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Evaluation of two International Reference Standards for antibodies to *Trypanosoma cruzi* in a WHO collaborative study

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Summary. Two freeze dried preparations of defibrinated human plasma containing anti-*Trypanosoma cruzi* antibodies, coded 09/186 and 09/188, were assessed for their suitability as International Reference Standards (IS) for the serodiagnosis of Chagas disease. A WHO collaborative study was undertaken by 24 laboratories from 16 countries. Preparations 09/186 and 09/188 were tested in 30 commercially available assays: enzyme immunoassays (EIA, *n*=16), one chemiluminescent immunoassay, indirect immunofluorescence assays (IFA, *n*=4), indirect hemagglutination assays (IHA, *n*=4), one particle agglutination assay and rapid immunochromatographic assays (RIC, *n*=4). In addition 10 in-house assays were used: EIA (*n*=2), IFA (*n*=5) and western blots (*n*=3). A radioimmunoprecipitation assay (RIPA) was used to characterize prototypes of 09/186 and 09/188 in a pilot study. Both preparations 09/186 and 09/188 were identified as sero-positive in all assays except for one laboratory. Based on the results of the collaborative study, the reactivity in EIA and ChLIA was used as a guide to establish the unitage mL^{-1}. The geometric mean endpoint titre estimated ranged from 2 to 37 for preparation 09/186, and from 3 to 59 for preparation 09/188. For 09/186, the overall Geometric mean (GM) was calculated as: 11 (Geometric Coefficients of variation [GCV] 104.7%; n=29) for assays using native antigen and 9 (GCV 89.4%; n=25) for assays using recombinant antigen. For 09/188, the overall GM was calculated as: 17 (GCV 101.3%; n=29) for assays using native antigen and 16 (GCV 125.1%; n=25) for assays using recombinant antigen.
It is proposed that preparations 09/186 and 09/188 are established as the 1st WHO IS for anti-*Trypanosoma cruzi* antibodies:
- Preparation 09/186, defined as the anti-*T.cruzi* antibody Standard representative of the region where *T.cruzi* II is predominant, with an arbitrary unitage of 1 TcII International Unit mL^{-1} for the undiluted concentration after reconstitution, according to the instructions for use.
- Preparation 09/188, defined as the anti-*T.cruzi* antibody Standard representative of the region where *T.cruzi* I is predominant, with an arbitrary unitage of 1 TcI International Unit mL^{-1} for the undiluted concentration after reconstitution, according to the instructions for use.
These standards , containing 0.5 mL freeze dried plasma, will be distributed together, with an assigned unitage of 0.5 IU per ampoule. The unitage to be associated with the endpoint titre can thus be taken as the reciprocal value of the titre, and this will inform the user of the relative strength of 09/186 and 09/188 in the assay of use.

INTRODUCTION

Chagas disease (Human American trypanosomiasis) is caused by an infection with the protozoan parasite *Trypanosoma cruzi* and was first described in 1909 (1, 2). It is estimated that around 10 million people are currently infected by *T. cruzi* in the world, and more than 10,000 people die from chronic clinical manifestations every year, mainly Chagasic cardiopathy. The endemic area of the disease is Latin America, from Mexico in the north to Argentina and Chile in the south. Nevertheless, during the last twenty years, implementation of large-scale vector control programmes in Central and South American countries, together with mandatory screening of blood donations for anti-*T. cruzi* antibodies, have resulted in an important reduction in the incidence and prevalence of the disease. On the other hand, in the past decades, mainly due to population mobility, Chagas disease cases have been increasingly detected in disease non-endemic countries in the Region of the Americas (Canada and the United States of America), the Western Pacific Region (mainly Australia and Japan) and the European Region (with reported cases in 16 countries) (3, 4).

