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Field Evaluation of a Rapid Immunochromatographic Assay for Detection of *Trypanosoma cruzi* Infection by Use of Whole Blood[▽]

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Laboratory and clinical diagnostic classification of seropositive individuals, followed by treatment and supportive therapy, is an established component of Chagas' disease control in areas where this disease is endemic. However, most Chagas' disease patients live in remote areas where neither equipped laboratories nor skilled human resources are widely available. Employing a rapid diagnostic test (RDT), when using whole blood samples, is the best option for Chagas' disease control. A high sensitivity and specificity for the Chagas Stat-Pak RDT (Chembio Diagnostic Systems, Inc., Medford, NY) has been reported for assays using serum and plasma, but its validity for the detection of antibodies to *Trypanosoma cruzi* infection in whole blood is unknown. This cross-sectional study measured the sensitivity and specificity of the Chagas Stat-Pak with whole blood, using conventional serological assays for comparison. The interobserver reliability in the interpretation of the Chagas Stat-Pak results and "ease-of-use" criterion needed to perform the Chagas Stat-Pak and conventional assays were also measured. The Chagas Stat-Pak yielded a high specificity (99.0%, 95% confidence interval [CI] = 98.4 to 99.4%) but a relatively low sensitivity (93.4%, 95% CI = 87.4 to 97.1%). The interobserver reliability was excellent (κ [$n = 1,913$] = 0.999, $P < 0.0001$), and the quantified ease-of-use criterion suggested that the RDT is simple to perform. Despite the attributes of the Chagas Stat-Pak, it is not an ideal diagnostic test for the population investigated in the present study due to its relatively low sensitivity and high cost. The RDT manufacturer is called upon to improve the test if the international community hopes to make progress in controlling Chagas infections in areas where this disease is endemic.

Chagas' disease, or American trypanosomiasis, an endemic disease from Mexico to southern Argentina, is caused by the *Trypanosoma cruzi* parasite. An estimated 13 million people are infected worldwide; of those, more than 14,000 die each year (24). Transmission of the disease to mammalian hosts occurs by infected feces of a blood-sucking triatomine insect through a break in the skin or through conjunctive or oral mucosa. Transmission through blood transfusion and pregnancy and delivery are also possible and, less frequently, transmission occurs via organ transplantation and laboratory accident (12). Oral transmission, especially in specific areas such as the Amazon Basin, has been reported (1).

Chagas' disease causes serious illness in humans. Many people become infected during childhood or adolescence. Usually, the early stage of infection is not severe. However, death during the early stage may occur, particularly in infants and the immunosuppressed. For about one-third of the persons who contract Chagas' disease, cardiac, digestive, and neurological sequelae may develop 10 to 20 years after the initial infection (5). Based upon a synthesis of prior studies, individuals developing these chronic symptoms may expect a decrease in life expectancy by an average of 9 years (5, 13–16, 21).

Bolivia reports one of the highest Chagas infection rates in

Latin America, with an area of endemicity covering 80% of its territory. In the 1980s it was estimated that 1 to 2 million people, or 22% of the Bolivian population, were infected with *T. cruzi* (23). Among recent Chagas' disease studies in Bolivia, Albarracin-Veizaga et al. (2) reported a seroprevalence of anti-*Trypanosoma cruzi* immunoglobulin G antibodies in 12.5% of individuals aged 10 years or older in a periurban area of Cochabamba municipality. Breniere et al. (4) reported a seroprevalence of 43.3% in a rural population within the Cochabamba Department, with a 21% prevalence of abnormal electrocardiograms. Médecins Sans Frontières (MSF), based upon the prevalence of Chagasic infections among young children and adolescents in the region of Sucre, Bolivia, to be ca. 4.0% (internal MSF project reports 2004 to 2007 [unpublished data]).

