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Technological advances in the molecular diagnosis of Chagas disease from the perspective of the Fio-Chagas Translational Programme

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An estimated 6 to 8 million people worldwide are infected with *Trypanosoma cruzi*, the parasite that causes Chagas disease (CD). Of those infected, an alarming 70% to 90% remain undiagnosed, making it one of the most under-recognised global health burdens. To celebrate the 25th anniversary of the Fiocruz Translational Programme in Chagas Disease (Fio-Chagas), this narrative review presents a chronological account of the development of polymerase chain reaction (PCR)-based assays for the molecular detection of *T. cruzi*. It highlights the significant contributions made by the Fio-Chagas Programme to the development and availability of a diagnostic product for the molecular diagnosis of CD. Following the proof of concept, the qPCR-based Biomol Chagas kit was manufactured using reagents produced by the Institute of Molecular Biology of Paraná (IBMP). After analytical and clinical validation, the kit was registered in 2022 by the Brazilian Health Regulatory Agency (ANVISA). Additional studies under field conditions were required for a cost-effectiveness analysis by the National Commission for the Incorporation of Technologies into the Brazilian Unified Health System (CONITEC). Currently, the kit has been incorporated into clinical studies across Latin America to diagnose congenital transmission, monitor the impact of antiparasitic treatment in chronic CD (CCD), and evaluate oral transmission in the Northern region of Brazil.

Key words: molecular diagnosis - Chagas disease - Fio-Chagas Translational Programme - PCR-based assays - Biomol Chagas kit

1980s-1990s: consolidation of polymerase chain reaction (PCR) as a diagnostic tool for Chagas disease (CD)

In the 1980s, the Department of Biochemistry and Molecular Biology at the Oswaldo Cruz Institute focused on understanding the genetic variability of *Trypanosoma cruzi* and its association with differences in biological traits, such as infectivity, tissue tropism, virulence, drug sensitivity, antigenic composition, and polymorphisms in zymodeme and schizodeme patterns.[1] A key characteristic of the members of kinetoplastida is the presence of a kinetoplast — a specialised region in the single mitochondrion that houses the largest amount of extranuclear DNA in eukaryotic cells. Known as kinetoplast DNA (kDNA), it accounts for 10-20% of total cellular DNA.[2] At the time, knowledge about the genetic composition of the kinetoplast was limited, with only a few studies using electron microscopy to observe the intricate kDNA network.[2,3]

The Department pioneered the manual Sanger sequencing protocol in Brazil. Using different strains and clones of *T. cruzi* [before the term discrete typing units (DTUs), was introduced], researchers conducted studies focusing on the manual sequencing of the minicircle molecules that constitute 95% of the kDNA network. The minicircles of *T. cruzi* are approximately 1,400 bp in length and exhibit a unique structural organisation. These molecules contain four highly conserved regions (located 90º apart) that are invariant across strains and clones of the parasite, interspersed with hypervariable sequence regions.[4] The identification of this molecular marker — present in thousands of copies (10,000-20,000) in the mitochondrial genome and highly conserved among diverse *T. cruzi* strains — was a significant milestone. This discovery established kDNA minicircles as potential targets for PCR development in CD diagnosis.

Before the advent of PCR for the diagnosis of sickle cell anaemia,[5] molecular hybridisation using specific probes targeting nuclear[6] or mitochondrial sequences of *T. cruzi*[7] had been tested for the detection of the parasite in clinical samples, sometimes in combination with xenodiagnosis. However, hybridisation procedures for detecting *T. cruzi* in chronic cases, even with multi-copy targets like kDNA, showed limited sensitivity and required the pre-amplification of the parasite or its kDNA. Therefore, the search for more reliable diagnostic methods for the chronic phase of Chagas disease advanced with the introduction of highly sensitive tests, such as the PCR amplification of *T. cruzi* DNA.

PCR specificity relies on the use of primers designed from known genome sequences ("target" sequences). The highly conserved sequences repeated four times within *T. cruzi* minicircles provided ideal PCR targets, as these sequences are highly abundant in the parasite's kDNA.[4] Sturm et al.[8] used the previously established consensus sequence to design primers complementary to the conserved blocks of the minicircles, demonstrat-

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ing their specificity for *T. cruzi* kDNA amplification using purified kDNA from different trypanosomatid species. Because of the genetic and structural similarities between the minicircles of *T. cruzi* and *Trypanosoma rangeli*, the primer sequences were adjusted for use with blood samples from *T. cruzi*-seropositive patients.[9] Notably, *T. rangeli* is not pathogenic to humans but shares some triatomine vector species, making primer adjustments necessary to avoid the risk of cross-reactivity. Other groups also explored nuclear satellite DNA as a PCR target, which consists of 195 bp sequences repeated 10^5 times in the nuclear genome of *T. cruzi*.[10,11,12,13]

To improve kDNA amplification for diagnostic use, Avila et al.[14] introduced a 6M guanidine-HCl solution containing 0.2M EDTA for the lysis and preservation of parasite DNA. This method facilitates the transport of blood samples from rural endemic areas to the laboratory without refrigeration. When kDNA is used as a target for PCR, the kinetoplast network must be cleaved to liberate the thousands of concatenated minicircles and distribute these target molecules homogeneously throughout the blood sample. Efficient kDNA cleavage was achieved by boiling guanidine-EDTA-blood lysates (GEB lysates) at 100ºC for 10 min, allowing DNA extraction from small aliquots (100-200 µL).[15] This boiling method increased PCR sensitivity, enabling the detection of a single parasite in 20 mL of GEB lysate (10 mL blood + 10 mL guanidine-EDTA). Such sensitivity is suitable for diagnosing chronic CD (CCD), where circulating parasite levels are low.

The kDNA-PCR approach was validated using blood samples from different geographic regions with varying endemicity. This molecular test, when applied to individuals with positive, negative, or indeterminate serology, demonstrated that PCR can effectively complement serological methods for the diagnosis of CD. The method showed high specificity, as no amplification products were observed in seronegative individuals. Compared to xenodiagnosis (the classical parasitological method), kDNA-PCR exhibited considerably higher sensitivity. For instance, a study involving 100 seropositive patients with CCD in a highly endemic area of Minas Gerais reported a sensitivity of 96.5%.[9] In that same area, xenodiagnosis sensitivity was also higher than usual (50-55%), likely due to the high parasitaemia caused by the *T. cruzi* strain circulating in the region.[16] Notably, until the advent of PCR, xenodiagnosis was considered the most sensitive parasitological technique available for diagnosing CD.[17]

PCR testing in a cohort of patients born in different endemic regions of Brazil and treated at Evandro Chagas Hospital (currently named the Instituto Nacional de Infectologia Evandro Chagas, INI) showed a sensitivity of 90% compared with serology, reflecting the mean sensitivity across infections from distinct locations.[18] Among a subgroup of patients with CCD who had been treated with benznidazole (Bz) and remained serologically positive, PCR could parasitologically distinguish between positive and negative patients. This offers an advantage over haemoculture and xenodiagnosis, positioning PCR as a complementary tool to serological tests for post-treatment follow-up.[18]

Initially, PCR protocols were not multiplexed. Therefore, when kDNA-PCR results were negative, a second PCR targeting the human β-globin gene[5] or another human constitutive marker was required to check for inhibitors and DNA quality, thereby helping to prevent false-negative results.

PCR-based assays to monitor treatment response

In the early 2000s, research into new drugs for CD was minimal compared to diseases attracting significant commercial investment, such as human immunodeficiency virus / acquired immunodeficiency syndrome (HIV/AIDS). The lack of reliable methods or a diagnostic gold standard for assessing etiological treatment efficacy in CD remains a major challenge.[19,20,21] Assessing cure is complex, often resulting in diverse and controversial findings regarding parasitological and clinical outcomes.[22,23] In chronic infections, because antibodies remain persistently positive for years or decades, serological tests cannot distinguish between a cleared infection and treatment failure in the short term. Molecular methods remain the gold standard for the early detection of treatment failure, but they do not constitute definitive tests of cure.[19,20,24] Given the fluctuation in parasitaemia during the long course of CD, a single negative post-treatment PCR result is no guarantee of parasitological cure; it likely indicates the absence of parasite DNA at that specific moment. Consequently, it cannot distinguish between the complete eradication of the parasite and the temporary suppression of detectable parasitaemia.[19] This diagnostic uncertainty stems directly from the underlying biology of the chronic phase of the disease, where an equilibrium between host immunity and tissue-sequestered parasites results in low and intermittent levels of parasitaemia that fundamentally impacts the performance of blood-based molecular assays. This scenario has led to a lack of consensus on how to determine treatment efficacy in chronic infections, making the development of early, reliable markers of cure a major priority in Target Product Profiles for CD.[25,26,27,28]

Therapeutic outcomes vary between countries due to distinct patient populations, the high genetic heterogeneity of *T. cruzi* (different DTUs - TcI to TcVI plus TcBat), and the variable sensitivity of parasitological and molecular methods used in conjunction with serology.[19] Ultimately, blood sample volume is a critical limiting factor in chronic patients with low levels of parasitaemia following treatment, and differences in PCR sensitivity may be explained by the intermittent presence and load of parasites at the time of collection.[29]

There is a consensus that the effectiveness of infection eradication depends on the time between diagnosis and treatment initiation; efficacy varies according to the disease phase.[30,31] Timely diagnosis and treatment are critical for preventing disease progression. However, the classic transmission of *T. cruzi* by triatomines is usually detected in the chronic phase, when parasitaemia and tissue parasitism are low. In this scenario, demonstrating treatment efficacy is difficult due to the lack of reliable cure criteria and the long-term persistence of specific antibodies even after parasite elimination.[32]

We were the first to propose using PCR as a complementary tool to serology for monitoring chemotherapy outcomes in a small cohort of seropositive patients with CCD who had been treated with Bz.[18] Today, PCR is routinely used to evaluate treatment outcomes in both acute and CCD, proving to be valuable for the early detection of treatment failure. The usefulness of parasitological and molecular tests lies primarily in positive results following treatment. A persistently positive PCR after treatment is considered therapeutic failure, and parasite load assessment by quantitative real-time PCR (qPCR) can be correlated with the treatment's impact on disease evolution.[19]

Contribution of the Fiocruz Translational Programme in CD to the development of qPCR

The Integrated Chagas Disease Programme (PIDC), later consolidated as the Fio-Chagas Network, is affiliated with the Vice-Presidency for Research and Technological Development at Fiocruz. Although launched in 2000, the Translational Programme was effectively established in 2006 as a result of scientific mobilisation and evolved into a collaborative, interdisciplinary, and translational model structured around thematic networks. Its primary goal was to promote coordination among basic, clinical, and applied research, anticipating the core approaches of translational science.

