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Aetiological treatment of congenital Chagas' disease diagnosed and monitored by the polymerase chain reaction

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***Objectives*: This prospective study focused on the evaluation of anti-parasitic therapy in congenital Chagas' disease, diagnosed and monitored by PCR and conventional diagnosis.**

***Materials and methods*: We studied 152 children born to seroreactive mothers, living in a non-endemic area. Fifty infants aged 0–6 months (GA) were diagnosed by microhaematocrit and PCR and 102 children aged 7 months to 17 years (GB) were diagnosed by serology and PCR. Forty treated patients were monitored for 2 or 3 years by PCR and conventional methods. A competitive-quantitative PCR was used to determine pre-therapy parasitic loads and follow their post-treatment evolution.**

***Results*: In GA, the sensitivities of the PCR and microhaematocrit were 100% and 82.4% and their specificities 97% and 100%, respectively. In GB, the sensitivity of the PCR was 73.8% with a specificity of 100%. Pre-therapy parasitic loads ranged from 12.5 to 125 000 and 12.5 to 125 parasite genomic equivalents/mL of blood in GA and GB, respectively. PCR turned negative in all treated pre-therapy PCR positive patients before or at the end of treatment, which was followed by their seronegativation in 10/10 GA, in 3/5 children initiating therapy at 7 months to 2 years of age but in 0/16 initiating therapy at an older age. Two out of the latter patients were occasionally PCR positive during post-treatment, suggesting no parasitological response. Out of nine pre-therapy PCR negative patients, four turned seronegative after treatment, suggesting that in undetermined patients, undetectable parasitic burdens may lead to better post-treatment prognosis.**

***Conclusions*: PCR was useful for sensitive diagnosis and therapy monitoring, allowing early detection of refractory cases.**

Keywords: *Trypanosoma cruzi*, congenital transmission, kinetoplastid DNA, competitive PCR, parasitological cure

Introduction

Chagas' disease whose aetiological agent is the protozoan *Trypanosoma cruzi*, affects about 20 million people in endemic countries from the United States to Argentina.[1–3] The infection may be acquired mainly through the haematophagous triatomid insect vector, blood transfusion and the trans-placental route.[2–4] Congenital Chagas' disease (CI) may be suspected from the offspring of any infected mother.[4,5] The prevalence of *T. cruzi* infection among pregnant women ranges from 2% to 51% in urban areas and from 23% to 81% in rural regions of Latin America.[4,5] In 1997, the subprogramme of control of pregnant women in Argentina examined 58 196 cases from 13 provinces, finding 9% seroreactivity to *T. cruzi*.[6] As a result of migration from endemic areas to Buenos Aires city, where the disease is not endemic, *T. cruzi* infection is detected in 6–8% of pregnant women delivering at public hospitals. In recent surveys, the likelihood of vertical transmission was estimated in 2.6% to 6.7%.[5–9]

Standard serodiagnosis of *T. cruzi* infection in infants born to seroreactive women has a low positive predictive value, because the presence of anti-*T. cruzi* IgG antibodies in the newborn may be due to passive transfer of IgG maternal antibodies, which in the non-infected infant would normally disappear around the sixth month of age.[4,5,7] Moreover, a small proportion of infected newborns are seronegative.[4,5] The detection of infant IgM antibodies against *T. cruzi* did not have a satisfactory performance in previous studies.[5,7] Therefore, the diagnosis of congenital Chagas' disease usually relies on

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microscopic observation of bloodstream trypomastigotes, which is more effective by the microhaematocrit concentration technique in infants under 6 months of age.[5,11] However, in those patients with undetectable parasitaemia, the aetiological treatment must be postponed towards the undetermined phase, when the infection can be precisely assessed by means of IgG based serodiagnosis.[5,10,12]

Anti-parasitic treatment is indicated in CI with greater success in newborns closer to delivery.[5,7,8,10] After aetiological treatment, the criterion of cure relies on serologic conversion to negative of the anti-*T. cruzi* antibody response,[10,13] but in patients initiating therapy at the undetermined phase, seroconversion usually occurs several years after treatment, requiring long-term follow-up.[10,13] In search of more sensitive laboratory tests to detect infection and evaluate treatment outcome in CI, the polymerase chain reaction (PCR) appeared as a promising laboratory tool.[14–24] Reconstitution experiments showed that PCR procedures were capable of detecting the equivalent of a single parasite cell in 10–20 mL of whole blood.[15,21] However, when PCR was applied to the analysis of clinical specimens, sensitivity was highly variable, depending on the epidemiological characteristics of the study populations, the volume of the clinical sample, the method used to isolate DNA, the parasite sequences and primers chosen for PCR as well as the thermo-cycling conditions.[14–24]

This prospective study is focused on the evaluation of anti-parasitic therapy in CI patients, living in Buenos Aires city, a non-endemic area for Chagas' disease, diagnosed and monitored by conventional and PCR-based assays.

Materials and methods

Patients

The study included children admitted to the Parasitology Laboratory of Ricardo Gutiérrez Children's Hospital, a tertiary care paediatric referral centre for diagnosis of Chagas' disease without a maternity service. It was approved by the Institutional Review Board, with the informed consent of the responsible adult.

For diagnostic purposes, children born to infected mothers were divided into two groups, according to their age at time of admission. Group A (GA): infants younger than 6 months of age; they underwent diagnostic screening by direct parasitological and serological methods. Group B (GB): children older than 6 months of age; they were serologically screened. Moreover, the study enrolled children born to non-infected mothers as a PCR control group (Group C, GC).

Diagnosis criteria

GA infants were diagnosed as infected, if they had parasites in blood. Those who had undetectable parasitaemia were re-tested by anti-*T. cruzi* IgG serological methods after their sixth month of life. GB children were considered infected if they presented reactivity by two serological tests.

