"Recent advances in Genetics and Molecular Diagnosis of Parasitic Protozoa".

Molecular diagnosis of *Trypanosoma cruzi,*

Review Article

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Abstract: Chagas disease, caused by the kinetoplastid protozoan *Trypanosoma cruzi*, affects millions of people, most of them neglected populations. The different phases of the disease, the transmission mode and the high genetic variability of the parasite determine that molecular detection methods display different degree of success. Molecular diagnostic tests may be employed during epidemiological surveys of transmission, blood bank screening, for early diagnosis of congenital transmission and acute infection due to oral transmission, transfusion or transplantation routes, reactivation due to immunosuppression and monitoring of treatment response in chronically infected patients receiving trypanomicidal chemotherapy. This manuscript summarizes the most widely used molecular tools to detect *T. cruzi* infection in different epidemiological and clinical scenarios.

Keywords: Chagas disease, *Trypanosoma cruzi*, quantitative Real Time PCR, loop-mediated amplification, molecular diagnosis.

1.Introduction Chagas disease (CD), caused by *Trypanosoma cruzi* is most likely "the most neglected of the neglected diseases" [WHO, 2012]. It has been treated as an endemic disease in tropical and subtropical areas of Southern and Central America, Mexico and Southern United States [Garcia MN et al., 2017], and is an

emerging global distress in non-endemic areas [Schmunis and Yadon, 2010]. Once vectorial and transfusional control have been achieved, perpetuation of infection occurs mainly through congenital transmission in endemic and non-endemic areas whereas in rural zones, outbreaks of oral infection are more significant [Alarcon de Noya et al., 2010; Shikanai-Yasuda, 2012]. The infection traverses an acute phase, evolving to an asymptomatic or symptomatic chronic phase, with different degrees of severity [Rassi A Jr et al. 2010].

Clinical molecular diagnosis of CD is important for: (i) early diagnosis of congenital transmission in newborns when presence of maternal anti-*T.cruzi* antibodies may deliver false positive results and microscopic observation lacks sensitivity, (ii) diagnosis of oral infections, (iii) early detection of infection in receptors of organs from CD donors, (iii) monitoring of reactivation in chronically infected patients immune-suppressed due to transplantation or AIDS and (iv) evaluation of treatment response, because detection of serological negative conversion in treated patients with a favorable outcome may take many years to occur.

2. Molecular detection and *T. cruzi* gene diversity. The genetic structure of *T. cruzi* populations is mainly a consequence of clonal propagation with rare events of genomic exchange [Tibayrenc M et al. 1986; Buscaglia and Di Noia, 2003; Sturm and Campbell, 2010]. Biological, biochemical and molecular markers demonstrated genetic polymorphism [Macedo et al. 2004; Miles et al. 2009]. Nowadays, natural populations are classified into six discrete typing units (DTUs TcI to TcVI), composed of sets of stocks genetically closer to one another than to any other one [Zingales et al. 2009; 2013]. In addition, Tcbat has been recently proposed as an independent DTU [Lima et al. 2015]. DTUs are identifiable by specific molecular markers, they depict particular geographical distribution, harbor different DNA content and gene dosage and may have preferential tropism for vector and reservoir species as well as tissue tropism within an infected host [Burgos et al. 2010; 2008; Miles et al. 2009; Lewis et al. 2009; Telleria et al. 2006; Vargas et al., 2004]. DTUs I to VI are all causative of CD [Zingales et al. 2013]. This genetic diversity must be taken into account when developing molecular diagnostic tests for worldwide applications.

3. Nucleic Acid amplification methods.

3.1. Polymerase Chain Reaction. Since the nineties, the Polymerase Chain Reaction (PCR) was proposed as the molecular tool of choice for sensitive detection of *T. cruzi* infection and monitoring of trypanocidal chemotherapy [Avila et al. 1993; Britto et al. 1995; Moser et al. 1989]. Later on, Real Time PCR was developed with the possibility of parasitic load quantification [Piron et al. 2007; Duffy et al. 2009, 2013]. Upon standardization, analytical and clinical validation, duplex TaqMan qPCR procedures directed to parasite highly repetitive sequences - the nuclear satellite DNA (SatDNA) or the minicircle molecule (kDNA) - plus an internal amplification control are being widely used for treatment monitoring [Ramirez et al. 2015; Duffy et al. 2013; Schijman et al. 2011]. Analytical sensitivity of kDNA qPCR is somewhat higher than that of SatDNA qPCR, with limits of detection of 0.234 and 0.698 parasite equivalents/mL, respectively. High concordance was observed between both methods in proficiency panels and clinical specimens [Ramirez et al., 2015]. Analytical sensitivity was more uniform among different DTUs for kDNA qPCR than for SatDNA qPCR, being the latter less sensitive for some TcI and TcIV strains, indicative of a lower gene dosage in their genomes [Ramirez et al., 2015; Duffy et al., 2009]. In regions where *T. rangeli* might be the cause of confounding diagnosis with *T. cruzi* [Guhl and Vallejo, 2003], kDNA qPCR could lead to false positive results due to *T. rangeli* because the minicircle region annealing with primers and probe is highly conserved and repeated in both trypanosomatid species. In fact, *T. rangeli* has been detected in infants in endemic areas for CD [Saldana et al. 2005]. Therefore, in these regions, a standardized qPCR assays based on SatDNA sequences should be indicated, because *T. rangeli* presents very low copy numbers of this sequence**.**

