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Comparison of four PCR methods for efficient detection of *Trypanosoma cruzi* in routine diagnostics

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ABSTRACT

Due to increased migration, Chagas disease has become an international health problem. Reliable diagnosis of chronically infected people is crucial for prevention of non-vectorial transmission as well as treatment. This study compared four distinct PCR methods for detection of *Trypanosoma cruzi* DNA for the use in well-equipped routine diagnostic laboratories. DNA was extracted of *T. cruzi*-positive and negative patients' blood samples and cultured *T. cruzi*, *T. rangeli* as well as *Leishmania spp.* One conventional and two real-time PCR methods targeting a repetitive Sat-DNA sequence as well as one conventional PCR method targeting the variable region of the kDNA minicircle were compared for sensitivity, intra- and interassay precision, limit of detection, specificity and cross-reactivity. Considering the performance, costs and ease of use, an algorithm for PCR-diagnosis of patients with a positive serology for *T. cruzi* antibodies was developed.

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1. Introduction

For decades after its discovery in 1909, Chagas disease (CD) was restricted to rural areas of South and Central America (Chagas, 1909; Dias et al., 2002; WHO, 2015). Urbanization, globalization and its facilitation of migration has transformed CD into an international health problem (Perez-Molina et al., 2012; Pinto Dias, 2013; Steverding, 2014; Vannucchi et al., 2014). In the last years, the number of infected individuals in previously non-endemic areas has significantly risen, amongst others in North America, the western Pacific region, and several European countries (Coura and Vinas, 2010; Jackson et al., 2010; Klein et al., 2012; Roca et al., 2011; WHO, 2010). Today, an estimated 6–7 million infections and 10,000 annual deaths are attributed to this life threatening disease worldwide (WHO, 2015).

CD is a chronic infectious disease caused by the protozoan flagellate *Trypanosoma cruzi* (*T. cruzi*). In endemic countries, CD is transmitted mainly through the contact with feces of different triatomine bugs. In non-endemic countries without vectorial transmission, the main risks for transmission are blood transfusions, organ transplantations and mother-to-child transmission, as women often migrate at young ages and later create families in their new homes (Cevallos and Hernandez, 2014; Perez-Ayala et al., 2011). CD presents itself in two phases: whereas the acute phase is dominated by unspecific or no symptoms at all, in the chronic phase cardiac and/or gastrointestinal symptoms can occur after a long asymptomatic interval (Rassi et al., 2010, 2012).

One of the first and very crucial steps in patient care is the diagnosis of the disease. Whereas serological methods remain the gold-standard for diagnosing CD because of their high sensitivity, performing a polymerase chain reaction (PCR) in addition can be important (Duarte et al., 2014; Godsel et al., 1995; Krautz et al., 1995; Krieger et al., 1992). Positive serological markers are only an indirect evidence of infection with *T. cruzi*, as they occur independently of the parasites' presence. The antibodies can persist in patients' blood for years even after successful treatment (Jackson et al., 2013; Sosa-Estani et al., 2009; Viotti et al., 2011). This has to be considered when monitoring treatment efficacy or quantifying parasite numbers in patients' blood (Avila et al., 1993). Other limiting factors of serology are the possible

transfer of antibodies from mother to child (Mallimaci et al., 2010; Piron et al., 2007), recently acquired infections (Grauert et al., 1993), and the possible cross-reactivity with *Leishmania spp. (L. spp.)* or *Trypanosoma rangeli (T. rangeli)* (Gomes et al., 2009; Wincker et al., 1994a). In such cases and when obtaining doubtful or inconsistent serological results, PCR methods can be helpful (Marcon et al., 2002).

In 2014, researchers started an initiative to combat CD in Germany (http://chagas.info/) (Navarro et al., 2017). During the process of creating a suitable laboratory in Germany, the establishment of a quick, reliant and economic diagnostic algorithm was needed. As detailed in this study, four distinct PCR methods for detection of *T. cruzi* in human blood were intensely evaluated. The comparison of results led to the first two officially accredited PCR methods for the detection of *T. cruzi* in a German routine laboratory.

2. Materials and methods

2.1. Samples

Samples consisted of 50 human EDTA blood samples that were previously tested positive by ELISA, IFAT and PCR for *T. cruzi* in the Department of Parasitology, National Microbiology Centre, Instituto de Salud Carlos III, Madrid, Spain (ISCIII) during 2010–2013. The PCR was similar to method A described below. More detailed information on the patients is shown in Table 3. None of them have received treatment prior to this study. Further, three negatively tested human EDTA-blood samples for *T. cruzi* and *L. spp.* as well as cultured epimastigotes of *T. cruzi* (DM28) (Grisard et al., 2014), *T. rangeli* and *T. brucei* (patient material, not further characterized) were used. Two *T. cruzi* negative blood samples were spiked with the above mentioned culture material. Additionally, *L. tropica-*, *L. donovani-*, *L. infantum-*, *L. braziliensis-* and *L. major-*positive patient samples were taken from symptomatic outpatients who were treated at the Division of Infectious Diseases and Tropical Medicine (DITM), Medical Center of the University of Munich (LMU), during 2007–2013. All human samples were taken from patients that had agreed on the use of their anonymized blood samples for research purposes. Ethical approval was obtained from the Ethical Committee of the Faculty of Medicine of the Ludwig-Maximilians-University (LMU) in Munich, Germany. All data were processed anonymously. Data were transferred to STATA, version 14, and analysis was performed. Linear regression models were used for a multivariate analysis of continuous variables. Comparison between two groups was calculated by Wilcoxon rank sum test for continuous variables and the χ^2 test or Fisher's exact test for categorical variables. Results were considered significant if p-values were <0.05.

