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The direct agglutination test as an alternative method for the diagnosis of canine and human visceral leishmaniasis

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Introduction. Visceral leishmaniasis is the most severe clinical form of leishmaniasis and is often fatal without proper treatment. Therefore, early and accurate diagnosis is important, but often difficult in endemic areas.
Objective. The aim was to evaluate a direct agglutination test as a potential visceral leishmaniasis diagnostic method in endemic areas of Venezuela
Materials and methods. The performance of the direct agglutination test, based on freeze-dried *Leishmania donovani* antigen was evaluated under laboratory conditions using serum samples of humans and dogs from several Venezuelan visceral leishmaniasis endemic areas: Nueva Esparta (Margarita Island), Lara, Anzoátegui and Trujillo Status. The study included confirmed visceral leishmaniasis patients (n=30), visceral leishmaniasis suspected subjects (n=4), healthy controls (n=19) and patients with other confirmed diseases (n=20). In addition, 24 serum samples from dogs with confirmed visceral leishmaniasis and 18 healthy control dogs were tested.
Results. All serum samples of visceral leishmaniasis patients, either active or recovered, were positive. They showed anti-*L. donovani* titers above 1:1600. Three out of four suspected visceral leishmaniasis cases were also positive, while serum samples from endemic controls and patients with other diseases had titers lower than 1:800. A sensitivity of 100% was obtained for all threshold levels under consideration and 100% specificity at the threshold titer of 1:800 (95% confidence interval: 91-100%). A 93% sensitivity (95% confidence interval: 76-99%) was observed in dog samples, with 100% specificity (95% confidence interval: 79-100%) at the threshold titer of 1:200.
Conclusion. The direct agglutination test seems suitable for use in epidemiological studies and for serological diagnosis of human visceral leishmaniasis and canine visceral leishmaniasis.
Key words: Leishmaniasis, visceral/diagnosis, agglutination tests, *Leishmania donovani*, humans, dogs, Venezuela.

Evaluación de una prueba de aglutinación directa como método alternativo para el diagnóstico de leishmaniasis visceral canina y humana en Venezuela

Introducción. La leishmaniasis visceral es la forma clínica más grave de la leishmaniasis. Ésta puede ser fatal si no se administra el tratamiento adecuado. Por ello, el diagnostico temprano es importante, pero a menudo difícil, en las áreas endémicas.
Objetivo. Evaluar el potencial de la prueba de aglutinación directa como un método para el diagnóstico de leishmaniasis visceral en zonas endémicas de Venezuela.
Materiales y métodos. La efectividad de la prueba de aglutinación directa con el antígeno congelación-descongelación de *Leishmania donovani* fue evaluada bajo condiciones de laboratorio usando muestras de sueros de humanos y perros provenientes de diferentes regiones endémicas de leishmaniasis visceral de Venezuela: Nueva Esparta (Isla de Margarita), Estados Lara, Anzoátegui y Trujillo. Se incluyeron pacientes con diagnóstico confirmado de leishmaniasis visceral (n=30), sospecha de leishmaniasis visceral (n=4), voluntarios sanos (n=19) y pacientes con otras enfermedades (n=20). Además, se evaluaron 24 muestras de suero de perros con leishmaniasis visceral y 18 controles.

Resultados. Todas las muestras de los pacientes con leishmaniasis visceral activa o curada fueron positivas. Mostraron títulos anti-*L. donovani* por encima de 1:1.600. Tres de cuatro casos con sospecha de leishmaniasis visceral también resultaron positivos a la prueba, mientras que los sueros controles y los de los pacientes con otras patologías dieron títulos por debajo de 1:800. Se obtuvo una sensibilidad de 100% a todos los puntos de corte considerados y una especificidad de 100% al punto de corte de 1:800 (intervalo de confianza de 95%, IC95%: 90,97%-100%). Las muestras de perros mostraron una sensibilidad de 92,59% (IC95%: 75,69%-99,09%) y 100% de especificidad (IC95%: 79,42%-100%) al punto de corte de 1:200.
Conclusión. En general, nuestros resultados indican que el uso de la prueba de aglutinación directa es apropiado para la realización de estudios epidemiológicos y para el diagnóstico serológico de leishmaniasis visceral humana y canina en las áreas rurales de Venezuela.

