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Short Report: Polymerase Chain Reaction for Chronic *Trypanosoma cruzi* Infection Yields Higher Sensitivity in Blood Clot Than Buffy Coat or Whole Blood Specimens

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Abstract. *Trypanosoma cruzi* polymerase chain reaction (PCR) is widely used, but sensitivity varies widely. We compared PCR using 121/122 primers targeting kinetoplast minicircle DNA in whole blood, buffy coat, and clot from Bolivian women. Sensitivity was significantly higher in clot (60.1%) than buffy coat (46.5%) or whole blood (40%). The use of clot could simplify specimen collection while improving sensitivity.

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, infects an estimated 8–10 million people in the Americas.[1,2] After an initial acute phase, lasting 60–90 days and marked by readily detectable parasitemia, infected individuals enter the chronic phase, which, in the absence of successful treatment, is life-long. In 20–30% of those infected, cardiomyopathy or gastrointestinal disease develops years to decades later.[3] The efficacy of drug treatment has been shown in randomized, double-blinded, placebo-controlled trials in children 6–12 years of age,[4,5] and recent data suggest that trypanocidal treatment in adults with early cardiac lesions may decrease disease progression.[6] Parasitemia in the chronic phase is undetectable by microscopy, and diagnosis relies on serologic tests. Because no single serologic test is sufficiently sensitive and specific, the accepted criteria for diagnosis of chronic *T. cruzi* infection consist of having positive results on two or more assays using different techniques or antigens (e.g., enzyme-linked immunosorbent assay [ELISA] and indirect fluorescent antibody [IFA]).[7]

Polymerase chain reaction (PCR) in the acute phase is highly sensitive, but published sensitivity in the chronic phase ranges from 40% to 100% and seems to depend on age and other characteristics of those tested, as well as PCR primers and methods.[8–11]

Our objective was to develop and evaluate a practical sensitive method for PCR diagnosis of chronic *T. cruzi* infection. *T. cruzi* PCR is most commonly performed on whole blood specimens.[8,10,11] Although some authors recommend mixing the fresh specimen with guanidine,[12] this reagent can no longer be shipped by air under new International Air Transport Association regulations, and its use complicates the logistics of specimen collection. A preliminary analysis showed no difference in sensitivity between whole blood in EDTA and whole blood mixed with guanidine (M. Calderon, unpublished data). Based on the literature, we hypothesized that circulating trypomastigotes would sediment in the buffy coat.[13] but might also be trapped with the cellular portion in clot. We therefore compared detection PCR in three types of specimens: whole blood in EDTA, buffy coat, and clot, from women in Santa Cruz, Bolivia.

Women admitted for delivery from November 13, 2006 to June 12, 2007 were asked to participate in serologic screening, as the first step in a study of congenital *T. cruzi* transmission. After informed consent, blood was collected into two 5-mL tubes with EDTA and one 5-mL tube without additives. One EDTA tube was centrifuged at 1,000*g* for 12 minutes, and the buffy coat (volume ~500 μL) was extracted; the other specimen was frozen as whole blood. The tube without additive was centrifuged at 1,000*g* for 5 minutes, and serum was drawn off to leave the clot (volume ~2 mL). All specimens were maintained at –20°C and shipped to the Universidad Peruana Cayetano Heredia, Lima, Peru. The study protocol was approved by ethical review boards of Johns Hopkins University Bloomberg School of Public Health, Asociacion Benefica PRISMA, Universidad Peruana Cayetano Heredia, Hospital Universitario Japones, and US Centers for Disease Control and Prevention.

Serum specimens were screened for antibodies to *T. cruzi* using a commercial ELISA (Chagatek, bioMérieux-Argentina, Buenos Aires, Argentina); the threshold for positive was calculated at 0.100 units above the mean absorbance of two negative control specimens on each plate, following the manufacturer's instructions. All specimens were also tested by IFA, using a titer of 1:32 as the positive cut-off.[14] Specimens positive by ELISA and IFA were considered confirmed positive for *T. cruzi* infection.[7] Specimens with discordant results were excluded.

Specimen aliquots (500 μL of whole blood or clot and 300 μL of buffy coat) were combined with buffer (10 mmol/L Tris HCl, pH 7.6, 5 mmol/L $MgCl_2$, 10 mmol/L NaCl), homogenized, and centrifuged. The supernatant was removed, and the pellet was resuspended in buffer and centrifuged. This procedure was repeated three times for buffy coat and whole blood and five times for clot. SDS and Proteinase K (Invitrogen, Carlsbad, CA) were added to reach concentrations of 0.5% and 0.5 mg/mL, respectively and the specimens were incubated for 2 hours at 56°C. DNA was extracted following a standard phenol-chloroform extraction protocol[11] and resuspended in 100 μL Tris HCl, 10 mmol/L, and EDTA 1 mmol/L. For the PCR reaction, the total volume was 25 μL (23 μL reaction mix and 2 μL DNA). This is equivalent to between 30 and 50 ng/μL DNA (human DNA, and, in positive specimens, parasite DNA).

