Molecular & Biochemical Parasitology 193 (2014) 71–74

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Short communication

Characterization of the small RNA content of *Trypanosoma cruzi* extracellular vesicles

Ethel Bayer-Santos[a,b], Fábio Mitsuo Lima[a], Jeronimo Conceição Ruiz[c], Igor C. Almeida[b,*], José Franco da Silveira[a,**]

[a] Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, São Paulo SP 04023-062, Brazil
[b] Border Biomedical Research Center, Department of Biological Sciences, University of Texas at El Paso, El Paso, TX 79968, USA
[c] Centro de Pesquisas Rene Rachou, Fundação Oswaldo Cruz, Minas Gerais, MG 30190-002, Brazil

ARTICLE INFO

Article history:
Received 2 December 2013
Received in revised form 11 February 2014
Accepted 15 February 2014
Available online 25 February 2014

Keywords:
Trypanosoma cruzi
Extracellular vesicles
Small noncoding RNA
High-throughput data analysis
Comparative transcriptome

ABSTRACT

A growing body of evidence in mammalian cells indicates that secreted vesicles can be used to mediate intercellular communication processes by transferring various bioactive molecules, including mRNAs and microRNAs. Based on these findings, we decided to analyze whether *Trypanosoma cruzi*-derived extracellular vesicles contain RNA molecules and performed a deep sequencing and genome-wide analysis of a size-fractioned cDNA library (16–40 nt) from extracellular vesicles secreted by noninfective epimastigote and infective metacyclic trypomastigote forms. Our data show that the small RNAs contained in these extracellular vesicles originate from multiple sources, including tRNAs. In addition, our results reveal that the variety and expression of small RNAs are different between parasite stages, suggesting diverse functions. Taken together, these observations call attention to the potential regulatory functions that these RNAs might play once transferred between parasites and/or to mammalian host cells.

Recent reports have described that the intercellular exchange of genetic material also occur in eukaryotes and multicellular organisms through several distinct pathways such as those dependent on nanotubes, extracellular vesicles (EVs), and nucleic acid-binding proteins [1]. The phenomenon of microRNA (miRNA) secretion and its role in intercellular crosstalk has recently gained increasing attention as some studies have provided evidence that membrane-bound vesicles released by several cell types can transfer miRNAs to neighboring or distant cells, playing a previously unrecognized role in modulating cell functions. Several mechanisms have been hypothesized describing the interactions of membrane-bound vesicles and recipient cells, these include receptor–ligand interactions, fusion of vesicles with target-cell membrane and vesicle endocytosis [2].

There are currently three types of EVs that are named according to the mechanisms by which they are released into the extracellular environment: (i) exosomes, which are small cup-shaped vesicles ranging around 20–100 ηm in diameter released by the exocytic fusion of multivesicular bodies (MVBs); (ii) ectosomes, which are large heterogeneous vesicles ranging around 100–1000 ηm in diameter released by the budding of the plasma membrane; and (iii) apoptotic bodies, which are larger vesicles (>1 μm in diameter) that originate from apoptotic cells [3]. In a previous report, we demonstrated that *Trypanosoma cruzi* releases at least two types of membrane vesicles (i.e., ectosomes and exosomes), generated by distinct pathways. We also showed that infective metacyclic forms release vesicles carrying virulence factors such as GP82 glycoproteins and mucins, while in contact with HeLa cells, thus suggesting that parasite-derived EVs could be used as carriers to deliver virulence and modulatory factors into host cells [4]. In the light of recent findings showing that mammalian cells are able to use membrane vesicles to transfer mRNAs and microRNAs to neighboring cells [5], together with the identification of a great number of proteins containing nucleic acid-binding domains in the proteomic analysis of *T. cruzi*-derived vesicles [4], we decided to analyze whether *T. cruzi*-secreted EVs also contain RNA molecules and perform their characterization.

