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Succinctus

Lack of evidence for integration of *Trypanosoma cruzi* minicircle DNA in South American human genomes

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ABSTRACT

Horizontal gene transfer involving kinetoplast DNA minicircles between *Trypanosoma cruzi* and its mammalian hosts has recently been proposed as a usual consequence of infection (Hecht et al., 2010). However, we have found no sequences longer than 29 bp perfectly matching minicircles of *T. cruzi* in the unassembled reads from Colombian and Peruvian human populations provided by the 1,000 Genome project (129 individuals in total, coverage from 1.4× to 36.3×, read length from 42 to 101 bp). The weak sequence matches that were identified are shared with a Finnish population used as a control from a non-endemic area.

Chagas disease is caused by infection with *Trypanosoma cruzi*, a kinetoplastid protozoan transmitted by reduviid bugs. The disease is widely distributed from Mexico to Argentina, being one of the three most frequent parasite-caused maladies together with malaria and schistosomiasis. The acute phase of the disease is often followed by a chronic stage, which is characterised by severe pathological alterations such as chagasic cardiomegaly, mega-colon or mega-oesophagus. There is currently no cure for the chronic stage and the pathogenesis of this stage has long been a matter of debate. Two alternative models, (i) a purely autoimmune mechanism and (ii) damage caused by parasite persistence, have been proposed (Bonney and Engman, 2008; Teixeira et al., 2011). The latter model, which has been recently gaining attention, involves an imbalanced immune response to low level parasitemia but does not rule out participation of an autoimmune component. An interesting but controversial theory, called lateral transfer of kinetoplast (k) DNA minicircles, has been put forward to explain the origin of the autoimmunity by proposing integration of the parasite's DNA into the host genome with subsequent expression of immunogenic aberrant gene products (reviewed in Teixeira et al., 2011). Elucidation of the pathogenesis of Chagas disease would have an impact on the choice of a proper strategy for treatment.

At the core of the issue is the presence or absence of live parasites (no matter how small their number) or integrated parasite DNA in patients with the chronic disease. The autoimmune model postulated that Chagas-specific mega-syndromes can develop regardless of the presence of parasites. That view was consistent with frequent failures to detect trypanosome infections in such patients using microscopy, xenodiagnosis or hemoculture (Ramírez et al., 2009). However, with the development of PCR-based assays as diagnostic and epidemiological tools for Chagas disease, it was demonstrated that the chronic phase is invariably characterised by very low parasitemia (Schijman et al., 2004). Early studies using PCR assays targeting multicopy kDNA minicircles reported 100% sensitivity in chronic Chagas disease patients (Avila et al., 1993), yet further results revealed some problematic issues with sensitivity and specificity. Recent large-scale studies indicate that at the chronic stage, sensitivity of serological methods is generally higher than that for PCR methods, namely almost 100% and 50–90%, respectively (Ramírez et al., 2009; Brasil et al., 2010; Schijman et al., 2011). In some studies using primers S35 and S36 (Sturm et al., 1989) or their modified versions (Castro et al., 2002; Hecht et al., 2010) for amplification of the conserved region (CR) of the kDNA minicircles (Fig. 1), a substantial fraction of controls, i.e. asymptomatic and seronegative individuals, originating mostly from central and South America, was PCR positive (Avila et al., 1993; Gomes et al., 1999; Castro et al., 2002; Hecht et al., 2010; Schijman et al., 2011).

The explanations used to address these supposedly false positives included: (i) high frequency of asymptomatic seronegative *T. cruzi* infections (Gomes et al., 1999; Castro et al., 2002), which are, however, currently considered to be rare (Ramírez et al., 2009; Brasil et al., 2010; Schijman et al., 2011); (ii) PCR contamination (Schijman et al., 2011); (iii) asymptomatic infection with *Trypanosoma rangeli* (Vallejo et al., 1999), a closely related species

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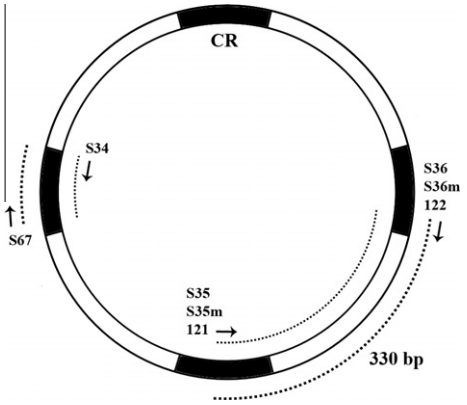

