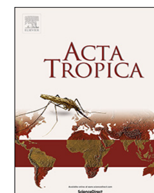
Acta Tropica 199 (2019) 105120

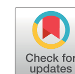
Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica



Role of nucleic acid amplification assays in monitoring treatment response in chagas disease: Usefulness in clinical trials



Elena Sulleiro[a], AQrturo Muñoz-Calderon[b], Alejandro G. Schijman[b,*]

[a] Department of Microbiology, Vall d'Hebron University Hospital, PROSICS Barcelona, Pº Vall d'Hebron 119-129, 08035 Barcelona, Spain
[b] Laboratorio de Biología Molecular de la Enfermedad de Chagas, Instituto de Investigaciones en Ingeniería Genética y Biología Molecular "Dr. Hector Torres" (INGEBI-CONICET), Vuelta de Obligado 2490; Second Floor, Ciudad de Buenos Aires, ZIP code 1428 Argentina

ARTICLE INFO

Keywords:
Trypanosoma cruzi
Chronic chagas disease
Quantitative real time PCR
Clinical trials
Treatment monitoring
Drug resistance

ABSTRACT

Chagas disease has become a global health problem due to migration of infected people out of Latin America to non-endemic countries. For more than 40 years, only the nitroimidazole compounds Benznidazole and Nifurtimox, have been used for specific treatment of *Trypanosoma cruzi* infection with disappointing results, specially due to the long duration of treatment and adverse events in the chronic phase. In the last years, ergosterol inhibitors have been also proposed for specific treatment. Different randomized clinical trials were performed for evaluating their treatment efficacy and safety.

One of the greatest concerns in clinical trials is to provide an early surrogate biomarker of response to trypanocidal chemotherapy. Serological response is slow and the classical parasitological tests have poor sensitivity and are time-consuming. Nowadays, PCR is the most helpful tool for assessing treatment response in a short period of time. Different protocols of PCR have been developed, being quantitative real time PCR based on amplification of repetitive satellite or minicircle DNA sequences plus an internal amplification standard, the mostly employed strategies in clinical trials. Standardized protocols and the use of an external quality assessment ensure adequate technical procedures and reliable data.

Clinical trials have shown a significant reduction in parasite loads, reaching undetectable DNA levels in bloodstream after specific treatment, however events of treatment failure have also been reported.

Treatment failure could be due to inadequate penetrance of the drugs into the affected tissues, to the presence of primary or secondary drug resistance of the infecting strains as well as to the existence of dormant parasite variants reluctant to drug action.

The early diagnosis of drug resistance would improve clinical management of Chagas disease patients, allowing dictating alternative therapies with a combination of existing drugs or new anti-*T. cruzi* agents.

The aim of this review was to describe the usefulness of detecting *T.cruzi* DNA by means of real time PCR assays, as surrogate biomarker in clinical trials for evaluating new drugs for CD or new regimens of available drugs and the possibility to detect treatment failure.

1. Introduction

Chagas disease (CD), caused by the kinetoplastid flagellate *Trypanosoma cruzi*, has been considered as "the most neglected of the neglected diseases" with research and development gaps related to diagnosis and treatment. Although CD has by tradition been treated as an endemic disease in the tropical and subtropical areas of South America, it has become a global concern due to migration of infected people to non-endemic countries. Complex interactions between the parasite and the mammalian host, as well as eco-epidemiological factors determine the infection outcome.

The infection passes through an acute and a chronic phase. During the acute phase, symptoms are variable and mostly decline spontaneously after weeks. Appropriate parasitical treatment can eradicate the parasite. In the chronic phase, approximately 70% of seropositive individuals are asymptomatic, whereas 30% develop cardiac and/ or digestive disease manifestations several years or decades later; out of them, 2–3% present severe forms that can evolve to sudden death. The factors governing the progression of chronic Chagas disease remain unknown and no prognostic markers are still available (Pérez-Molina and Molina, 2018).

