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Articulos Originales

Differential patterns between Leishmaniasis and Chagas disease employing Trypanosoma cruzi epimastigotes.

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Abstract: In various regions of Latin America, T. cruzi and Leishmania infection overlap, so that mixed infections are being reported in circulation, and therefore specific diagnostic tests should be performed to avoid cross-reactions between these two pathologies.
Objective: To determine fluorescence patterns that allow differentiation between Leishmaniasis, Chagas, and mixed infection using T. Cruzi epimastigotes.
Methods: Indirect immunofluorescence technique was used with T. cruzi epimastigotes (autochthonous TcV) as figured antigen against a panel of serum samples coded as A, B, C and D, corresponding to: patients with Leishmaniasis (A), Mixed Leishmania and Chagas infection (B), Chagas disease (C) and without either infection (D).
Results: Different fluorescence intensity patterns at membrane and nucleus level of T. cruzi epimastigotes (autochthonous TcV) were observed in the four sample panels.
Conclusions: Immunofluorescence (IF) with T. cruzi epimastigote antigens has been shown to be useful in the differentiation between Chagas disease, Leishmaniasis and/or mixed infections by both parasites in those areas where the coexistence of both is common.

Keywords: leishmaniasis, chagas, Trypanosoma cruzi, epimastigotes, Fluorescent Antibody Technique Indirect.

Resumen: En diferentes regiones de Latinoamérica la infección por T. cruzi y Leishmania se superponen, por lo cual se reportan infecciones mixtas circulantes, debido a esto; deben realizarse pruebas diagnósticas específicas para evitar reacciones cruzadas entre estas dos patologías.
Objetivo: determinar patrones de fluorescencia que permitan la diferenciación entre Leishmaniasis, enfermedad de Chagas e infección mixta empleando epimastigotes de T. cruzi.
Métodos: se empleó la técnica de Inmunofluorescencia Indirecta utilizando epimastigotes de T. cruzi (TcV autóctono) como antígeno figurado frente a un panel de muestras de suero codificados como A, B, C y D correspondientes a pacientes con infección por: Leishmaniasis (A), Infección mixta por Leishmania y Chagas(B), Enfermedad de Chagas (C) y sin ninguna de las dos infecciones (D).

Resultados: en los cuatro paneles de muestras se observaron diferentes patrones de intensidad de fluorescencia a nivel de membrana y núcleo de los epimastigotes de T. cruzi (TcV autóctono).
Conclusiones: la técnica de Inmunofluorescencia (IFI) con antígenos de epimastigotes de T. cruzi a demostrado utilidad en la diferenciación entre enfermedad de Chagas, Leishmaniasis y/o infecciones mixtas por ambos parásitos en aquellas zonas donde la coexistencia de ambas es habitual.
Palabras clave: leishmaniasis, chagas, Trypanosoma cruzi, epimastigotes, Técnica del Anticuerpo Fluorescente Indirecta.

Introducción

Chagas disease, also called American Trypanosomiasis, is a disease caused by the protozoan parasite Trypanosoma cruzi. The World Health Organisation (WHO) estimates that there are between 6 and 7 million people infected by Trypanosoma cruzi, which causes Chagas disease, in 21 countries of the American continent, meaning that approximately 13% of the Latin American population is at risk of contracting the disease [2]. Bolivia, in this context, suffers one of the highest infection rates of Chagas disease with a seroprevalence of more than 50% [3]. This disease was established almost exclusively in rural areas. Currently, because of population migrations, not only from rural areas but also across continents, there has been a change in the epidemiological profile of Chagas disease [-6]. The World Health Organisation?s report on Leishmaniasis indicates that there are about 1,500,000 people affected by various forms of the disease worldwide. It is estimated that around 350 million people are at risk of infection and illness each [7-9]. In Bolivia, Leishmaniasis is less common than Chagas disease, but it affects people in five of Bolivia?s nine departments [10].

In different regions of Latin America, T.cruzi and Leishmania infection overlap [10-12]. This is the case in some regions of Brazil, the Yungas in Bolivia and northern Argentina, where both infections coexist [10, 14]. Previous reports indicate the existence of mixed circulating infections in both reservoirs and humans, with the presence of overlapping ecological niches of T. cruzi and Leishmaniasis. [10, 14], causing the development of mixed infections, which is why conventional diagnostic tests should be applied with caution as they can be cross-reactive [14]. Hence the importance of performing different serological tests such as ELISA, HAI and specific IF for the diagnosis of the chronic phase of Chagas disease and smear and/or culture for the diagnosis of American tegumentary Leishmaniasis [15]. For the serological diagnosis of Leishmaniasis and Chagas disease, Indirect Immunofluorescence (IIF) was usually performed. This is a relatively low-cost technique due to the fact that the antigenic substrate can be prepared in any medium-complex laboratory [16], furthermore, this technique has a very good diagnostic sensitivity and specificity, particularly for Chagas disease [17]. However, the use of this serological technique for the diagnosis of Leishmaniasis is currently in doubt, as experts do not recommend the use of IF when

Chagas disease and Leishmaniasis are both present [15, 18], especially in tropical and subtropical regions because of possible cross-reactions between the two pathologies at low titres, given that the aetiological agents of these two diseases have a very close common ancestry [19, 20,] therefore, it is to be expected that they share a significant number of antigenic characteristics. Thus, patients with either infection or mixed infection may be misdiagnosed as a result of serological cross-reactions when mixtures of uncharacterised antigens are used [21, 22].