3.2.Loop mediated amplification. Thanks to a complex design of primers, auto-strand displacement DNA synthesis and Bst DNA polymerase, LAMP is able to amplify large amounts of DNA within 30–60 minutes of incubation. The reaction occurs between 60 and 65°C, so it can be done without the use of a thermocycler [Mori et al. 2001; Notomi et al. 2010]. Visualization of amplification can be addressed by the naked eye and followed in real-time by measuring turbidity or fluorescence using intercalating dyes. In-tube visualization may be achieved using manganese loaded calcein which starts fluorescing upon complexation of manganese by pyrophosphate during DNA synthesis. LAMP reagents are stable at room temperatures up to 37°C, avoiding the need of a cold chain [Njiru et al. 2003; Poon et al. 2006]. A first LAMP procedure was based on

the 18s rDNA gene with an analytical sensitivity of 100fg of DNA per test and cross reactivity with Leishmania sp and was evaluated in triatomine feces [Thekisoe et al. 2010]. The use of this target in a LAMP protocol tested in human blood reached a detection level of 50 parasites/mL, which allowed detection of congenitally infected patients [Rivero et al. 2017]. A novel prototype kit for detection of *T.cruzi* satDNA in human blood samples developed by Eiken Company containing dried reagents on the inside of the caps in microtubes reached high analytical accuracy, without cross-reactivity with Leishmania sp or *T. rangeli* [Besuschio et al. 2017]. Analytical sensitivity was $1x10^{-2}$ fg/μL of CL Brener (Tc VI) and Sylvio X10 (Tc I) DNAs and detected all DTUs, with some variations in the "time to threshold", due to the heterogeneity in copy numbers of satellite repeats [Duffy et al. 2009]. It also detected $1x10^{-2}$ parasite equivalents/mL in spiked EDTA blood extracted with commercial columns or rapid Boil& Spin method and $1x10^{-1}$ par.eq/mL in spiked heparinized blood using fiberglass columns for DNA extraction. It rendered high sensitivity in acute, congenital and reactivated patients and less sensitivity in chronic Chagas disease, in high concordance with qPCR performed in the same samples [Besuschio et al., 2017; Flores, M et al. personal communication].

4. Clinical specimens used for molecular diagnosis of CD. The type of clinical sample for diagnosis depends on the clinical setting. Although most nucleic acid amplification SOPs were developed for whole peripheral blood samples treated with Guanidine hydrochloride-EDTA stabilizing buffer, PCR based on frozen EDTA blood alone or mixed with different stabilizing agents, PAXgene Blood DNA tubes, blood spots in filter paper have been reported [Moreira et al. 2016, Sanchez et al. 2016; Wei et al; 2016; Braz et al. 2008]. In addition, heparinized blood was evaluated for LAMP [Besuschio et al, 2017]. Blood clots and serum samples showed adequate sensitivity in some settings [Moreira et al. 2016; Russomando et al., 1992;]. Umbilical cord blood and tissue and heel prick blood are also candidate specimens for early diagnosis of congenital transmission [Mora et al., 2005]. Clinical samples other than blood have been tested too. In the acute experimental model of Guinea pigs, DNA was detected in urine, suggesting it could be valuable for acute human detection [Yagashira et al. 2003]. PCR detected parasite DNA in amniotic fluid from an infected mother with a premature delivery [Nilo et al. 2000]. In chronic CD, *T. cruzi* DNA was amplified using different PCR methods in a proportion of gum samples from cases with diverse degrees of gingival inflammation [Añez et al. 2011]. In HIV coinfection, reactivation can be detected in cerebral spinal fluid or

brain biopsies [Burgos et al. 2005; Bern 2012] and in transplanted patients reactivation can be detected in endomyocardial biopsies or skin chagomas [Diez et al., 2007; Burgos et al. 2010]. Nevertheless, for diagnostic purposes, whole blood persists as the sample of choice.