CI was diagnosed if the child (a) was born to an infected mother, (b) had never had a transfusion and (c) had never lived in an endemic area.

Serodiagnosis

We carried out an indirect haemagglutination (IHA) test (Lab Polychaco, Buenos Aires, Argentina), an ELISA employing a whole parasite lysate (Wiener, Rosario, Argentina) and a passive particle agglutination test (PPA) (Bayer, Buenos Aires, Argentina), as recommended by the manufacturers. IHA and PPA tests were considered reactive if the IgG antibody titre was ≥1:16; ELISA test was considered reactive if the ratio of positivity was >1.2.

Parasitological diagnosis

The microhaematocrit (MH) test was carried out as previously published.[11]

Nucleic acid extraction

Two millilitres of peripheral blood was collected from each paediatric patient and immediately mixed with one volume of 2× lysis buffer containing 6 M guanidine hydrochloride (Sigma, St Louis, USA) and 200 mM EDTA, pH 8.0 (GE).[15] The resulting GE-blood lysate (GEB) was boiled[23] and stored at 4°C. DNA was purified from 100 µL aliquots of GEB, as previously reported.[19]

Polymerase chain reaction

A hot-start PCR procedure, targeted to the 330 bp minicircle fragment of the *T. cruzi* kinetoplastid (kDNA) genome[14] was carried out in 50 µL reactions using PCR tubes containing wax beads (Molecular BioProducts, San Diego, CA, USA). The 12 µL lower mixture carried 2 µL of 25 mM $MgCl_2$, 5 µL of 2.5 mM of each deoxynucleotide triphosphate (dNTP) (Promega, WI, USA), 1.5 µL of 50 µM of primers 121 [5′-AAATAATGTACGG G(T/G)GAGATGCATGA-3′] and 122 (5′-GGTTCGATTGGGGTTGGTGTAATATA-3′) and 1.2 µL of 10× *Taq* DNA polymerase buffer (Gibco-BRL, Rockville, MD, USA). The 33 µL upper mixture carried 4 µL of 25 mM $MgCl_2$, 3.8 µL of 10× *Taq* DNA polymerase buffer, 1.25 units of *Taq* DNA polymerase (Gibco-BRL) and 5 µL of specimen DNA. Amplification was carried out in a MJR PTC-100 thermocycler (MJ Research, Watertown, MA, USA) as follows: one step of 3 min denaturation at 94°C; five cycles at 64°C for 40 s, 72°C for 1 min, 94°C for 40 s; five cycles at 63°C for 40 s, 72°C for 1 min and 94°C for 40 s; 27 cycles at 62°C for 40 s, 72°C for 1 min and 94°C for 40 s; with one final extension step at 72°C for 10 min. PCR products were analysed by agarose gel electrophoresis followed by Southern hybridization. Hybond-N plus membranes (Amersham, Little Chalfont, Buckinghamshire, UK) were pre-hybridized in 4% skimmed milk, 1 M sodium chloride–0.1 M sodium citrate (SSC) and 0.1% sodium dodecyl sulphate (SDS) buffer at 55°C for 2 h and hybridized overnight with a ^{32}P-labelled kDNA probe obtained by heminested-PCR, as previously reported.[24] Washings were carried out in 0.3 M sodium chloride–0.03 M sodium citrate–0.2% SDS at room temperature (for 20 min ×2), and in 0.1× SSC–0.1% SDS at 55°C for 1 h.

Each PCR test run included no more than six samples, one DNA preparation from an infected human blood sample as a positive control, one DNA preparation from blood of a healthy subject as a negative control, an amplification reaction without DNA as a negative PCR mixture control and an amplification reaction with 1 fg of total *T. cruzi* DNA as a weak positive PCR control.

Specimen DNA purification, assembling of reagent mixtures, cycling, gel electrophoresis and hybridization procedures were carried out in different laboratory working areas. A blood specimen was considered as PCR positive if kDNA-PCR followed by hybridization was positive on both DNA preparations. PCR analysis was carried out blinded to the serological and parasitological diagnosis.

Quantitative competitive PCR assays

Construction of a quantification standard: In order to set up a quantitative competitive PCR assay (QC-PCR),[25] a quantification standard (QS) was constructed by cloning a competitor DNA fragment in the pGEMT-easy plasmid vector (Promega). The competitor template was created by generating an internal deletion of a kDNA-PCR product, using a gene SOEing approach.[26] It binds primers 121 and 122, yielding a PCR product of 278 bp [GenBank accession number AF239913, see Figure 3(a)], distinguishable from the 330 bp *T. cruzi* kDNA in agarose gels. To carry

out the QC-PCR assays, the QS was linearized by restriction enzyme digestion and quantified. The QS standards consisted of 10-fold serial dilutions ranging from 5 to 5×10^6 copies/μL.

QC-PCR assay conditions: QC-PCR master mixtures contained the same concentrations of reagents as hot-start PCR mixtures, except primers (5 μM final concentration), in a final volume of 95 μL. Five microlitres of each QS standard was added to a series of PCR tubes containing 5 μL of specimen DNA. Cycling parameters were as follows: one step of 2 min denaturation at 94°C; five cycles at 66°C for 1 min, 72°C for 1 min, 94°C for 1 min; 30 cycles at 64°C for 1 min, 72°C for 1 min and 94°C for 1 min; with one final extension step at 72°C for 10 min. The amount of PCR products generated by the competitor and target DNAs was compared after electrophoresis to detect the equivalency point, as previously reported.[26] The parasite load was expressed in parasite equivalents/mL or $\log_{10}$ parasite equivalents/mL of blood, assuming that each parasite cell harbours 10 000 minicircles with four copies each of the 330 bp kDNA template.[27,28]

Analytical sensitivity of the PCR assays

To assess the analytical sensitivity of kDNA-PCR and quantitative-PCR tests, we carried out titration assays with 10-fold serial dilutions up to 0.025 parasite genomic equivalents/mL from an artificial blood specimen spiked with a known amount of cultured parasites.[28] The blood was collected in GE buffer, boiled for 15 min, left to stand at room temperature overnight and distributed in 100 μL aliquots. Four 100 μL volume replicates were processed for PCR and QC-PCR amplification procedures, as detailed above.

