INTRODUCTION

Chagas disease is a parasitic infection caused by Trypanosoma cruzi. It is mainly found in Central and South America, where the World Health Organization (WHO) estimates that the population at risk is approximately 60 million people and that 9–12 million people are infected. However, the infection is no longer limited to Latin America, because immigration to North America and Europe has meant that Chagas disease is now a major burden to health services in these areas. In Europe, Spain is the main host country for South American immigrants, many from endemic areas for Chagas disease. In 2008, Spain received over 1,700,000 immigrants from Latin American countries endemic for Chagas disease. Of these immigrants, 5.2% were potentially infected with T. cruzi, and 17,390 may develop Chagas disease. T. cruzi can be non-vector transmitted by vertical transmission, organ transplant, or transfusion of infected blood and its components.

During the acute phase of Chagas disease, parasitological or molecular methods are the main diagnostic tools for detecting T. cruzi in blood samples. However, these approaches are not effective in the chronic and undetermined phases of infection, which are characterized by lower parasitemia and the presence of specific antibodies in blood. Therefore, immunological methods are much more reliable for diagnostic purposes.

Although numerous tests are available for diagnosing Chagas disease, the WHO has recommended carrying out at least two assays in parallel. Consequently, a subject is considered infected when the results of the two serological tests are positive. Several serological assays and antigens have been proposed and evaluated for use as confirmatory or supplementary tests of T. cruzi infection. However, consensus has not been reached to date as regards establishing a reference technique, and no single test is considered the gold standard for unequivocal diagnosis of infection by this parasite.

Although most manufacturers of serological tests for Chagas disease claim sensitivity and specificity levels close to 100%, the occurrence of inconclusive and false-positive results is a recurrent problem, particularly when serological titers are near the cutoff point. This variability between different methods and laboratories is probably caused by the use of different strains of T. cruzi, different antigenic fractions, and different procedures, the immunogenetic capacity of the parasite strain, and the variation in humoral response that each individual develops during infection.

Furthermore, in patients who are coinfected with other pathogens, especially Leishmania, the possibility of cross-reactivity frequently limits the specificity of serological results. This is considered a major drawback for the serological diagnosis of Chagas disease in areas endemic for both T. cruzi and Leishmania spp. The use of recombinant antigens only partially resolves the problem of cross-reactions.

The use of a qualitative method, such as Western blot (WB) analysis, has an advantage over other serological techniques in that it can be used to identify antibodies that recognize different polypeptide fractions in the complex antigenic mixtures of parasite antigens. Hence, it can perform better than other immunological techniques.

Given that no commercial WB test is currently available, we evaluated the performance of a WB method that uses a crude antigen of T. cruzi epimastigote antigen as an alternative approach to confirm T. cruzi infection and detect cross-reactivity with Leishmania.

MATERIAL AND METHODS

Panel of sera. We studied a panel of 137 cryopreserved sera from the Laboratory of Parasitology Collection at the Universitat de Barcelona, Barcelona, Spain, and 10 validated Chagasic sera provided by the Instituto Carlos III (Madrid, Spain). These samples were as follows: patients with chronic Chagas disease (N = 37; cohort I); Spanish patients with visceral leishmaniasis (VL) caused by L. infantum (N = 27; cohort II); Colombian patients with cutaneous leishmaniasis (CL) caused by L. panamensis (N = 28; cohort III); healthy subjects who were seronegative for T. cruzi and Leishmania (N = 55), 28 from a region with endemic Chagas disease and leishmaniasis (Colombia; EA; cohort IV) and 27 from a non-endemic area for Chagas disease and leishmaniasis (NEA; Minorca, the Balearic Islands, Spain; cohort V). The study
was approved by the Ethical Committee of Research of the University of Barcelona (Barcelona, Spain).

The diagnosis of chronic Chagas disease was performed using two enzyme-linked immunosorbent assays (ELISAs): one with commercial recombinant antigens (cutoff according to the manufacturer’s instructions [ratio > 1]; BioELISA Chagas; Biokit S.A., Lliçà d’Amunt, Catalonia, Spain) and the other consisting of an in-house ELISA with crude antigen from \textit{T. cruzi} epimastigotes. The results were quantified in units (U), and the cutoff was established as previously described\textsuperscript{14} at 20 U. Samples were considered positive if the results of both assays were positive.