Initially, the PIDC comprised three networks: I. Medicines, therapeutics, and clinical trials; II. Vector taxonomy and ecology of *T. cruzi* transmission cycles; III. Diagnosis, clinical course, and pathogenesis. During the 2006 meeting, these networks were redefined, splitting Network III into Diagnosis (III) and Physiopathogeny (IV). One of the main goals of Network III was to develop and validate a qPCR-based molecular diagnostic test. Implementing qPCR to measure bloodstream parasite load and track its evolution during treatment is especially useful for monitoring responses to prolonged therapies, as a sustained decrease in parasite load indicates therapeutic efficacy.[33,19]

Participation in PIDC (Fio-Chagas) meetings provided ongoing opportunities for diagnostic network members to discuss persistent gaps in developing accurate diagnostic tests to monitor CD treatment efficacy. Notably, in 2000, the Laboratory of Molecular Biology and Endemic Diseases at the Oswaldo Cruz Institute — owing to its pioneering use of PCR as a diagnostic tool — became responsible for installing and managing one of the first real-time PCR platforms in Latin America, which gave rise to the first multi-user technological platform at Fiocruz. Mastering this new technique enabled researchers to perform qPCR assays targeting the kDNA of *T. cruzi*.

However, limitations in the precise quantification of parasite load were identified, mainly due to the high variability in minicircle copy numbers among parasite strains and isolates, even between clones of the same lineage.[34] Although kDNA targets provide excellent analytical sensitivity, nuclear satellite DNA (satDNA) became the preferred target for qPCR assays. This shift occurred because satDNA offers greater consistency in copy numbers across *T. cruzi* lineages and superior robustness for quantitative applications, despite its lower abundance in TcI, which has 5- to 10-fold fewer satDNA copies.[35]

The first study to evaluate real-time PCR as an accurate and sensitive method for quantifying tissue parasite burden in mice infected with *T. cruzi* assessed both satDNA and kDNA minicircles using SYBR Green.[36] Subsequently, a Spanish group developed a real-time PCR assay using a TaqMan probe targeting *T. cruzi* satDNA to detect parasite DNA in the blood of CD patients.[37] Using this same molecular target, Duffy et al.[35] established a SYBR Green-based real-time PCR targeting conserved motifs in these repetitive satellite sequences to monitor disease reactivation in heart transplant patients under immunosuppressive therapy, as well as post-treatment outcomes in paediatric patients. The inclusion of a standardised amount of plasmid containing a heterologous sequence allowed for the normalisation of DNA extraction yields and the detection of false negatives due to reaction inhibition.[35]

Building on the support of PIDC Diagnostic Network III and the experience gained from real-time quantification assays applied to clinical trials, the team sought partnerships to develop and validate the materials and calibrators for qPCR, laying the groundwork for a prototype molecular diagnostic kit.

International consensus for PCR-based protocols

About 20 years ago, Latin American researchers acknowledged the need to standardise and validate PCR-based assays for CD diagnosis and management across laboratories and countries. Previously, each laboratory used its own protocol and technology, hampering the reliable comparison of results among investigator groups. An integrated effort by World Health Organization (WHO)/Training in Tropical Diseases (TDR) and Pan American Health Organization (PAHO) led to a 2008 symposium in Buenos Aires, where researchers from different countries gathered to establish a "Consortium for standardization and validation of the clinical use of PCR for *T. cruzi* DNA detection in CD".[38] The meeting defined best PCR practices, their clinical applicability and interpretation (with attention to diverse parasite lineages), as well as the technology's limitations.

The resulting information led to the formulation of a Standard Operating Procedure (SOP) for PCR, currently used as a diagnostic tool for acute CD cases. These include the early detection of congenital transmission and recent infections in children, the investigation of food-borne outbreaks, the identification of infection in organ recipients from donors with CD, and the monitoring of reactivation in immunosuppressed patients. Additionally, PCR has been frequently used to monitor treatment outcomes and to evaluate new drug candidates in clinical trials.[31]

Once again, in 2011, a collaborative initiative led by WHO/TDR and PAHO, with coordination from Argentina and Brazil, marked a significant advancement in standardising and validating quantitative molecular diagnostic approaches for CD.[39] The primary focus of this multinational effort was the detection and quantifi-

cation of parasite loads in peripheral blood samples from patients with acute and CCD. This initiative enabled the establishment of harmonised analytical qPCR protocols and their validation through interlaboratory assays across 14 countries. It also enabled the rigorous evaluation of TaqMan duplex qPCR assays that could serve as reliable surrogate biomarkers for both diagnosis and treatment monitoring. A key element of the duplex qPCR involved the incorporation of a normalised amount of plasmid containing a heterologous sequence prior to DNA extraction from blood samples. The plasmid served as an internal amplification control, allowing laboratories to monitor the entire diagnostic procedure and ensure the accuracy and reproducibility of results. This approach was fundamental in supporting analytical validation and ensuring consistency among participating laboratories.[39]

Thus, the 2008 Buenos Aires symposium established a consortium primarily focused on the standardisation and validation of conventional qualitative PCR methods, although a limited number of real-time PCR assays were also evaluated to assess the presence or absence of DNA in the samples. In contrast, the subsequent international initiative in 2011 was specifically dedicated to the harmonisation, analytical validation, and multicentre evaluation of qPCR methods for *T. cruzi* detection and quantification.

In a global context, molecular diagnosis is currently the gold standard for the early detection of acute cases of CD. Its use directly addresses non-vectorial transmission pathways — congenital transmission, blood transfusion, and organ transplantation — which drive the urbanisation of CD in the United States and Europe.[30] In this regard, reliable qPCR tests become necessary for screening initiatives aimed at the early identification and treatment of vertically acquired *T. cruzi* infections in neonates, ensuring timely, curative intervention.[25] Transmission via blood transfusion and organ transplantation is also highly relevant in non-endemic settings, making hemovigilance for CD systematic in many endemic and non-endemic countries.[24]

Roadmap for the development of the Biomol Chagas kit

Once the foundations for the molecular diagnosis of CD had been consolidated — namely, (i) the investigation of the best molecular targets for the detection and quantification of *T. cruzi* from different DTUs by qPCR;[35,37,40] (ii) the validation of assays in blood and tissue samples from patients with CD from different endemic areas and with varying parasitic loads;[39,41] and (iii) a consensus definition of the potential applications and limitations of molecular detection of *T. cruzi* DNA and parasite load quantification by qPCR[26,27] — there was a natural path towards developing *in vitro* diagnostic (IVD) kits for the molecular diagnosis of CD using human blood samples.

In the late 2000s, several research groups were involved in a large clinical study evaluating the efficacy of Bz in patients with CCD. The BENEFIT study (Benznidazole Evaluation For Interrupting Trypanosomiasis) was an international clinical trial designed to evaluate the use of Bz in patients with established CCD, particularly those with cardiac involvement.[42] The BENEFIT trial enrolled patients with chronic Chagas cardiomyopathy from Argentina, Bolivia, Brazil, Colombia, and El Salvador, providing a multinational cohort that subsequently revealed important geographic differences in PCR positivity and treatment responses among participants from different endemic regions. The study included more than 2,800 patients, and its main objective was to determine whether antiparasitic treatment at this advanced stage of the disease could reduce major clinical events, such as death, cardiac arrest, the need for transplantation, or the progression of heart failure. To achieve this objective, conventional PCR was used as the primary tool to evaluate the outcome of antiparasitic treatment.[42]

However, molecular diagnosis experts participating in the BENEFIT study recognised the need and opportunity to validate qPCR assays for detecting and quantifying parasite load in guanidine-EDTA-preserved blood samples from the study participants. Thus, in 2013, they published a paper[41] describing a methodological substudy aimed at validating these qPCR assays. For this purpose, the authors used pre-treatment samples from the BENEFIT study, enabling the evaluation of molecular test performance in a large multicentre cohort, with standardised procedures for sample collection, processing, and storage. Overall, the study demonstrated that qPCR is a sensitive and reproducible tool for detecting parasitaemia in chronic patients, even at low levels, and is particularly useful as a parasitological endpoint in studies like BENEFIT. This work by Moreira et al.[41] had a significant impact by contributing to the international standardisation of the molecular diagnosis of CD, enabling comparisons between studies and strengthening the use of qPCR in clinical and translational research. Additionally, it showed for the first time that parasite loads in patients from Brazil were significantly lower than those in patients from Colombia and Argentina. This suggests that such differences could reflect epidemiological and biological factors, such as the genetic diversity of circulating *T. cruzi*, variations in infection duration, the natural history of the disease, and differences in the host immune response.[41]

The leadership in this study,[41] together with the accumulated evidence and the international consensus on qPCR, led Fiocruz researchers to propose the development of a kit for the molecular diagnosis of CD using blood samples from patients with chronic disease. At that time, the main motivation was to create a tool to monitor patients undergoing antiparasitic treatment, although other applications, such as diagnosis in newborns (to monitor vertical transmission) and monitoring immunosuppressed patients (such as transplant recipients and HIV/*T. cruzi*-co-infected individuals), were also envisioned.

The proof of concept for the kit's development was extensively discussed in meetings of the PIDC diagnostic network. One of the key coordinators among the groups involved in CD diagnostics was Marco Aurélio Krieger, who was then affiliated with the Carlos Chagas Institute (Fiocruz Paraná) and coordinated the PDTIS (Technological Development Programme for Health

Inputs) diagnostic network. PDTIS was a strategic initiative aimed at strengthening Brazil's diagnostic capacity, especially for infectious and neglected diseases. This programme organised a network of laboratories distributed across different Fiocruz units and partner institutions with the goal of integrating technical expertise, infrastructure, and innovation in diagnostics. The network worked on the development, validation, and application of tests for several diseases of public health interest, including CD, leishmaniasis, tuberculosis, and arboviruses. One of the pillars of PDTIS was to support the creation of national diagnostic kits, from the research phase through multicentre validation.

In 2011, following the initial presentation of the project to develop a molecular diagnostic kit for CD at the annual PDTIS diagnostic network meeting, it was formally incorporated into the programme's project portfolio as project RID 34. Over the next three years, the project was closely monitored by the network, while the technological development of the prototype kit was carried out using inputs produced by the Institute of Molecular Biology of Paraná (IBMP). This development started from scratch, including defining the composition of a proprietary mastermix for the product and establishing the ideal concentrations of key reagents — such as $MgCl_2$, dNTPs, ROX, primers, and TaqMan probes — in a multiplex format. In summary, the main milestones achieved during the project's development within PDTIS between 2012 and 2014 included: (i) the development of a real-time multiplex qPCR assay with an initial evaluation of its performance in the molecular diagnosis and quantification of parasite load using blood samples from CD patients; (ii) the establishment of an exogenous Internal Amplification Control (IAC) in the multiplex qPCR targeting *T. cruzi* satDNA; (iii) the standardisation of the multiplex qPCR reaction using IBMP-produced reagents; and (iv) the analytical and clinical validation of the prototype kit using *T. cruzi* reference strains and samples from patients with CCD. By the end of 2014, although the prototype kit had been validated, uncertainty remained about how it would transition into a commercial product.