Laboratory and clinical diagnostic classification of seropositive individuals, particularly during childhood and adolescence, is paramount to providing effective treatment and supportive therapy. In Bolivia, under the direction of the Bolivian National Chagas Programme, the conventional serological assays for diagnostic classification of *T. cruzi* infection are the conventional enzyme-linked immunosorbent assay (ELISA), the indirect hemagglutination assay (IHA) and as a confirmation assay, the recombinant ELISA. Diagnosis with these conventional assays is routinely conducted in laboratories based in large urban centers.

However, most Chagas' disease patients live in periurban

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and rural areas where neither equipped laboratories nor skilled human resources are widely available. Specific constraints in remote areas result in delays, losses to patient follow-up, and high healthcare costs. These include complex diagnostic protocols requiring at least two patient visits to the health center or laboratory, lack of or difficulty in accessing health centers, inadequately equipped laboratories (no electricity, refrigeration, or conventional serological assays), lack of skilled human resources, and low diagnostic confirmation processing speed. Moreover, conventional assays when used in remote areas may lose their published high sensitivity and specificity achieved when conducted under reference laboratory conditions (3).

The Chagas Stat-Pak (Chembio Diagnostic Systems, Inc., Medford, NY) is a rapid immunochromatographic assay for detection of antibody to *T. cruzi*. According to the manufacturer, this simple (one-step) rapid diagnostic test (RDT) can be conducted with serum, plasma, or whole blood samples and is performed in precisely 15 min. Neither refrigeration, nor a laboratory structure, nor specialized human skills are required for the performance of this test. In addition, in dry and constant room temperatures specimens can be stored for extended periods of time for result checking and documentation (10).

The high sensitivity and specificity of the Chagas Stat-Pak RDT, when assayed with serum and plasma, is well established in previous studies (10, 11). In a blinded study (10), the Chagas Stat-Pak RDT identified infected and uninfected individuals with a sensitivity of 98.5% and a specificity of 94.8%. The study also independently evaluated serum from four Latin American countries and found a sensitivity of 100% and a specificity of 98.6%. A third set of tests comparing sera with plasma and eluates from filter paper yielded results identical to those obtained with serum. The performance of the Chagas Stat-Pak RDT was also compared to a standard ELISA diagnostic assay in the serodiagnosis of Chagas' disease in Central America (11). Of 3,400 blood donor samples, 4.6% were positive in both assays. Three sera of 2,084 samples from reference laboratories were negative with the RDT but positive with the ELISA (99.8% agreement). Agreement of 100% between the two tests was observed with 339 additional sera from patients with cardiopathies and 175 sera from potential blood donors in emergency surgical cases. The Chagas Stat-Pak showed 99.6% and 99.9% sensitivity and specificity, respectively, for assays using 5,998 serum samples.

Rationale and objectives. The above-cited studies demonstrate the validity of Chagas Stat-Pak RDT for the detection of antibodies to *T. cruzi* in serum and plasma, but the sensitivity and specificity of the RDT when whole blood is tested is unknown. A highly sensitive and specific RDT used for diagnostic or screening procedures, performed using whole blood by trained nonlaboratory workers, would circumvent constraints associated with conventional serological assays and bolster Chagas' disease control efforts. To our knowledge, the present study is the first attempt to validate the performance of the Chagas Stat-Pak RDT under field conditions using whole-blood samples.

This cross-sectional study describes the demographic characteristics of the study population; evaluates the ability of the Chagas Stat-Pak RDT, when using whole blood, to correctly classify individuals as either positive or negative for Chagas

infection, using conventional serological assays for comparison; measures interobserver reliability in the interpretation of the Chagas Stat-Pak RDT results; and compares the "ease-of-use" criterion needed to perform the Chagas Stat-Pak RDT with the ease-of-use criterion of the conventional assays.

MATERIALS AND METHODS

Study location and participants. The present study was conducted in the Valle Hermoso district located within the city of Sucre, Bolivia. The district has an approximate population of 46,570 persons at an elevation of 2,790 m and is located approximately 710 km southwest of the capital, La Paz. The study sampling frame consisted of individuals aged 9 months to 17.9 years. The district has an estimated population of 19,120 persons within this target age group.