2.2. DNA purification

Patients' blood samples were treated with a mixture of 6 M guanidine hydrochloride, 0.2 M EDTA, pH 8.00 (guanidine-EDTA) in the ratio of 1:1 and left incubating for at least 12 hours as previously described (Avila et al., 1991; Britto et al., 1993; Duffy et al., 2013; Wincker et al., 1994b). Afterwards, the samples were boiled for 15 minutes, which partially disrupts the kDNA minicircle (Virreira et al., 2003). DNA extraction was carried out using the High Pure PCR Template Preparation kit (Roche Diagnostics Corp., Indianapolis, IN, USA) as described in the manufacturers' instructions. The purified DNA template was stored at ≤−20 °C. DNA extraction of cultured material was performed as described in the manufacturers' instructions without pre-treatment with guanidine hydrochloride.

2.3. DNA amplification

After extensive literature research and the recommendations of ISCIII, two conventional and two real-time PCR methods were elected for closer evaluation (Norman et al., 2011; Piron et al., 2007; Schijman et al., 2011): Two were conventional PCR methods, method A recommended by the ISCIII and method B by Schijman et al. (2011). Primers for method A target the variable region of the kDNA minicircle (Wincker et al., 1994a) and for method B a repetitive Sat-DNA sequence (Cummings and Tarleton, 2003) of the *T. cruzi* genome. Methods C and D were real-time PCR tests, both targeting repetitive Sat-DNA sequences. Method C was amongst the best performing methods in the work of Schijman et al. (2011), method D was a commercial kit (Dia.Pro - Diagnostic Bioprobes Srl, Sesto San Giovanni, Italy). For methods A and B the Professional Standard thermal cycler (Biometra GmbH, Göttingen, Germany) was used, for methods C and D the CFX C1000 Real-Time thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers are listed in Table 1. Amplification was performed as detailed below:

Method A: The PCR mix contained 1× AmpliTaq Gold Buffer, 2 mM MgCl₂, 0.33 µM of each of the kDNA specific primers 121 (Sturm et al., 1989), and 122 (Wincker et al., 1994a, 1994b), 0.2 mM deoxyribonucleotide triphosphates (dNTPs), 2.5 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 10 µl template DNA and water, adding up to a final volume of 75 µl. The ~330 base pair (bp) fragment was amplified under the following conditions: 5 min at 95 °C; 35× (1 min at 94 °C, 1 min at 64 °C, 1 min at 72 °C); 10 min at 72 °C.

Method B: The PCR mix contained 1× AmpliTaq Gold Buffer, 3 mM MgCl₂, 0.5 µM of each of the Sat-DNA specific primers TczF and TczR (Cummings and Tarleton, 2003), 0.25 mM dNTPs, 1.5 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 10 µl template DNA and water, adding up to a final volume of 60 µl. The ~182 bp fragment was amplified under the following conditions: 3 min at 94 °C; 40× (45 s at 94 °C, 1 min at 68 °C, 1 min at 72 °C); 10 min at 72 °C (Schijman et al., 2011). PCR products for methods A and B were analyzed by electrophoresis in a 2% agarose gel in 1× TAE buffer stained with GelRed (Biotium, Hayward, CA, USA).

Method C: The PCR mix contained 1× FastStart Universal Probe Master (ROX) (Roche Diagnostics Corp., Indiana, USA), 0.75 µM of each of the Sat-DNA specific primers cruzi1, cruzi2 and TaqMan probe cruzi 3, 2 µl template DNA and water, adding up to a final volume of 20 µl. Cyling conditions of the ~166 bp fragment were as follows: 95 °C for 15 min, 45× (95 °C for 10 s, 54 °C for 1 min). Fluorescence was measured at the end of each cycle at 54 °C (Schijman et al., 2011).

Method D was performed with the commercially available kit TCRUZIDNA.CE (Diagnostic Bioprobes Srl, Sesto San Giovanni, Italy) following the manufacturer's instructions. Data of method C and D were analyzed with CFX Manager Software Version 2.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The threshold was set to 50 relative fluorescent units (RFU) and thus sufficiently low to be within the exponential growth region of the amplification curve.

2.4. Controls

Adequate positive and negative controls were included in each run to detect possible contamination. After each DNA purification, the negative extraction control (water) was examined.

2.5. Selected definitions

The following terms were used to evaluate the performance of each PCR method:

Intra-assay precision (Intra). To evaluate the intra-assay precision, one known positive and one known negative sample were tested three times in one run on the first day of evaluation.

Inter-assay precision (Inter 1 and Inter 2). To evaluate the inter-assay precision, the same positive and negative samples used to determine the intra-assay precision were tested once on the second day (Inter 1) and once on the third day (Inter 2) of evaluation.

Limit of detection (LOD). After DNA amplification of cultured *T. cruzi*-strain DM28 as described above for methods A, B, C, and D, an electrophoresis of the amplicons in a 2% agarose gel in 1× TAE buffer stained