In 2015, a meeting was held involving researchers from the Oswaldo Cruz Institute (IOC) — who were responsible for developing the prototype — representatives from IBMP, and a team from the Institute of Immunobiological Technology of Fiocruz (Bio-Manguinhos). The main objective of this meeting was to decide the future of the kit. At that time, potential producers still faced many uncertainties, particularly regarding the kit's commercial market. It was then decided that IBMP would manufacture the kit, which would be named NAT (Nucleic Acid Test) Chagas.

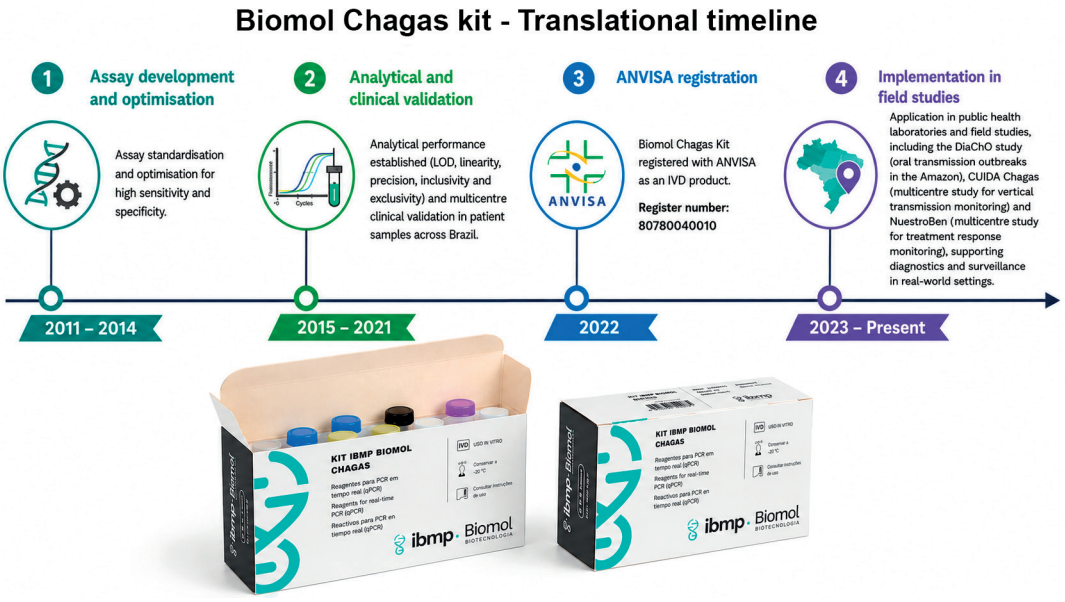
However, the following years were marked by considerable uncertainty regarding the future of the NAT Chagas kit. IBMP, a public-private (mixed capital) company closely integrated with Fiocruz, was consolidating its position in the national molecular diagnostics market. As a result, during the Zika epidemic (2015-2016), and the subsequent yellow fever epidemic (2017-2018), IBMP focused its efforts on developing and producing kits to meet the primary demands of the Brazilian Ministry of Health. Consequently, little progress was made on the NAT Chagas kit during this period.

However, the fate of the kit began to change in 2018 during the XV Meeting of the Translational Research Programme in Chagas Disease (Fio-Chagas 2018). This meeting, held at the Itaboraí Palace (a Fiocruz unit in Petrópolis, Rio de Janeiro, Brazil), was attended by Fiocruz researchers, CD specialists from various fields, and external guests, including Dr Pedro Albajar Viñas from the WHO Department of Control of Neglected Tropical Diseases in Geneva, who is responsible for the WHO's CD control programme. The President of Fiocruz, Dr Nísia Trindade Lima, was also in attendance. Throughout the event, a broad consensus emerged among participants regarding the urgency of finalising the kit, which specialists regarded as an imperative tool for advancing the diagnosis of CD. In this context, Dr Nísia requested that the IOC researchers coordinating the project draft a letter detailing the current status of the kit. She intended to forward this letter to the Vice President of Production and Innovation in Health to jointly discuss with the IBMP the necessary steps to complete the kit and register it with ANVISA (the Brazilian Health Regulatory Agency).

Indeed, this initiative propelled the subsequent stages of development and validation of the prototype kit. In the following years, IOC researchers and the IBMP team continued working to complete all the necessary steps to finalise the product, including the production of pilot batches, scale-up, and the establishment of consistent batch production. These efforts were associated with analytical and clinical validations using samples from *T. cruzi* reference strains and other hemoparasites, as well as samples from patients with CD. Finally, in 2022, the dossier containing the main results of this development was submitted to ANVISA. A few months later, in July 2022, the kit was registered by ANVISA under registration number 80780040010. The IBMP Biomol Chagas kit, with its translational timeline from assay development to implementation in field studies, is represented in Figure.

The performance of the Biomol Chagas kit

The main results of the validations performed with the IBMP Biomol Chagas kit to obtain regulatory approval were published in 2023.[43] In that study, the kit demonstrated a dynamic range from 10^4 to 0.5 parasite equivalents/mL and a limit of detection (LOD_{95}) of 0.16 parasite equivalents/mL of blood. The LOD_{95} was estimated using DNA extracted from GEB samples spiked with known quantities of *T. cruzi* (Y strain-TcII) and analysed with the Biomol Chagas kit and an in-house assay. The DNA recovered from these spiked samples was amplified by qPCR for five consecutive days, yielding a total of 60 replicates for each parasite concentration. In addition, the kit detected *T. cruzi* strains belonging to all six discrete typing units (DTUs - TcI to TcVI), performing similarly to the in-house qPCR assay evaluated in parallel. Because the kit demonstrated similar performance in detecting parasites from different DTUs, no major differences in sensitivity are expected among CD patients from different countries. Indeed, in studies currently being conducted in



Translational timeline of the Biomol Chagas kit from assay development to field study implementation. This timeline summarises the major milestones in the development and translational pathway of the IBMP Biomol Chagas Kit, a real-time polymerase chain reaction (qPCR) assay for the molecular detection of *Trypanosoma cruzi*. LOD: limit of detection; ANVISA: Brazilian Health Regulatory Agency; IVD: *in vitro* diagnostics; DiaChO: oral Chagas diagnosis; CUIDA Chagas: communities united for innovation, development, and care for Chagas disease.

various endemic countries — such as Argentina, Bolivia, and Brazil — the kit has shown excellent performance compared to in-house PCR (unpublished data). However, these studies must still be completed to ensure the kit's widespread use throughout the endemic region.

Clinical validation was performed using guanidine-EDTA blood samples from 50 participants from the Northeast, Southeast, South, and Central-West regions of Brazil, including 32 individuals who were seropositive and 18 who were seronegative for CD. All positive patients had CCD, with or without cardiomyopathy, and originated from different Brazilian states, where they were infected with *T. cruzi* strains from distinct DTUs. The results obtained with the IBMP Biomol Chagas kit showed 100% agreement with the in-house qPCR assay. Furthermore, when qPCR results were compared with serology as the reference method, a sensitivity of 59.4% and a specificity of 100% were observed.[43] The estimated sensitivity of qPCR relative to the serological reference standard should be interpreted cautiously, as seropositive patients with CCD do not consistently exhibit detectable circulating *T. cruzi* DNA; thus, this value does not necessarily reflect an intrinsic limitation of the molecular assay. This relatively low sensitivity can be explained by the biological characteristics of chronic *T. cruzi* infection, which features low and intermittent parasitaemia. Consequently, strategies such as serial blood sampling, processing larger blood volumes, optimising DNA extraction methods, and implementing highly sensitive technologies, including digital PCR, are being explored to improve parasite detection.[44,45,46]

Although the kit was registered for detecting *T. cruzi* DNA in blood, it was also used in this study to quantify parasite load using a standard curve based on synthetic parasite DNA, similar to the positive control provided with the kit. In experiments with the in-house qPCR assay, the parasite load ranged from 0.15 to 112.64 satDNA copies/µL, with a median of 8.11 satDNA copies/µL. In comparison, parasite loads ranging from 0.12 to 158.09 satDNA copies/µL were observed using the IBMP Biomol Chagas kit, with a median of 5.35 satDNA copies/µL. When the parasite load values obtained by both assays were compared, no significant difference was found.[43] However, it is important to note that, using the same quantification approach, Muñoz-Calderón et al.[47] reported LOD_{95} values of 0.903 satDNA copies/µL [95% confidence interval (CI): 0.745-1.497] for the Dm28c strain (TcI), and 0.667 satDNA copies/µL (95% CI: 0.113-3.927) for the CL Brener clone (TcVI). This indicates that the parasite loads detected in most of the patients were above the analytical detection limits of the assay.

However, it was still necessary to validate the kit in other scenarios where molecular diagnosis shows great promise for improving CD detection, such as in the investigation of vertical transmission and oral transmission outbreaks of *T. cruzi*. To this end, a collaboration was established with researchers from the Hospital de Niños Dr Ricardo Gutiérrez (Buenos Aires, Argentina) and the Fundação de Medicina Tropical Dr Heitor Vieira Dourado (Manaus, Brazil). Although these data have not yet been published, the kit showed high agreement with the results obtained using the reference molecular assay (the in-house qPCR). Thus, the kit, initially validated for samples from chronic patients, also demonstrated high potential for accurate molecular diagnosis in acute CD settings.

For a new diagnostic kit to be adopted by Brazil's Central Public Health Laboratories (LACENs) within the Brazilian Public Health System (SUS), it must first

be evaluated by National Commission for the Incorporation of Technologies into the SUS (CONITEC). In 2022, when validation results were presented at the Chagas-Leish meeting held during the 57th Congress of the Brazilian Society of Tropical Medicine (SBMT), the SBMT board issued a Public Motion and sent it to the Ministry of Health. This document highlighted the need to implement molecular diagnosis for CD in settings where qPCR assays offer clear advantages over current parasitological methods. The IBMP Biomol Chagas kit — the only kit registered in Brazil for the molecular diagnosis of CD — was featured in the document as a highly important and urgent tool for advancing CD diagnosis in the country. Upon receiving the motion, Ministry of Health technicians met to review the kit. While the Ministry recognised the importance of implementing the kit within the SUS, it requested additional field studies involving larger sample sizes to support a cost-effectiveness analysis before CONITEC could consider the kit for incorporation.