PCR amplifications were performed using the 121/122 primer set targeting the kinetoplast minicircle (5′-AAATAA-

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TABLE 1

Performance of PCR in clot, buffy coat, and whole blood specimens from women in Santa Cruz, Bolivia, relative to *T. cruzi* infection status as indicated by results of two serologic assays

Specimen type	Specimens processed by PCR (*N*)	Seropositive specimens* [*N* (%)]	PCR sensitivity† (95% CI)	PCR specificity† (95% CI)
Clot	516	148 (28.7%)	60.1% (53.7–68.6)‡	100% (99.0–100)
Buffy coat	208	71 (34.1%)	46.5% (36.2–59.7)‡	100% (97.3–100)
Whole blood	520	150 (28.8%)	40.0% (32.9–48.7)‡	100% (99.0–100)

* Positive by both ELISA and IFA (see text for explanation).
† Performance relative to confirmed positive or negative serologic results.
‡ *P* values for pairwise comparisons of PCR sensitivity in clot versus buffy coat, 0.0237; clot versus whole blood, < 0.0001; buffy coat versus whole blood, 0.233.

TGTACGGGKGAGATGCATGA-3′ and 5′-GGTTCGATTGGGGTTGGTGTAATATA-3′).[10,11,15,16] A total volume of 25 μL was used for each reaction with the following concentrations: 2.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.2 μmol/L of each primer, 0.025 U/μL Taq polymerase, 1 mg/μL bovine serum albumin (BSA), 1× PCR buffer, and 2 μL of the sample DNA. The reactions were heated to 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and for 72°C for 30 seconds. A final extension at 72°C was carried out for 7 minutes. Positive and negative controls, as well as a control for amplification of human β-globulin, were included. Specimens showing the characteristic 330-bp minicircle amplification product were considered positive; those with a β-globulin band but no 330-bp band were considered negative. The technicians who performed the PCR assays were blinded to the serologic results.

Performance characteristics of the PCR assays were determined relative to confirmed serologic status. A multivariable log link model was used to estimate 95% confidence intervals (CIs) and compare PCR sensitivity in whole blood versus clot, whole blood versus buffy coat, and clot versus buffy coat. Generalized estimating equations were included to account for repeated measures on the same individuals. Analysis was performed in SAS 9.1 (SAS Institute, Cary, NC) and Stata 9.1 (StataCorp, College Station, TX).

A total of 526 pregnant women were screened for the study; their mean age was 24.6 years (range, 13–45 years). One hundred fifty-two (28.9%) women had positive results and 371 had negative results on both serologic assays. Two specimens were positive by ELISA but negative by IFA, whereas one was positive by IFA but negative by ELISA; the three discordant specimens were excluded from further analysis. Not all specimens had PCR performed, and buffy coat specimens were only collected during the latter half of the study; the number tested by PCR totaled 520 whole blood, 516 clot, and 208 buffy coat specimens.

The sensitivity of PCR was significantly higher in clot (60.1%), compared with buffy coat (46.5%) or whole blood (40%) (Table 1). The specificity was 100% for all specimen types. Agreement between PCR results for different specimen types was good but not excellent: κ statistics for the pairwise comparisons were 0.78 for clot versus buffy coat, 0.69 for clot versus whole blood, and 0.73 for buffy coat versus whole blood. Despite its better sensitivity, even PCR in clot did not detect all specimens positive by PCR. Of 97 specimens positive by PCR in any specimen type, PCR in clot detected 89 (92%), whereas PCR in whole blood detected 60 (63%); 6 specimens were positive in whole blood but negative in clot, whereas 34 were positive in clot but not whole blood. PCR in buffy coat was positive for 33 (75%) of the 44 PCR-positive specimens for which this specimen type was collected; 4 specimens were positive in buffy coat but not clot, whereas 9 were positive in clot but not buffy coat.

Because screening for chronic Chagas disease always involves collection of serum for serologic testing, blood clot is also collected but is usually discarded. Our results showed that blood clot could be a valuable resource for the diagnosis of *T. cruzi* infection by PCR; indeed, in our laboratory, detection PCR was more sensitive in clot than in whole blood or buffy coat. Use of clot could greatly simplify specimen collection and decrease the amount of blood drawn from study participants.

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