Advances in the Diagnosis of Chagas Disease Using Methods Based on Molecular Biology: PCR

Louis V. Kirchhoff

University of Iowa and Department of Veterans Affairs Medical Center
Iowa City, Iowa, USA

ABSTRACT
Chagas disease (American trypanosomiasis) is a major cause of morbidity and death in Latin America. An estimated 16-18 million persons are infected with the causative protozoan parasite, Trypanosoma cruzi, and 45,000 individuals are thought to die of the illness each year [1]. Accurate diagnosis of Chagas disease is necessary to permit appropriate treatment and monitoring, as well as for donor screening in blood banks, but unfortunately making a definitive diagnosis of T. cruzi infection is often problematic. In this article I review the problems related to Chagas disease diagnosis, the work that has been done on developing diagnostic assays based on the polymerase chain reaction (PCR), and the extent to which these PCR tests currently are used in Chagas disease diagnosis.

DIAGNOSIS OF T. cruzi INFECTION
Acute Chagas disease, in its vector-borne, transfusional, and congenital forms, is usually diagnosed by parasitologic methods. In this phase of the illness, which can last six to eight weeks after the initial infection, the parasites often can be seen by microscopic examination of anticoagulated blood [2]. In many patients with acute Chagas disease, however, parasitemias are low and microscopic examination does not yield positive results. Such patients pose a difficult challenge. Assays for anti-T. cruzi IgM have not been standardized and are not widely available. In addition parasitologic tests, such as xenodiagnosis and hemoculture, have sensitivities of only about 50% and usually take several weeks before positive results become available. This lag is a major problem, because it is presumed that the earlier a patient is treated, the greater the probability of a parasitologic cure. An additional problem is the fact that it is difficult to obtain sufficient blood from newborns and small children for xenodiagnosis or hemoculture, thus making the diagnosis of congenital and pediatric Chagas disease in patients without patent parasitemias even more difficult. Because of these problems, a rapid and accurate test would be useful in many patients with acute Chagas disease.

Chronic Chagas disease is, for the most part diagnosed by detecting IgG antibodies that react specifically with T. cruzi antigens. Detecting the parasite generally is not necessary. Several sensitive serodiagnostic tests have been used widely for decades in Latin America, such as indirect hemagglutination, indirect immunofluorescence, and enzyme-linked immunosorbent assays [3,4]. The occurrence of false-positive results, however, has been a persistent shortcoming of these assays. This problem typically arises with specimens from persons having histories of illnesses such as leishmaniasis, malaria, toxoplasmosis, syphilis, and other parasitic and nonparasitic diseases. There are two major consequences of this lack of specificity. The first is that although the serologic assays are effective tools for detecting blood donors infected with T. cruzi, the price of this sensitivity is high, as many uncontaminated units of blood are discarded in endemic countries because of false positive serologic results. Secondly, many persons with false positive serologic results suffer psychologically and economically because of the diagnosis.

As an approach for dealing with the shortcomings of the currently available serologic tests, most authorities in Latin America recommend that samples be tested in two or three serologic tests. This approach is useful in clinical situations where patients are only accepted as being infected with T. cruzi if they are positive in more than one serologic assay. The number of persons incorrectly labeled as having the illness is thus reduced. In blood banks, moreover, using more than one test certainly optimizes the sensitivity of the overall process. The disadvantage of this approach, however, is that the number of discarded units is vastly increased, because any unit found to have even a low positive titer in a single test is not transfused.

The practice of doing more than one serologic test carries with it a large economic and logistical burden, both in clinical settings and blood banks. An accurate and rapid primary or confirmatory test is sorely needed. The fact that xenodiagnosis and hemoculture are laborious and take weeks to complete limits their widespread
use for this purpose. Tests based on western-blotted *T. cruzi* antigens have been under development for years, but none has reached the level of an acceptable confirmatory test. A highly sensitive and specific radioimmune precipitation assay I described more than a decade ago [5] has been used extensively in the United States as a confirmatory assay [6,7]. Its complexity and the lack of easy availability of radiiodine in the endemic countries preclude its use there and relegate it to a role as a reference test. Thus, it is clear that better diagnostic tools are needed for chronic Chagas disease, as is the case in the acute form of the illness.