The use of the Biomol Chagas kit in the field

The need to commercialise kits for the molecular diagnosis of CD has long been discussed as a necessary step towards implementing PCR in routine diagnostic laboratories.[26,27,48] Although developed decades ago, in-house conventional and real-time PCR assays remain largely restricted to specialised centres, as they are more difficult to implement and validate in lower-complexity laboratories. In contrast, diagnostic kits are generally designed to facilitate use, including all the controls necessary for testing. Moreover, because they are manufactured under Good Manufacturing Practices (GMP), they offer high reproducibility and strict quality control, which are essential for their proper implementation in routine diagnostic laboratories or clinical trials. The Biomol Chagas kit was developed to broaden access to standardised, quality-assured molecular diagnostics beyond specialised reference centres across Latin America.

Beyond Latin America, the globalisation of CD has created an increasing demand for sensitive molecular diagnostic tools in non-endemic settings — including the United States, European countries, and Australia — particularly for blood safety programmes, congenital screening initiatives, and the clinical management and monitoring of chronically infected individuals, in whom parasitaemia is typically low and serology remains the primary diagnostic approach.[30]

Currently, the market for molecular diagnostic kits for CD includes several options, such as the Biomol Chagas (IBMP, Brazil), the RealStar Chagas PCR Kit 1.0 (Altona Diagnostics, Germany), the *T. cruzi* DNA Test (Wiener, Argentina), and the VIASURE *Trypanosoma cruzi* Real Time PCR Detection Kit (CerTest, Spain). All these kits were developed to amplify the most studied molecular targets of *T. cruzi* described in the literature, such as nuclear satellite DNA (satDNA) and kinetoplast DNA (kDNA). Nevertheless, their commercialisation remains limited, as regulatory approval in most countries is still pending. In Brazil, the only kit registered with ANVISA for the molecular diagnosis of CD is the Biomol Chagas kit (IBMP). In addition to pending regulatory approval in many countries, the widespread implementation of commercial PCR assays for CD is challenged by economic constraints, limited laboratory infrastructure, insufficient technical expertise, and unequal access to molecular diagnostic platforms in resource-limited endemic settings.

Given the need to use standardised kits instead of in-house qPCR assays to ensure greater reproducibility and quality control, several clinical studies conducted in endemic countries in Latin America have begun to adopt the Biomol Chagas kit. The CUIDA Chagas study (Communities United for Innovation, Development, and Care for CD)[49] is an international initiative coordinated by Fiocruz that aims to test, treat, and care for people affected by CD in Latin America, operating in countries such as Bolivia, Brazil, Colombia, and Paraguay. The study incorporated the Biomol Chagas kit for the detection of *T. cruzi* DNA in blood samples from newborns with suspected congenital transmission and adults with CCD. Its use in CUIDA Chagas has improved diagnostic sensitivity, particularly in settings where parasitaemia is low and conventional parasitological methods have limitations.

The NuestroBen study,[50] coordinated by the Drugs for Neglected Diseases Initiative (DNDi), focuses on evaluating the effectiveness of Bz treatment under different therapeutic regimens, with an emphasis on the longitudinal follow-up of patients and the identification of early markers of therapeutic response. Conducted in endemic settings, the study aims to generate robust evidence on the impact of antiparasitic treatment, especially in the chronic phase of the disease, where assessing cure remains a major challenge. In this context, the study incorporated the Biomol Chagas kit to detect *T. cruzi* DNA in peripheral blood samples from patients with CCD. The application of the kit has enabled the monitoring of parasitaemia over time, serving as an important tool to assess treatment response, identify therapeutic failures, and detect possible cases of parasite persistence.[50]

The Biomol Chagas kit is also being evaluated in the context of the oral transmission of *T. cruzi*, a highly relevant epidemiological scenario in Brazil today. DiaChO (Oral Chagas Diagnosis) — a diagnostic accuracy study coordinated by Fiocruz and registered with ReBEC (RBR-34sj2yx) — aims to improve the diagnosis of patients involved in oral outbreaks of CD. This is achieved through the comparative validation of qPCR (Biomol Chagas - IBMP) and LAMP (Loopamp Chagas - Eiken) kits. The study uses samples from patients in the acute phase of the disease, individuals in the recent chronic (post-acute) phase, and food products associated with oral outbreaks in northern Brazil. The trial is being conducted in the states of Pará, Amapá, and Amazonas, where oral transmission, frequently linked to *açaí* consumption, shows the highest prevalence in the country. In this study, the performance of molecular diagnostic kits is compared with that of conventional parasitological and serological tests to assess their potential to improve clinical sensitivity and enable early detection of orally transmitted CD. Early diagnosis facilitates the timely initiation of antiparasitic treatment, potentially preventing disease progression and reducing mortality rates. The integration of

LAMP into the DiaChO study was motivated by its operational advantages for outbreak investigation, such as isothermal amplification without a thermocycler, simplified laboratory infrastructure, rapid turnaround time, and the potential to expand molecular testing in decentralised, resource-limited settings. Meanwhile, qPCR serves as the reference standard for comparative evaluation.

Although these studies are still ongoing, their results already demonstrate the advantages and economic feasibility of using the kit for the molecular diagnosis of CD. Though the kit is intended for laboratories with the infrastructure required to perform PCR assays, samples can be transported at room temperature after stabilisation with guanidine-EDTA buffer, which can be supplied with the kit. This facilitates the implementation of the methodology within the SUS. The Biomol Chagas kit could also be integrated into routine molecular diagnostic services through the LACEN network and blood banks, which already use qPCR- or RT-qPCR-based kits to detect influenza A, influenza B, coronavirus disease 2019 (COVID-19), HIV, hepatitis B, hepatitis C, *Plasmodium*, Zika virus, dengue virus, chikungunya virus, and yellow fever virus. This would represent a major advance in the diagnosis of CD in Brazil.

After decades of advances in the molecular diagnosis of CD, the development of the Biomol Chagas kit represents a success story of resilience, in which the Fio-Chagas Translational Programme played a fundamental role at every stage of its development and validation, as well as in supporting its implementation in routine practice within the SUS.

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To the Fio-Chagas Translational Programme in Chagas Disease, especially the members of the Diagnostic Network, for fostering an environment of discussion and encouragement for the development of diagnostic tools for this neglected disease. They also thank the Technological Development Programme for Health Inputs (PDTIS) at Fiocruz for its support and regular follow-up during the proof-of-concept phase of the kit's development. The authors pay tribute to Marco Aurélio Krieger (*in memoriam*), Vice President of Research, Production and Innovation in Health at Fiocruz from 2017 to 2025, for initiating — together with the team from the Institute of Molecular Biology of Paraná (IBMP) — the development of the Biomol Chagas kit, as well as the subsequent production of pilot batches, scale up, and presentation of the main results to ANVISA. They also express their gratitude to Dr Carlos Morel, who, beginning in the 1990s within the former Department of Biochemistry and Molecular Biology (DBBM), led the project to develop the PCR assay for the molecular diagnosis of Chagas disease in collaboration with Dr Patrick Wincker. Finally, they acknowledge Maria Angelica Cardoso for her indispensable technical support throughout all stages of the standardization and development of the PCR assays.

AUTHORS' CONTRIBUTION

CB and OCM participated in conceptualisation, investigation, data curation, validation, original draft preparation, critical revision of the intellectual content, and the approval of the final version of the manuscript. The authors declare no conflicts of interest.

DATA AVAILABILITY

The data supporting this study are available within the article.

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OPEN PEER REVIEW

Memórias do IOC thanks the anonymous reviewers for their contribution to the peer review of this work.

FIRST REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

This historical narrative celebrates the 25th anniversary of the Fio-Chagas Translational Program by chronicling the development of PCR-based molecular diagnostics for Trypanosoma cruzi, culminating in the regulatory approval and field deployment of the IBMP Biomol Chagas Kit. The authors — both long-standing leaders in this field, trace the arc from foundational kDNA minicircle sequencing in the 1980s through international consensus-building, qPCR validation in the BENEFIT trial sub-study, and the eventual ANVISA registration in 2022. The manuscript is well-organized, historically accurate, and provides important institutional memory for the Chagas disease research community. This is a valuable commemorative contribution that will be of interest to the Chagas disease research community. The narrative is coherent, and the authors are authoritative voices on this subject. The main weaknesses are (1) limited critical reflection on setbacks and unresolved gaps, (2) some missing quantitative context that would strengthen the historical framing, and (3) minor structural and referencing issues. These are readily addressable and do not require major revision.

Comments

Lines 1–12. The abstract is very brief and does not mention the Biomol Chagas Kit by name, even though it is the central product described in the manuscript. Consider adding one sentence specifying the kit and its regulatory status.

Line 6. The phrase 'historical narrative presents a timeline' is slightly redundant. Consider: 'This narrative presents a chronological account of...'

Lines 20–28: The description of kDNA structure and minicircle organization is technically sound, but the sentence 'These molecules were composed of heterogeneous hypervariable sequence regions interspersed with four direct repeats of conserved nucleotide sequences (~120 bp), each containing smaller blocks of nearly identical composition (~18 bp), arranged in the same orientation' is dense for general readers of MIOC. Consider adding a brief explanatory clause or a simple parenthetical to clarify why this structure is diagnostically useful.

Lines 52–58: The explanation of why T. rangeli necessitated primer adjustment would benefit from a one-sentence clarification that T. rangeli is non-pathogenic to humans but shares triatomine vectors, to help readers who are not specialists in trypanosomatid biology understand the diagnostic risk of cross-reactivity.

Line 70: '96.5% sensitivity was achieved in a highly endemic area of Minas Gerais', please specify the sample size and patient population (seropositive chronic patients, confirmed acute, etc.) for this figure, or direct readers to the specific reference for these details

Lines 113–116. The statement that 'research into new drugs for Chagas disease was minimal compared to non-neglected diseases like AIDS' is imprecise. AIDS is not a neglected disease; the intended contrast is presumably with well-funded diseases. Suggest revising to: 'compared to diseases attracting significant commercial investment, such as HIV/AIDS.'

Lines ~123–128. The discussion of differences in PCR sensitivity due to intermittent parasitemia and blood volume is important but abrupt. A single bridging sentence connecting this to the chronic phase biology would improve the flow.

Insufficient critical discussion of persistent diagnostic limitations. The manuscript reads largely as a success story, but the field continues to face well-known unresolved challenges that should be acknowledged more explicitly in a historical narrative of this scope.