Fig. 1. Arrangement of four conserved regions (black segments) in a *Trypanosoma cruzi* minicircle, primer annealing sites (arrows) and amplification products (dashed lines). PCR assays for kinetoplast DNA minicircles usually use primer pairs S35–S36, 121–122, S35m–S36m (used in Hecht et al., 2010), amplifying across one or more variable regions and producing the main product of 330 bp. The S34–S67 product spanning only the CR is also shown.

with similar geographic distribution and hosts and, finally, (iv) a widespread integration of minicircles into the host germline genome, mostly into LINE-1 (long interspersed elements) repeats, in accordance with the lateral kDNA transfer hypothesis (Hecht et al., 2010), and their inheritance by uninfected progeny. The latter alternative invokes an unusually high incidence of minicircle integration events, as well as vertical transmission. Hecht et al. (2010) investigated five families, each sired by a Chagas disease patient: among 28 descendants in the first generation, five individuals were infected with *T. cruzi* and 11, or approximately 40%, were supposed to have minicircle integration without infection. These data could not be confounded by congenital disease transmission which is estimated to be of much lower incidence (2–10%) (Sánchez Negrete et al., 2005).

It is reasonable to anticipate that the question of host genome modification by the parasite DNA in chronic patients will ultimately be resolved by the analysis of high-coverage genome sequences of Chagas disease patients and their offspring, compared with non-travelling persons from non-endemic regions. Although this is yet to be achieved, analysis of available human genomes may be illuminating. Taking into account the very high infection rates in human populations affected with *T. cruzi*, which are currently at the level of several percent and were up to 40–80% before the introduction of modern insecticides and housing improvements (Aufderheide et al., 2004), as well as ~40% kDNA integration and inheritance rate reported by Hecht et al. (2010), the influx of integrated *T. cruzi* minicircles into the human population is expected to affect several percent of individuals per generation. This level would make the events of minicircle integration, in principle, easily detectable in population genomic surveys. To investigate this issue, we took advantage of the 1000 Genomes Project which provides low-coverage genomic reads for 27 human populations. Results of this analysis are reported herein (1000 Genomes Project Consortium, 2010).

Sequence similarity searches were done with the NCBI BLAST 2.2.25+ package (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/2.2.25/ BLASTN algorithm, option 'task' set to 'blastn'). Alignment of SOLiD colorspace reads to a colorspace reference sequence was done with SHRiMP 2.2.2 (http://compbio.cs.toronto.edu/shrimp/). Matches to human genomic repeats in reads longer than 50 bp were found with RepeatMasker (Smit, A.F.A., Hubley, R., Green, P. RepeatMasker Open-3.0. 1996–2010 http://www.repeatmasker.org). K-mer counts in read datasets were obtained with Jellyfish 1.1.2 (http://www.cbcb.umd.edu/software/jellyfish/).

Partial and complete *T. cruzi* kDNA minicircle sequences (381 entries) were extracted from GenBank™ (30 April, 2011 release). Two human populations analyzed in the 1000 Genomes Project were regarded as likely to include Chagas disease patients and/or their descendants: Colombians from Bogota and Peruvians from Lima, 129 individuals in total (Table 1). Finns, 77 individuals, were used as a European control group likely lacking any contact with *T. cruzi*. The reference human genome build 37.2 and alternative assemblies were also analyzed.

Raw sequence reads supplied by the 1000 Genomes Project underwent non-stringent sequence quality checks (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/README.sequence_data). Most individuals have been sequenced with Illumina GAII or HiSeq2000. Twenty-five individuals in Colombian and Peruvian populations have been sequenced with ABI SOLiD (Table 1). For the purpose of maximum sensitivity, individual minicircle sequences were used as queries against unassembled human sequence reads at a high *E*-value cut-off = 0.01. The cut-off value used corresponded to a minimum hit length of 25–26 bp with 100% identity or that of 29 bp with >90% identity. At this sensitivity level, even reads including short minicircle fragments, e.g. reads that overlap boundaries of integrated minicircle sequences, can be detected, and regions of accidental similarity to amplification primers can be found.