The Chagas disease vector, a haematophagous triatomine bug, principally found in the Americas, is infected when it sucks blood from infected mammals, including humans. The parasite multiplies in the hindgut of the insect and divides into metacyclic trypomastigotes, a highly infectious form that is transmitted when humans put in contact the contaminated faeces of the vector with any skin break, including the bite, or the eye or oral mucous membranes. In disease-endemic, but also in non-endemic areas, the parasite can be transmitted by ingestion of contaminated food, blood transfusion, congenital transmission, organ transplantation or laboratory accident (5). Chagas disease starts with an acute phase, which lasts for 6–8 weeks, and it follows with a chronic phase that persists for life without successful anti-parasitic treatment and, consequently, without parasitological cure. In most of the acute cases symptoms are absent or mild but can include fever, headache, enlarged lymph glands, pallor, muscle pain, difficulty in breathing, swelling and abdominal or chest pain. In less than 50% of people bitten by a triatomine bug, first visible characteristic signs can be a skin lesion or a purplish swelling of the lids of one eye. During the chronic phase, the parasites are hidden mainly in the heart and digestive muscle. Up to 30% of patients suffer from cardiac disorders and up to 10% suffer from digestive (typically enlargement of the oesophagus or colon), neurological or mixed alterations. In later years the infection can lead to sudden death or heart failure caused by progressive destruction of the heart muscle.

Laboratory diagnosis of the acute phase is based on the microscopic detection of the trypomastigote form of the parasite. Two to four months after the acute phase, most infected individuals enter the chronic phase with no or limited parasitemia, hence the laboratory diagnosis relies on the detection of IgG antibodies against *T. cruzi* (6). The application of biochemical and molecular techniques has revealed great genetic diversity among *T. cruzi* isolates and two major groups have been identified: *T. cruzi* I (TcI) and *T. cruzi* II (TcII) (7, 8). TcI circulates in domestic and sylvatic cycles and is predominantly found north of the Amazon basin (9, 10, 11, 12, 13) and TcII is associated with the domestic cycle and is predominant in Southern Latin American countries (14, 15, 16, 17, 18, 19, 20). In fact, since the first publication on the subject by Carlos Chagas in 1924 (21), several studies have described regional differences both in terms of parasitological, serological and clinical characteristics, together with anti-parasitological treatment response (5, 22, 23, 24). However, confirmation of the *T. cruzi* genotype from infected donors in the chronic phase remains difficult as parasitaemia is usually very low or absent.

Available assay methods for serodiagnosis of Chagas disease are the enzyme immunoassay (EIA) (25), the indirect immunofluorescence assay (IFA) (26, 27), the indirect haemagglutination assay (IHA) and particle agglutination assay (PAG) (28), chemiluminescent immunoassay (ChLIA) (29), and rapid immunochromatographic assay (RIC) (30). Western blot (31) and RIPA (32, 33) are used as supplemental assays. These tests usually employ a mix of antigens, mainly obtained from the epimastigote form of the parasite, but also from the trypomastigote and amastigote forms, either as a lysate, purified proteins, recombinant antigens or synthetic peptides (34). Most commercial tests have been developed with antigens of parasite forms of *T. cruzi* II, in South America.

The first "WHO Consultation on International Biological Reference Preparations for Chagas Diagnostic Tests" was convened by the WHO Programme on Blood Products and Related Biologicals/Quality and Safety: Medicines Team, in July 2007 (18). The objective of the Consultation was to discuss the type of materials needed to support the development of global reference standards for use in the quality control of Chagas disease diagnostic tests, based on the detection of antibodies to *T.cruzi*. During the consultation, the development of International Biological Reference Preparations for antibodies against *T. cruzi* was agreed using samples obtained from disease endemic countries, one from the region where *T. cruzi* I prevails (i.e. Mexico), and another one from the region where *T. cruzi* II prevails (i.e. Brazil). It was considered that the availability of internationally agreed reference preparations would contribute to facilitating the control of the analytical sensitivity of in-house and commercially available tests by manufacturers, regulators, blood establishments, reference and diagnostic laboratories. Furthermore, it would facilitate development of new tests and support harmonization of international regulations.

The suitability of materials selected for the production of two reference standards derived from plasma obtained from healthy blood donors, positive for *T. cruzi*, living in areas where Chagas disease is endemic and who are at a high risk of coming into contact with triatomine insects was discussed at the second "WHO Consultation on International Biological Reference Preparations for Chagas Diagnostic Tests", in January 2009. The composition of the global reference standards, intended use and the design of a WHO collaborative study (CS) to calibrate the proposed preparations were also considered. There was also consensus to use samples of medium reactivity, suitable for the various immunoassay formats.