Therapeutic regimen and treatment follow-up

Patients were treated with nifurtimox (Lampit, Bayer, Buenos Aires, Argentina) at 10–15 mg/kg/day or benznidazole (Radanil, Roche, Buenos Aires, Argentina) at 5–8 mg/kg/day in two daily doses for 60 days. The criterion of cure was the negativation of IgG anti-*T. cruzi* antibodies.[10]

In order to evaluate the treatment outcome according to age groups, the CI patients were divided into three groups, GI (younger than 3 months), GII (between 7 months and 2 years old) and GIII (older than 3 years old). GI infants were followed up by MH, PCR and IgG based serological tests before treatment (T0), at 30 days (T1), at the end of treatment (T2), and 6 months (T3), 1 year (T4) and 2 years (T5) after treatment. GII and GIII children were monitored by PCR and serological tests before treatment (T0), at the end of treatment (T2), and 6 months (T3), 1 year (T4), 2 years (T5) and 3 years (T6) after treatment.

Statistical analysis

All results were analysed using the χ^2 statistical test, and P values <0.05 were considered to be significant.

Results

Detection limit and reliability of the kDNA-PCR assay

The kDNA-PCR test allowed detection of 2.5 parasite genome equivalents/mL of blood in agarose gels or 0.25 parasite genome equivalents/mL of blood after Southern hybridization, in 100% of the tested replicates. Figure 1 shows kDNA-PCR products detected in ethidium bromide-stained agarose gels (Figure 1a) and following Southern-blot hybridization (Figure 1b) from clinical specimens and controls. In lane 4, there is a negative PCR result obtained from the blood specimen of a non-infected patient from GA. Lane 5 depicts the

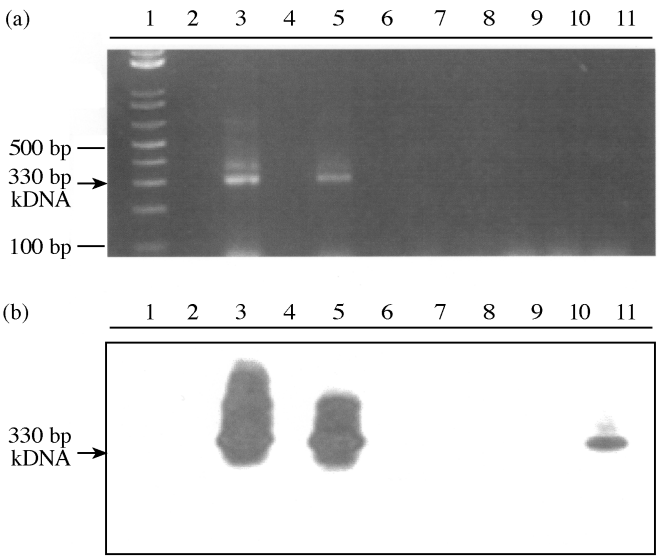

Figure 1. (a) Ethidium-bromide-stained 3% agarose gel and (b) Southern-blot analysis of *T. cruzi* kDNA-PCR products from human blood specimens and controls. Lane 1, 100 bp DNA ladder molecular weight marker; lane 2, PCR negative control; lane 3, *T. cruzi* DNA positive control; lane 4, GA non-infected patient; lane 5, PCR positive GA CI patient; lanes 6 to 10, treatment monitoring of patient of lane 5 at T1, T2, T3, T4 and T5, respectively (see profile F in Figure 4); lane 11, GB CI patient.

330 bp kDNA amplicon visualized in the agarose gel, obtained from one MH negative blood sample of a GA infected patient, who turned to PCR negative following treatment with nifurtimox (lanes 6–10, Figure 1 and see profile F, Figure 4). Lane 11, shows the kDNA-PCR result obtained from the blood sample of a GB infected patient, that was only detectable following Southern hybridization (Figure 1b).

In order to evaluate the reliability of the kDNA-PCR results in clinical specimens, we carried out PCR assays in two subsequent blood samples collected from the same infected subject before initiating aetiological treatment. Pre-therapy blood samples from GA were collected, separated by a period of 7–20 days, whereas blood samples from GB were withdrawn separated by a period of 30–45 days. Accordingly, 16 congenital Chagas' disease patients, four GA and 12 GB were tested (Figure 2). Among GA cases, three profiles were obtained (A–C, Figure 2). Profile A is composed of one case who was positive by PCR, MH and serodiagnosis in both tested specimens. Profile B is composed of two patients, who were PCR and serological positive in both tested specimens but MH positive in only one of them. Profile C is of one PCR positive, MH positive and seronegative case in both samples. Accordingly, in GA, the PCR and serological findings were concordant in 4/4 paired specimens, whereas the MH results were concordant in 2/4 paired specimens (GA, Figure 2). Among the 12 GB tested cases, three profiles were obtained (D–F, Figure 2). Profile D is composed of five cases who were seropositive and PCR positive in both specimens. Profile E is of five patients seropositive but PCR negative in both samples. Profile F corresponds to two patients seropositive in both samples but PCR positive in only one of them. Then, in GB the PCR results were concordant in 10/12 paired specimens (83.3%), whereas the serological findings were 100% concordant (GB, Figure 2).