Lines 304–340. The sensitivity of qPCR in chronic Chagas disease remains a fundamental limitation (reported at 59.4% for the Biomol Chagas Kit in the validation study, as the authors mention. However, the manuscript does not discuss the biological reasons underlying this limitation, namely, intermittent and low-level parasitemia or what strategies are being pursued to address it.

Lines 100, 103–108. The long-standing absence of a cure criterion based on parasitological or molecular endpoints is mentioned but deserves a more substantive discussion, particularly in light of the PCR-negative-does-not-equal-cure caveat noted on lines. Readers would benefit from understanding the current consensus on how PCR negativity should be interpreted during post-treatment follow-up.

The observation that Brazilian patients show significantly lower parasite loads than Colombian and Argentine patients (Lines 253–260) is a striking and important finding, but it is presented without a hypothesis or discussion of its implications for kit performance across different endemic settings. This should be briefly addressed.

Missing context on the current diagnostic landscape and unmet needs. For a historical narrative targeting an international readership, additional context would strengthen the framing and motivation:

Lines 16–40. The Introduction does not situate the magnitude of the Chagas disease burden or the scale of underdiagnosis that makes molecular diagnostics necessary. A brief statement of disease burden (estimated global infections and the proportion undiagnosed) would provide essential context. This is especially relevant given that this narrative is intended to celebrate translational progress.

Lines 370–378. The manuscript notes that regulatory approval for commercial Chagas PCR kits in most countries outside Brazil is pending, a significant limitation to global uptake. A sentence on the barriers that exist (regulatory, economic, infrastructure) would be informative and consistent with the narrative's translational framing.

The role of the U.S. and non-Latin American contexts is absent. Chagas disease is now recognized as a significant imported and locally transmitted infection in the United States, Europe, and Australia. The manuscript focuses exclusively on Latin American endemic settings and Brazilian regulatory frameworks. While this is appropriate for the Fio-Chagas anniversary context, at least a brief acknowledgment of molecular diagnostic needs in non-endemic settings (e.g., blood banking, congenital screening in the U.S. and Europe) would broaden the manuscript's relevance to the journal's international audience. This is especially pertinent given that T. cruzi infection in non-endemic countries is largely chronic and serology-confirmed, precisely the setting where qPCR is most needed.

qPCR Development (lines 138–200)

Line 148. 'The first real-time PCR thermocycler of Latin America, this is a bold historical claim. If documented, please provide a citation or institutional record. If not citable, soften the claim: 'one of the first real-time PCR platforms established in Latin America.'

Lines 155–162. The limitation of kDNA minicircle copy number variability among strains as a barrier to precise quantification is mentioned but could be slightly expanded. A single sentence noting that satellite DNA (satDNA) was ultimately preferred for quantification due to its greater copy-number consistency across DTUs would help readers understand why the field transitioned away from kDNA targets for qPCR.

Lines 204–210. The 2008 Buenos Aires symposium is described as establishing a 'Consortium for standardization and validation of the clinical use of PCR (conventional, qualitative).' The parenthetical '(conventional, qualitative)' implies the 2008 consensus focused only on conventional PCR, whereas qPCR standardization came later with the 2011 initiative. This distinction is made implicitly but could be stated more explicitly for clarity.

Line 220. The phrase 'marked a significant advancement' is slightly vague. Specify what was advanced: harmonized analytical protocols, ring-trial validation across 14 countries, or selection of TaqMan duplex qPCR as the reference method.

Lines 268–272. The BENEFIT trial is introduced as a clinical trial in 'several Latin American countries,' but the specific countries are not named. Given that the manuscript later notes that parasite loads differed between Brazil, Colombia, and Argentina (a key finding from the BENEFIT sub-study), naming the participating countries would be consistent and informative.

Lines 310–320: The role of the Zika and Yellow Fever epidemics in delaying kit development is explained honestly and adds valuable institutional context. This is one of the strongest passages for illustrating the challenges of translational work in a public health institution. No changes needed, this is commended.

Lines 324–332: The account of the Fio-Chagas 2018 meeting and President Lima's involvement is historically important. However, the phrase 'strong pressure for the completion of the kit' is informal for a scientific manuscript. Consider: 'broad consensus among participants on the urgency of finalizing the kit.'

Lines 343–347. The LOD of 0.16 parasite equivalents/mL is an impressive analytical figure. Please clarify whether this was determined using spiked samples, reference strains, or clinical samples, as LOD claims can differ substantially depending on the validation matrix.

Lines 354–360. The reported 59.4% sensitivity relative to serology as the reference method is appropriately contextualized in the manuscript. However, I recommend adding a brief clarification that serology represents an imperfect reference standard for qPCR evaluation, given that chronic-phase patients with confirmed seropositivity do not consistently exhibit detectable circulating parasite DNA. This important caveat would prevent readers from misinterpreting the 59.4% sensitivity as an intrinsic limitation of the molecular assay, when it may instead reflect the biological reality of intermittent parasitemia in chronic Chagas disease.

Lines 370–376. The claim that parasite loads in the validation study ranged up to 158.09 satDNA copies/µL is presented without units normalized to blood volume (copies/mL or equivalents/mL). Please ensure consistency with the units used in the LOD statement (parasite equivalents/mL).

Lines 405–410. The statement that in-house assays 'are still largely restricted to more specialized centers' is true, but it does not acknowledge the important role that reference laboratories in Latin America play in current Chagas disease diagnosis. A brief acknowledgment of the existing infrastructure and of how the Biomol Chagas Kit is intended to expand capacity beyond these centers would be appropriate.

Lines 432–440. The DiaChO study, which evaluates LAMP alongside qPCR for outbreaks of oral Chagas transmission, is an exciting development. A brief note on the theoretical advantages of LAMP in outbreak settings (no thermocycler required, simpler infrastructure) would help readers understand why this comparative study was designed.

Lines 453–462: The final paragraph proposing integration of the Biomol Chagas Kit into the SUS LACENs network via the existing qPCR infrastructure for Influenza, COVID-19, HIV, etc., is one of the manuscript's strongest translational statements and is well-argued. No changes needed.

Recommendation: Accepted with Minor Revision. This is an important historical narrative from two of the field's most authoritative researchers. The manuscript will make a lasting contribution to the documentation of translational science in Chagas disease. The requested revisions are primarily clarifications and contextual additions rather than substantive restructuring. The authors are encouraged to:

- Add a brief critical reflection on the persistent 59.4% sensitivity limitation and its biological basis
- Clarify or soften the 'first real-time PCR thermocycler in Latin America' claim with a citation or qualifier
- Add one sentence of global/non-endemic context for molecular diagnosis
- Ensure parasite load units are consistent throughout.

All other comments are optional suggestions that the authors may address at their discretion.

REVIEWER #2

The manuscript provides a comprehensive historical overview of the contributions made by the Oswaldo Cruz Foundation (Fiocruz) and the Oswaldo Cruz Institute to the development, optimization, and validation of molecular methods for the diagnosis of Chagas disease. It traces the institution's trajectory in advancing molecular diagnostics, including significant achievements in the identification and evaluation of molecular markers of treatment failure, as well as its role in the development and implementation of domestically manufactured diagnostic kits. Overall, the study offers valuable insight into the institution's longstanding contribution to strengthening diagnostic capacity, improving patient follow-up, and fostering technological innovation for the diagnosis and treatment of Trypanosoma cruzi infection.

Handling Editor's Comments

Dear Authors,

Thank you for submitting your manuscript entitled "Technological advances in the molecular diagnosis of Chagas disease from the perspective of the Fio-Chagas Translational Program" to Memórias do Instituto Oswaldo Cruz.

The manuscript was evaluated by two reviewers. One reviewer recommended acceptance in its current form, whereas the second reviewer recommended minor revision. After considering both reports, I agree that the manuscript is suitable for publication after minor revision.

The manuscript provides a valuable historical and translational account of the development of PCR-based molecular diagnosis for Chagas disease within the Fio-Chagas Translational Program, culminating in the development, regulatory approval, and field application of the Biomol Chagas Kit. The reviewers recognized the relevance of the contribution, the authority of the authors, and the importance of preserving this institutional and scientific memory for the Chagas disease research community.

Before acceptance, please revise the manuscript addressing the following points:

Add a brief critical discussion of the persistent limitations of qPCR sensitivity in chronic Chagas disease, particularly in relation to intermittent and low-level parasitemia, and clarify the interpretation of PCR-negative results during post-treatment follow-up.

Clarify the statement regarding the first real-time PCR thermocycler in Latin America. If this claim is supported by a citation or institutional record, please provide it. Otherwise, please soften the wording.

Ensure consistency in the units used to report parasite load and limit of detection throughout the manuscript.

Add brief contextual information on the relevance of molecular diagnosis beyond Latin American endemic settings, including its potential role in non-endemic countries.

Revise the abstract to explicitly mention the Biomol Chagas Kit and its regulatory status, since this product is central to the manuscript.

In addition, I encourage the authors to consider including a high-resolution figure illustrating the Biomol Chagas Kit, or a composite figure showing the kit together with a concise timeline of its development, validation, ANVISA registration, and field implementation. Given the historical and translational nature of the article, such a figure would strengthen the visual presentation and help readers understand the concrete technological product emerging from the Fio-Chagas trajectory.

The remaining reviewer comments should be addressed where appropriate, especially those related to clarification of technical terminology, sample size/context for reported performance estimates, and minor wording improvements.

Please submit a revised version of the manuscript together with a point-by-point response to the reviewer's comments.

Sincerely,

Fred Luciano Neves Santos

Guest Editor

Memórias do Instituto Oswaldo Cruz

Reviewer: 1

This historical narrative celebrates the 25th anniversary of the Fio-Chagas Translational Program by chronicling the development of PCR-based molecular diagnostics for Trypanosoma cruzi, culminating in the regulatory approval and field deployment of the IBMP Biomol Chagas Kit. The authors — both long-standing leaders in this field, trace the arc from foundational kDNA minicircle sequencing in the 1980s through international consensus-building, qPCR validation in the BENEFIT trial sub-study, and the eventual ANVISA registration in 2022. The manuscript is well-organized, historically accurate, and provides important institutional memory for the Chagas disease research community.

This is a valuable commemorative contribution that will be of interest to the Chagas disease research community. The narrative is coherent, and the authors are authoritative voices on this subject. The main weaknesses are (1) limited critical reflection on setbacks and unresolved gaps, (2) some missing quantitative context that would strengthen the historical framing, and (3) minor structural and referencing issues. These are readily addressable and do not require major revision.