The CS was designed to validate and characterize the antibody reactivity of the above proposed candidate preparations (subsequently coded 09/186 and 09/188) including both, screening tests and supplementary tests. Efforts were made to ensure that most commercially available serodiagnostic assays for Chagas disease be represented. The primary aims of the CS were to:

1) Characterize the reactivity of the two candidate ISs in IFAs, IHAs, western blots, PAG, RIPA and RIC, currently in use.
2) Assess the suitability of the two freeze-dried preparations 09/186 and 09/188 as candidate International Standards (ISs) for the quality control of EIAs;

The results of the WHO collaborative study are presented in this Report. The list of participants in the collaborative study and at the above Consultations are attached in Appendix 2 and 3.

MATERIALS AND METHODS

Selection of blood donor plasma samples. Plasma samples from blood donors with previous positive serology in anti-*T. cruzi* antibody detection tests were provided by the Hidalgo State Centre of Blood Transfusion, Mexico, and the Fundação Pró-Sangue Hemocentro de São Paulo, Brazil, for the preparation of the candidate reference standards calibrated in this study. Two positive plasma units were selected at the Hidalgo State Centre from male blood donors (43 and 48 years old). The reactivity of these samples was evaluated by IHA, IFA, EIA and WB at the Instituto de Diagnóstico y Referencia Epidemiológicos, Mexico Federal District. Three positive plasma units were collected at the Fundação Pró-Sangue from blood donors original from the following endemic areas: Minas Gerais, Brazil (female, 31 years old); Canela, Chile (male 58 years old) and Pernambuco, Brazil (female, 51 years old). EIA and IFA tests were used for confirmation of the donors as anti-*T.cruzi* positive together with evidence of high risk of *T. cruzi* infection in a questionnaire to assess the epidemiological profile and autochthonous endemic origin. Plasma samples obtained from Mexico were considered to represent the TcI predominant region and plasma samples obtained in Brazil and Chile the TcII predominant region. Due consent was obtained from all blood donors.

Efforts to isolate the parasite from blood donor samples were unsuccessful to date and hence the *T. cruzi* genotype could not be confirmed. This difficulty to isolate the parasite in hemoculture was expected given the very low or absent parasitemia in the chronic phase of the Chagas disease.
Leishmania chagasi is known to be the most important agent that can cause cross reaction with anti-*T.cruzi* assays (35, 36). Donor samples and the two seropositive pools of defibrinated human plasma were tested negative by RIC IT-LEISH® (DiaMed).

Preparation and characterization of the candidate bulk materials. All plasma units were defibrinated at the Fundação Pró-Sangue by adding 0.5 mL of a 200 mM $CaCl_2$ solution to a 100 mL unit of plasma. The mixture was incubated at 37°C for 2 hours and for 24 hours at 4°C and the fibrin clot was removed by centrifugation at 6000 *g* for 30 min. $CaCl_2$ was removed from the supernatant by dialysis using a cellulose membrane with a cutoff of 12 kDa (Sigma, Germany). The defibrinated plasma was filtered through a 5 µm pore size membrane (Sigma) to remove residual fibrin particles. As a preservative, Bronidox (5-Bromo-5-nitro-1,3-dioxane, Henkel Chemicals, Germany) was added to a final concentration of 0.05%.

Two seropositive pools of defibrinated human plasma, representative of the latent (chronic) stage of infection with *T. cruzi* in Brazil and Mexico were prepared. The candidate material representing the TcI predominant region was constituted of 200 mL of defibrinated plasma from each of the two selected Mexican donations and diluted with two units of 200 mL of negative defibrinated plasma each from the TcI predominant region to prepare a total volume of 800 mL. The candidate material representing the TcII predominant region was prepared by mixing 80 mL, 120 mL and 60 mL of defibrinated plasma selected from two Brazilian and one Chilean donation. The 260 mL pool of positive units was diluted in 1,240 mL of negative defibrinated plasma originated from seven seronegative Brazilian donors to obtain a total volume of 1,500 mL.

Both candidate bulk materials ("TcI" and "TcII") were tested with 3 different EIAs and an IFA for confirmation of positive reactivity for detection of anti-*T.cruzi* antibodies. In order to know whether lyophilisation could affect antibody concentration titres, a pilot study was also carried out.

Approximately 50 mL of each of the “TcI” and “TcII” bulk materials were freeze-dried (FD) at the National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK, and tested in a pilot study to assure that the antibody concentration in each preparation was in an appropriate range for multiple assay types and multiple laboratories and to ensure the reactivity of the original native liquid materials would remain after freeze drying and reconstitution.