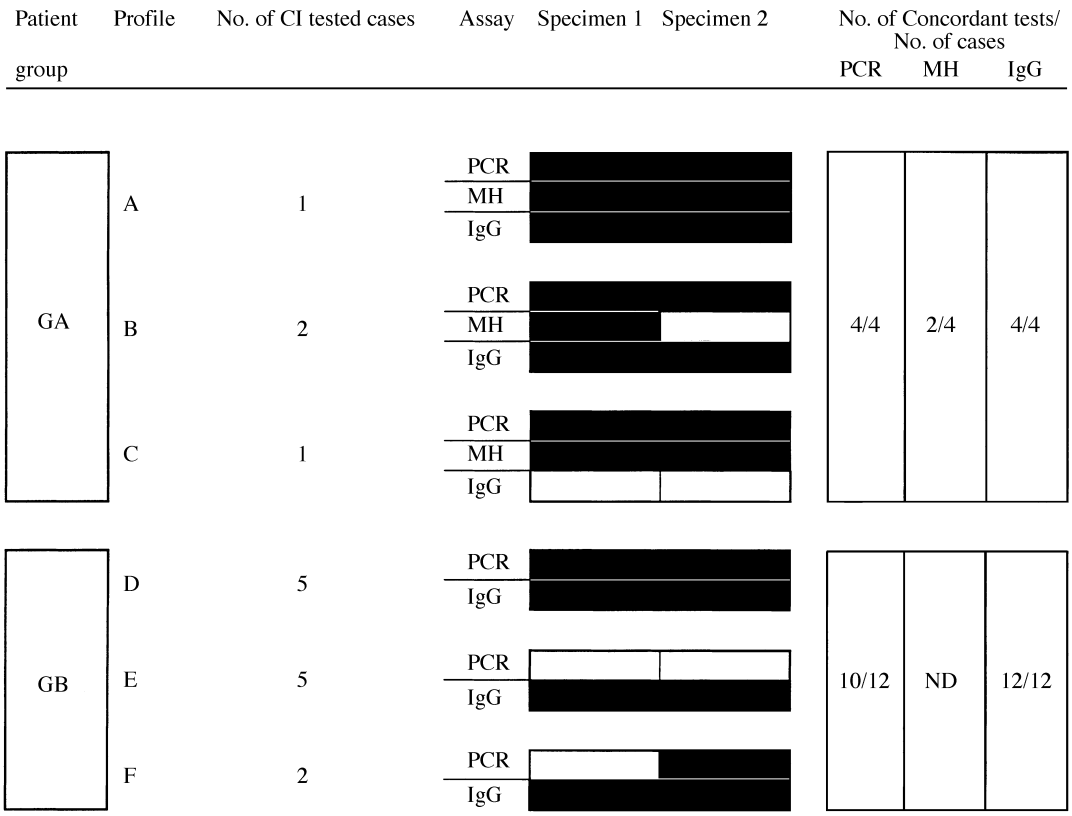

Figure 2. Concordance of results obtained by kDNA-PCR and conventional diagnostic methods in two serial pre-therapy peripheral blood specimens from GA and GB CI patients. Filled boxes, positive findings; open boxes, negative findings; IgG, conventional serodiagnosis; MH, microhaematocrit.

Diagnosis of congenital Chagas' disease

During 1998–2000, 1606 paediatric individuals were admitted, 473 classified as GA and 1133 as GB. Based on diagnosis criteria, 18 out of 473 GA infants (3.8%) and 81 out of 1133 GB children (7.1%) were congenitally infected.

The PCR was carried out in 152 children born to infected mothers; 50 were GA, 17 infected (CI) and 33 non-infected (NI), whereas 102 were GB, 61 CI and 41 NI (Table 1). Moreover, 22 infants born to non-infected mothers (GC) were included as a PCR control group (Table 1).

PCR was positive in 62 out of 78 CI patients and in one of 74 NI patients born to infected mothers (Table 1). Accordingly, among children born to seropositive mothers, the sensitivity of the PCR was 79.5%, its specificity was 98.6% with a positive predictive value (PPV) of 98% and a negative predictive value (NPV) of 82% (GA + GB, Table 2). PCR was negative in all GC tested patients born to non-infected mothers (specificity 100%) (Table 1).

PCR-based detection of congenital infection in GA

The PCR was positive in 17/17 congenitally infected (CI) and in 1/33 non-infected (NI) (Table 1). Accordingly, in GA, the sensitivity of the PCR was 100% with a specificity of 97%. The PPV and NPV were 94% and 100%, respectively (GA, Table 2).

The MH was positive in 14/17 CI and in 0/33 NI (Table 1). Two of the three CI cases with MH negative findings presented a parasitological positive result in another blood specimen not tested by PCR, and hence were diagnosed as congenital Chagas' disease (data not shown). The third one was diagnosed as infected after his sixth month of age by the positivity of anti-*T. cruzi* IgG antibodies. Thus, the sensitivity of the MH was 82.4% with a specificity of 100%. Its PPV was 100% and the NPV was 92%. Out of the 17 infected patients, 14 presented anti-*T. cruzi* IgG antibodies (82.4%); the remaining three seronegative cases were diagnosed by means of their positive MH tests. Out of the 33 non-infected subjects, 19 were seropositive at time of diagnosis (57.6%), becoming seronegative when re-tested after their sixth month of life.

PCR-based detection of congenital infection in GB

The PCR was positive in 45/61 seropositive patients and negative in all seronegative cases (Table 1). The sensitivity of the PCR among infected GB was 73.8%, its specificity was 100% with a PPV of 100% and a NPV of 72% (GB; Table 2).