To obtain the *T. cruzi* EVs, epimastigotes and metacyclic trypomastigotes (clone Dm28c) were cultivated as described previously with minor modifications [4]. Epimastigotes forms were collected at exponential growth phase (~2.5×10^7 cells/mL), while metacyclic trypomastigotes were collected from the culture supernatant of TAU3AAG medium after 72 h of differentiation (5×10^6 cells/mL). To obtain the sufficient amount of EVs-derived small RNAs for

* Corresponding author. Tel.: +1 915 747 6086.
** Corresponding author. Tel.: +55 11 5571 1095.
E-mail addresses: icalmeida@utep.edu (I.C. Almeida), jose.franco@unifesp.br, jose.franco@pq.cnpq.br (J.F. da Silveira).

http://dx.doi.org/10.1016/j.molbiopara.2014.02.004

library construction, we combined the total RNA extracted from two distinct biological preparations. Briefly, two distinct preparations of 3×10^9 epimastigotes or metacyclic trypomastigotes (1×10^8 parasites/mL) were incubated for 12 h in DMEM without fetal bovine serum (FBS) or in TAU3AAG medium, respectively. Parasite viability was assessed by propidium iodide incorporation and showed that more than 98% of cells were viable. After the incubation, cells were removed by centrifugation at $3000 \times g$ for 10 min (1st pellet), and the supernatant was filtered in 0.45-μm syringe filters and ultracentrifuged at $100{,}000 \times g$ for 2 h (2nd pellet). The pellets containing cells (1st pellet) and EVs (2nd pellet) were mixed with 1 mL of Tri Reagent (Sigma) and RNA extraction was performed as described by the manufacturer with minor modifications. To improve the recovery of small RNAs, 30 μg of glycogen (Invitrogen) was added followed by isopropanol precipitation for 16 h at −20 °C.

Total RNAs extracted from two distinct biological replicates were mixed 1:1 (1 μg each) before small RNA isolation and library generation. Such procedure was performed for all samples (eVes, mVes and mCell) so each one of them was composed of two distinct biological replicates, thus results presented in the manuscript are an average of independent biological replicates. Small RNAs were sequenced by LC Sciences (Houston, TX). Briefly, the small RNA fraction of 16–40 nt was isolated from total RNA of epimastigote- and metacyclic-derived vesicles (eVes and mVes, respectively), and metacyclic trypomastigote parental cells (mCell) in a 15% Tris–borate–EDTA–urea polyacrylamide gel. Fig. 1A shows a denaturing formaldehyde agarose gel displaying the differences observed between total RNA of eVes and total RNA of parental eCell from which vesicles were isolated. It is interesting to note that eVes seem to be composed of a wide variety of RNA molecules, including rRNA, mRNAs, and small RNAs. The profile of mVes is quite similar to the eVes in its RNA composition (data not shown). However, as we were unable to perform the characterization of all types of RNA molecules contained in these *T. cruzi*-derived EVs, we decided to focus on the small noncoding RNAs, which have the potential to be regulatory molecules.

A small RNA library was generated using the Illumina Truseq™ Small RNA Preparation kit, which is specifically designed to isolate small RNAs having 5′ phosphate and 3′ hydroxyl ends. The purified cDNA library was used for cluster generation on Illumina's Cluster Station and sequenced on Illumina GAIIx. Raw sequencing reads were obtained using Illumina's Pipeline v1.5 software, following sequencing image analysis by pipeline Firecrest module and base-calling by pipeline Bustard module. Sequencing data analysis was performed by a proprietary pipeline script (LC Sciences). After the raw sequence reads were extracted from image data, a series of digital filters was employed to remove unmappable/low quality reads and adaptors. Those remaining filtered reads were grouped and used to map with the reference database that was composed of *T. cruzi* transcripts from TriTrypDB 4.0 (http://tritrypdb.org/tritrypdb/). Filtered unique reads were aligned against the reference database using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). One mismatch was allowed in a seed alignment, the seed length was set to 20 and the strategy of local alignment was used for mapping. The unique reads were clustered into seven classes (rRNA, tRNA, snRNA, snoRNA, coding-sequences (CDS), pseudogene, and unspecified) based on their products annotated in the NCBI Gene database. Normalization of sequence counts in each sample was performed by the DESeq method.

All unique reads were distributed into seven categories using genome annotations (Table 1) and results revealed some differences between EVs derived from both developmental forms (eVes vs. mVes) as well as between EVs and cells (mVes vs. mCell). The length distribution of all unique reads separated according to their

Table 1
Classes of small RNAs.