5. Quality control assurance in molecular diagnosis of CD. Quality controls are fundamental for reliable molecular diagnosis. False negative results can arise because of PCR inhibitors in the samples, which require the use of internal controls to detect inhibition and enable discriminating true from false negative results. On the other side, inadequate laboratory conditions may favor carry-over contamination leading to false positive results. A first External Quality Assurance system has been recently implemented to evaluate the performance of molecular biology laboratories involved in qPCR based follow-up in clinical trials of chronic Chagas disease cohorts [Ramirez et al. 2017]. Proficiency testing panels containing seronegative blood samples spiked with 1, 10 and 100 par. eq./mL of four *T. cruzi* stocks, belonging to different DTUs, as well as negative controls were analyzed simultaneously, blinded to sample distribution, at 4-month intervals in different laboratories. Moreover, randomly selected blood samples from patients were sent to the reference laboratory for retesting analysis. Laboratories applied a same SOP [Duffy et al. 2013] with a high degree of agreement, within and between laboratories, of qualitative results of proficiency testing panels for all *T. cruzi* stocks. No significant differences were found between qualitative and quantitative qPCR results, when clinical samples were retested [Ramirez et al. 2017].

6. Application of Nucleic Acid amplification in epidemiological and clinical scenarios.

6.1. Oral infection. Oral transmission is the most important route of infection in Brazilian Amazon and Venezuela [Shikanai-Yasuda and Carvalho, 2012; Alarcón de Noya et al. 2010]. Other South American countries have also reported outbreaks associated with food consumption [Ramirez JD et al. 2013; Blanchet et al. 2014]. In most outbreaks molecular methods were fundamental for specific diagnosis. A real-time PCR method has been also developed for detection of *T. cruzi* in açai pulp, to determine innocuity of this food regarding oral transmission [De Souza et al. 2017].

6.2. Congenital infection: Current assays for early detection of congenital cases fail to diagnose more than half of infected neonates and 10 month follow-up for serological diagnosis is poor. Molecular strategies in

newborns/neonates could enable earlier diagnosis and circumvent loss to follow-up [Besuschio et al. 2017; Cura et al. 2017; Bua et al. 2013; Schijman et al. 2003; Mora et al. 2005]. A recent study carried out in Bolivia showed a good cumulative sensitivity of qPCR done in cord blood and peripheral blood at one month of age and revealed that infants with clinical signs had higher parasite loads [Messenger et al. 2017]. It is still necessary to test standardized real-time procedures using lower volumes of peripheral blood or using cord or heel prick blood, ideally collected in solid supports such as filter paper. A kit prototype based on duplex TaqMan Real-Time PCR (qPCR) that starts from 1 mL of cord or peripheral blood mixed with a DNA stabilizer solution has been built and is currently under field validation [Schijman et al. unpublished results].

6.3. Prediction of congenital transmission in chronically infected pregnant women: Treating infected women of childbearing age prevents congenital Chagas disease [Sosa-Estani et al. 2009; Fabbro et al. 2014; Moscatelli et al., 2015; Alvarez et al., 2017]. A prospective study involving 144 seropositive pregnant women demonstrated that 18.8% of mothers with a positive PCR result transmitted the infection (16 infected children out of 85 pregnancies), while uninfected children were detected among 74 pregnancies when maternal PCR was negative [Murcia et al. 2017]. Out of a cohort of treated mothers, 92.1% had negative PCR results, compared with 32.2% of untreated ones. No infected children were detected from previously treated mothers, compared with 13.2% among untreated ones. Thus, PCR screening of *T. cruzi*-infected pregnant women is a useful tool for predicting the risk of congenital transmission.

6.4. Acute infection by organ transplantation. Early detection of *T. cruzi* transmission allows prompt treatment of donor-derived infections [McCormack et al. 2012; Cura et al. 2013]. The use of organs from seropositive donors could potentially be expanded if nucleic acid amplification-based monitoring of recipients is established in organ transplant units, with a substantial impact in shortening the waiting period on the transplant lists. PCR was able to detect acute infection in transplanted recipients between 13 and 224 days earlier than conventional serological assays and between 28 and 47 days earlier than the “Strout” method [Cura et al. 2013]. LAMP also detected parasite DNA in samples from transplanted patients [Besuschio et al, 2017].

6.5. Chronic Chagas disease. In the chronic phase, nucleic acid amplification based techniques are less sensitive than serological tests due to the low and intermittent burden of bloodstream parasites, then serological methods dictate diagnosis.