Estimation of the parasitic load by competitive PCR

In order to characterize the bloodstream parasitic load in patients at time of diagnosis as well as during post-treatment follow-up, we constructed a quantification standard to carry out competitive PCR assays (QC-PCR, Figure 3a). The QC-PCR assay was capable of quantifying DNA samples containing above 500 copies/μL of the 330 bp kDNA template. Because the QC-PCR reaction contained 5 μL of DNA which corresponded to 1/200 of the DNA content/mL in the original blood specimen, the detection limit of QC-PCR was 12.5 parasite genomic equivalents/mL, assuming that each parasite

Table 1. Characteristics of paediatric groups. Comparison of conventional serology, microhaematocrit and PCR for diagnosis of CI

	Group characteristics				No. positive/no. tested (%)		
Group (no.)	diagnosis	no.	M/F	mean age (range)	serology	MH	PCR
GA (50)	CI	17	6/11	2.5 months (12 days–6 months)	14/17 (82.4)	14/17 (82.4)	17/17 (100)
	NI	33	12/21	1.9 months (8 days–6 months)	19/33 (57.6)	0/33 (0)	1/33 (3)
GB (102)	CI	61	31/30	7.4 years (8 months–17 years)	61/61 (100)	ND	45/61 (73.8)
	NI	41	16/25	5.2 years (8 months–10 years)	0/41 (0)	ND	0/41 (0)
GA + GB (152)	CI	78	37/41	5.8 years (12 days–17 years)	75/78 (96.1)	14/17 (82.3)	62/78 (79.5)
	NI	74	28/46	2.9 years (8 days–10 years)	19/74 (25.7)	0/33 (0)	1/74 (1.3)
GC (22)	NI	22	10/12	5.1 years (8 months–15 years)	0/22 (0)	ND	0/22 (0)

ND, not done.

Table 2. Evaluation of the PCR for detection of *T. cruzi* DNA in CI

Group	Sensitivity	Specificity	Positive predictive value	Negative predictive value
GA	100	97 (91.1–102.8)	94 (83.9–105)	100
GB	73.8 (62.7–84.8)	100	100	72 (60.3–83.6)
GA + GB	79.5 (70.5–88.4)	98.6 (96–101.3)	98 (95.3–101.5)	82 (74–90)

95% confidence intervals in parentheses.

cell harbours 10 000 minicircles, with four copies each of the 330 bp kDNA template.[27,28]

To estimate the bloodstream parasitic load in pre-therapy samples of CI patients of different age groups, we carried out QC-PCR assays in PCR positive DNA samples from 13 CI patients, seven GA and six GB (Figure 3b). The analysis of GA revealed parasitic loads ranging from around 12.5 parasite genome equivalents/mL (1.09 $\log_{10}$) (case 1, Figure 3b) to 125 000 parasite genome equivalents/mL (5.09 $\log_{10}$) (cases 6 and 7, Figure 3b). In contrast, the parasitic load of GB cases ranged from around 12.5 parasite genome equivalents/mL (1.08 $\log_{10}$) to 125 parasite genome equivalents/mL (2.09 $\log_{10}$) (cases 8 to 13, Figure 3b).

Monitoring of anti-parasitic therapy

To evaluate the outcome of 40 patients undergoing anti-parasitic treatment, they were classified in three age groups, 10 GI (<3 months of age), six GII (7 months to 2 years old) and 24 GIII (>3 years old). Cure was achieved in 100% of GI cases, in 66.7% of GII cases and in 12.5% of GIII cases ($P = 0.0000$). Out of the treated GI patients, six different profiles of treatment outcome are observed (GI, profiles A to F, Figure 4). Profile A is composed of two seronegative infants who were PCR and MH positive at T0; PCR and MH became negative from T1; the serology remained negative through 2 years of follow-up, indicating cure. Profile B corresponds to one serologically positive infant who was PCR and MH positive at T0. Profile A and B patients became parasitological and serological negative from T1 onwards. Profiles C and D are similar to profile B, except that these patients cured at longer post-treatment periods, at T2 and T3, respectively. Profile E is composed of two seropositive patients who were PCR and MH positive at T0 but became MH negative at T1 and PCR and seronegative at T2. The evolution of the parasitic loads during treatment was estimated by QC-PCR in one patient with profile E (case 7, Figure 3b); his parasitaemia decreased from around 125 000 parasitic genome equivalents/mL at T0 to 12.5 parasite genome equivalents/mL at T1 (case 7, Figure 3c) being undetectable from T2. The parasitic loads of the other profile E case was not assessed by QC-PCR because the PCR positivity at T1 was detected following Southern hybridization, which is under the detection limit of QC-PCR. Profile F is composed of two serologically positive