Two community sensitization teams, consisting of two health workers each, were sent to the Valle Hermoso district to notify the populace about the study and recruit eligible individuals. Each team visited all community centers, schools, and other groups in their designated area and went door-to-door to inform the community about the study. In addition, all primary health facilities were requested to send potentially eligible individuals for screening.

Children or adolescents were eligible to participate if they met the age criteria, resided within Valle Hermoso district, and the parent or guardian who accompanied them to an MSF-sponsored health clinic signed an informed consent. No eligible individuals who agreed to participate were excluded from the study. The enrollment of study participants began on 18 April 2007 and ended on 12 July 2007, a duration of 12 weeks.

Ethics approval and data analysis. This study was approved by the MSF International Ethics Review Board, the Ethics Committee of San Simón de Cochabamba University, in agreement with the Bolivian National Chagas Programme. The study complied with all relevant federal guidelines and institutional policies.

Data for each study participant were entered in Microsoft Excel and analyzed with SPSS 12.0 (SPSS, Inc., Chicago, IL).

Diagnostic test procedures. Once enrolled, 10 μ l of whole blood was collected from the study participant using a disposable 10- μ l Microsafe tube; the amount needed for the Chagas Stat-Pak RDT. The Chagas Stat-Pak RDT was then performed and, precisely 15 min later, the result was independently interpreted by two healthcare workers. Each recorded his or her interpretation in separate registers. If there was discordance, a third party was sought to derive consensus about the test result, and a final result was recorded in the patient's record along with the original interpretations of the two health workers.

The Chagas Stat-Pak RDT is performed using a specific antibody-binding protein that conjugates to dye particles and antigens bound to the test membrane. A whole-blood sample was applied to the sample well and flowed laterally across the membrane. If the sample contained antibodies to *T. cruzi*, the complex bound to the antigens on the membrane, producing a pink and/or purple line (i.e., the test was considered positive or reactive). If the sample did not contain antibodies to *T. cruzi*, the complex remained unbound, and no line appeared (i.e., the test was considered negative or nonreactive) (10). Figure 1 presents an image of reactivity versus nonreactivity demonstrated by the Chagas Stat-Pak RDT.

Next, for each study participant, 3.5 ml of whole blood was obtained by venipuncture and stored in a dry tube. The specimens were transferred twice daily, via a temperature-monitored cold box (2 to 8°C), to the MSF Sucre laboratory for same-day centrifugation and serum extraction in order to perform the conventional assays. Conventional assays were performed less than 6 h after sample collection.

Conventional assays, administered serially starting with conventional ELISA (Chagatest ELISA; Wiener Laboratorios, S.A.I.C., Argentina) and then Chagas IHA (HAI Chagas Polychaco; Laboratório Lemos, S.R.L., Argentina) and, in the case of discordance between the two, recombinant ELISA (Chagatest ELISA recombinante, v.3.0; Wiener Laboratorios, S.A.I.C., Argentina), were used for diagnostic classification and served for comparison for the Chagas Stat-Pak RDT. In accordance with the Bolivian National Chagas Programme and World Health Organization technical guidance, in order to be considered Chagas' disease positive, two out of the three assays had to be positive (i.e., conventional ELISA plus Chagas IHA, Chagas IHA plus recombinant ELISA, or conventional ELISA plus recombinant ELISA) (23). Conventional ELISA and recombinant ELISA assay interpretation was performed according to the manufacturer recommendations. Chagas IHA assay interpretation was considered positive if agglutination covered $\geq 50\%$ of the bottom well of the titration plate at a titer of ≥ 8 . The absence of agglutination or agglutination covering $< 50\%$ of the bottom well at a titer ≥ 8 was considered negative.