An *E*-value produced by the BLAST program (a number of higher- or similar-scoring hits expected by chance in a database) takes into account database size, but not database complexity (http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html#ref6). The actual probability of finding a hit by chance in a database, *P*-value, was roughly estimated by dividing a number of distinct 25-mers in a readpool of an individual by the maximum possible number of DNA 25-mers, 4^{25}. All hits were approximated with 25-mers, as shortest hits selected for analysis were 25-mers with 100% identity. *P*-values, which are almost equal to *E*-values when approaching zero ($P\text{-value} = 1 - e^{-E\text{-value}}$), were $\sim 10^{-6}$ for low- and moderate-coverage samples of Colombian and Finnish populations (Supplementary Table S1).

BLAST searches with complete minicircle sequences produced no long identical or nearly identical matches in sequencing reads of the three human populations: the longest match with 100% identity was 29 bp (data not shown), but read lengths were generally 50–100 bp (Table 1). As minicircle variable regions comprise a pool of almost random sequences, a closer analysis was focused on the minicircle CR, defined according to Sturm et al. (1989). The reference human genome has a 46 bp match with 87% identity to the 5′-part of the CR of a single minicircle variant (Accession No. AB434518), located within THE1B-int retrotransposon of the ERVL-MaLR family, LTR (long terminal repeats) class. In the three populations analyzed, 172/206 individuals contained up to 13 reads matching this region (alignment length 42–51 bp, average identity 87%). These reads were assembled into a contig of 163 bp in length with a perfect match in the reference human genome, referred to as "frequent hit" in Supplementary Fig. S1 and Table S2. Thus, searching for possible population-specific matches not occurring in the reference genome required setting the identity threshold to 88%. At this threshold, hits to the minicircle CR were found in reads of all three populations analyzed (see hit statistics for populations in Table 1, individual hits in Supplementary Table S1, and alignment of individual hits in Supplementary Fig. S1). Reads bearing hits to the CR were aligned with BLASTN to the reference human genome at *E*-value cut-off = 0.001 (Supplementary Table S1): alignment length was 26–137 bp (average 68 bp); identity 75–100% (average 92%). Sixteen Illumina reads out of 89 tested had no matches in any reference assembly, suggesting sequencing errors.

Although most individuals sequenced had an average sequencing coverage 5× to 7×, which is far from the standard 30×

Table 1
Sequencing statistics for populations studied and minicircle conserved region hit statistics for an identity threshold of 88%.

Population	Colombian	Colombian	Peruvian	Peruvian	Finnish
Sequencing technology	Illumina HiSeq, GAII	ABI SOLiD 3	Illumina (mostly HiSeq)	ABI SOLiD 4	Illumina (mostly GAII)
Error rate for sequencing technology in unfiltered reads,%		2–4 in colorspace (Magi et al., 2010)	0.7–0.9 (Minoche et al., 2011) 6.5–11.8 within regions with Q2 (Minoche et al., 2011)	2–4 in colorspace (Magi et al., 2010)	1.6 (Minoche et al., 2011) 14.2 within regions with Q2 (Minoche et al., 2011)
Individuals	54	12	50	13	77
Total bases, Gbp	1155	581	1027	591	1172
Genome coverage min	4.4	9.1	1.4	5.8	2.7
Genome coverage max	21.5	19.6	14.8	36.3	19
Average genome coverage	7.1	16.1	6.8	15.2	5.1
Read length min, bp	91	49	90	42	37
Read length max, bp	101	50	101	50	160
Average read length, bp	99	50	98	47	89
Individuals with hits	25	7	15	10	29
Number of hits	32	16 [a]	19	18 [a]	38
Hits per individual min	1	1	1	1	1
Hits per individual max	3	5	2	4	4
Hits per individual, average	1.3	2.3	1.3	1.8	1.3
Average coverage in individuals with hits	6.2	15	5.9	18.7	5.0
Alignment length min, bp	26	25	27	27	25
Alignment length max, bp	43	38	44	40	42
Average alignment length, bp	35	34	35	34	34

[a] Only SOLiD reads aligning to the reference human genome in basespace (Supplementary Table S1) were counted (for details on the treatment of SOLiD reads see Supplementary Data S1).