RNA	eVes		mVes		mCell	
	# of reads	%	# of reads	%	# of reads	%
rRNA	2,373,222	55.80	2,717,445	76.46	984,404	57.95
tRNA	1,496,361	35.18	234,005	6.58	337,837	19.89
CDS	56,245	1.32	187,876	5.29	80,085	4.71
snoRNA	3068	0.07	18,129	0.51	67,930	4.00
Pseudogene	14,125	0.33	31,433	0.88	13,970	0.82
snRNA	413	0.01	2954	0.08	972	0.06
Unspecified	48	0.00	419	0.01	77	0.00
Nohit	466,011	10.96	473,791	13.33	255,694	15.05
Total reads	4,252,955	100.00	3,554,196	100.00	1,698,747	100.00

categories and a summary of all data obtained and analyzed in the present work can be found in Tables S1A-I and S2A-G, respectively. rRNA-derived small RNAs were the most abundant class in the three samples (56–76%), followed by tRNA-derived small RNAs that were the second most abundant class in eVes (35%) and mCell (20%), and present in lower proportion in mVes fraction (6%). Reads that could not be aligned to the genome (no hit) were the second most abundant class in mVes (13%) and the third in eVes (11%) and mCell (15%). Small RNAs derived from coding-sequences were slightly more abundant in metacyclic-derived samples (5%) as compared to epimastigotes (1%), and snoRNA-derived small RNAs were mainly found in the mCell sample (4%). Several studies have shown that RNAs are selectively packed into membrane vesicles as the RNA profiles in these vesicles do not fully reflect the RNA profiles observed in the parental cells [5]. A list of small RNAs found in the EVs of epimastigotes and metacyclic trypomastigotes containing their relative differential expression is shown in Table S3A-G. Thus, in accordance with these results we found several sequences that are differentially expressed in EVs derived from both parasite forms (eVes vs. mVes) as well as differentially expressed in EVs and parental cells (mVes vs. mCell) (Table S3), which supports the differential packaging of these small RNAs into parasite-derived EVs. It is worth mentioning that since there are no replicates, significance could not be assessed (Table S3).

In accordance with previous results showing that rRNA-derived small RNA fragments are present in almost all small RNA sequence libraries, these molecules constituted the most abundant class in the three libraries analyzed in our work (eVes, mVes, and mCell). Although these results might suggest that these small RNAs are randomly degraded products, more recent studies in mammals and yeast have revealed that rRNA-derived small RNAs are functional molecules processed by special enzymes [6] and they might play important roles in heterochromatin assembly and transcription regulation [7]. Moreover, in a previous work we have shown the presence of a relatively high percentage (6-11%) of ribosomal proteins and nucleic acid-binding proteins in the *T. cruzi* secretome [4]. Thus, the rRNA-derived small RNAs could originate from the turnover of ribosomes and/or they could be also associated with polysomes carrying mRNAs which may be mobilized into secreted vesicles [5].

Similarly to what was obtained in a different study analyzing *T. cruzi* small RNAs [8], in our work around 11–15% of the sequenced reads could not be aligned to the genome (no hit) using the default alignment procedures. One possible reason is due to genomic differences between the reference clone CL Brener used as database and the clone Dm28c used to construct the cDNA library. tRNA-derived small RNA fragments (tsRNA) have been identified in other *Trypanosoma* spp. and experimental evidence revealed that these fragments were usually synthesized when cells or organisms were under stress conditions [8–11]. In our study, the median length of tsRNA was of 32 nt (Fig. 1B), which is consistent with the current

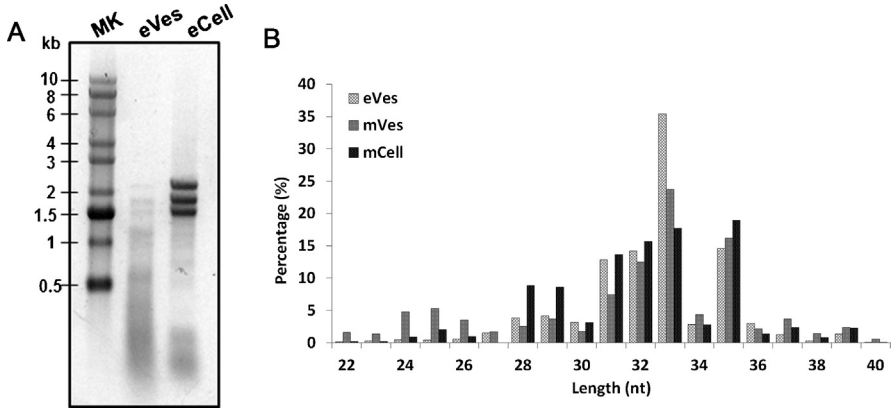

Fig. 1. Profile of the small RNAs of *T. cruzi* extracellular vesicles. (A) Denaturing formaldehyde agarose gel (1%) showing the distinct RNA band pattern between epimastigote-derived vesicles (eVes) and parental epimastigote cells (eCell). (B) Length distribution of tRNA-derived small RNAs.