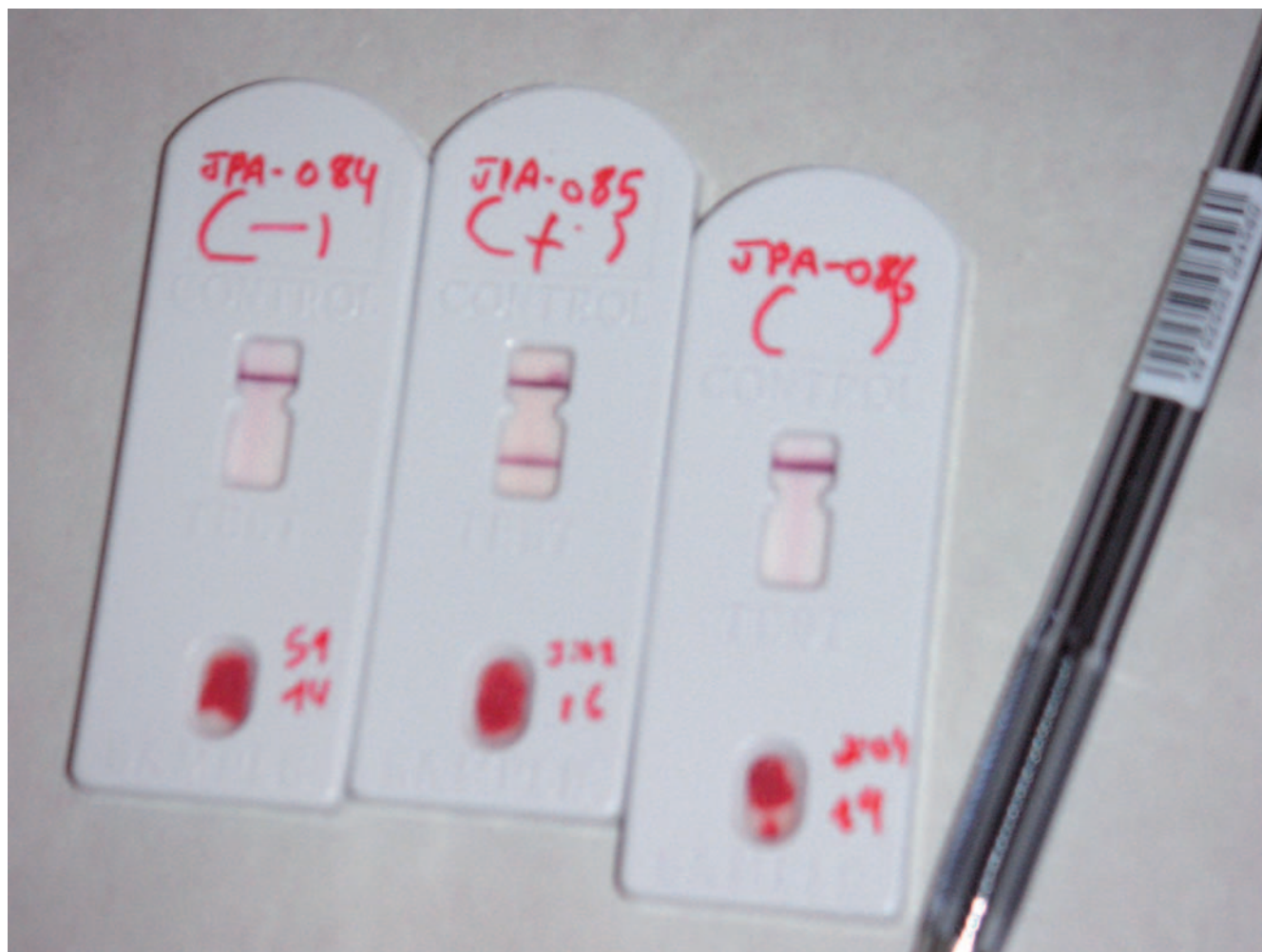


FIG. 1. Reactivity versus nonreactivity of the Chagas Stat-Pak RDT. From left to right, the first and third tests are nonreactive, while the second is reactive. All three tests produce a control line (top), while a pink/purple line on the second test (bottom) appeared after the *T. cruzi* antibody complex bound to the antigens on the membrane.