* Corresponding author.
E-mail addresses: esulleir@vhebron.net (E. Sulleiro), amunoz@dna.uba.ar (A. Muñoz-Calderon), schijman@dna.uba.ar (A.G. Schijman).

https://doi.org/10.1016/j.actatropica.2019.105120
Received 26 March 2019; Received in revised form 23 July 2019; Accepted 30 July 2019
Available online 31 July 2019
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2. Parasitical drugs

For more than 40 years, only two nitro-heterocyclic compounds have been used for specific treatment of *Trypanosoma cruzi* infection: Nifurtimox (NFX) produced firstly by Bayer since 1967 (Lampit®) and recently also by Gador (Furtimox®) and Benznidazole (BZ) produced by Roche since 1972, Rochagan® and Radanil®) and discontinued recently, until LAFEPE and ELEA companies have started their production (Coura and de Castro, 2002). Although these drugs are far from optimal activity, they are effective against infections in the acute phase while their usefulness in preventing or alleviating symptoms in the chronic stage is controversial (Urbina, 2010). Numerous clinical trials have reported up to 80% of parasitological cure in the acute phase (Bahia-Oliveira et al., 2000) and 60–70% of cure in early chronic phase in children up to 14 years of age (de Andrade et al., 1996; Sosa-Estani et al., 1998, 2009, Yun et al., 2009; Sales Junior et al., 2017). For this reason, the major limitation on the use of both drugs is low anti-parasitic activity in the chronic phase, with only 5–20% of patients considered cured, as assessed after 20 years or more of follow-up (Cancado, 2002; Pinto Días, 2006).

These marked differences in efficacy of the nitroderivatives between the acute and chronic stages of Chagas disease are unknown (Cancado, 2002). Possible factors involved are probably related to the presence of different parasite lineages in distinct geographic regions (Carranza et al., 2009, Zingales et al. 2012, Brenière et al., 2017; Rodrigues-dos-Santos et al., 2018) with differential drug susceptibility (Filardi and Brener, 1987; Murta et al., 1998; Toledo et al., 2003; Muñoz-Calderón et al., 2012) and differences in genetic background, immune response among human populations (Yun et al., 2009), associated in turn with the observed geographic differences in therapeutic outcome (Rassi and Luquetti, 1992; Yun et al., 2009).)

Another factor to be taken into account is the time elapsed between the onset of infection and drug treatment, because cases (mostly asymptomatic) treated closer to the acute phase period might display a more rapid cure. Additionally, with treatment regimens that extend over several months, failures to complete the therapeutic schedule are more likely to occur.

3. Early Biomarkers of cure or treatment failure

One of the greatest concerns in clinical trials is to provide an early surrogate biomarker of response to trypanocidal chemotherapy.

The negativization of two serological tests using crude and recombinant antigens is the current criteria of cure in Chagas disease. However, in the advanced chronic phase, the antibodies decrease is slow, taking years to complete and requires a long follow-up (Gomes et al., 2009). In a meta-analysis made by Sguassero et al. in, 2015, the negativization of conventional serological tests (ELISA, IFI, HIA) was 10% after 48 months of follow-up. In the long-term, the rate of negativization increased up between 20% and 45% (Sguassero et al., 2015).

On the other hand, negativization of parasitological tests in peripheral blood, hemoculture, xenodiagnosis and/or polymerase chain reaction (PCR), do not ensure the absence of infection. Indeed, a negative result cannot be taken as criteria of cure, while a positive result is a useful marker of treatment failure.

Hemoculture and xenodiagnosis have poor sensitivity, are laborious, time-consuming and only available in reference research institutions with special laboratory biosecurity conditions. In contrast, PCR is a helpful tool for the early detection of treatment failure and to reduce the time of follow-up (Britto CC, 2009). Actually, the PCR is the leading test for assessing responses to treatment in a short period of time (Pinazo MJ et al., 2015).

