veterinary medical importance. In Brazil, the disease is endemic and is expanding rapidly into municipalities that have not yet diagnosed cases. Control strategies are widely used in order to reduce the incidence of canine disease and consequently the disease in humans. However, despite efforts to control the disease, the incidence of human and canine VL is still high. This fact can be attributed to the low sensitivity of the diagnostic tests used in epidemiological surveys and the length of time between the dogs' diagnosis and treatment. Another critical factor for the permanence of the disease is the fact that in areas endemic for VL, more than 80% of infected dogs are asymptomatic and may not be properly diagnosed by serological tests, due to the low number of antibodies produced by the immune system of the dog host or because it is the initial stage of the disease. Although the clinical disease is not observed in these animals, they are carriers of *Leishmania* and, therefore, are a relevant source of the parasite reservoir. Therefore, serological tests capable of diagnosing early infections in positive dogs, especially asymptomatic ones, remain an important issue for VL control.

Serological methods are important tools in the diagnosis of VL, since this disease is characterized by a large production of host-specific antibodies and the stimulation of polyclonal B cells. Serological techniques, such as the enzyme-linked immunosorbent assay (ELISA) and the rapid immunochromatographic test (ICT) are relatively simple, easy to perform, have quick and low-cost results, and can be performed manually or automated. The ICT still has the advantage of being independent of technology and qualified labor, which makes this test an excellent tool for mass screening, mainly in the field. These techniques allow the use of different types of antigens, such as crude or soluble extract of *Leishmania* ssp. or recombinant proteins. Recombinant molecules with varying sensitivity and specificity have emerged as an alternative to improve the quality of this type of diagnosis. Recombinant molecules are an integral part of the currently available immunological tests for the disease and could form the basis for new diagnostic tools. In any case, it is necessary to find the most sensitive targets to the point of reducing the existing variability of the humoral response of patients or dogs. More molecules that are specific are also needed, that is, molecules composed only of portions that are relevant in the antigen-antibody interaction exclusive for leishmaniasis. This will lead to the development of better and more accurate tests.

Over the years, several recombinant proteins from *L. infantum* and *L. donovani* have been characterized and evaluated, proving to be useful in the discrimination of VL. In this thesis, a new recombinant and synthetic antigen called

KDDR-plus was characterized, which is predominantly based on the repetitive portion found in the kinesin protein of *L. infantum*, being composed of 15.3 repetitions in a sequence of 39 amino acids. Initially, rKDDR-plus was used as an antigenic target in the serological diagnosis of VL, showing promising results in ELISA assays, both in human diagnosis and in the recognition of canine sera. In the serological ELISA assays, using human sera, the sensitivity and specificity of the test was 98% and 100%, respectively, and when evaluating canine sera, the sensitivity was 97% and the specificity 98%, indicating its potential for use in diagnosis VL. Subsequently, the performance of a rapid test was evaluated in a point of care format based on the rKDDR-plus antigen. The rapid lateral flow test (Lateral Flow Test) called ICT/KDDR-plus was evaluated against human and dog sera infected with *Leishmania*. Among the characteristics of the evaluated test, it is noteworthy that it has a low manufacturing cost, easy production on a large scale, relatively long shelf life without the need for refrigeration, the low volume of sample required for execution and obtaining results in a few minutes.

Another promising recombinant protein, characterized and tested in this study, is called Dyn-1, which stood out for its excellent ability to identify dogs with asymptomatic infections. rDyn-1 is a GTP (guanosine triphosphate) binding protein from *L. infantum* (LinJ.29.2310) selected from the ImmunonomeBase database due to its similarity with proteins associated with host defense processes. This protein was able to recognize sera from asymptomatic dogs, as well as from symptomatic ones. In serological ELISA assays, the rDyn-1 protein was able to recognize 100% of dogs considered asymptomatic.

Detailed screenings of the complete sequences of the proteins were carried out to expose the peptides responsible for the most relevant characteristics in both proteins. The peptides present in the rDyn-1 protein responsible for the ability to identify symptomatic, as well as asymptomatic ones, and the peptides present in the rKDDR-plus protein responsible for the ability to discriminate dogs that have *L. infantum* infection without showing cross-reaction with other organisms, were identified. In order to identify potential immunologically competent targets specific selection criteria were defined to identify targets with the highest score. For this purpose, a selection of epitopes of the recombinant Dyn-1 and KDDR-plus proteins was carried out, through screening in immunoblotting assays with pools of canine sera infected with *L. infantum* (asymptomatic and symptomatic) and non-infected (healthy dogs) and dogs infected with *T. cruzi*, *Babesia* sp. or *Ehrlichia* sp. for the selection of potential candidates for VL diagnosis. Thus identifying a peptide mixture composed of amino acid sequences of two recombinant proteins (Dyn-1 and KDDR-plus) with potential application in the diagnosis of CanL.

However the use of recombinant proteins has improved the sensitivity and specificity of the diagnosis. However, a new generation of antigens has been gaining prominence for further improving the diagnostic accuracy of immunological methods. Greater sensitivity and specificity can be achieved using multiepitope antigens, that is, multiple grouped peptide sequences composed of several fragments of more exposed epitopes unique to the molecule of interest. A judicious selection of the central antigenic parts of the native proteins generating short peptide fragments could refine and reduce the variability of the humoral response of patients and dogs. These

molecules still have the advantage of being simpler and cheaper to produce than whole recombinant proteins. In this perspective, the search and improvement of new targets for the diagnosis of VL is the way to quickly and effectively control the disease. Because, tests that present satisfactory sensitivity minimize the number of false negative cases, and tests with high specificity, avoid false positive results. The correct diagnosis allows the immediate initiation of treatment, when indicated, and helps in the epidemiological surveillance of dogs on a larger scale, helping to make clinical and epidemiological decisions, thus promoting better disease control actions. Therefore, from the results obtained so far in this study, it is expected to actually contribute to the control of VL, through the development of more efficient tests, and the encouragement of the production of new technologies not only for VL, but also for other infectious diseases.