All individuals found to be Chagas' disease positive, according to conventional assays, were offered Benznidazole by the MSF medical team. Benznidazole is the standard first-line treatment for Chagasic infections in Bolivia. Side effects for benznidazole were reported in 37.3% of treated patients.

Laboratory quality control. Quality control protocols were applied at both health clinic and laboratory levels. Prior to the initiation of the study, spectrophotometers and micropipettes were calibrated and thoroughly cleansed by the level III reference laboratory of the Ministry of Health in Sucre according to manufacturer-recommended standard procedures. Internal quality control measures consisted of a weekly analysis of the Chagas Stat-Pak performance with positive and negative serum controls in the MSF Sucre laboratory according to the test procedure instructions of all respective package inserts. The procedural routines of the laboratory and health workers was assessed on a weekly basis by following a "good practices" checklist. Positive and negative serum controls were included for each series of conventional assays each time a new microtitration plate was used in the laboratory. External quality control was carried out by the level III reference laboratory of the Ministry of Health in Sucre, analyzing a panel of serum samples (positive and negative) sent from the MSF Sucre laboratory every fourth week.

Measurements of diagnostic test performance. The sensitivity and specificity of the Chagas Stat-Pak RDT were calculated. Sensitivity was defined as the proportion of study participants positive for Chagas' disease according to the conventional assays that were correctly identified as positive by the Chagas Stat-Pak RDT. Specificity was defined as the proportion of study participants

without Chagas' disease according to the conventional assays that were correctly identified as negative by the Chagas Stat-Pak RDT.

The interobserver reliability in the interpretation of the Chagas Stat-Pak results was measured through the calculation of a kappa statistic. This measurement estimated the extent to which the interpretation of the RDT varied when the test was read independently by two trained, nonlaboratory health-care workers.

The ease-of-use criterion to perform the Chagas Stat-Pak RDT and the conventional assays were quantified and compared. At the completion of the study, both the trained, nonlaboratory health workers who conducted RDT tests and the laboratory workers who conducted the conventional assays completed a questionnaire which, in addition to itemizing and scoring general characteristics of the tests, assessed their perceptions about ease of use in performing the respective tests. Questionnaire items included documentation of the number of doubtful and invalid tests and assessment of ease of performance, ease of interpretation, quality of the instruction sheet, ease of opening the package, and ease of identifying reagents. Each questionnaire item was given an individual score (range, 1 to 5), a higher score indicating a more favorable response. A total of 51 points was possible.

The questionnaire is a modification of a questionnaire designed by Epicentre (Paris, France) to evaluate an RDT for the diagnosis of uncomplicated *Plasmodium falciparum* malaria (7). An MSF laboratory technician and coauthor (L.F.) established content validity of the questionnaire by reviewing the document for appropriateness and representativeness of the contents.

TABLE 1. Characteristics of the study population screened for Chagas' disease using the Chagas Stat-Pak RDT and conventional serological assays in Sucre, Bolivia, from April to June 2007

Demographic category	No. of subjects (n = 1,913)	% Total
Age		
9 mo to 4.9 yr	331	17
5 yr to 17.9 yr	1,582	83
Gender		
Male	930	49
Female	983	51

RESULTS

Demographics. A total of 1,913 study participants were enrolled. Table 1 presents the characteristics of the study population. The mean age of participants was 9.3 years (standard deviation, 4.4) and the median age, 9.0 years (interquartile range, 6 to 13). Only 331 (17%) of the participants were under 5 years of age compared to 1,582 (83%) participants aged 5 to 17.9 years. Males were slightly under-represented (49%) compared to females (51%) (male/female ratio of 0.95).