Given the need to differentiate between these two pathologies in a tropical region of the department of Cochabamba, where both pathologies are found, because of the population migration flows in recent years from Chagas endemic areas to tropical regions endemic for Leishmaniasis, this descriptive study was carried out to determine the differential diagnosis of Leishmaniasis, Chagas disease and mixed infection using T. cruzi epimastigotes (autochthonous TcV) following the technique of Luis Vásquez Huerta et al [16].

Materials and methods

Ninety-seven blood serum samples belonging to people with absence of Chagas disease and/or Leishmaniasis infection and people infected with T. cruzi and Leishmania spp. were studied, arranged as follows: Serum samples from patients with Leishmania spp Infection (n=23), Serum samples from patients with mixed Leishmania spp and T. cruzi Infection (n=11), Samples with T. cruzi Infection (n= 30) and Serum samples from patients with no evidence of both infections (n= 33).

Said panels were prepared from samples collected at the immunology laboratory of the LABIMED service and the laboratory of the San Francisco de Asís hospital in the municipality of Villa Tunari.

The serum panels were organised into four groups (A, B, C, D), summarised in table 1, making a total of 97 samples obtained.

Analytical procedures

a. Obtaining T. cruzi epimastigotes

Trypanosoma cruzi (TcV) parasites in their epimastigote form were donated by Dr. MC Torrico, which were obtained from cultures in the Parasitology laboratory of the LABIMED service, Faculty of Medicine, Universidad Mayor de San Simón.

b. Indirect immunofluorescence (IFI)

For the Indirect Immunofluorescence technique (IF), a positive control and a negative control were used. The antigen used was Trypanosome epimastigotes obtained from cultures (Donation), which were fixed on slides and preserved in the freezer. The antigen-antibody reaction was performed on these slides.

In a microtitre plate, 1/16, 1/32 and 1/64 dilutions of each serum to be evaluated and of the controls were made. Once homogenised, these were dispensed in the area of the circle corresponding to the plate. Once the plate was incubated, washed with PBS-Tween and dried, 15 uL of

fluorescein-labelled anti-human IgG conjugate (Biomerieux) was added, incubated for 30 minutes, the slides were washed again and left to dry for the readings.

c. Immunofluorescence microscopy observation

During the observation of the samples, emphasis was placed on the intensity, presence and absence of fluorescence on the surface and nucleus of the epimastigotes. Readings were recorded as: Positive for Chagas(+). When the surface or edges of the parasites fluoresced a deep apple-green colour. Positive for Leishmania. Recorded, when the core of the parasites fluoresced a deep apple-green colour, according to ref16.

Mixed: when the surface and nucleus both fluoresced bright green; Negative (-) If the parasites appear dull and dark and finally Indeterminate (I) If a faint inhomogeneous fluorescence is observed on either the surface or nucleus of the parasites.

d. HAI technique for Chagas

For indirect haemagglutination, the HAI Chagas Polychaco kit which employs ram red blood cells was used. The dilution with which the procedure was started was 1/8, for which in a microtitre plate 70 ?L of the serum diluent was placed in the first wells and 25 ?L of diluent in the second, third and fourth wells. To the first wells, 10 ?L of the Serum to be evaluated were also placed, in addition to the positive and negative control Serum. Se realizó diluciones sucesivas transfiriendo 25 uL de los sueros a evaluar, desde la dilución 1/8 hasta el 1/64, desechando los últimos 25uL. Subsequently, 25uL of the antigen suspension was added to each well. The plate was stirred and allowed to settle for 60 minutes until it was ready for reading.

Statistical analysis

The statistical analysis of the results was carried out using Frequency Distributions. Ethical considerations Permission was obtained from patients for the collection of blood samples for the purpose of this study in both laboratories.

Results

The blood serum panels (Table 1) represent subjects with defined diagnostic characteristics.

The samples included in panel A, corresponding to 23 individuals, were identified as Leishmania spp infection based on clinical and laboratory diagnostic results. The samples in panel B belonged to 11 subjects with mixed infection by Leishmania spp and T. cruzi, based on the laboratory diagnostic results. Likewise, the samples included in panel C corresponded to 30 people with Trypanozoma cruzi infection. Finally, the samples in panel D belonged to 33 people with no evidence of any of the indicated infections.