R: We thank the reviewer for their generous and encouraging comments. We are pleased that the manuscript successfully captures the historical arc of the Fio-Chagas Translational Program and the development of the IBMP Biomol Chagas Kit. Your recognition of the manuscript's value as an important institutional memory for the Chagas disease community is highly appreciated. The weaknesses pointed out were considered in its new version.

Comments

Lines 1–12. The abstract is very brief and does not mention the Biomol Chagas Kit by name, even though it is the central product described in the manuscript. Consider adding one sentence specifying the kit and its regulatory status.

R: Thank you for this important comment. At the end of the abstract we included:

"Following the proof-of-concept, the qPCR-based Biomol Chagas kit was manufactured using reagents produced by the Institute of Molecular Biology of Paraná. After analytical and clinical validation, the kit was registered in 2022 by the Brazilian Health Surveillance Agency (ANVISA). Additional field-condition studies were required for a cost-effectiveness analysis by the National Commission for the Incorporation of Technologies into the Brazilian Unified Health System (CONITEC). Currently, the kit has been incorporated into clinical studies in Latin America to diagnose congenital transmission, monitor the impact of antiparasitic treatment in chronic Chagas disease, and evaluate oral transmission in the Northern Region of Brazil."

Line 6. The phrase 'historical narrative presents a timeline' is slightly redundant. Consider: 'This narrative presents a chronological account of...'

R: Ok, done.

Lines 20–28: The description of kDNA structure and minicircle organization is technically sound, but the sentence 'These molecules were composed of heterogeneous hypervariable sequence regions interspersed with four direct repeats of conserved nucleotide sequences (~120 bp), each containing smaller blocks of nearly identical composition (~18 bp), arranged in the same orientation' is dense for general readers of MIOC. Consider adding a brief explanatory clause or a simple parenthetical to clarify why this structure is diagnostically useful.

R: Thank you for this significant critique that made us rewrite the sentence more concisely.

"These molecules contain four highly conserved regions (located 90° apart) that are invariant across strains and clones of the parasite, interspersed by hypervariable sequence regions."

Lines 52–58: The explanation of why T. rangeli necessitated primer adjustment would benefit from a one-sentence clarification that T. rangeli is non-pathogenic to humans but shares triatomine vectors, to help readers who are not specialists in trypanosomatid biology understand the diagnostic risk of cross-reactivity.

R: Thank you for your comment. The following sentence was included for clarification.

"It should be noted that *T. rangeli* is not pathogenic to humans but shares some triatomine vector species, making primer adjustments necessary to avoid the risk of cross-reaction."

Line 70: '96.5% sensitivity was achieved in a highly endemic area of Minas Gerais', please specify the sample size and patient population (seropositive chronic patients, confirmed acute, etc.) for this figure, or direct readers to the specific reference for these details

R: Ok, sample size and patient population were specified.

Lines 113–116. The statement that 'research into new drugs for Chagas disease was minimal compared to non-neglected diseases like AIDS' is imprecise. AIDS is not a neglected disease; the intended contrast is presumably with well-funded diseases. Suggest revising to: 'compared to diseases attracting significant commercial investment, such as HIV/AIDS.'

R: We agree with your suggestion, the sentence was revised.

Lines ~123–128. The discussion of differences in PCR sensitivity due to intermittent parasitemia and blood volume is important but abrupt. A single bridging sentence connecting this to the chronic phase biology would improve the flow.

Insufficient critical discussion of persistent diagnostic limitations. The manuscript reads largely as a success story, but the field continues to face well-known unresolved challenges that should be acknowledged more explicitly in a historical narrative of this scope.

R: Thank you for your valuable analysis. We acknowledge the insufficient critical discussion of diagnostic restrictions and agree that balancing the historical success narrative with persistent, unresolved limitations strengthens the manuscript.

To resolve these issues, we have made the following revisions to the manuscript:

"Evaluating therapeutic efficacy in chronic *T. cruzi* infection is severely hindered by a lack of early surrogate biomarkers of treatment response or cure.[20] Because antibodies remain persistently positive for years or decades, serological tests cannot distinguish between cleared infection and treatment failure in the short term. Molecular methods remain the gold standard for early detecting treatment failure, but do not constitute definitive tests of cure.[19-20,24] Considering the fluctuation in parasitemia during the long course of Chagas disease, there is no guarantee that a single "flash" of negative posttreatment PCR means parasitological cure, it may probably indicates the absence of parasite DNA in the blood at that moment, not being able to distinguish between sterilizing cure and temporary suppression of detectable parasitemia.[19] This diagnostic uncertainty stems directly from the underlying biology of the chronic phase of disease, where an equilibrium between host immunity and tissue-sequestered parasites results in low and intermittent parasitemia that fundamentally impact the performance of blood-based molecular assays. Consequently, this scenario leads to a lack of consensus on how to state treatment efficacy in chronic infection, turning the development of early reliable markers of cure a major priority identified in Target Product Profiles for Chagas disease.[25-27]

Lines 304–340. The sensitivity of qPCR in chronic Chagas disease remains a fundamental limitation (reported at 59.4% for the Biomol Chagas Kit in the validation study, as the authors mention. However, the manuscript does not discuss the biological reasons underlying this limitation, namely, intermittent and low-level parasitemia or what strategies are being pursued to address it.

R: We agree that the biological basis for the limited sensitivity of qPCR in chronic Chagas disease deserves further discussion. In chronically infected individuals, parasitemia is typically low, fluctuating, and often intermittent, which inherently limits the probability of detecting circulating parasite DNA in a single blood sample. We have therefore revised the manuscript to explicitly address these biological constraints and to discuss current strategies aimed at improving molecular detection, including optimization of blood processing and DNA extraction procedures, the analysis of larger blood volumes, serial sampling, and the development of ultrasensitive molecular approaches such as digital PCR.

Thus, the following text was added to the manuscript: "The estimated sensitivity of qPCR using serology as the reference standard should be interpreted cautiously, as seropositive patients with chronic Chagas disease do not consistently exhibit detectable circulating T. cruzi DNA; therefore, this value does not necessarily reflect an intrinsic limitation of the molecular assay. This relatively low sensitivity can be explained by the biological characteristics of chronic T. cruzi infection, due to low and intermittent parasitemia; consequently, strategies such as serial blood sampling, processing of larger blood volumes, optimization of DNA extraction methods, and the implementation of highly sensitive technologies, including digital PCR, are being explored to improve parasite detection (Parrado et al., 2019; Farani et al., 2025; Ramírez and Moreira, 2019)".

Lines 100, 103–108. The long-standing absence of a cure criterion based on parasitological or molecular endpoints is mentioned but deserves a more substantive discussion, particularly in light of the PCR-negative-does-not-equal-cure caveat noted on lines. Readers would benefit from understanding the current consensus on how PCR negativity should be interpreted during post-treatment follow-up.

R: We agree that this issue deserves further discussion, particularly because no parasitological or molecular marker has yet been validated as a definitive criterion of cure in Chagas disease. This diagnostic uncertainty stems directly from the underlying biology of the chronic phase of disease, where an equilibrium between host immunity and tissue-sequestered parasites results in low and intermittent parasitemia that fundamentally impact the performance of blood-based molecular assays. Current consensus indicates that a positive PCR result after treatment constitutes evidence of therapeutic failure, whereas a negative PCR result should be interpreted cautiously, as it does not necessarily indicate parasite clearance because of the scarce parasitemia characteristic of chronic T. cruzi infection. We have revised the manuscript to clarify this point and to highlight that, according to published Target Product Profiles and expert consensus documents, there remains an unmet need for validated biomarkers capable of providing an early and reliable assessment of treatment efficacy.

So, the following text was added to the manuscript:

"Evaluating therapeutic efficacy in chronic T. cruzi infection is severely hindered by a lack of early surrogate biomarkers of treatment response or cure (Pinazo et al. 2015). Because antibodies remain persistently positive for years or decades, serological tests cannot distinguish between cleared infection and treatment failure in the short term. Molecular methods remain the gold standard for early detecting treatment failure, but do not constitute definitive tests of cure (Britto 2009, Pinazo et al., 2015, Rassi et al., 2012). Considering the fluctuation in parasitemia during the long course of Chagas disease, there is no guarantee that a single "flash" of negative posttreatment PCR means parasitological cure, it may probably indicates the absence of parasite DNA at that

moment, not being able to distinguish between the complete eradication of the parasite and temporary suppression of detectable parasitemia (Britto 2009). This scenario leads to a lack of consensus on how to state treatment efficacy in chronic infection, turning the development of early reliable markers of cure a major priority identified in Target Product Profiles for Chagas disease (Porrás et al., 2015; Alonso-Padilla et al., 2020)."

The observation that Brazilian patients show significantly lower parasite loads than Colombian and Argentine patients (Lines 253–260) is a striking and important finding, but it is presented without a hypothesis or discussion of its implications for kit performance across different endemic settings. This should be briefly addressed.

R. As discussed in Moreira et al (2013), it is possible to hypothesize that individuals from Argentina, Brazil and Colombia were infected with distinct DTUs or even presented mixed infections. This in part could reflect the remarkable differences in parasite loads observed, mainly those between the Brazilian patients compared to the Argentinean and Colombian ones. Even so, as reported by Moreira et al. (2023), the BioMol Chagas kit demonstrated similar performance in detecting parasites from different DTUs. Therefore, no major differences in sensitivity are expected among Chagas disease patients from different countries. Indeed, in studies currently being conducted in various endemic countries—such as Argentina, Bolivia, and Brazil—the kit has shown excellent performance compared to in-house PCR (unpublished data). However, these studies must still be completed to ensure the kit's widespread use throughout the endemic region.

Regarding this subject, the following sentence was added to the manuscript:

"As the kit demonstrated similar performance in detecting parasites from different DTUs, no major differences in sensitivity are expected among Chagas disease patients from different countries. Indeed, in studies currently being conducted in various endemic countries—such as Argentina, Bolivia, and Brazil—the kit has shown excellent performance compared to in-house PCR (unpublished data). However, these studies must still be completed to ensure the kit's widespread use throughout the endemic region."

Missing context on the current diagnostic landscape and unmet needs. For a historical narrative targeting an international readership, additional context would strengthen the framing and motivation:

R. We thank the reviewer for this important point of view. A paragraph was included at the end of the chapter regarding the establishment of International Consensus for PCR-Based Protocols:

"In a global context, molecular diagnosis is currently the gold standard for early detection of acute cases of Chagas disease. It's use directly addresses non-vectorial transmission pathways - congenital, blood transfusion, and organ transplantation - which drive the urbanization of Chagas disease in the United States and Europe (Villa et al., 2007). In this regard, reliable qPCR tests become necessary for screening initiatives aimed at early identifying and treating vertically acquired T. cruzi infections in neonates, to ensure timely, curative intervention (WHO 2021). Transmission via blood transfusion and organ transplantation is also highly relevant in non-endemic settings, making hemovigilance for Chagas disease systematic in many endemic and non-endemic countries (Rassi et al., 2012).