view of bisectional cleavage of mature tRNA (~70–80 nt) near the anticodon. Most tRNA isoacceptors were found to be precursors of tsRNA identified in parasite-derived EVs, but specific isoacceptors were detected in higher levels as compared to others, suggesting that tsRNA are differentially packed into membrane vesicles (Table 2 and Table S3). Consistent with results published by Reifur et al. [10], we found that the most abundant tsRNAs in metacyclic forms (mCell) were $tRNA^{Glu}$ (42%), $tRNA^{Thr}$ (21%), and $tRNA^{Val}$ (15%). Moreover, contrasting with tsRNA identified in mCell, the most abundant tsRNAs found in mVes were quite diverse and were composed of $tRNA^{Glu}$ (33%), $tRNA^{Thr}$ (17%), $tRNA^{Gly}$ (10%), $tRNA^{Val}$ (9%), and $tRNA^{Phe}$ (9%). In addition, Garcia et al. [9] showed that $tRNA^{Glu}$ (60%) and $tRNA^{Asp}$ (35%) were the most abundantly expressed tsRNAs in epimastigote cells (eCell), while the most abundant tsRNAs identified in eVes by the present study comprised $tRNA^{Glu}$ (49%), $tRNA^{Val}$ (31%), $tRNA^{Thr}$ (12%), and $tRNA^{Asp}$ (3%). Taken together, these data reinforce the differential packaging of small RNAs into parasite-derived EVs and show the difference in vesicle composition between the two parasite developmental forms.

In eukaryotes, tRNA cleavage has been proposed as a mechanism to down-regulate or inhibit protein synthesis during differentiation and under oxidative stress, as well as under nutrient starvation. In *T. cruzi*, the machinery required for RNA interference (RNAi) seems to be either entirely lost or extensively simplified as the parasite is unable to use dsRNA to trigger degradation of target mRNAs [12]. In this context, tsRNAs have gained special attention as it was shown that these molecules can associate with Argonaute/Piwi protein family members, leading to the assembly of specific ribonucleoprotein complexes involved in silencing gene expression [6,13,14]. It is interesting to note that RNA-binding proteins constitute an important fraction of *T. cruzi* secretome [4] and these proteins are known to play an essential role in the control of gene expression in trypanosomes, modulating RNA processing, stability, turnover, and translation [15]. Moreover, additional experimental evidence demonstrated that the tRNA-maturing enzyme tRNase Z was able to down-regulate expression of human genes by degrading mRNAs under the direction of small-guide tsRNA [16,17]. The tRNase Z belongs to the metallo-lactamase family and it was reported that trypanosomatid genomes encode for at least 4 metallo-lactamases [9]. Furthermore, tsRNAs were reported to be recruited to cytoplasmic granules that partially colocalized with reservosomes [9], which suggests that they could be packed into vesicles for secretion.

Table 2
Small RNAs mapped on tRNA isoacceptors.

tRNA type	eVes		mVes		mCell	
	# of reads[a]	%	# of reads[a]	%	# of reads[a]	%
Ala	4997	0.0	2520	2.0	2190	1.0
Arg	8009	0.0	2686	2.0	5405	1.0
Asn	14	0.0	26	0.0	17	0.0
Asp	67,610	3.0	9113	7.0	20,542	5.0
Cys	274	0.0	339	0.0	351	0.0
Glu	1,028,208	49.0	43,706	33.0	182,381	42.0
Gln	2954	0.0	1988	2.0	2515	1.0
Gly	39,870	2.0	12,713	10.0	38,617	9.0
His	11,475	1.0	3287	2.0	11,926	3.0
Ile	80	0.0	107	0.0	62	0.0
Leu	2490	0.0	4180	3.0	2302	1.0
Lys	145	0.0	303	0.0	329	0.0
Met	1270	0.0	785	1.0	626	0.0
Phe	15,584	1.0	12,129	9.0	3354	1.0
Pro	9972	0.0	1490	1.0	2656	1.0
Ser	96	0.0	259	0.0	171	0.0
Thr	255,589	12.0	22,855	17.0	93,032	21.0
Trp	1448	0.0	331	0.0	4760	1.0
Tyr	257	0.0	320	0.0	314	0.0
Val	650,606	31.0	12,364	9.0	65,375	15.0
	2,100,948	100.0	131,501	100.0	436,925	100.0

[a] Normalized values derived from Table S3 were used.