Palabras clave: leishmaniasis visceral/diagnóstico, pruebas de aglutinación, *Leishmania donovani*, humanos, perros, Venezuela

Visceral leishmaniasis is an infection caused by parasites of the genus *Leishmania*, species *L. infantum* (= *L.chagasi*) and *L. donovani.* These parasites are transmitted by an insect vector pertaining to the genera *Lutzomyia* or *Phlebotomus* (Diptera: Psychodidae) from human to human (anthroponotic transmission) or from an animal reservoir to humans (zoonotic transmission) (1). Visceral leishmaniasis is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia. If left untreated, the fatality rate can be as high as 100% (2). Therefore, early diagnosis is essential.

Approximately 500,000 new cases of human visceral leishmaniasis occur annually, particularly in East Africa, Brazil and India. In America, it is widely distributed, from the northern provinces of Argentina to the United States (3); more than 90% of the human cases are reported from Brazil.

Visceral leishmaniasis is of increasing importance in Venezuela. During the period 1995-2003, 378 cases of human visceral leishmaniasis were reported in widely distributed foci. Seventy percent of these cases occured in children between 0-10 years of age; the 1-2 year-old age group was the most affected (4,5). One of the active areas of visceral leishmaniasis in Venezuela is Margarita Island. A recent study revealed an average infection rate of 21.6% in domestic dogs in eight endemic communities on the island (6).

The diagnosis of human visceral leishmaniasis is difficult. The principal symptoms of it may be indistinguishable from those of other parasitic diseases, such as malaria, schistosomiasis and syndromes associated with liver and spleen enlargement (7). The clinical indicators of visceral leishmaniasis can be confirmed by direct detection of parasites. However, patient sampling is invasive, parasite identification requires skilled microscopy, and parasite isolation is expensive and difficult. Moreover, diagnostic molecular techniques are not readily applicable under field conditions. Because of these limitations, a number of indirect immunological methods have been developed. These include the following: 1) fluorescent antibody test (IFAT) (8,9); 2) ELISA method using whole killed parasites (10,11) or defined leishmanial antigens such as K39 (12); 3) immunochromatography (13), and 4) direct agglutination test (DAT).

DAT is still the first line diagnostic tool in many developing countries (14-16), as it is a simple test with high sensitivity, specificity and reproducibility, easy to perform and does not require specialized equipment (17-19). However, the DAT has not been validated in Venezuela. Therefore, the performance of DAT was evaluated by using freeze-dried *L. donovani* antigen in detecting anti-*Leishmania* antibodies in sera from dogs and VL patients. Serum samples from human patients suffering from other diseases and apparently healthy controls (human and canine samples) were also included in order to determine the sensitivity and specificity of the test.

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Materials and methods

Human serum samples

Serum samples, stored in the serum collection of the "Instituto de Biomedicina, Universidad Central de Venezuela" (Caracas, Venezuela), were used to determine the sensitivity and specificity of the DAT under laboratory conditions. The patient samples were collected in four endemic areas: Nueva Esparta (Margarita Island), Lara, Anzoátegui and Trujillo States. The following groups of samples were used: 1) sera from patients with active clinical and parasitologically proven visceral leishmaniasis (n=26), 2) sera from recovered patients (n=4), 3) serum samples of patients clinically suspected of visceral leishmaniasis (n=4), 4) healthy blood donors from an endemic area who had no history of visceral leishmaniasis and no clinical symptoms of leishmaniasis (n=11), 5) non- endemic area control subjects (n=8), 6) sera from patients with other confirmed diseases (n=20). The latter group included 5 disease categories: Chagas' disease (n=4), leprosy (n=4), dengue (n=4), giardiasis (n=4), and allergies (n=4).

Canine serum samples

Forty-two serum samples were collected in endemic areas, from two different groups: 1) dogs with clinical signs of VL. All these dogs were parasitologically confirmed as VL, (CanVL, n=26), and 2) healthy control dogs (n=16).