Sets of eight vials (two FD and two liquid for each of the two preparations) were distributed to 5 laboratories: 3 using EIA, IHA and IFA, one using TESA blot and one using RIPA. The results were reported to the NIBSC who analysed the results. The reactivity of the liquid candidate bulk materials is shown in Table 1. The antibody potency of the reconstituted FD preparations relative to liquid prior to FD, shown in Table 2 was characterized by EIA (n=4), IHA (n=3), one PAG and IFA (n=2), which did not reveal loss of potency after FD. The reactivity of the preparations, either in their native liquid form or as reconstituted FD preparations, was also characterized in TESA blot and RIPA. Results are presented in Table 3, and Figs 1 and 2. In TESA blot, freeze-drying resulted in slightly lower band intensities for the TcII bulk material diluted 1:800 - 1:1600 (Fig. 1). Reactivity in TESA blot was observed with bands of a MW of 150-160kDa and with bands of 95 kDa. In TESA blots of the TcI bulk material, freeze drying had no effect on band intensity (Fig. 1). This TcII bulk material was tested in RIPA either in its liquid form or as a reconstituted FD preparation (Fig. 2). Both preparations reacted strongly with the 72 and 90 kDa antigens, even up to 1:100 dilution. Reactivity to these bands is considered to be the primary readout for a positive reaction. Counts per minute (CPM) were also determined prior to PAGE and subsequent autoradiography. The CPM for the liquid preparation and the reconstituted FD preparation is given in Table 3. CPM level remained stable after freeze drying and correlated well with the intensity of the bands observed on the autoradiograph shown in Fig. 2. Based on autoradiographs and CPM, the reconstituted lyophilized "TcII" sample appears virtually identical to the native liquid preparation. Any quantitative differences in CPM reflect normal sample variations seen in RIPA. The ”TcI” bulk material was tested in RIPA either in its native liquid form or as a reconstituted FD sample (Fig. 2). Based on autoradiographs and CPM, the reconstituted lyophilized "TcI" sample appeared virtually identical to the native sample (as was the case for the ”TcII” material) and quantitative differences in CPM reflect normal variation seen in RIPA. Taken together, these results supported a decision to formulate the proposed candidate materials as freeze dried preparations.

Preparation of the proposed reference standard 09/186. Approximately 1.4 L of the defibrinated plasma pool representing the *T. cruzi* II region were sent to the NIBSC for filling and freeze-drying. The pool was thawed and dispensed in 0.5 mL aliquots into glass ampoules, coded 09/186, and a total of 2181 ampoules were produced.

The mean fill weight for 93 ampoules was 0.5152 g (coefficient of variation (CV) of 0.20%). On the same day, freeze-drying under vacuum was started and completed after four days. Ampoules were back filled with pure N_2 (moisture content <10 ppm). Residual moisture measured by the Karl-Fischer method for 6 ampoules was 0.04% (CV of 13.87%). Sixty eight ampoules were rejected during the production process, 40 ampoules were held for accelerated degradation studies and 1962 ampoules were stored at -20^{o}C.

Preparation of the proposed reference standard 09/188. Approximately 0.8 L of defibrinated plasma pool representing the *T. cruzi* I region were sent to NIBSC for filling and freeze-drying. The pool was thawed and dispensed in 0.5 mL aliquots into glass ampoules coded 09/188, and 1307 ampoules were produced.

The mean fill weight for 52 ampoules was 0.5156 g (CV of 0.41%). On the same day, freeze-drying under vacuum was started and completed after four days. Ampoules were back filled with pure N_2 (moisture content <10 ppm). Residual moisture measured by the Karl-Fischer method for 6 ampoules was 0.09% (CV of 8.28%). Thirty three ampoules were rejected during the production process, 40 ampoules were held for accelerated degradation studies and 1168 ampoules were stored at -20^{o}C.