Lines 16–40. The Introduction does not situate the magnitude of the Chagas disease burden or the scale of underdiagnosis that makes molecular diagnostics necessary. A brief statement of disease burden (estimated global infections and the proportion undiagnosed) would provide essential context. This is especially relevant given that this narrative is intended to celebrate translational progress.

R: We appreciate and agree with your valuable feedback. Two phrases were now included at the beginning of the abstract.

"An estimated 6 to 8 million people worldwide are infected with *Trypanosoma cruzi*, the parasite that causes Chagas disease. Of those infected, an alarming 70 to 90% remain undiagnosed, making it one of the most hidden global health burdens."

Lines 370–378. The manuscript notes that regulatory approval for commercial Chagas PCR kits in most countries outside Brazil is pending, a significant limitation to global uptake. A sentence on the barriers that exist (regulatory, economic, infrastructure) would be informative and consistent with the narrative's translational framing.

R: We thank the reviewer for this valuable suggestion. We agree that, beyond regulatory approval, several factors currently limit the global implementation of commercial PCR assays for Chagas disease, including economic constraints, limited laboratory infrastructure, and insufficient access to molecular diagnostic platforms in many endemic regions. We have now added a sentence to the manuscript to address these translational barriers.

"In addition to pending regulatory approval in many countries, the widespread implementation of commercial PCR assays for Chagas disease is challenged by economic constraints, limited laboratory infrastructure, insufficient technical expertise, and unequal access to molecular diagnostic platforms in resource-limited endemic settings."

The role of the U.S. and non-Latin American contexts is absent. Chagas disease is now recognized as a significant imported and locally transmitted infection in the United States, Europe, and Australia. The manuscript focuses exclusively on Latin American endemic settings and Brazilian regulatory frameworks. While this is appropriate for the Fio-Chagas anniversary context, at least a brief acknowledgment of molecular diagnostic needs in non-endemic settings (e.g., blood banking, congenital screening in the U.S. and Europe) would broaden the manuscript's relevance to the journal's international audience. This is especially pertinent given that T. cruzi infection in non-endemic countries is largely chronic and serology-confirmed, precisely the setting where qPCR is most needed.

qPCR Development (lines 138–200)
R: We thank the reviewer for this important comment. We agree that the initial version of the manuscript primarily emphasized the Latin American and Brazilian contexts, reflecting the historical and institutional perspective of the Fio-Chagas anniversary. However, Chagas disease has become a global health issue due to population mobility, with increasing recognition of both imported and autochthonous cases in the United States, Europe, and other non-endemic regions. We have now included a statement acknowledging the growing need for sensitive molecular diagnostic tools in non-endemic settings, particularly for applications such as blood donor screening, congenital Chagas disease surveillance, and the management of chronically infected individuals, where qPCR may provide important complementary information beyond serological diagnosis.

Thus, the following sentence was added to the manuscript:

"Beyond Latin America, the globalization of Chagas disease has created an increasing demand for sensitive molecular diagnostic tools in non-endemic countries, including the United States, Europe, and Australia, particularly for blood safety programs, congenital screening initiatives, and the clinical management and monitoring of chronically infected individuals in whom parasitemia is typically low and serology remains the primary diagnostic approach (Villa et al., 2007)."

qPCR Development (lines 138–200)

Line 148. 'The first real-time PCR thermocycler of Latin America, this is a bold historical claim. If documented, please provide a citation or institutional record. If not citable, soften the claim: 'one of the first real-time PCR platforms established in Latin America.'

R. Done. The sentence was modified, as suggested.

Lines 155–162. The limitation of kDNA minicircle copy number variability among strains as a barrier to precise quantification is mentioned but could be slightly expanded. A single sentence noting that satellite DNA (satDNA) was ultimately preferred for quantification due to its greater copy-number consistency across DTUs would help readers understand why the field transitioned away from kDNA targets for qPCR.

R. We thank the reviewer for this valuable suggestion. We agree that a brief explanation regarding the transition from kDNA to satDNA targets in quantitative PCR assays would improve the manuscript's clarity for a broader readership. We have therefore added a sentence highlighting the selection of nuclear satellite DNA as the ideal target for qPCR because of its comparatively greater copy-number stability across T. cruzi lineages.

Thus, the following sentence was added to the manuscript: "Although kDNA targets provide excellent analytical sensitivity, nuclear satellite DNA (satDNA) became the preferred target for qPCR assays due to its comparatively greater consistency in copy numbers across T. cruzi lineages and its robustness for quantitative applications, despite its lower abundance in TcI (5- to 10-fold fewer satDNA copies)".

Lines 204–210. The 2008 Buenos Aires symposium is described as establishing a 'Consortium for standardization and validation of the clinical use of PCR (conventional, qualitative).' The parenthetical '(conventional, qualitative)' implies the 2008 consensus focused only on conventional PCR, whereas qPCR standardization came later with the 2011 initiative. This distinction is made implicitly but could be stated more explicitly for clarity.

R. We agree that the original wording may have suggested that the 2008 Buenos Aires initiative was exclusively focused on conventional PCR. Although the primary objective of the 2008 consortium was the standardization and validation of conventional qualitative PCR methods, the international workshop also included a limited evaluation of real-time PCR assays applied solely to assess for the presence or absence of DNA in the samples . However, systematic harmonization, analytical validation, and multicenter assessment of quantitative real-time PCR (qPCR) methods were subsequently addressed in the dedicated international initiative launched in 2011. We have revised the text to clarify this distinction.

The following sentence was added to the manuscript: "The 2008 Buenos Aires symposium established a consortium primarily focused on the standardization and validation of conventional qualitative PCR methods, although a limited number of real-time PCR assays were also evaluated to assess for the presence or absence of DNA in the samples; in contrast, the subsequent international initiative initiated in 2011 was specifically dedicated to the harmonization, analytical validation, and multicenter evaluation of quantitative real-time PCR (qPCR) methods for T. cruzi detection and quantification."

Line 220. The phrase 'marked a significant advancement' is slightly vague. Specify what was advanced: harmonized analytical protocols, ring-trial validation across 14 countries, or selection of TaqMan duplex qPCR as the reference method.

R. We thank the reviewer for the comment. The text has been modified to clarify what was considered a significant advance.

"The primary focus of this multinational effort was the detection and quantification of parasite loads in peripheral blood samples from patients with acute and chronic Chagas disease. This initiative enabled the establishment of harmonized analytical qPCR protocols, validation through interlaboratory assays in 14 countries to rigorously evaluate the performance of TaqMan duplex qPCR assays that could serve as reliable surrogate biomarkers for both diagnosis and treatment monitoring."

Lines 268–272. The BENEFIT trial is introduced as a clinical trial in 'several Latin American countries,' but the specific countries are not named. Given that the manuscript later notes that parasite loads differed between Brazil, Colombia, and Argentina (a key finding from the BENEFIT sub-study), naming the participating countries would be consistent and informative.

R. We agree that explicitly naming the countries participating in the BENEFIT trial would provide greater context and facilitate the interpretation of subsequent findings regarding geographic differences in parasite loads. We have therefore revised the manuscript to specify the countries involved in the trial and to better contextualize the observed inter-country variability in parasitemia among patients with chronic Chagas cardiomyopathy.

Thus, the following sentence was added to the manuscript: "The BENEFIT trial enrolled patients with chronic Chagas cardiomyopathy from Argentina, Bolivia, Brazil, Colombia, and El Salvador, providing a multicountry cohort that subsequently revealed important geographic differences in PCR positivity and treatment responses among participants from different endemic regions."

Lines 310–320: The role of the Zika and Yellow Fever epidemics in delaying kit development is explained honestly and adds valuable institutional context. This is one of the strongest passages for illustrating the challenges of translational work in a public health institution. No changes needed, this is commended.

R. We sincerely thank the reviewer for this positive evaluation and for recognizing the relevance of this section. We are pleased that the discussion on the impact of the Zika and Yellow Fever epidemics on the development timeline of diagnostic kits was considered valuable for illustrating the translational challenges faced by public health institutions.

Lines 324–332: The account of the Fio-Chagas 2018 meeting and President Lima's involvement is historically important. However, the phrase 'strong pressure for the completion of the kit' is informal for a scientific manuscript. Consider: 'broad consensus among participants on the urgency of finalizing the kit.'

R. Done. The sentence was modified, as suggested.

Lines 343–347. The LOD of 0.16 parasite equivalents/mL is an impressive analytical figure. Please clarify whether this was determined using spiked samples, reference strains, or clinical samples, as LOD claims can differ substantially depending on the validation matrix.

R. We agree with the referee. So, we added complementary information detailing the experimental strategy used for the determination of the LOD, including the type of samples and materials used in the validation experiments, as follows: "The LOD95 was estimated in DNA extracted from GEB samples spiked with known quantities of T. cruzi (Y strain—TcII) and analyzed with the NAT Chagas and the in-house assay. The recovered DNA from these spiked samples were amplified by qPCR for five consecutive days, for a total of 60 replicates for each parasite concentration."

Lines 354–360. The reported 59.4% sensitivity relative to serology as the reference method is appropriately contextualized in the manuscript. However, I recommend adding a brief clarification that serology represents an imperfect reference standard for qPCR evaluation, given that chronic-phase patients with confirmed seropositivity do not consistently exhibit detectable circulating parasite DNA. This important caveat would prevent readers from misinterpreting the 59.4% sensitivity as an intrinsic limitation of the molecular assay, when it may instead reflect the biological reality of intermittent parasitemia in chronic Chagas disease.

R. We thank the reviewer for this insightful comment. We agree that the sensitivity of qPCR estimated using serology as the reference standard should be interpreted with caution, particularly in chronic Chagas disease. Serological assays identify exposure and chronic infection but do not necessarily reflect the presence of detectable circulating parasite DNA at the time of sampling. Therefore, the observed sensitivity of 59.4% likely reflects, at least in part, the biological characteristics of chronic T. cruzi infection, including low and intermittent parasitemia, rather than an intrinsic limitation of the molecular assay. We have added the following statement to the manuscript to clarify this important caveat:

"The estimated sensitivity of qPCR using serology as the reference standard should be interpreted cautiously, as seropositive patients with chronic Chagas disease do not consistently exhibit detectable circulating T. cruzi DNA because of the typically low and intermittent nature of parasitemia; therefore, this value does not necessarily reflect an intrinsic limitation of the molecular assay. This relatively low sensitivity can be explained by the biological characteristics of chronic T. cruzi infection, due to low and intermittent parasitemia; consequently, strategies such as serial blood sampling, processing of larger blood volumes, optimization of DNA extraction methods, and the implementation of highly sensitive technologies, including digital PCR, are being explored to improve parasite detection (Parrado et al., 2019; Farani et al., 2025; Ramírez and Moreira, 2019)."