4. Polymerase chain reaction based methods for treatment monitoring

Since its first development, the PCR directed to *T.cruzi* DNA was applied to treatment monitoring, but different protocols were described showing different results of sensitivity, specificity and accuracy after treatment and during follow-up (Britto C et al., 1995). Many factors contribute to PCR variability, such as the volume of blood collected, conditions of sample conservation and transportation, DNA extraction procedure, molecular targets, sets of primers and probes as well as thermo-cycling conditions. The best results were obtained using methods based on the amplification of repetitive satellite DNA (SatDNA) or minicircle DNA (kDNA) sequences (Schijman et al., 2011).

The implementation of real time quantitative PCR (qPCR) assays using fluorescent dyes as Sybr Green or TaqMan fluorogenic probes have shown good results for monitoring treatment response (Schijman AG, 2018). The qPCR technology adds the possibility of quantifying the parasite load and the use of an internal control of DNA integrity and/or PCR inhibition in the same reaction, when using TaqMan probes in multiplexed reactions. Due to the need of an accurate measurement of parasitic loads, Ramirez et al. carried out an analytical validation of qPCR methods targetting the above mentioned sequences, for quantification of *T. cruzi* DNA in blood samples (Ramírez JC et al, 2015). Both targets showed a high concordance in sensitivity and parasitic load values. Indeed, these validated methods, which use the parasite sequences of choice in duplex TaqMan qPCR format coupled to the amplification of an internal control, are the ones mostly employed for treatment monitoring in clinical trials (Moreira OC et al., 2013; Morillo C et al., 2015; Molina I et al., 2014; Torrico F et al., 2018).

Aiming to enhance the sensitivity of qPCR for monitoring treatment response in clinical trials, many strategies have been proposed: the use of a multiplex qPCR using paired kDNA and satDNA, the analysis of serial blood samples to increase the number of replicates and the exploration of automatization of DNA extraction procedures (Seiringer P el al., 2017; Álvarez et al., 2016; Abras A et al., 2018; Parrado R et al., 2018).

Besides, to ensure the quality of laboratory data, an External Quality Assurance (EQA) system has been recently designed and implemented to evaluate the performance of PCR laboratories involved in Chagas disease diagnosis and treatment monitoring. The system involves the evaluation of proficiency testing panels containing negative controls and different dilutions of seronegative human blood spiked with known quantities of parasite cells obtained by culturing. These specimens are blindly analyzed and the results evaluated by an external laboratory (Ramirez JC et al., 2017).

The perspective of the production and validation of commercial kits for molecular diagnosis and monitoring of T.cruzi infection would improve the performance and reproducibility of home-brewed molecular tests performed at present by different laboratories in multi center studies.

The Table 1 shows published clinical trials dedicated to Chagas disease that employed PCR methodology to detect parasite DNA in bloodstream as surrogate marker of treatment response.

Nowadays, new trials have been started to evaluate new schemes of Benznidazole (Multibenz, BENDITA), Nifurtimox (CHICOstudy) and Fexinidazole with promising preliminary results (unpublished data). All of them use qPCR for monitoring treatment response and in most of them, EQA programs have been implemented to enhance the quality of the laboratory results.

All of these studies have shown a significant reduction in parasite load to undetectable DNA levels during specific treatment. However, it has been observed a relapse in parasitic loads after the end of the treatment with a significant difference between cohorts treated with Benznidazole and ergosterol inhibitors. The rates of failure of Benznidazole reached 46% in the BENEFIT study and 39.6% in the study performed by Galvao et al. after three years of follow-up (Morillo

Table 1
Clinical trials using PCR as biomarker.