**PCR ASSAYS FOR DETECTING INFECTIOUS AGENTS**

In the late 1980's, assays based on PCR were developed for a variety of detection challenges, including several infectious diseases [8]. PCRs are similar to xenodiagnosis and hemoculture in that multiplication is involved, but instead of having entire parasites multiply to the point of being visually detectable, as is the case in the latter two methods, PCRs only multiply (amplify) one minute element of the parasite. Although intricate at a molecular level, the end result of a successful PCR is quite simple. In a typical PCR, billions of copies of a relatively short stretch of DNA are synthesized, and the number of identical copies is so large that when separated electrophoretically from other components of the reaction mixture, the copies can be seen in an agarose gel as a single diagnostic band of a known size. PCR succeeds as a tool for detection of an infectious agent such as *T. cruzi* because it is a specific process in which only a known DNA sequence of the sought-after organism is amplified, even though the target sequence represents only an infinitesimally small portion of the DNA in the reaction mixture. Moreover, a PCR gives visually interpretable results because the amplification process is logarithmic. In an ideal reaction with 100% efficiency and 30 cycles of amplification, 230 or 1.07 x 10^8 copies are made of each copy of the target DNA sequence in the reaction mixture. In typical PCRs many thousands of copies of the target DNA sequence are present initially and as a consequence a truely enormous number of copies are synthesized.

**DIAGNOSIS OF *T. cruzi* INFECTION WITH PCR ASSAYS**

PCR technology also has been applied to the challenge of developing better assays for diagnosing *T. cruzi* infection. In the summer of 1989, seminal papers on the use of PCR for diagnosing *T. cruzi* were published by Larry Simpson's group at the University of California [9] and by my group at the University of Iowa [10]. Both of the approaches described are based on the amplification of DNA sequences of which there are large numbers of copies in each parasite. In the method presented by the California group, a highly-conserved 330-base pair segment of *T. cruzi* kinetoplast minicircles is amplified using a primer pair designated S35-S36. Every *T. cruzi* parasite has about 30,000 minicircles and each minicircle has four amplifiable 330-base pair segments. Thus each organism has roughly 120,000 amplifiable copies of the target sequence, and in contrived experiments the authors were able to detect 1/1,000th of a parasite genome. In the procedure described by the Iowa investigators, a 188-base pair nuclear repetitive DNA sequence, of which each parasite has ~100,000 copies, is amplified by a primer pair called TCZ1-TCZ2 [11]. In experiments done in vitro, as little as 1/200th of the DNA of a single parasite gave a positive result.

Although various primer pairs have been used for amplification of *T. cruzi* DNA in PCR-based assays, none has been shown in head-to-head comparisons to form the basis of an assay more sensitive for *T. cruzi* detection than those based on the primer pairs described in the original 1989 publications. This reality is consistent with the fact that these subsequently-defined primer pairs amplify DNA sequences of which there are far fewer copies present in each parasite than the 120,000 and 100,000 copies of the target DNA sequences amplified by S35-S36 and TCZ1-TCZ2, respectively. In terms of the relative sensitivity of the assay based on the latter two primer pairs, in contrived experiments in the only direct comparison published to date, the TCZ1-TCZ2 assay showed slightly greater sensitivity [12]. Overall, however, I think the two assays can be considered comparably sensitive. In terms of specificity, moreover, assays based on these two primer pairs did not give false positive results when DNAs from several mammalian and triatomine species, as well as from a variety of infectious microorganisms including protozoans, were used as templates. The only exception to this group of negative results was that in my hands the S35-S36 assay produced a broad and relatively weak band centered around 330 base pairs when DNA from *Trypanosoma rangeli* was used as template. This phenomenon was not observed with the TCZ1-TCZ2 assay [13]. Despite these two elements favoring the use of the TCZ1-TCZ2 primer pair, for historical reasons the majority of the studies done in humans to date have employed the S35-S36 primer pair.