Diagnosis of VL and CL were confirmed by the detection of parasites in bone marrow or skin tissue by direct smear observation and/or culture. The serology of \textit{Leishmania} was performed by ELISA and WB.\textsuperscript{15,16} Healthy subjects (cohorts IV and V) were seronegative for Chagas disease and leishmaniasis.

\textbf{\textit{T. cruzi} antigen.} The antigen used was a total extract of epimastigotes from \textit{T. cruzi} (Maracay strain) cultured in liver infusion tryptose (LIT) medium with 10% heat-inactivated fetal calf serum at 28°C and collected during the exponential growth phase. Cells were washed three times in phosphate-buffered saline (pH 7.4). They were then counted and adjusted to a concentration of $3 \times 10^6$ epimastigotes/mL in sample buffer (0.5 M Tris·HCl, pH 6.8, 0.01 M ethylenediaminetetraacetic acid [EDTA], 5% sodium dodecyl sulphate [SDS], 5% 2-mercaptoethanol, 0.0125% bromophenol blue) and boiled for 5 minutes. The antigen was stored at −40°C until use.

Antigen electrophoresis was carried out on 0.1% SDS/12% polyacrylamide gels using molecular mass protein standards (standard low range; Bio-Rad) in a Mini-Protean II (Bio-Rad) at 100 V for 1.5 hours, and the sheets were blocked with 1% skimmed milk and 0.2% Tween 20. They were then incubated on the strips for 1.5 hours at 37°C. Bound immunoglobulins were developed by incubation with a 1:1,000 dilution of protein A peroxidase conjugate (Pierce Protein Research Products, US) for 1 hour at room temperature. After three additional washes with TS–0.05% Tween 20 and one wash with TS, the substrate (0.05% H$_2$O$_2$ and 0.3% 4-chloro-1-naphthol in methanol dissolved in TS 500) was added. The color was allowed to develop at room temperature, and the reaction was stopped with tap water after 30 minutes. The presence of bound antibodies was analyzed using the Gel Doc System (Bio-Rad).

\textbf{Statistical analysis.} The Student $t$ test and Pearson correlation coefficient analysis were performed using the PASW Statistics program. Differences were considered significant at $P < 0.05$.

\section*{RESULTS}

In our immunoblot analysis, the 37 sera from Chagasic patients revealed a homogeneous polypeptide pattern in the \textit{T. cruzi} epimastigote antigen in which at least 43 reactive bands were recognized, the molecular masses of which ranged from 16 to 100 kDa. The mean number ($x \pm SD$) of polypeptide fractions detected by sera from patients with Chagas disease was 22.68 ± 3.95. A direct correlation was observed between the number of bands identified in the WB and the ELISA values ($r = 0.85$ for the in-house ELISA with whole epimastigote antigens and $r = 0.82$ for the commercial ELISA with recombinant antigens).

The mean number of bands detected by sera from other cohorts was 12.19 ± 4.51 for the VL group, 6.79 ± 3.29 for the CL group, 1.89 ± 1.73 for healthy subjects from endemic areas, and 1.15 ± 1.19 for healthy subjects from non-endemic areas. As expected, differences in the number of bands detected between sera from patients with Chagas disease and those patients from other cohorts were significant in all cases (Student $t$ test, $P < 0.001$).