Description of study samples. Each participating laboratory received two sets of samples comprising seven coded ampoules (A, B, C, D, E, F and H). Sample G was retracted during the study. A brief description of each sample, the study codes, batch codes and the reactivity in various immunoassays prior to freeze-drying are given in Table 1. Duplicates of 09/186 (A and E) and 01/576 (B and F) were included in the sets to provide an independent measure of within-laboratory variability. All samples tested negative for antibodies to HIV 1/2 and Hepatitis C, and Hepatitis B surface antigen. Ethical approval was obtained from the Human Materials Advisory Committee at NIBSC approving the processing of the candidate reference standards (09-002SR). Finally, all study samples were distributed as FD preparations on dry ice by courier to the participants of the CS. Additional samples of 09/186 and 09/188 were used to analyse the stability of the preparations by two participant laboratories.

Serodiagnostic tests. The tests used by the participants are summarized in Table 4. They were assigned a code in roman numbers, which is used to present results without disclosing their identity in the study. The participants were asked to analyse the samples with tests they currently use for blood screening, investigation or serodiagnosis. A total of 42 different tests were used. Thirty tests are commercially available: EIA (*n*=16), IFA (*n*=4), IHA (*n*=4), RIC (*n*=4), one PAG and one ChLIA. Some tests were marketed under different trade names in different countries in which case they were grouped together for the analysis of the results. Eleven in-house tests were used: EIA (*n*=2), IFA (*n*=5), western blots (*n*=3) and one RIPA. The RIPA, which has a qualitative and a quantitative aspect, was used only used in the pilot study to confirm results of screening tests such as IFA, HAI, PAG and EIA.

Two types of methods were distinguished: titration methods (IFA, IHA and PAG) and EIAs including ChLIA. Titration methods yield an endpoint titre and participating laboratories that use these were requested to perform four independent runs for each method. Since EIAs and ChLIA produce a numerical response such as an absorbance or a fluorescence value, participating laboratories were requested to report the results of six sequential doubling dilutions for each sample. The antigenic composition for recombinant EIAs, ChLIA, native EIAs, IHA, PAG and IFA tests included in the study is given in Table 5.

Study Protocol. The protocol distributed to the study participants is attached in Appendix 4. Data sheets were provided to ensure that all required information was recorded. Instructions for the reconstitution and testing of samples were also provided. Participants were informed that the EIAs would be the primary tests for the calibration of the candidate standards and were requested to prepare six sequential dilutions (2x, 4x, 8x, 16x, 32x and 64x) of the reconstituted samples with the diluent

provided by each test manufacturer. The participants were requested to test the samples on at least two different days. Two fresh sets of dilutions from the same sample were run on each of the two days so that four data points were generated for each sample and respective sequential dilutions.

IFA, IHA and PAG methods were performed following the manufacturer's sample dilution procedure. Each sample was tested four times on at least two separate days (2 assays on day 1 and two assays on day 2). The participating laboratories reported IHAs and IFAs titres as the reciprocal of the highest sample doubling dilutions that were positive, performed according to the test kit manufacturer's procedure.

Participating laboratories that used commercially available tests followed procedures described by the manufacturer while laboratories using in-house tests followed standard procedures.

Participating laboratories and assay codification. Twenty-four participants from 16 countries, representing national reference laboratories (*n*=10), research laboratories (*n*=5), blood establishments laboratories (*n*=5), diagnostic laboratories (*n*=3) and one national regulatory authority took part in the collaborative study. The participating laboratories were from the following countries: Argentina (*n*=2), Bolivia, Brazil (*n*=5), Chile, Costa Rica, Ecuador, France, Honduras, Japan, México (*n*=2), Panamá, Paraguay, Spain, Switzerland, United Kingdom and United States of America (*n*=3). A full list of participants and their affiliation is given in Appendix 2. One laboratory did not participate in the CS but performed the supplemental RIPA test in the pilot study.

The participants returned raw data to NIBSC for analysis together with a description of the methods used and the manufacturer's instructions. Throughout the study, participating laboratories were identified by a randomly assigned code number to maintain confidentiality, followed by a letter representing the number of the assay performed (e.g. 10c means laboratory 10; 3rd assay). The same letter does not correspond to the same assay among the laboratories (e.g. 10c and 9c mean assay 3 for both laboratories but would not necessarily be the same test).

Data analysis. For the EIA methods, endpoint titre estimates were calculated by linear regression using the $\log_{10}$-transformed optical densities and cut-off values supplied by the participants. These have also been expressed in mIU mL^{-1} taking the potency of each candidate standard to be 1 IU mL^{-1}. The endpoint titre is defined as the reciprocal of the highest dilution of a sample that gives a reading above the cut-off.