Since the publication of the two original papers in 1989, nearly 100 articles have appeared in the indexed literature that deal with one aspect or another of *T. cruzi* detection by PCR, and a sizable portion of these relate to the issue of Chagas disease diagnosis in humans. In an effort to develop sensitive assays, the authors of these articles have taken a variety of experimental approaches. Parameters that have been varied include the serologic and parasitologic characteristics of the patients studied, the method of extracting DNA from their blood, the conditions of the reaction, the method of detection of amplification products, and as noted, the target DNA sequence to be amplified [14,15]. One of the thorny issues that has plagued these efforts to develop an accurate PCR assay is the fact that there is no definitive, readily-available serologic test to use as a basis of comparison.
If one chooses to study, for example, a group of patients composed of serologically positive and negative individuals, given the limitations of the available assays, inevitably some misclassified persons will be included. Typically, most of the incorrectly diagnosed persons will be false positives, but depending on the specific assay used, an occasional false negative person may be included as well. In any event, the consequence of these misclassifications will be to underestimate both the sensitivity and the specificity of the experimental PCR test being evaluated. If, on the other hand, one were to limit the study group exclusively to individuals who previously had positive xenodiagnoses or hemocultures and persons from non-endemic areas with negative serology, the sensitivity of the experimental PCR assay likely would be exaggerated because such parasitologically positive persons presumably have higher parasitemias than other seropositive persons who were negative in one or the other of the two parasitological tests.

Putting aside this and other methodologic problems, it is clear that a general pattern of results regarding the performance of PCR assays for diagnosing Chagas disease has emerged during the 13 years they have been under study. Overall, and disappointingly, the sensitivities of the PCR tests used in the major studies have fallen in the range of 80-95%. Notably, in one of the earlier investigations, 100% of the 95 persons in the study group, which included three with negative serology, were found to be positive by PCR assay [11]. In two studies done in Northeastern Brazil, however, where the prevalence of Chagas disease in the general population is relatively low, the sensitivities of the PCR assays employed were only 44.7% and 59.4% [16,17]. To some degree, these latter relatively low sensitivities may be the result of the low overall prevalence of \textit{T. cruzi} infection in the region, which inevitably would increase the percentage of false positive study participants in the groups determined to be serologically positive. In a group of nine key studies of this issue done in the 1990s, the sensitivities of the PCR assays used to study primarily serologically positive specimens ranged from 44.7% to 100%, with most results falling a bit over 90% [18-24]. In summary, there is no doubt that a lack of sensitivity is a problem for PCR tests for \textit{T. cruzi} infection.

There are several reasons that may underlie the inability of PCR tests to detect \textit{T. cruzi} in persons who are, in fact, infected with the parasite. First on the list would be the fact that parasitemias are low in chronically infected people and may actually be intermittent. In either event, a sample taken at one point in time could simply not contain any parasites. At the level of the processing of blood samples, inadequate extraction or dispersal of the amplifiable DNA sequences could be a problem, as they are naturally tied up in chromosomes (nuclear repetitive sequence, TCZ1-TCZ2 primer pair) or the concatenated network of mini- and maxicircles (kinetoplast DNA, S35-S36 primer pair).

Finally, issues pertaining to the reaction mixture, such as variable Taq polymerase activity, improperly diluted buffers, etc., may at times account for false negative results. In terms of specificity, the occurrence of false positive results, caused by the contamination of reaction mixtures with amplicons produced in earlier runs, has been recurrent problem in some laboratories. Physical separation of the areas in which PCRs are set up from those in which reaction products are processed is an important step towards avoiding this problem. In addition, the exclusive use of barrier pipet tips and disposable supplies also helps to reduce the risk of contamination [12].

**PERSPECTIVES**

Thirteen years have elapsed since the first two descriptions of PCR-based assays for detection \textit{T. cruzi} infection were published, and to my knowledge such tests are not commercially available and are not used outside of research environments. Thus their impact has been minimal. What factors have impeded the widespread clinical use of PCR-based assays? As noted, a lack of sensitivity is a significant problem, as is the recurrent problem of contamination of reaction mixtures with amplicons and consequent false positive results. The issue of inappropriate technology is also important, as extraction of DNA from clinical samples, amplification, gel electrophoresis, and interpretation of results are beyond the technical level of many clinical laboratories in endemic countries.