6.6 Treatment monitoring. Molecular methods are useful tools for treatment monitoring [Pinazo et al., 2015; Padilla et al, 2017; Viotti et al, 2015; Schijman et al. 2017]. Blood based qPCR techniques are being consistently used to detect therapeutic response or failure in clinical trials with traditional and novel drugs, that were administered with different regimens and combinations [Moreira et al. 2016; Molina et al. 2014; Morillo et al., 2015; Alvarez et al., 2016; Riarte et al.,]. Most trials in chronic CD have shown a lower efficacy of ravuconazole and posaconazole in comparison to benznidazole [Morillo et al.2017; Torrico et al., 2017]. Nevertheless, conclusions regarding the efficacy of these novel drugs could be somewhat biased because of pharmacodynamical issues, rather than due to a truly defficient trypanomicidal action [Urbina, 2017]. Bloodstream levels of these drugs in patients appear below those required to erradicate intracelular parasites in experimental models [Urbina, 2015]. Besides, clearance of parasitic loads exerted by drugs can be transient and lead to misleading conclusions when follow-up is performed at the short term, such it has been observed in some studies [Santos et al., 2016; Torrico et al., 2017]. Ideally, molecular methods used for monitoring of chronic patients should be performed for several years after treatment to confirm or discard available data.

6.7. Chagas disease reactivation due to organ transplantation The use of immunosuppressive drugs in organ transplantation has increased clinical significance and predispose patients to reactivation of chronic infection [Bern, 2012]. Among transplanted organs, heart transplantation leads to a higher proportion of reactivation cases than other ones. Molecular methods performed in peripheral blood and endomiocardial biopsies allow anticipating clinical signs of CD reactivation by several months [Diez et al. 2007; Burgos et al. 2010: Da Costa et al., 2016].

6.8. Chagas disease reactivation due to HIV coinfection. Quantitative PCR distinguished groups of HIV/*T. cruzi* coinfected patients with and without Chagas reactivation. Indeed the highest parasitemia was observed in coinfected patients with clinical reactivation (median 1428.90 T. cruzi/mL), followed by coinfected patients without reactivation (median 1.57 *T. cruzi*/mL) and patients with Chagas disease without HIV (median 0.00 *T.*

cruzi/mL) [De Freitas et al. 2011]. Therefore, this strategy could be used as a criterion for recommending pre-emptive therapy in patients with chronic CD with HIV infection or immunosuppression [Perez Molina et al. 2011; Almeida E. et al. 2010].

***7. T.cruzi* DNA persistence and clinical implications** In situ polymerase chain reaction analysis was used in the murine model to disclose a correlation between the persistence of parasites and the presence of disease in muscle tissue [Zhang and Tarleton, 1999; Schijman et al. 2004]. Recently, molecular amplification of parasitic mRNAs was set up to address persistence of active parasites in tissues, which may aid in unraveling parasitic tissue tropism and efficacy of trypanocidal drugs [Juiz et al. 2017].

8. Final Remarks Estimation of the biological significance of molecular based outcomes, in particular showing clearance of bloodstream parasitic loads after treatment has been seldom explored. Due to the intracellular live forms of the parasite, the predictive value of a negative bloodstream-based PCR or LAMP outcome to assess cure remains to be determined. Although trypanocidal treatment aim should be pathogen eradication, it is difficult to assert if in CD, particularly in the adult chronic phase, this will be the case. Although posttreatment reduction of the pathogen burden in the chronic phase could not yet be associated to a better clinical outcome, it is clear than it prevents vertical transmission.

Target product profiles (TPPs) for molecular diagnosis of CD have been proposed and focused to acute and congenital transmission, chronic phase and assessment of response to anti-parasitic treatment [Pinazo et al. 2014; Porras et al. 2015]. These TPPs considered minimal and optimal needs related to patients´epidemiological and clinical groups, assay clinical sensitivity and specificity, sampling volume and types of clinical specimens, conservation, shipping and storage conditions, infrastructure needed and operators` technical skills, usefulness of qualitative or quantitative reports and genotyping.

The need of point-of-care diagnostic tests has been highlighted; POC assays should contain stable materials for sample collection and the ability to perform the assays in prevailing climatic conditions. For public health applications, diagnostic assays should include low cost, simple manufacturing and distribution procedures, sustainable production and supply requirements. Disposal should be in conformity with biosafety standards and suitability by health care and target populations.

9. Conclusions

Standardized and validated PCR and LAMP methodologies are now available laboratory tools with high sensitivity and specificity that can improve current diagnosis of CD, specially in the following scenarios: (i) early diagnosis of congenital, transfusional, oral infections, monitoring of reactivation in immunosuppressed *T.cruzi* infected patients and follow-up of treatment response. The development of commercial kits based on these standardized methods is in progress; their evaluation in prospective field studies will allow predict their potential in improving diagnosis in the clinical practice and benefit public health diagnostics polices.

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