Direct agglutination test

The DAT was performed as described previously (14,20,21). Briefly, serum samples were diluted in physiological saline (0.9% NaCl) containing 0.8% β-mercaptoethanol. Two-fold dilutions of the sera were made, starting at a dilution of 1:100 and going up to a maximum serum dilution of 1:102,400. Freeze-dried DAT antigen (stained *L. donovani* promastigotes) produced by KIT Biomedical Research (The Netherlands) was reconstituted with physiological saline according to the manufacturer's instructions (21). Reconstituted (50 µL) antigen solution (concentration of 5×10^7 parasites per mL) was added to each well of the microwell plate containing 50 µL of diluted serum. The results were read visually after 18 hours of incubation at ambient temperature. The end-point titer was taken as the last well where agglutination was seen. This agglutination shows as blue mats or enlarged blue dots in comparison to the compact blue dots present in the negative control wells.

Statistical analysis

The sensitivity and the specificity of the DAT were calculated using the following formulas: Sensitivity = TPx100/(TP+FN) and Specificity = TNx100/(TN+FP), where TN represents true negative, TP true positive, FN false negative and FP false positive (22). Predictive values were calculated using GraphPad INSTAT-3™ software, version 3.02 (GraphPad Software, San Diego, CA), by analysis of 2x2 contingency tables. Sensitivity, specificity and predictive values of the DAT at 4 dilutions (1:100, 1:200, 1:400 and 1:800) were estimated with exact binomial 95% confidence intervals (95% CI).

Ethical aspects

The study was approved by the research ethics committee of the Biomedicine Institute.

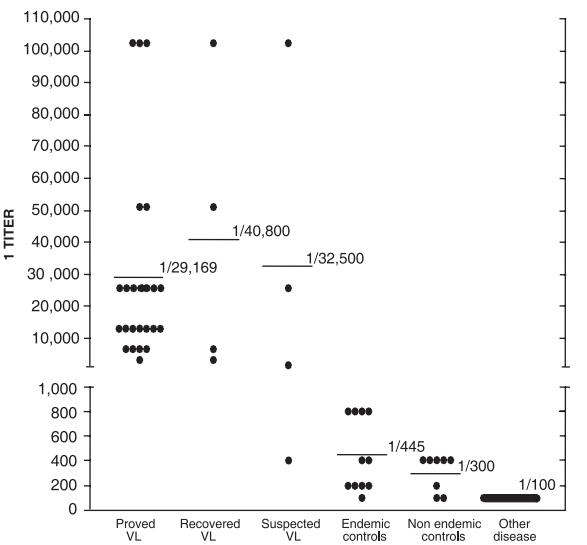

Figure 1. Direct agglutination test (DAT) results for the detection of human anti-*Leishmania* antibodies in various patient and control groups. VL: visceral leishmaniasis, endemic area controls, non-endemic area controls. Cross bars indicate mean titer values for each group.

Results

Human DAT titers

The distribution of DAT titers for human serum samples is shown in figure 1. All serum samples from visceral leishmaniasis patients had titers >1:1,600. All serum samples tested from the recovered cases also showed DAT titers above 1:1,600. Similarly, 3 out of 4 samples from visceral leishmaniasis-suspected individuals showed positive titers (titer>1:1,600), and one sample had a titer of 1:400. Testing of serum samples from endemic controls and patients with other diseases resulted in DAT titers below 1:800.

Sensitivity and specificity

The DAT performance depends on the titer threshold titer that is selected. Table 1 shows sensitivity and specificity calculated with 95% CI for values ranging from 1:100 to 1:800. Sensitivity was 100% (no false results) for all titers considered. The specificity gradually increased across the dilution range to reach 100% specificity at a titer of 1:800 (95% CI: 91.0%-100%). A negative value of 100% was obtained at all dilutions. In contrast, the positive predictive values of 91.0% and 100% were obtained at titers of 1:400 (95% CI: 77.9%-97.41%) and 1:800 (95% CI: 91.0%-100%), respectively.