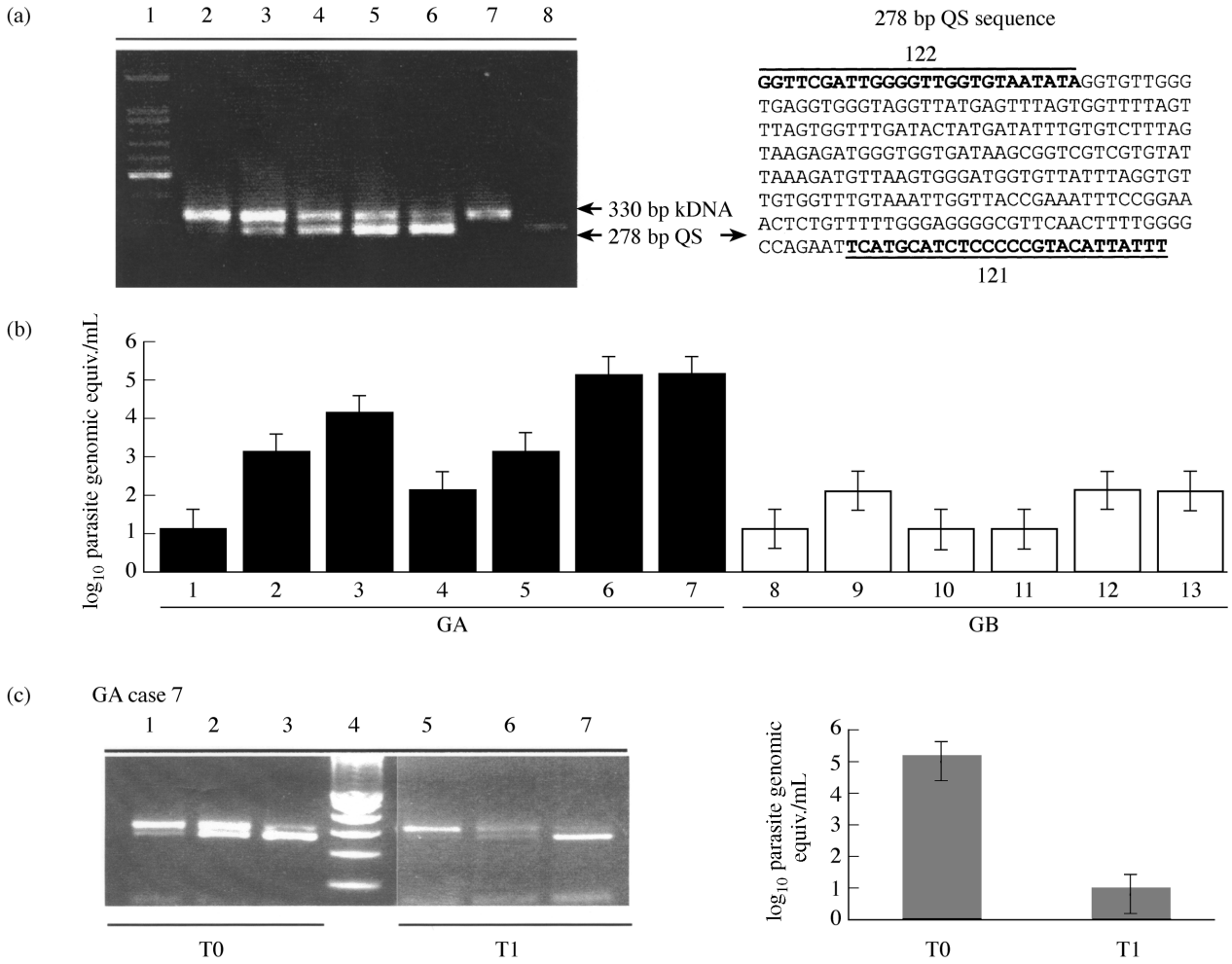

Figure 3. (a) Left-hand panel: agarose gel electrophoresis showing a competitive quantitative PCR test with five-fold dilutions of the quantification standard (QS). Lane 1, 100 bp DNA ladder molecular weight marker; lane 2, 250 copies; lane 3, 1250 copies; lane 4, 6250 copies; lane 5, 31 250 copies; lane 6, 156 250 copies; lane 7, 330 bp kDNA amplicon; lane 8, 278 bp competitor template. The equivalency point is observed in lane 4. Right-hand panel: nucleotide sequence of the 278 bp competitor template (QS). (b) Bloodstream parasitic loads obtained in pre-therapy samples of CI patients from GA and GB groups. (c) Bloodstream parasitic loads obtained during treatment follow-up of CI patients. Left-hand panel: agarose gel electrophoresis showing QC-PCR results from patient 7 (Figure 3b) at T0 and T1. Lanes 1–3, QC-PCR with 1/100 diluted DNA from case 7 and 10-fold dilutions of QS, at T0. Lanes 1–3, 2.5×10^4 to 2.5×10^6 copies of QS; lane 4, 100 bp DNA ladder molecular weight marker; lanes 5–7, 2.5×10^2 to 2.5×10^4 copies of QS at T1. Right-hand panel: bloodstream parasitic load of patient 7 at T0 and T1 (profile E, Figure 4).

patients, MH negative but PCR positive at T0. They were PCR negative from T1 (Figure 1, lanes 5 to 10) and seronegative from T2 (Figure 4).

In GII, four different profiles were observed; profiles G, H and J were cured during follow-up. Profile G is composed of two T0-PCR positive patients, who became PCR negative at T2 and seronegative at T4. Profile H is similar to profile G, except that seroconversion to negative was detected at T5. Profile I is composed of two T0-PCR positive cases, which turned PCR negative at T2, remaining seropositive through follow-up. Profile J is one T0-PCR negative patient, who persisted PCR negative during follow-up and became seronegative at T3.

Out of the 24 GIII patients, six profiles were distinguished (GIII, profiles K to P, Figure 4). Sixteen cases (profiles K–M) were PCR positive at T0, whereas eight cases (profiles N–P) were PCR negative at T0. Cure was achieved in 3/8 T0-PCR negative cases (37.5%) but in none of the 16 T0-PCR positive ones ($P = 0.049$). Profile K includes 14/24 GIII patients (58.3%) who remained seropositive through follow-up, whereas the PCR was negative when tested at T2 onwards. Profiles L and M are of two cases which presented sporadic PCR positive findings after treatment in the absence of seronegativation. Profile N encompasses three T0-PCR negative patients who were cured before T4. They persisted as PCR negative during follow-up. Profile O is composed of four T0-PCR negative patients who remained PCR negative but seropositive until T6. Profile P is one T0-PCR negative case, which showed a sporadic PCR positive result at T5, persisting as seropositive through follow-up.