coverage, the observed low number of hits per individual, ranging from 1.3 to 2.3 (Table 1), lack of sequence conservation among minicircle-matching reads of a single individual (Supplementary Fig. S1), and very low base quality values encountered in most of those reads (Supplementary Table S1) suggest again that most minicircle hits passing the 88% identity threshold result from sequencing errors, which is not unusual for second generation sequencing data (see error rates in Table 1). The average hit count per individual in Illumina datasets is 1.3, whereas theoretical probability of a genomic 25-mer with coverage 1 according to Poisson distribution is <0.1 for individuals with coverage >4.7× and read length 100 bp (Supplementary Table S1). In contrast, for the 'frequent hit' having a single 100% match in the reference human genome (Supplementary Table S1), and thus representing an example of a genuine genomic sequence, the average hit count per individual sequenced with Illumina is 3.3. Moreover, quality scores in minicircle-matching reads were generally very low: 69 of 89 Illumina reads had at least one base in the alignment range with Phred quality 2, a value marking regions of especially low quality (corresponding to an arbitrarily assigned theoretical error rate of 63%). The actual error rate in regions with Phred quality 2 is 6.5–14.2%, depending on the sequencing run conditions and instrument model (Minoche et al., 2011). SOLiD reads demonstrated the same quality pattern: 173 of 186 minicircle-matching reads had at least one base in the alignment range with the lowest possible quality value (Supplementary Table S1).

The majority of minicircle-matching reads aligned to the 5′-part of the CR (Supplementary Fig. S1), while the most invariable part of the CR, with annealing sites for S35 and S36 primers, had a few matches. Primer S35, 25 bp in length (Sturm et al., 1989; Avila et al., 1993; Schijman et al., 2011), also labelled as primer 121 elsewhere (Castro et al., 2002; Schijman et al., 2011), had only eight hits with 10–13 identical bp in the whole dataset analyzed; modified primer S35 (named S35m herein), 20 bp in length (Hecht et al., 2010), had three hits with 15–17 bp identical at the 3′ end; versions of primer S36 (original version of 21 bp (Sturm et al., 1989; Schijman et al., 2011); primer 122 of 26 bp (Castro et al., 2002; Schijman et al., 2011); primer S36m of 19 bp (Hecht et al., 2010)) had three hits with 17–18 bp identical at the 3′ end (Supplementary Table S1, Fig. S1). Extremely rare frequency and low quality values (Supplementary Table S1) of these reads suggest that human genomes lack any matches to minicircle-specific primers, and therefore low specificity of PCR protocols observed by Schijman et al. (2011), as well as by other authors, cannot be explained by the genomic data.

The lengths and identities of BLASTN hits observed in the Colombian and Peruvian populations, although acceptable from the statistical point of view (*P*-values of $\sim 10^{-6}$), were too small (no more than 29 bp of perfectly matching sequence) to be used as evidence of recent minicircle integration events of the kind suggested by Hecht et al. (2010). In addition, the hits cannot be attributed to remnants of more ancestral integration events, since virtually identical hits were found in the European control population (Table 1, Supplementary Table S1). Therefore, the origin of the sequence similarity regions is most likely accidental. Our study indicates that high length and similarity thresholds should be used when looking for integrated *T. cruzi* minicircle sequences in genomic read datasets, and strict filtering of low-quality reads is mandatory.

Prevalence of Chagas disease in South America differs greatly among countries, as well as between rural and urban areas. Prevalence remained fairly constant around 40% in archaeological samples from northern Chile and southern Peru dating from ~9,000 B.P. (years before present time) to ~450 B.P. (Aufderheide et al., 2004), never dropped below 3% country-wide in studies conducted in the 1970s and 1980s, and often exceeded 20% in rural areas (Moncayo, 2003; Aufderheide et al., 2004). However, more recently the prevalence of Chagas disease decreased significantly due to vigorous control measures (Moncayo, 2003). Recent estimates of country-wide prevalence in Colombia were 3.1% in 1999 (Guhl and Vallejo, 1999), and 1.5% in children up to 14 years old in 2000–2002 (Guhl, 2007). In Peru prevalence was 2.4% in Lima blood donors in 1993 (Guhl, 2007), 2–3% in an endemic urban location in 2011 (Hunter et al., 2012) and 7.6% in an endemic rural location in 2010 (Delgado et al., 2011).