Lines 370–376. The claim that parasite loads in the validation study ranged up to 158.09 satDNA copies/µL is presented without units normalized to blood volume (copies/mL or equivalents/mL). Please ensure consistency with the units used in the LOD statement (parasite equivalents/mL).

R. Authors agree that consistency in reporting parasite load units is important to facilitate data interpretation. In the clinical validation study by Moreira et al. (2023), parasite loads in clinical samples were quantified using a satDNA standard curve and were therefore expressed as satDNA copies/µL rather than Par. Eq./mL. To facilitate comparison with analytical sensitivity data reported using the same quantification approach, we have added a

statement comparing the parasite loads observed in our clinical validation study with the LOD95 reported by Muñoz-Calderón et al. (2021), who also expressed analytical sensitivity as satDNA copies/uL using synthetic DNA standards.

The following sentence was added to the manuscript: "Even though, it is important to note that, using the same quantification approach, Muñoz-Calderón et al. (2021) reported LOD95 values of 0.903 satDNA copies/µL (95% CI: 0.745–1.497) for the Dm28c strain and 0.667 satDNA copies/µL (95% CI: 0.113–3.927) for the CL Brener clone, indicating that the parasite loads detected in the vast majority of patients were above the analytical detection limits of the assay.

Lines 405–410. The statement that in-house assays 'are still largely restricted to more specialized centers' is true, but it does not acknowledge the important role that reference laboratories in Latin America play in current Chagas disease diagnosis. A brief acknowledgment of the existing infrastructure and of how the Biomol Chagas Kit is intended to expand capacity beyond these centers would be appropriate.

R. We fully agree that reference laboratories throughout Latin America have played a pivotal role in the implementation, standardization, and clinical application of molecular diagnostics for Chagas disease. These laboratories have generated much of the evidence supporting the use of PCR-based methods and continue to provide high-quality diagnostic and reference services. We have revised the manuscript to acknowledge their important contribution and to clarify that the Biomol Chagas Kit was developed not to replace these reference centers, but rather to facilitate the broader implementation of standardized molecular diagnostics by expanding access beyond highly specialized laboratories through a commercially validated assay.

The following sentence was added to the manuscript: "Although molecular diagnosis of Chagas disease has been successfully implemented by reference laboratories across Latin America, which have played a central role in the standardization and clinical validation of PCR-based methods, access to these assays remains largely concentrated in specialized centers. The Biomol Chagas Kit was developed to support the broader dissemination of standardized and quality-assured molecular diagnostics, facilitating the implementation in additional clinical and public health laboratories beyond these reference centers".

Lines 432–440. The DiaChO study, which evaluates LAMP alongside qPCR for outbreaks of oral Chagas transmission, is an exciting development. A brief note on the theoretical advantages of LAMP in outbreak settings (no thermocycler required, simpler infrastructure) would help readers understand why this comparative study was designed.

R. We thank the reviewer for this comment. We agree that briefly highlighting the theoretical advantages of LAMP provides important context for the rationale of the DiaChO study. Although qPCR remains the reference molecular method because of its high analytical performance and quantitative capability, LAMP offers several operational advantages for outbreak investigations, including amplification under isothermal conditions without the need for a thermocycler, simplified laboratory infrastructure, rapid turnaround time, and potential applicability in decentralized settings. We have revised the manuscript to clarify that these characteristics motivated the inclusion of LAMP alongside qPCR in the DiaChO study.

Thus, the following sentence was added to the manuscript: "The inclusion of LAMP in the DiaChO study was motivated by its potential operational advantages for the investigation of oral Chagas disease outbreaks, including isothermal amplification without the need for a thermocycler, simplified laboratory infrastructure, rapid time-to-result, and the possibility of expanding access to molecular diagnosis in decentralized or resource-limited settings, while qPCR serves as the reference method for comparative evaluation".

Lines 453–462: The final paragraph proposing integration of the Biomol Chagas Kit into the SUS LACENs network via the existing qPCR infrastructure for Influenza, COVID-19, HIV, etc., is one of the manuscript's strongest translational statements and is well-argued. No changes needed.

R. We sincerely thank the reviewer for this positive assessment. We appreciate the recognition of the translational perspective presented in the final section of the manuscript. We are pleased that the proposed integration of the Biomol Chagas Kit into the existing SUS/LACEN molecular diagnostic network was considered a well-supported and practical strategy to expand access to molecular diagnosis for Chagas disease in Brazil.

Recommendation: Accepted with Minor Revision. This is an important historical narrative from two of the field's most authoritative researchers. The manuscript will make a lasting contribution to the documentation of translational science in Chagas disease. The requested revisions are primarily clarifications and contextual additions rather than substantive restructuring. The authors are encouraged to:

• Add a brief critical reflection on the persistent 59.4% sensitivity limitation and its biological basis

R. Done

• Clarify or soften the 'first real-time PCR thermocycler in Latin America' claim with a citation or qualifier

R. Done

• Add one sentence of global/non-endemic context for molecular diagnosis

R. Done

• Ensure parasite load units are consistent throughout.

R. Done

All other comments are optional suggestions that the authors may address at their discretion.

Reviewer: 2

The manuscript provides a comprehensive historical overview of the contributions made by the Oswaldo Cruz Foundation (Fiocruz) and the Oswaldo Cruz Institute to the development, optimization, and validation of molecular methods for the diagnosis of Chagas disease. It traces the institution's trajectory in advancing molecular diagnostics, including significant achievements in the identification and evaluation of molecular markers of treatment failure, as well as its role in the development and implementation of domestically manufactured diagnostic kits. Overall, the study offers valuable insight into the institution's longstanding contribution to strengthening diagnostic capacity, improving patient follow-up, and fostering technological innovation for the diagnosis and treatment of Trypanosoma cruzi infection.

R. We thank the reviewer for this thoughtful and encouraging assessment of our manuscript. We also appreciate the recognition of Fiocruz's longstanding contributions to the development, optimization, validation, and translation of molecular diagnostic methods for Chagas disease. We are particularly pleased that the reviewer acknowledged the manuscript's emphasis on the integration of scientific research, technological innovation, and public health, as well as the efforts to strengthen diagnostic capacity and improve patient management.

Handling Editor's Comments

Dear Authors,

Thank you for submitting your manuscript entitled "Technological advances in the molecular diagnosis of Chagas disease from the perspective of the Fio-Chagas Translational Program" to Memórias do Instituto Oswaldo Cruz.

The manuscript was evaluated by two reviewers. One reviewer recommended acceptance in its current form, whereas the second reviewer recommended minor revision. After considering both reports, I agree that the manuscript is suitable for publication after minor revision.

The manuscript provides a valuable historical and translational account of the development of PCR-based molecular diagnosis for Chagas disease within the Fio-Chagas Translational Program, culminating in the development, regulatory approval, and field application of the Biomol Chagas Kit. The reviewers recognized the relevance of the contribution, the authority of the authors, and the importance of preserving this institutional and scientific memory for the Chagas disease research community.

Before acceptance, please revise the manuscript addressing the following points:

Add a brief critical discussion of the persistent limitations of qPCR sensitivity in chronic Chagas disease, particularly in relation to intermittent and low-level parasitemia, and clarify the interpretation of PCR-negative results during post-treatment follow-up.

Clarify the statement regarding the first real-time PCR thermocycler in Latin America. If this claim is supported by a citation or institutional record, please provide it. Otherwise, please soften the wording.

Ensure consistency in the units used to report parasite load and limit of detection throughout the manuscript.

Add brief contextual information on the relevance of molecular diagnosis beyond Latin American endemic settings, including its potential role in non-endemic countries.

Revise the abstract to explicitly mention the Biomol Chagas Kit and its regulatory status, since this product is central to the manuscript.

R. We thank the Editor for the careful evaluation of our manuscript and for the constructive comments and suggestions. We believe that these recommendations have substantially improved the quality, clarity, and overall impact of the manuscript. All the points raised by Reviewer 1 and by the Editor were addressed during the revision process and are discussed in detail in our point-by-point responses to the reviewers. **Briefly**, we have expanded the discussion on the biological limitations affecting qPCR sensitivity in chronic Chagas disease and clarified the interpretation of PCR-negative results during post-treatment follow-up; revised the statement regarding the introduction of the first real-time PCR thermocycler in Latin America, softening the wording where appropriate; harmonized and clarified the presentation of parasite load and limit of detection units throughout the manuscript; incorporated additional context on the growing importance of molecular diagnosis in non-endemic countries and revised the abstract to explicitly mention the Biomol Chagas Kit and its regulatory status, highlighting its central role in the translational history presented in this review.

In addition, I encourage the authors to consider including a high-resolution figure illustrating the Biomol Chagas Kit, or a composite figure showing the kit together with a concise timeline of its development, validation, ANVISA registration, and field implementation. Given the historical and translational nature of the article, such a figure would strengthen the visual presentation and help readers understand the concrete technological product emerging from the Fio-Chagas trajectory.

The remaining reviewer comments should be addressed where appropriate, especially those related to clarification of technical terminology, sample size/context for reported performance estimates, and minor wording improvements.

R. We thank the Editor for this valuable suggestion. Following this recommendation, we have prepared a high-resolution figure illustrating the Biomol Chagas Kit together with a timeline highlighting the major milestones in its translational development, including assay development and optimization, analytical and clinical validation, ANVISA registration, and its implementation in field studies. We believe that this figure enhances the visual

presentation of the manuscript and better illustrates the technological trajectory and public health impact of the Biomol Chagas Kit within the Fio-Chagas program.

Please submit a revised version of the manuscript together with a point-by-point response to the reviewer's comments.

Sincerely,
Fred Luciano Neves Santos
Guest Editor
Memórias do Instituto Oswaldo Cruz

SECOND REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

The manuscript is accepted for publication

REVIEWER #2

Numerous changes and minor issues have already been addressed in the revised manuscript following the comments provided by the other reviewer. These revisions have improved the overall coherence and cohesion of the manuscript and have also strengthened the reference section by incorporating additional relevant citations.

DECISION: ACCEPT SUBMISSION | RECEIVED 10 MAY 2026 | ACCEPTED 10 SEPTEMBER 2026