Sensitivity and specificity. In the present study, the Chagas Stat-Pak RDT correctly detected 113 of the 121 positive results identified by the conventional assays, yielding 93.4% sensitivity (95% confidence interval = 87.4 to 97.1%). Likewise, of the 1,792 negative results according to conventional assays, 1,774 were correctly categorized by the RDT, yielding 99.0% specificity (95% confidence interval = 98.4 to 99.4%). Percentages were calculated using the results presented in Table 2.

Interobserver reliability. A total of four trained, nonlaboratory health workers, working in pairs, interpreted the 1,913 Chagas Stat-Pak test results. These individuals concurred on 1,911 of the test results, yielding 99.9% overall agreement (n = 1,913, kappa = 0.999, P < 0.0001).

Ease-of-use criterion. A total of six individuals conducted the diagnostic tests and completed ease-of-use criterion for each of the tests they performed. Two individuals were laboratory workers who performed the three conventional assays, and four were trained, nonlaboratory health workers who performed the Chagas Stat-Pak RDT only.

Each questionnaire item was scored according to the scheme outlined in the Materials and Methods section of the present study. Of a total of 51 possible points, the RDT scored the highest with 39.25 points (77%), followed by the Chagas IHA assay with 34 points (67%). The conventional ELISA and recombinant ELISA each scored 30 points (59%).

Doubtful tests were reported for ≤1.0% of the RDTs performed but >1.0% for the three conventional assays. The ease of performance was reported to be “very simple” for the RDT but “difficult” for each of the conventional tests. Likewise, the interpretation of the RDT was reported to be “very simple” compared to “simple” for the conventional assays. The quality of the RDT instruction sheet was reported as “above average” but inferior to the “excellent” quality of the instruction sheets for the conventional assays. Trained, nonlaboratory health workers found opening the RDT package cumbersome and gave this survey item an “average” score only. The RDT test

TABLE 2. Two-by-two table of Chagas' disease classification as defined by using the Chagas Stat-Pak and conventional serological assays in Sucre, Bolivia, from April to June 2007

Stat-Pak results	No. of results (n = 1,913) obtained by conventional serology:		
	Positive	Negative	Total
Positive	113	18	131
Negative	8	1,774	1,782
Total	121	1,792	1,913

did not require identification of reagents, while laboratory workers reported the labeling for the identification of conventional assay reagents to be excellent.

DISCUSSION

Laboratory and clinical diagnostic classification of seropositive individuals, followed by treatment and supportive therapy, is an established component of Chagas' disease control in areas of endemicity. The use of a valid RDT, when whole-blood samples are used, performed by trained nonlaboratory health-care workers is the best option for supporting this disease control component. Effective laboratory-based solutions applied to rural or periurban areas of endemicity are not widely available or likely to be anytime in the near future (20).

Overall, the Chagas Stat-Pak RDT is advantageous to the conventional assays. The RDT takes fewer steps to perform and is easier to interpret. The RDT can be stored up to 30°C, but the three conventional assays require refrigeration from 2 to 8°C. RDT results are available precisely 15 min after blood application while the Chagas IHA, and the two ELISAs require 3 and 4 h, respectively, to complete. The RDT does not require additional materials such as a spectrophotometer, micropipettes, an incubator, a centrifuge, or parafilm that are necessary to perform the conventional assays. Further, the RDT has the advantage of dispensing the blood specimen directly onto the test device, whereas the conventional assays require a buffer solution to dispense in a test tube.

The conventional assays retain some advantages over the RDT. The stability of the result is less than 30 min for the RDT and the ELISAs, while the constancy of Chagas IHA results is more than 24 h. In Bolivia, the RDT costs between 1 and 2 U.S. dollars (USD) per test. This is more expensive than the conventional assays, which cost less than 1 USD per test. An RDT price of 1 USD remains “out of reach” for many impoverished people affected by the disease.

A 99% Chagas Stat-Pak RDT specificity suggests that, had the RDT been used as a diagnostic assay in the absence of conventional testing, 1% of screened individuals would have been treated unnecessarily. The standard first-line treatment, benznidazole, is well tolerated among children and adolescents but can have serious side effects that include allergic dermatopathy, peripheral neuropathy, and granulocytopenia (6).