	Clinical trial	Patients	Trypanocidal drug	PCR protocol	PCR pre-treatment	Parasitological Outcomes	EQA program
Galvão et al., 2003	Randomized, double-blind, placebo-controlled	- 111 children 7-12 years - Brasil	- Benznidazole (7-5 mg/kg daily/60 d) - Placebo	- PCR + hybridization - kDNA - Single result: One extraction + one amplification	Yes, both positive and undetectable	Conversion rates of *T.cruzi* DNA in peripheral blood at 3 years follow up	No
BENEFIT Morillo et al., 2015	International multicenter, randomized, double-blind and placebo-controlled	- 2854 Chronic Chagas cardiomyopathy - Adult - Argentina, Bolivia, Brazil, Colombia, and El Salvador	- Benznidazole (5 mg kg daily/ 60d)was modified to the administration of a fixed dose of 300 mg per day and a variable duration of therapy (between 40 and 80 days) on the basis of the patient's weight, thereby preserving the total dose - Placebo	- PCR - kDNA - Single result: One extraction + one amplifiction	Yes, both positive and undetectable	Conversion rates of *T.cruzi* DNA in peripheral blood at the end of treatment, 2, 5 or more years of follow up	No
TRAENA Riarte et al. unpublished	Randomized, double-blind and placebo-controlled	- Symptomatic and asymptomatic chronic Chagas disease. - Argentina	- Benznidazole - Placebo	- qPCR - SatDNA - TaqMan dúplex qPCR	Yes, both positive and undetectable	Significant reduction of parasitic load at 12-24 months after treatment	No
STOP-Chagas Morillo et al., 2017	Prospective, multicenter randomized placebo-controlled	- 120 Asymptomatic Chagas disease - Argentina, Chile, Spain,Colombia, - Guatemala, and Mexico	- Posaconazole 400 mg b.i.d./60d - Benznidazole 200 mg + placebo b.i.d./60d - Benznidazole 200 mg + posaconazole 400 mg b.i.d./ 60d - -Placebo 10 mg b.i.d./60d	- Real time PCR - kDNA - 12 PCR result: each DNA sample was analyzed 3 times with 3 PCR, with 4 - technical replicates	Yes, all positive	Conversion rates of *T.cruzi* DNA in peripheral blood at the end of treatment, 90, 120, 150, 180, and 360 days) days of follow up	No
CHAGASAZOL Molina et al., 2014	Prospective, randomized Not placebo controlled	- 78 symptomatic and asymptomatic chronic Chagas disease. - Spain (mainly olivian)	- Benznidazole 150 mg b.i.d./60d - High-dose Posaconazole 400 mg b.i.d./60d - Low-dose Posaconazole 100 mg b.i.d./60d	- Real time PCR - satDNA - Two PCR results: One DNA extraction + duplicate amplification	Yes, all positive	Conversion rates of *T.cruzi* DNA in peripheral blood at the end of treatment, 4, 6 and 12 months follow up	Yes
E-1224 Torrico et al., 2018	Proof-of-concept, double-blind, randomized	- 231 symptomatic and asymptomatic chronic Chagas disease. - Bolivia	- High-dose E1224 (4000 mg/8 w) - Low-dose E1224 (2000 mg/8 w) - Short-dose E1224 (2400 mg/4 w + placebo/4 w), - Benznidazole - (5 mg/kg per day/60d) - Placebo (8 w).	- -q PCR - satDNA - Nine PCR results: Three DNA extraction + three amplification for each one	Yes, all positive	Conversion rates of *T.cruzi* DNA in peripheral blood at the end of treatment, 4, 6 and 12 months follow up	Yes

qPCR: real time quantitative DNA. kDNA: kinetoplastid DNA. satDNA: satellite DNA. EQA: External Quality Assurance Program.
b.i.d.: twice a day. d: days. w: weeks.

et al., 2015; Galvão et al., 2003). However, in Chagasazol and STOP-Chagas studies, which used posaconazole, the positive results of PCR increased faster arriving to 90% of positivity during the first year of follow-up (Molina et al., 2014; Morillo et al., 2017). Among the ergosterol inhibitors, only after a regimen of 4000 mg/week for 8 weeks of E1224, a prodrug of ravuconazole, sustained parasitological response was obtained at 12 months post-treatment period (Torrico et al., 2018).

With respect to clinical follow-up, there was not a significant difference in PCR results between symptomatic and asymptomatic chronically infected patients (Molina et al., 2014, Riarte A, unpublished data). Only in the BENEFIT study, in which all patients included suffered cardiac involvement, it was shown that Benznidazole could reduce parasitic loads without significant effect on clinical progression along 5 years of follow-up (Morillo et al., 2015).