A standard pattern of reactivity for sera from patients with Chagas disease was constructed with those bands that were recognized by 100% of sera from cohort I and by less than 100% of sera from the other cohorts.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Proteins & Cohort I Chagas disease ($N = 37$; %) & Cohort II VL ($N = 26$; %) & Cohort III CL ($N = 28$; %) & Cohort IV healthy EA ($N = 28$; %) & Cohort IV healthy NEA ($N = 27$; %) \\
\hline
16 & 81.1 & 30.8 & 0 & 0 & 0 \\
18 & 89.2 & 11.5 & 0 & 0 & 0 \\
20 & 64.9 & 100 & 3.6 & 0 & 0 \\
21 & 5.4 & 100 & 0 & 0 & 0 \\
22 & 83.8 & 3.8 & 0 & 0 & 0 \\
23 & 8.1 & 100 & 0 & 0 & 0 \\
24 & 35.1 & 100 & 0 & 0 & 0 \\
25 & 86.5 & 23.1 & 0 & 0 & 0 \\
26 & 24.3 & 26.9 & 17.9 & 7.1 & 11.1 \\
27 & 91.9 & 7.7 & 0 & 0 & 0 \\
28 & 100 & 23.1 & 3.6 & 7.1 & 0 \\
29 & 30 & 11.5 & 21.4 & 0 & 0 \\
31 & 45.9 & 3.8 & 0 & 0 & 0 \\
32 & 100 & 34.6 & 21.4 & 0 & 0 \\
33 & 21.6 & 23.1 & 0 & 3.6 & 0 \\
34 & 48.6 & 46.2 & 0 & 0 & 0 \\
35 & 0 & 34.6 & 7.1 & 0 & 3.7 \\
36 & 67.6 & 53.8 & 10.7 & 10.7 & 7.4 \\
38 & 100 & 19.2 & 35.7 & 21.4 & 0 \\
39 & 100 & 7.7 & 32.1 & 0 & 0 \\
40 & 100 & 38.5 & 7.1 & 10.7 & 14.8 \\
42 & 2.7 & 7.7 & 7.1 & 3.6 & 7.4 \\
44 & 73 & 57.7 & 3.6 & 14.3 & 0 \\
46 & 5.4 & 100 & 7.1 & 0 & 7.4 \\
48 & 100 & 38.5 & 14.3 & 3.6 & 14.8 \\
52 & 24.3 & 65.4 & 92.9 & 7.1 & 0 \\
56 & 40.5 & 84.6 & 78.6 & 7.1 & 7.4 \\
58 & 0 & 100 & 78.6 & 3.6 & 0 \\
60 & 94.6 & 88.5 & 10.7 & 10.7 & 3.7 \\
62 & 37.8 & 50 & 28.6 & 10.7 & 0 \\
64 & 2.7 & 7.7 & 0 & 0 & 0 \\
66 & 75.7 & 73.1 & 82.1 & 28.6 & 3.7 \\
68 & 5.4 & 11.5 & 0 & 0 & 0 \\
70 & 5.4 & 38.5 & 0 & 3.6 & 0 \\
72 & 51.4 & 23.1 & 10.7 & 14.3 & 11.1 \\
76 & 94.6 & 76.9 & 46.4 & 3.6 & 7.4 \\
80 & 5.4 & 3.8 & 0 & 0 & 0 \\
82 & 10.8 & 7.7 & 7.1 & 3.6 & 0 \\
86 & 89.2 & 84.6 & 3.6 & 3.6 & 7.4 \\
88 & 5.4 & 7.7 & 3.6 & 3.6 & 0 \\
90 & 56.8 & 0 & 0 & 0 & 0 \\
92 & 16.2 & 3.8 & 7.1 & 0 & 0 \\
96 & 100 & 76.9 & 32.1 & 0 & 11.1 \\
98 & 2.7 & 15.4 & 0 & 3.6 & 0 \\
100 & 70.3 & 0 & 0 & 3.6 & 0 \\
\hline
\end{tabular}
\caption{Reactivity of the polypeptide fractions of \textit{T. cruzi} epimastigotes with the sera from patients with Chagas disease and leishmaniasis and healthy subjects.}
\end{table}

\* Percentage of sera that reacts with the antigen fraction.
50% of sera from any other cohort (Table 1). This resulted in a homogeneous standard band pattern consisting of six antigenic bands corresponding to 28, 32, 38, 39, 40, and 48 kDa that were recognized simultaneously for all Chagasic patients, regardless of their ELISA titer values (high, intermediate, or low) (Table 1 and Figure 1). In contrast, sera from patients with VL and CL showed a heterogeneous pattern of reactivity in which distinct antigenic fractions of \textit{T. cruzi} epimastigote antigen were recognized, mainly the polypeptides of 20, 21, 23, 24, 34, 36, 44, 46, 48, 52, 56, 58, 60, 62, 66, 76, 86, and 96 kDa for VL cases and 52, 56, 58, 66, and 76 kDa for CL cases (Figure 2). The number of bands in the established standard pattern of reactivity for Chagas disease that were simultaneously recognized by sera from cohorts II to V was never higher than four (Table 2). Accordingly, a serum may be considered positive for Chagas disease when it reacts simultaneously against at least five antigenic bands of the standard pattern (28, 32, 38, 39, 40, and 48 kDa).