With respect to sensitivity, the Chagas Stat-Pak RDT, when whole blood is used as the sample medium, compares less favorably with the whole-blood RDT diagnosis of other parasitological vector-borne diseases. The rK39 RDT (DiaMed-IT-

Leish) had a reported 100% sensitivity for the diagnosis of visceral leishmaniasis (17), and the Paracheck-Pf RDT (Orchid Biomedical Systems, Goa, India) had a reported 99% sensitivity for the diagnosis of uncomplicated *P. falciparum* malaria (18).

In the present study, the Chagas Stat-Pak RDT, using whole blood, failed to identify eight positive individuals identified through conventional assays. The 93.4% sensitivity of the RDT found in the present study fell short of the Chagasic infection technical guidance provided by the World Health Organization, which states that an ideal test should be easy to perform in a single step, be fast and inexpensive, require no special equipment or refrigeration of reagents, and have a sensitivity and specificity of 100% (22). The false-negative rate of 6.6% found in the present study suggests that a substantial number of individuals remain undiagnosed, resulting in missed treatment opportunities and increased likelihood of chronic-stage disease sequelae.

In the Valle Hermoso district in Sucre, Bolivia, the Chagas Stat-Pak was not an ideal screening or diagnostic test. The sensitivity of the RDT, when conducted under field conditions and using whole-blood samples, was lower than the sensitivity reported in Chagas Stat-Pak validation studies that used serum and plasma samples (10, 11). It was noted that four of eight false-negative RDTs turned positive when the RDT was read at 20 min rather than the manufacturer-recommended 15 min. If interpreted at 20 min, the Chagas Stat-Pak RDT test would have had an improved sensitivity of 96.7%. This observation does not qualify as "official" test results because the package insert recommendation that interpretation should occur precisely 15 min after sample application was not adhered to. Future validation studies should include interpretation readings at 5-min intervals from 15 to 30 min to ensure that adequate time is allotted for test reactivity.

Cross-reactivity may occur between the sera of patients infected with *T. cruzi* and the sera of patients infected with *Leishmania* spp. when conventional assays are used for the diagnosis of Chagasic infections (8, 19). This phenomenon may have accounted for some or all of the eight false-negative individuals. Persons with negative RDT tests may be true negatives for Chagas' disease but may have another disease in which the antibodies cross-reacted with Chagas' disease antigens in the conventional assays. Had cross-reactivity been assessed, an increased sensitivity for the Chagas Stat-Pak RDT may have been demonstrated.

Considering the RDTs reported high specificity (99%), its high ease-of-use criterion score compared to conventional assays, and its excellent interobserver reliability (99.9% overall agreement) with a kappa statistic suggesting this excellence is beyond chance alone (9), the results are encouraging. However, in light of the importance of using whole blood under field conditions, the relatively low sensitivity in comparison to the sensitivity of the RDT when serum or plasma is used hinders the ability of this test to give dependable results under the conditions outlined here. To date, due to a lack of other options, MSF continues to use whole blood with the Chagas Stat-Pak RDT when screening a population where the disease is endemic, with final diagnostic classification being dependent upon conventional serological assays.

Conclusion and recommendations. Although the present study cites numerous advantages that the Chagas Stat-Pak RDT holds over the conventional assays, future progress for the control of Chagasic infections in areas of endemicity in Latin America necessitates research and development initiatives to improve the RDT. In remote areas, the use of whole blood as a sample medium is crucial since it simplifies the test procedures performed by trained, but nonlaboratory health-care workers. Thus, the sensitivity and specificity of an RDT that uses whole blood should approach the sensitivity and specificity of conventional assays that are conducted under reference laboratory conditions and use plasma or serum. If possible, the RDT cost should be set as low as possible to increase accessibility for impoverished people in high-prevalence regions.

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