Table 1. Characteristics of blood serum panels based on laboratory tests performed.

Sample panel	Laboratory tests		Interpretation
	Type of test	Results	
Panel A (n=23)	E.P.D	Positive	Leishmania spp infection
	Diagnostic culture	Positive	
	H.A.I	Negative	
	I.F.I	Negative	
	ELISA	-	
Panel B (n=11)	E.P.D	Positive	Mixed Leishmania spp; and T. cruzi infection
	Diagnostic culture	Positive	
	H.A.I	Positive	
	I.F.I	Positive	
	ELISA	Positive	
Panel C (n=30)	E.P.D	-	T. cruzi infection
	Diagnostic culture	-	
	H.A.I	Positive	
	IF.I	Positive	
	ELISA	Positive	
Panel D (n=33)	E.P.D	-	Free of both infections
	Diagnostic culture	-	
	H.A.I	Negative	
	I.F.I	Negative	
	E.P.D	Negative	

EPD= Direct parasitological examination ; HAI= Iindirect Hemagglutination for Chagas disease;
IFI= Indirect immunofluorescence for Chagas disease; ELISA= Enzyme-linked immunoassay for Chagas disease.

Table 1.
Characteristics of blood serum panels based on laboratory tests performed.

Microscopic observation of the reaction between the T. cruzi epimastigotes used as figured antigen for the immunofluorescence microscopy technique with the serum samples of Panel ?A?, corresponding to persons with Leishmania spp. infection (Figure 1 and Table 2), shows the presence of an exclusively nuclear fluorescence pattern that is visualised as internal fluorescence radiating towards the periphery.

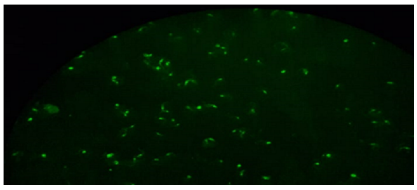

Figure 1
Nuclear fluorescence pattern gives the T. cruzi epimastigotes (used as a figured antigen)a marked fluorescence intensity that is seen as small bulbs with green fluorescent illumination in the centre and irradiation towards the periphery. The nuclear fluorescence pattern corresponds to samples from individuals identified as Leishmania spp. infection (panel A).

Serum sample panels	Fluorecensent pattern							
	Nuclear				Membrane			
	Fluorescence intensity							
	(-)	(+)	(++)	(+++)	(-)	(+)	(++)	(+++)
A (n=23)	-	12(52)	5(22)	6(26)	-	-	-	-
B (n=11)	-	9(82)	1(9)	1(9)	-	9(82)	1(9)	1(9)
C (n=22)	-	-	-	-	-	-	2(9)	20(91)
D (n=33)	25(76)	8(24)	-	-	25 (76)	-	-	-

A= Serum samples from persons infected with Leishmania spp; B= Serum samples from persons with mixed leishmania spp and T. cruzi infection; C= Serum samples from persons with T. cruzi infection; D= Serum samples from people without either of the two infections studied.

Table 2.
Serum samples according to the fluorescence pattern they produce with Trypanosoma cruzi epimastigotes. Data expressed in frequency (%).

Panel ?B? corresponding to individuals identified with mixed infection by Leishmania spp and T. cruzi, observed by fluorescence microscopy, showed a pattern of nuclear and membrane fluorescence with different degrees of intensity (Figure 2 and Table 2).

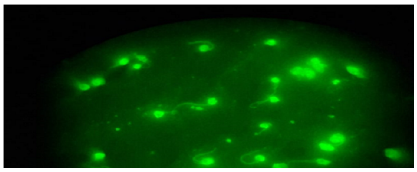

Figure 2.
Core/membrane fluorescence pattern gives the T. cruzi epimastigotes used as a figured antigen a marked fluorescence intensity that is seen as small bulbs with green fluorescent illumination. The core/membrane fluorescence pattern corresponds to samples from individuals identified as coinfected with Leishmania spp and Trypanosoma cruzi. (Panel B).

The behaviour of the samples in panel ?C?, from individuals identified as T. cruzi infection, showed a membrane fluorescence pattern exclusively with intensity of mostly three crosses (Figure 3 and Table 2).

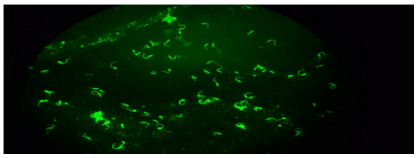

Figure 3.
Membrane fluorescence patterns give the T. cruzi epimastigotes (used as a figured antigen) a peripheral fluorescence intensity that defines the outline of the parasites. The membrane fluorescence pattern corresponds to samples from individuals identified as Trypanosoma cruzi infection. (Panel C).

f panel ?D?, 24% of the samples showed nuclear fluorescence, corresponding to individuals without either of the two infections studied and with permanent residence in the tropical area. (Table 2).