Canine DAT titers, sensitivity and specificity

The DAT results for canine anti-*Leishmania* antibody are summarized in figure 2. Twenty two out of 26 dogs with visceral leishmaniasis had titers above 1:400, while all canine control titers were 1:200 or lower. However, the sensitivity was variable (Table 2). A 96.2% sensitivity was obtained using

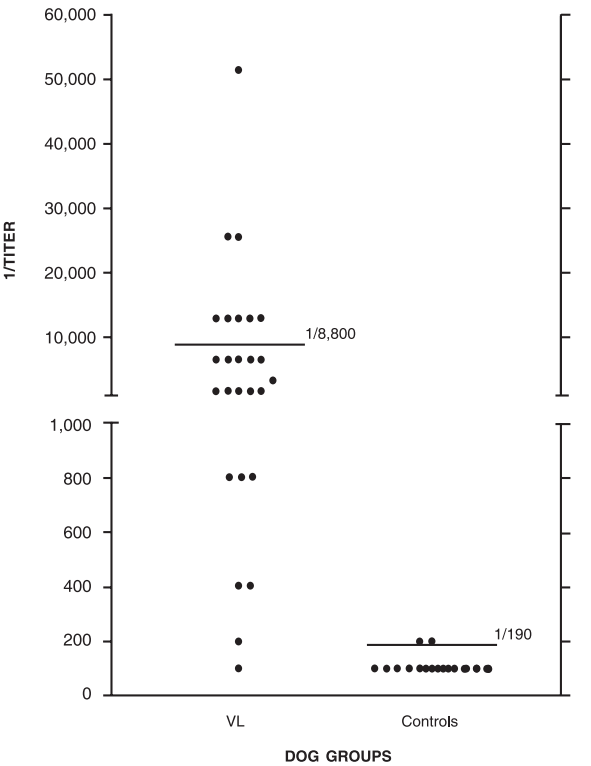

Figure 2. Direct agglutination test (DAT) results for the detection of canine anti-*Leishmania* antibodies in various dog groups. VL: visceral leishmaniasis. Cross bars indicate mean titers for each group.

Table 1. Sensitivity, specificity, positive/negative predictive values, and likelihood ratios of the direct agglutination test (DAT) for human anti-*Leishmania* antibodies at different cut-off titers.

	Cut-off titer			
	1/100	**1/200**	**1/400**	**1/800**
Sensitivity (%)*	100 (90.97-100)	100 (90.97-100)	100 (90.97-100)	100 (90.97-100)
Specificity (%)	58.97 (42.07-74.44)	71.79 (55.09-85.02)	89.74 (75.77-97.14)	100 (90.97-100)
Positive predictive value (%)*	70.91 (57.11-82.39)	78 (94.05-88.49)	90.70 (77.88-97.41)	100 (90.97-100)
Negative predictive value (%)*	100 (85.19-100)	100 (87.66-100)	100 (90.01-100)	100 (90.97-100)
Likelihood ratio*	2.438	3.545	9.750	-

*Values in parentheses are 95% confidence intervals.

Table 2. Sensitivity, specificity, positive/negative predictive values, and likelihood ratios of the direct agglutination test (DAT) for canine anti-*Leishmania* antibodies at different cut-off titers.

	Cut-off titer			
	1/100	**1/200**	**1/400**	**1/800**
Sensitivity (%)*	96.15 (80.38-99.90)	92.59 (75.69-99.09)	84.00 (63.92-95.46)	73.08 (52.19-88.43)
Specificity (%)*	87.50 (61.65-98.45)	100 (79.42-100)	100 (79.42-100)	100 (79.42-100)
Positive predictive value (%)*	92.59 (75.69-99.09)	100 (86.29-100)	100 (83.90-100)	100 (82.37-100)
Negative Predictive Value (%)*	93.33 (68.06-99.83)	88.89 (65.32-98.63)	80 (56.33-94.27)	69.57 (47.10-99.99)
Likelihood ratio	7.69	-	-	-

*Values in parentheses are 95% confidence limits.

a cut-off titer of 1:100 (95% CI: 80.4%-99.9%). Interestingly, the specificity was optimal (100%: 95% CI: 79.4%-100%) at cut-off titers of 1:200, 1:400 and 1:800. Using a threshold titer of 1:200 or higher (1:400 and 1:800), the predictive positive values were 100% (95% CI: 79.4%-100%). The negative predictive value was 88.9% (95% CI: 65.3-98.6) at the threshold titer of 1:200.