One limitation of the molecular techniques outcomes in clinical trials of established chronic infections is the short time of follow-up (1–7 years). It would be necessary to perform such monitoring for several years to confirm primary results.

Factors exposed above concerning suboptimal efficacy of available drugs, bring with them the existence of *T. cruzi* strains refractory to treatment distributed throughout South America, though the extent to which this reflects an acquired resistance or natural variation in sensitivity is unknown (Filardi and Brener, 1987; Castro et al., 2006).

5. Treatment failure and drug resistance

Treatment failures have been reported for both drugs used for treatment of Chagas disease (Britto et al., 1999; de Lana et al., 2009; Fernandes et al., 2009). However, the difficulties of finding resistant trypanosomes in the field and the subsequent administration of chemotherapy make it difficult to attribute these failures to the presence of drug-resistant parasites and not to the effectiveness of the pharmacological treatment.

In some organisms (Trichomonas, Giardia and Entameba), resistance to nitroimidazoles is explained by a deficiency in nitro group-reducing enzymes, but there are no similar studies in *T. cruzi*. For this reason, unlike other tripanosomiasis, distinguishing between treatment failure due to drug resistance or due to other reasons is even more challenging in the case of Chagas disease. Furthermore, the toxicity of the available drugs in combination with the need for prolonged treatment regimens has an inevitable impact on patients´ compliance, providing a selective environment for the development of drug-resistant parasites.Can primary drug resistance be present in the transmission cycles?

Up to now, there are no signs for a spread of drug-resistant *T. cruzi* strains in the field. However, because of the biological and genetic diversity of different populations (Andrade et al., 2004; Zingales et al., 2012), naturally occurring drug-resistant parasites have been isolated (Filardi et al., 1987; Murta et al., 1998, 1999). This biological and genetic diversity could also explain the character of susceptibility of Tulahuen (DTU VI) and Y (DTU II) strains to NFX and on the other hand, the resistance of Sonya and Colombiana (DTU I) strain (Neal and van Bueren, 1988).

Therefore, any point mutation originated could change the general activity and/or the substrate specificity, inducing drug-resistance (Murta et al., 2006). Additionally, it should be added that wild populations of *T. cruzi* may contain both susceptible and resistant forms to chemotherapeutic agents so that the destruction of susceptible forms by drugs leads to the selection and proliferation of resistant subpopulations (Murta and Romanha, 1998).

These resistant forms could enter again the cycle of transmission of the disease and thus resistant epimastigotes may transmit that property to the corresponding amastigotes (Nirdé et al., 1995). In this way, drug resistance can be extended to the infective stage of *T. cruzi*. (Marra, 1969; Nozaki et al., 1996).

6. Genes associated with generation of drug resistance

The mode of action of nitroheterocyclic compounds appears to involve the metabolic activation of the compounds initiated through the reduction of the nitro groups mediated by a mitochondrial NADH-dependent type-I nitroreductase (NTR I), which uses FMN as a cofactor (Hall and Wilkinson, 2012). Another oxidoreductase that uses FMN as a cofactor and is involved in phenomena of resistance is the Old Yellow Enzyme (OYE), initially described by Warburg and Christian in, 1932. These enzymes can reduce nitro esters, nitroaromatics, or α,β-unsaturated compounds (Yanto et al., 2010) and among its diverse functions they are associated with detoxification, oxidative stress response, and specific metabolic pathways such as ergot alkaloid biosynthesis (Toogood et al., 2010).

Equally important is the broad-spectrum of resistance, named Multidrug Resistance (MDR) phenotype. This MDR phenotype determined by cross-resistance to structurally and functionally unrelated drugs could be a limiting factor to the treatment of parasitosis. Among the factors related to the onset of the MDR phenotype, the best characterized is the overexpression of membrane transporters, mostly from the ATP-Binding Cassette (ABC) protein superfamily (Gottesman, 2002). In this context, the expression of ABC transporters has been related to chemotherapy failure. The most known ABC transporters are ABCB1 and ABCC1, involved in the multidrug resistance phenotype in cancer, given their participation in cellular detoxification. Twenty seven ABC genes have been identified in the *T.cruzi* genome. Nonetheless, only four ABC genes have been characterized: ABCA3, involved in vesicular trafficking; ABCG1, overexpressed in strains naturally resistant to Benznidazole, and P-glycoprotein 1 and 2, whose participation in drug resistance is controversial (Franco et al., 2015; Zingales et al., 2015).