The post-treatment parasitic loads in patients with profiles L, M and P could not be determined by QC-PCR because the kDNA-PCR positive results were achieved following Southern hybridization, suggesting parasitic burdens below 2.5 parasite equivalents/mL.

Discussion

This longitudinal study applied PCR techniques for detection of *T. cruzi* infection and evaluation of treatment outcome in congenital Chagas' disease. To our knowledge, this is the first prospective PCR

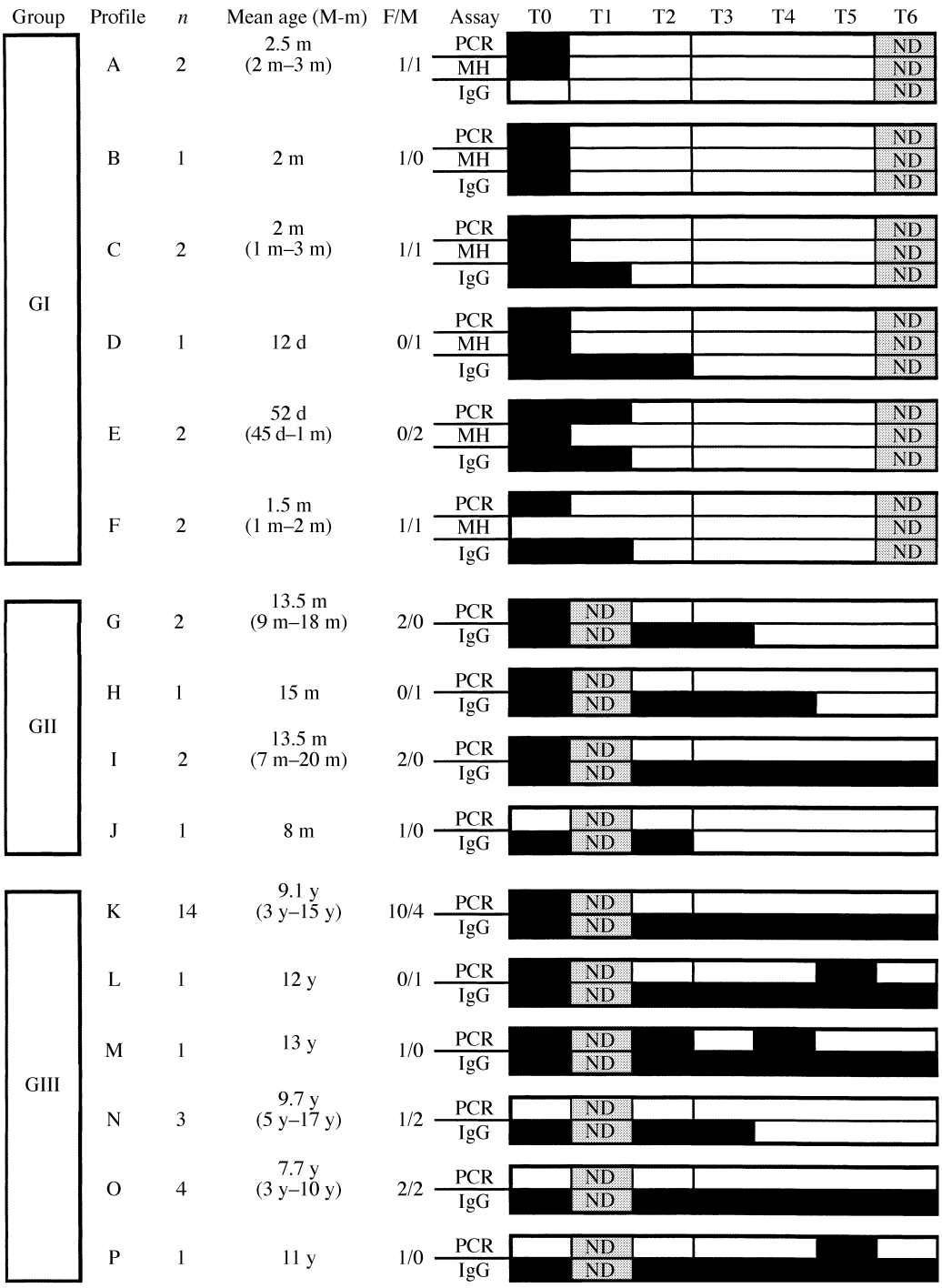

Figure 4. GI infants were followed up by MH, PCR and IgG based serodiagnosis before treatment (T0), at 30 days (T1), at the end of treatment (T2), and 6 months (T3), 1 year (T4) and 2 years (T5) after treatment. GII and GIII children were monitored by PCR and serological tests before treatment (T0), at the end of treatment (T2), and 6 months (T3), 1 year (T4), 2 years (T5) and 3 years (T6) after treatment. M–m, maximum–minimum ages; F/M, female/male; ND, not done; m, month(s); d, days; y, years; filled boxes, positive findings; open boxes, negative findings.

study in a cohort of paediatric patients with congenital Chagas' disease from a non-endemic area.[17,18] This epidemiological scenario allowed us to follow the natural history of congenital infection without risks of vectorial re-infections.