In summary, it is safe to admit that before disease control measures implemented in the last two to three decades, and before urbanisation in the second half of the 20th century that started

to change housing conditions and resulted in significantly lower contacts with the vectors, the historic prevalence of *T. cruzi* infection in South America was more than 10%. Data of Hecht et al. (2010) on heritable minicircle integration are difficult to interpret quantitatively as only five families were studied, but according to this study, as much as 40% of children of the *T. cruzi*-infected individuals may have heritable minicircle integration in at least one chromosome. Therefore, the horizontal gene transfer (HGT) rate resulting from *T. cruzi* infection in South America can be inferred at 5% per generation (~10% prevalence and ~50% inheritance rate). Integrations into LINE-1 repeats reported by Hecht et al. (2010) can be considered selectively neutral, and the elimination rate for integrated sequences is not known, but is probably lower than the supposed HGT rate. Under the simplest model of forward and reverse mutations in a single locus without selection and other factors, equilibrium allele frequencies are defined as: $p_t + 1 = p_t(1 - u) + (1 - p)t(v) = p_t$, where p_t and $(1 - p)_t$ are wild-type and mutant allele frequencies in generation t; u and v stand for forward and reverse mutation rates, respectively. If $u = 5v$, then equilibrium frequency of a mutant allele $(1 - p)_{eq} = u/(u + v) = 0.8333$, and if $u = 0.05$, then $1 - p = 0.32$ after 10 generations ($1 - p = 0.075$ after 10 generations for $u = 0.01$). Thus, although rigorous mathematical models for the minicircle HGT cannot be constructed as most variables cannot be estimated accurately, it is apparent that very high frequencies of integrated minicircles would have been reached even in the immigrant South American population during no more than 500 years, or 25 generations, of contact with the pathogen, and indigenous populations would have been saturated with pathogen sequences after at least 9,000 years, or 450 generations, of contact (Aufderheide et al., 2004). However, we found no evidence of minicircle integration in 129 South American individuals sequenced at low or moderate coverage. Although, due to sequencing biases, no readpool can represent an individual's genome completely, and hence absence of a sequence in the readpool cannot be proven, our study suggests that the minicircle HGT may occur at a much lower rate than that inferred from the work of Hecht et al. (2010).

Nevertheless, we believe that screening the ever growing human genomic dataset for kDNA minicircle sequences of *T. cruzi* should be continued as the conditions for HGT are in place in this host–parasite system. *Trypanosoma cruzi* is one of a few pathogens that live in the cytosol of host cells, so a lysed trypanosome releases thousands of minicircles directly into the host nucleo-cytoplasmic compartment. Short sequence matches between the minicircle CR and the human genome do exist and those may drive recombination. Some integration events may be heritable, since *T. cruzi* multiplies in many tissues at the acute stage of Chagas disease, probably including germ line cells, and minicircle DNA has also been revealed in these organs in chronic patients (Olivares-Villagómez et al., 1998). HGT is facilitated by the high presence of the parasite in affected human populations, with infection rates reaching up to 80% in some rural areas before the introduction of modern insecticides and housing (Aufderheide et al., 2004). Minicircle sequences were amplified from individual heart macrophages allegedly free of the parasite, with only one minicircle variable region detected per macrophage (Elias et al., 2003). Minicircle integration was not investigated in that study as host-trypanosome DNA junctions were not analyzed. A case of HGT possibly implicating *T. cruzi* has been found recently (Gilbert et al., 2010). A triatomine bug, *Rhodnius prolixus*, and some of the animals that it bites, i.e. squirrel monkey and opossum, harbour very similar and unique DNA transposons. It was proposed that *T. cruzi* could be the transmitter of these mobile elements (Gilbert et al., 2010), although its genome retained no traces of these events.

In conclusion, the population genomic data currently available do not favour heritable integration of kDNA minicircles into the human genome as a usual consequence of Chagas disease, although this scenario cannot be fully excluded. Whole genome sequence data obtained from *T. cruzi*-infected cells, as well as from Chagas disease patients and their descendants, can ultimately resolve the exciting possibility of minicircle HGT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2012.04.001.

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