Other studies have also shown that resistance to NFX can occur independently of resistance to BZ (Filardi and Brener, 1987). Additional mechanisms which give rise to resistance against nitroheterocyclic drugs must exist. Identifying these, using the full complement of post-genome technologies, must be regarded as a priority in Chagas disease research.

7. Nitroheterociclic resistance and clinical cohorts of patients under treatment

There are numerous in vitro studies on the susceptibility of strains of *T. cruzi* that have contributed significantly to the knowledge of this disease. Andrade et al., 1983 and 1992, carried out comparative analyses of numerous strains of T. cruzi, resulting in two groups of trypanosomes, one susceptible and the other resistant to BZ, which allowed to establish a correlation with clinical-therapeutic relevance. On the other hand, Murta et al., 1998, working with a greater number of strains, obtained a response to chemotherapy in a characteristic way for each group. Meanwhile, Moreno et al., 2010 developed a methodology to quantify BZ susceptibility in haemoculture-derived isolates from patients obtained before and after BZ treatment. Comparison of the in vitro BZ resistance of the pre-treatment parasites from patients considered cured and from those experiencing therapeutic failure showed no correlation with therapeutic outcome.

Most recently, Quebrada Palacio et al., 2018 studying the behavior of the parasites and performing comprehensive drug sensitivity testing, demonstrated substantial intra-DTU phenotypic diversity within TcV, a similar finding to that described by Mejia et al., 2012 for TcI isolates.

In general terms, it can be said that there is still a great knowledge gap concerning the genetics and biology of infective parasitic populations in patients under treatment. The early diagnosis of drug resistance using as source clinical samples would improve the clinical management of Chagas disease patients, allowing dictating alternative therapies with a combination of existing drugs or new anti-*T. cruzi* agents. However, this is a difficult task, due to the low parasitic load frequently

observed during the chronic phase of the disease.

8. Final remarks

Currently available diagnostics and trypanomicidal chemotherapy are suboptimal for adequate control and treatment programs. The design of novel drugs for Chagas disease is still challenging due to different parasitic-host features, such as the parasite life cycle that includes intracellular forms which can be in a dormant stage (Sánchez-Valdéz et al., 2018), its genetic diversity associated with different tissue tropism (Zingales et al., 2014), the propagation of the infection by different routes in diverse geographic areas where human ethnicities display diversity of genetic and immunological backgrounds.

The production of commercial kits to facilitate and improve monitoring of parasite response by means of PCR techniques or novel strategies such as Loop isothermal amplification or the recombinase polymerase assay (RPA) for amplification of DNA/RNA-based biomarkers (Besuschio et al., 2017; Jimenez-Coello et al., 2018), the search for improved nucleic acid extraction protocols adapted to different types and volumes of clinical samples, the validation of different stabilizing agents to allow adequate sample conservation especially in rural endemic areas, the development of technical procedures allowing evaluation of parasite persistence in target tissues even after etiological treatment, such as sensitive detection of amastigote forms using Reverse transcription polymerase chain reaction (RT-PCR) of amastigote-specific genes are some challenges to face in the future in order to validate the detection of parasite nucleic acids as surrogate markers of treatment outcome.

Such tasks require a strong coordinated action between international non-profit Organizations, Foundations, Pharmaceutical Companies and Academia to promote sufficient investments devoted to develop and evaluate better solutions for treating this neglected disease (Reithinger et al., 2009).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Proyect PICT-2014 -1188 to AGS funded by FONCyT, Ministerio de Ciencia, Tecnologìa e Innovacion Productiva, Argentina.

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