Discussion

Visceral leishmaniasis is an important endemic disease in Venezuela. Its incidence has increased in recent years (4). Margarita Island is currently the most active focus. Unfortunately, a high percentage of affected people are children under four years old (5). Although direct detection of the parasite from patient samples is the optimum method for confirmation of visceral leishmaniasis, sampling is very uncomfortable for the patients, especially for young children. In addition, techniques to identify the parasite can be time consuming, expensive and difficult; other indirect methods, such as ELISA, require specialized equipment. These considerations have encouraged an evaluation of the performance of DAT for visceral leishmaniasis serological diagnosis in humans and canines in Venezuela.

The test showed very high sensitivity and specificity for human sera, similar to previous reports (20-23). Positive and negative predictive values of 100% were obtained at a cut-off titer of 1:800. In addition, sera from recovered visceral leishmaniasis patients were positive. This conforms to the results of previous studies, showing high antibody titers to *Leishmania donovani* seven years after healing (14,25). Moreover, similar observations have been reported for recombinant K39-based ELISAs, (5,14) However, these techniques do not discriminate between active infection and past infection. An *L. infantum* infection may be associated with dermal lesions as well in susceptible individuals (children, inmunocompromised patients and others); sympatric transmission has been described (26-28). In the current study, DAT was positive in a patient with diffuse cutaneous leishmaniasis (data not shown). Nevertheless, the clinical manifestations of American cutaneous leishmaniasis have been well characterized and significantly differ from those observed for human visceral leishmaniasis (29). Therefore, using similar conditions, DAT can be used for serological diagnosis of human visceral leishmaniasis in combination with the clinical evaluation of the patients in endemic areas where American cutaneous leishmaniasis and visceral leishmaniasis coexist.

Dogs are important reservoirs in the infectious cycle of *L. infantum* in the Mediterranean basin and in Latin America, including Venezuela (6,30), and probably plays an important role in visceral leishmaniasis transmission. Hence, the presence of seropositive dogs in human dwellings is considered as a possible risk factor for *L. infantum* infection (31-33). Consequently, early and accurate diagnosis of canine leishmaniasis is needed to

aid in the control of human and canine visceral leishmaniasis and to reduce the transmission from the reservoir to humans. As for human visceral leishmaniasis, the diagnosis of canine visceral leishmaniasis is a difficult task because the clinical signs are variable, the histopathology is non-specific and parasites are rarely seen (19).

The DAT was shown to constitute a good alternative for the diagnosis of canine visceral leishmaniasis, especially in that it can detect infection at early stages (34). Using a dilution titer of 1:200, the sensitivity and specificity of DAT were 92.6% (two false negative results) and 100%, respectively. These results were similar to those found in other studies (35-37). Oskam *et al.* (37), in the Old World (37), reported 100% sensitivity and 98.8% specificity (titer threshold of 1:640). Da Silva observed 100% sensitivity and 91% specificity in Belo Horizonte (Minas Gerais, Brazil) (35). In contrast, another study conducted in Para, Brazil showed only 71.4% sensitivity (38). The lower sensitivity of the latter study may have been due to the use of a different antigen or to particular characteristics of the parasites circulating in the endemic area where it was carried out.

Under the current study conditions, the combination of sensitivity and specificity was optimal at 1:800 for humans and 1:200 for dogs. This is considered the recommended thresholds to be used in future studies.

The direct agglutination test (DAT) is recommended as a suitable tool for the sero-diagnosis of human and canine visceral leishmaniasis in Venezuelan endemic areas. It can provide valuable information to the public health authorities in a rapid, timely interval.

Conflict of interests

The authors declare that they have no competing interests.

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