Analysis of samples with no evidence of T cruzi or Leishmania spp infections, grouped according to whether they corresponded to people living in the tropical area (TA) or peri-urban area of the city of Cochabamba (PA), showed absence of membrane (M0) and nuclear (N0) fluorescence in 38% of the TA samples and in 100% of the PA samples. The presence of nuclear fluorescence with intensity one cross (N1) was observed in 62% of the TA samples (Figure 4).

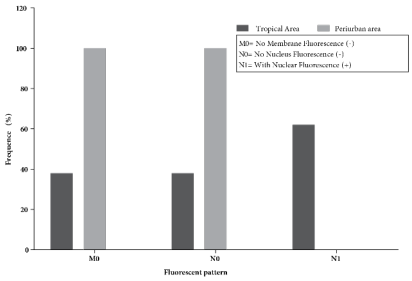

Figure 4

. Frequency of serum samples according to the fluorescence pattern produced by the reaction of fluorochrome-labelled antibodies reacting with Trypanosoma cruzi epimastigotes, from people without evidence of any of the infections evaluated (Leishmania spp and T. cruzi). AT= panel of serum samples from people living in tropical areas (n= 13); AP= panel of serum samples from people living in the peri-urban area of the city of Cochabamba (n=20).

Discussion

Leishmaniasis and Chagas disease are widely distributed in both rural and tropical areas of Latin America. Both are considered by the WHO as tropical diseases of major importance, within the ?Neglected or Forgotten Diseases?23. These two diseases are transmitted by different species of protozoa of the order Kinetoplastidae, [R 22] , 25.

Bolivia has been mainly characterised by high rates of mucocutaneous leishmaniasis (MCL) and cutaneous leishmaniasis (CL)4. The increase in cases of leishmaniasis in recent years is mainly attributed to new human settlements due to population migration, mainly from Chagas disease endemic areas to tropical regionss [5, 6] , which produces changes in the behaviour of the condition due to the coexistence of both pathologies; this is the case in the tropical region of Cochabamba. Hence the importance of detecting both illnesses in areas where they coexist, due to the negative repercussions this could have on the initiation of treatment for leishmaniasis with N-methylglumine antimoniate (Glucantime®), since one of the adverse effects of this first-line drug is cardiotoxicity [26] .

The immunological diagnosis of Leishmaniasis and Chagas disease presents problems regarding cross-reactions with different species of Leishmania spp and Trypanosoma rangeli [10, 12, 27] especially in co-endemic areas, For this reason, conventional immunofluorescence assays frequently encounter this challenge, given the type of antigen used when detecting anti-T.cruzi antibodies in people co-infected with both trypanozomatids. [10, 11, 27, 28] . In this study, in order to determine the differential diagnosis between Leishmaniasis and Chagas disease, T. cruzi epimastigotes were used as antigens14and as shown in Figures 1 and 2, a nuclear fluorescence pattern was observed with the serum corresponding to people with leishmaniasis (panel A), while for mixed infections a nuclear and peripheral fluorescence pattern was observed (panel B); on the other hand, a peripheral pattern, i.e. fluorescence at the membrane level, was observed with the serum corresponding to people diagnosed

with Chagas disease (panel C) (Figure 3), which is consistent with the results of a previous study [14, 29, 30].

On the other hand, when identifying the samples from the tropical area (AT) or the peri-urban area of Cochabamba (AP) without evidence of either of the two conditions (panel D), the presence of nuclear fluorescence with an intensity of one cross (N1) was observed in 8/13 (62%) of the samples from individuals residing in the tropical area (Figure 4), an aspect that was not observed in the serum samples from the residents of the peri-urban area. The presence of fluorescence in this population group from the tropics could be due to the fact that the immune system of the individuals residing in the tropical area promoted both direct and indirect elimination by phagocytosis of the aggressor microorganism, subsequently developing memory antibodies that were detected by immunofluorescence, which could be one of the reasons why these individuals did not present visible clinical lesions. Therefore, factors such as the species and virulence of Leishmania together with the immune and nutritional response of the host could be responsible for these results.

Immunofluorescence (IF) with antigens from T. cruzi epimastigotes has proven useful in differentiating between Chagas disease, Leishmaniasis and/or mixed infections by both parasites in tropical areas where the two coexist as a result of people migrating from Chagas-endemic areas. Therefore, this technique, with emphasis on microscopic observation of immunofluorescence patterns, could be of interest as well as being technically and economically feasible as an alternative to conventional tests. Acknowledgements To Dr. MC Torrico and Lic. Amilcar Flores for their collaboration in the development of this work. Conflict of interest: he authors declare that there is no conflict of interest.

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