**DISCUSSION**

WB analysis using a range of strains and antigenic preparations has been used to diagnose Chagas disease in Central and South America.\textsuperscript{12,17–23} The main limitation of this technique is that it is difficult to perform interlaboratory comparisons, mainly due to the heterogeneity of the strains and antigenic preparations used. In addition, the size of the slab gels and the concentration of polyacrylamide used as well as the different molecular weight markers all introduce additional variations in band separation and the establishment of respective molecular weights.

Although some slight differences in antigen molecular weights were found when our method was compared with other studies that shared the same source of antigens (crude epimastigote antigens) and similar electrophoretic conditions,\textsuperscript{12,24,25} our results confirm a similar pattern of reactivity in Chagasic sera.

Various criteria of positivity have been used to detect Chagas disease in WB analysis—for example, serum recognition

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**TABLE 2**

<table>
<thead>
<tr>
<th>Number of bands</th>
<th>Cohort II VL</th>
<th>Cohort III CL</th>
<th>Cohort IV healthy EA</th>
<th>Cohort V healthy NEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>Percent</td>
<td>Number</td>
<td>Percent</td>
<td>Number</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>5</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>7.4</td>
<td>1</td>
<td>3.5</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>11.1</td>
<td>3</td>
<td>3.5</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>33.3</td>
<td>11</td>
<td>39.3</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>25.9</td>
<td>3</td>
<td>10.7</td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>22.2</td>
<td>12</td>
<td>7.1</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>28</td>
<td>28</td>
<td>27</td>
</tr>
</tbody>
</table>

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**Figure 1.** Profiles recognized in WB with \textit{T. cruzi} epimastigote antigen by sera from Chagasic patients. Lines 1–13 = high ELISA titers;\textsuperscript{*} lines 14–19 = intermediate ELISA titers;\textsuperscript{†} Patients with inconclusive or borderline ELISA titers;\textsuperscript{‡} Line 20 = Chagas disease was confirmed by PCR; line 21 = patient diagnosed and treated in country of origin 3.5 years earlier; line 22 = a second blood extraction gave positive ELISA results.\textsuperscript{*} High titers: ELISA in house values > 80 U and ELISA BioKit \textit{r} > 4. \textsuperscript{†} Intermediate titers: ELISA in house values between 40 and 80 U and BioKit \textit{r} between 2 and 4. \textsuperscript{‡} Low or borderline titers: ELISA in house between 20 and 40 U and BioKit \textit{r} between 1 and 2. \textsuperscript{\$} Inconclusive when only one of the sera was positive for one of the ELISAs.

**Figure 2.** Profiles recognized in WB with \textit{T. cruzi} epimastigote antigen by sera from (A) patients with visceral leishmaniasis, (B) patients with cutaneous leishmaniasis, and (C) healthy subjects from endemic and non-endemic areas.
of at least 3 bands from a group of 7 bands (14, 19, 27, 30, 34, 37, and 75 kDa) or at least 5 bands from a set of 10 (25, 30, 33, 37, 39, 45, 52, 70, 75, and 92 kDa). Using an excreted–secreted antigen (TESA), it was considered that serum was positive when the 150- to 160-kDa antigen fractions were recognized. Unlike the other criteria mentioned, we found that the homogeneous and repetitive patterns of at least five of six bands of 28, 32, 38, 39, 40, and 48 kDa correlated with Chagas disease.

Antigenic cross-reactivity with Leishmania is a major drawback of conventional serological techniques for the diagnosis of T. cruzi infection. The bands recognized by the sera of patients with Chagas disease and American CL, mucocutaneous leishmaniasis, and VL using homologous and heterologous antigens have been addressed previously, with different results. In our study, individual bands from the standard pattern were recognized by sera from patients with VL or CL, but the general pattern of reactivity of these sera was very different from the pattern of sera from Chagasic patients; in no case did it include more than four bands of those bands included in the standard pattern for Chagas disease.

In summary, our results show that WB is an efficient alternative method for the diagnosis and confirmation of T. cruzi infection when the criteria defined in the present study are applied. This finding is particularly relevant when other serological tests are discordant or borderline. Given that the homogeneous pattern observed in Chagasic sera is easily distinguishable from the heterogeneous pattern shown by leishmanial sera, cases of cross-reactivity with Leishmania can be differentiated using WB.

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REFERENCES