As noted, one of the major potential uses of a highly sensitive and specific PCR assay for detecting \textit{T. cruzi} infection would be in screening donated blood. Ideally, such an accurate test would be useful for identifying donated units giving false positive serologic studies, thus making them available for transfusion. Given the less than optimal sensitivities of the PCR assays for \textit{T. cruzi} detection developed to date, it is clear that this approach cannot be used to reliably separate \textit{T. cruzi}-contaminated units from those which are serologically false positive. Given what is known about the sensitivities of the PCR assays, I think it is unlikely that blood bank authorities will ever feel comfortable transfusing units that are PCR-negative but serologically positive, even if in only one test. The solution to the problem of false positive serologic results and the consequent discarding of uncontaminated units will more likely be found in the development of improved serologic assays, perhaps based on recombinant antigens, with higher levels of specificity than the currently available kits.

More accurate tests are also needed for diagnosing chronic Chagas disease in clinical settings, especially assays with better levels of specificity. A useful role for PCR assays would then be in testing persons with
inconsistent or borderline serology. Those who turned out to be PCR-positive would be accepted as such and have specific treatment, if appropriate, and long-term monitoring. Given the sensitivities of the PCR assays, persons with negative PCR test results, however, would have to be followed as possibly being infected and perhaps retested. Nonetheless, this general approach would be useful, as the infection status of a sizable number of persons would be resolved by its application.

Lastly, the detection of persistent *T. cruzi* infection in persons who have been treated with benznidazole or nifurtimox, the two largely ineffective agents available for treating patients with Chagas disease, is problematic [26]. Presently it is difficult to determine if a patient has been cured, as antibody titers suggesting infection may persist for years and the sensitivities of hemoculture and xenodiagnosis, which as noted are low even in untreated patients, can be suppressed by the treatments. Thus, in this case as well there is a potential role for PCR assays for *T. cruzi* infection, although again it must be kept in mind that, given the sensitivities of the assays, the procedure would only be useful in identifying patients in whom treatment had failed [27,28]. Another issue is that the usefulness of detecting persistent infection in treated patients is limited. While this is a challenging diagnostic problem that has defied development of a satisfactory solution, since it is not recommended that patients in whom treatment has failed be given another round of therapy, it is not useful in terms of patient management to know which patients are still infected. On the other hand, an accurate PCR-based assay could play a major role in the evaluation of new drugs for treating *T. cruzi* infection, and in many other research contexts as well.

The niche then for PCR-based assays for detection of *T. cruzi* infection may lie in patients with acute Chagas disease in whom microscopic examination has not produced positive results. Although definitive data regarding the sensitivities of PCR assays in acutely infected persons have not been published, I think it is fair to assume that since parasitemias are higher in acute disease than in chronic infections, that the sensitivities of PCR tests will be higher in the former than in the latter. Given that the number of acute cases of Chagas disease has fallen markedly in the last decade due to the successes of vector control programs, and since the patients with this phase of the illness are dispersed and often in less accessible areas, there would not appear to be a concentrated market that would drive the development of such a PCR-based assay by private industry. A more concentrated target group for testing, however, would be newborns at risk for congenital disease. In endemic areas it would be useful to implement effective programs for prenatal screening and then use a PCR test to identify congenitally infected infants. This would be particularly useful because early treatment is indicated in such patients.

Finally, we can reasonably ask what areas of research could be pursued in an effort to develop better and more useful PCR-based assays for detecting *T. cruzi* infection that would increase its sensitivity to the point of allowing its use in donated blood. As noted, optimization of extraction and dispersion procedures, with the goal of increasing sensitivity, is a clear route to improved tests, although it must be pointed out that much work already has been done along these lines [14,15]. It is certainly possible, moreover, that further improvement in the sensitivities of PCR based assays may not be possible because of the extremely low and variable parasitemias in chronically infected persons. Utilization of procedures to avoid contamination of samples and reagents with amplicons from previous runs would reduce the problem of false positive results, and considerable research has been done in this area [29]. Finally, the commercial development of PCR-based colorimetric assays for *T. cruzi* infection would go a long way toward increasing the accessibility of this technology in endemic countries.

### References

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