Although no more than 2 mL of peripheral blood was withdrawn from each paediatric patient, the starting volume of blood was appropriate because of the high analytical sensitivity of the PCR assay. The incorporation of a hybridization step increased the sensitivity to 0.25 equivalents of a parasitic genome/mL; therefore any 2 mL of blood sample containing at least one intact parasite or 0.5 genomic equivalents should be kDNA-PCR positive, which is above the limit of detection of conventional parasitological methods. Indeed, in 17 infected infants below 6 months of age, the sensitivity of the PCR (100%) was higher than that of the MH (82.4%) (GA, Table 2). This

was corroborated by the higher concordance obtained by PCR (100%) with respect to MH (50%) when two subsequent pre-therapy blood specimens were tested in four infected GA patients (Figure 2), as well as by the finding of two pre-therapy PCR and MH positive patients presenting PCR positive but MH negative results after 30 days of treatment (Figure 4, profile E, T1). On the other hand, the latter finding indicated that a therapeutic regimen of 60 days was necessary to achieve parasite clearance in the above mentioned patients, which was accompanied by their serological conversion to negative, demonstrating cure (Figure 4).

The positivity of PCR at time of diagnosis varied between age groups. In GA, it was 100%, whereas in GB it lowered to 73.8% ($P = 0.0042$), probably because of the lower parasite burden at the undetermined phase of the infection. This was in agreement with: (i) the lower values of parasitic loads quantified by QC-PCR (Figure 3b), (ii) the finding of 16.7% discordant PCR tests between serial pre-therapy blood samples in GB infected patients (Figure 2), and (iii) the fact that whereas in GA, the PCR positivity was always detected directly in agarose gels, 13% of the PCR positive GB cases were identified only after Southern hybridization, revealing very low parasitaemias, between 0.25 and 2.5 parasitic genome equivalents/mL (example in Figure 1, lane 11).

One non-infected GA patient who was serologically and PCR positive at time of diagnosis was negative by both assays in the second specimen collected when the patient was more than 7 months old (GB). Because the gold standard to assess infection in GB is the positivity of anti-*T. cruzi* IgG antibody response,[5,10] it was assumed that the PCR positive finding in the first specimen was a false positive PCR result [see GA (NI) in Table 1]. However, PCR was negative among 100% of non-infected GB cases as well as among 100% of control paediatric individuals born to seronegative mothers (GC, Table 1).

Nifurtimox and benznidazole proved to be effective anti-parasitic drugs in previous studies in Argentina.[5,10,13] We did not observe differences in toxicity, clinical, serological and PCR outcome between both drugs in our patients (data not shown).

In our cohort, cure was achieved early after treatment in those infants who started therapy in their first months of life (GI, Figure 4), in accordance with previous studies.[6,7,10] Interestingly, in all 10 monitored GI patients, the PCR became negative earlier (profiles C, D and F, Figure 4) or at the same stage (profiles B and E, Figure 4) as serology did.

Among patients at the undetermined phase, short-term evaluation of cure presents a certain degree of difficulty since conventional serology usually remains positive for many years after treatment.[10,13] In fact, in our cohort, seronegativation occurred in 66.7% of infants aged 7–20 months of life (GII) and in only 12.5% of cases who initiated therapy at an older age (GIII) ($P = 0.023$) (Figure 4). Interestingly, out of GIII patients, the three cases that became seronegative during follow-up were PCR negative in their pre-therapy samples, persisting PCR negative up to T6 (profile N, Figure 4). This observation leads to the hypothesis that undetermined or chronic chagasic patients with undetectable bloodstream parasitic burdens might have a better treatment outcome than chronic patients with patent parasitaemias. Furthermore, two of the three cases with profile N were brother and sister, suggesting that the replicating properties and/or the drug susceptibility of a given parasite strain may also play a role in the efficacy of chemotherapy.[29]

The PCR provided a helpful clinical tool for early detection of treatment failure. In three patients who initiated therapy at the undetermined phase, the PCR was sporadically positive after treatment (profiles L, M and P in Figure 4). However, no correlation was found in the anti-*T. cruzi* IgG antibody titres between these post-treatment PCR positive cases and those PCR negative uncured patients (profiles I, K and O in Figure 4). Because these patients live in a non-endemic area, the post-treatment recurrence of *T. cruzi* DNA detection may be explained by the inefficacy of the drug to penetrate all infected tissues. The relapse of *T. cruzi* DNAaemia was also observed in 20% of adult patients with chronic Chagas' disease treated with benznidazole in an endemic area under surveillance.[28,30] Consequently, PCR appears remarkably useful for early detection of refractory cases to therapy, who should enter clinical trials evaluating different therapeutic regimens as well as novel anti-parasitic drugs with better pharmacokinetic-pharmacodynamic properties.[31] The dissociation between post-treatment persistent negative PCR results with positive conventional serology after 3 years of follow-up observed in 20 out of 30 undetermined Chagas' disease patients (GII + GIII, Figure 4) is in agreement with previous findings in treated chronic chagasic patients, presenting reactivity by conventional serology with clearance of lytic antibodies.[32] In fact, a strong correlation has been reported between the detection of lytic antibodies and positive PCR results, suggesting that PCR could be effective in evaluating parasitological cure.[33] Nevertheless, the definite clinical significance of the observed persistent PCR negativation in uncured patients will be assessed through extended follow-up, which is currently under way.

The implementation of a quantitative PCR assay to determine the bloodstream parasitic load and follow its evolution during treatment (Figure 3c) could be particularly useful as an indicator of response in prolonged therapeutic regimens,[31] as it is mandatory in the patient management of certain viral infections.[25,34] Moreover, the parasitic load might be a useful epidemiological tool to estimate patients' infectivity, regarding the risk of transmission.

The data reported here, using conventional as well as molecular biology based laboratory tools to demonstrate the efficacy of chemotherapy in congenital Chagas' disease, reinforce the need to screen all pregnant women living in or emigrating from endemic areas, in order to provide their newborns with an early accurate diagnosis for more successful treatment outcome.[5,7,10]

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