Small RNAs derived from CDS (1–5%) and pseudogenes (1%) comprised a minor portion of the libraries and could simply represent degradation products derived from mRNAs due to the constitutive polycistronic transcription and post-transcription gene expression regulation of these organisms. However, these small RNAs might also represent functional molecules that form sense-antisense dsRNAs by complementary bases thus generating natural antisense siRNAs (NAT-siRNAs), which can display regulatory functions as already shown in *Trypanosoma brucei* [18]. NATs can be divided into two types: *cis*-NATs in which the two transcripts derive from the same genomic locus but opposite strands, and *trans*-NATs in which the transcripts derive from different genomic loci. Small RNAs derived from pseudogenes were assigned to multigene families that are enriched in subtelomeric regions such as the surface glycoprotein GP63 (50% of eVes, 23% of mVes and 17% of mCell), *trans*-sialidase (18% of eVes, 23% of mVes and 34% of mCell), and retrotransposons hot spot (RHS) (4% of eVes, 10% of mVes and 7% of mCell), suggesting that these subtelomeric pseudogenes might also display additional roles apart from increasing the chance of homologous recombination and antigenic variability.

Although the functional meaning of the presence of these small RNA molecules in *T. cruzi*-secreted EVs still need to be elucidated, results from mammalian cells [5,19] and two recent reports from *Plasmodium falciparum* [20] and *T. cruzi* [21] showing that these parasites are able to use membrane vesicles to transfer genetic information between different cells, make us intrigued

about the possible functions that these small RNAs might play once transferred to mammalian host cells and/or to other parasites. Garcia-Silva et al. [21] showed that epimastigotes submitted to nutrient starvation secrete extracellular vesicles carrying small tRNAs and TcPIWI-tryp proteins as cargo, which could be transferred to other parasites and to mammalian cells, thus increasing metacyclogenesis and susceptibility of mammalian cells to infection [21]. These findings make us speculate whether these vesicles could be used as vehicles to exchange genetic material between parasites, and between parasites and host cells as suggested by Hecht et al. [22]. Further work will be needed to clarify the molecular mechanisms that underlay these vesicles-mediated exchange events and their further implications in host–pathogen interactions and quorum sensing.

Acknowledgments

This work was supported by the National Institutes of Health (NIH) grants R01AI070655-A5 and R01AI070655-A5S1 (to I.C.A.), and 5G12MD007592 (to BBRC/UTEP), and by grants from FAPESP (2011/51475-3) and CNPq (Brazil) to J.F.S. and E.B.S. We would like to thank Dr. Marjorie Mendes Marini e Souza for her help with data analysis. We are thankful to the Biomolecule Analysis and Genomic Analysis Core Facilities at BBRC/UTEP (NIH/NIMHD grant 5G12MD007592).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2014.02.004.

References

[1] Belting M, Wittrup A. Nanotubes, exosomes, and nucleic acid-binding peptides provide novel mechanisms of intercellular communication in eukaryotic cells: implications in health and disease. J Cell Biol 2008;183(7):1187–91.
[2] Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. Trends Cell Biol 2009;19(2):43–51.
[3] Gyorgy B, Szabo TG, Pasztoi M, Pal Z, Misjak P, Aradi B, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci 2011;68(16):2667–88.
[4] Bayer-Santos E, Aguilar-Bonavides C, Rodrigues SP, Cordero EM, Marques AF, Varela-Ramirez A, et al. Proteomic analysis of *Trypanosoma cruzi* secretome: characterization of two populations of extracellular vesicles and soluble proteins. J Proteome Res 2013;12(2):883–97.
[5] Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9(6):654–9.
[6] Li Z, Ender C, Meister G, Moore PS, Chang Y, John B. Extensive terminal and asymmetric processing of small RNAs from rRNAs, snoRNAs, snRNAs, and tRNAs. Nucleic Acids Res 2012;40(14):6787–99.
[7] Cam HP, Sugiyama T, Chen ES, Chen X, FitzGerald PC, Grewal SI. Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. Nat Genet 2005;37(8):809–19.
[8] Franzen O, Arner E, Ferella M, Nilsson D, Respuela P, Carninci P, et al. The short non-coding transcriptome of the protozoan parasite *Trypanosoma cruzi*. PLoS Negl Trop Dis 2011;5(8):e1283.
[9] Garcia-Silva MR, Frugier M, Tosar JP, Correa-Dominguez A, Ronalte-Alves L, Parodi-Talice A, et al. A population of tRNA-derived small RNAs is actively produced in *Trypanosoma cruzi* and recruited to specific cytoplasmic granules. Mol Biochem Parasitol 2010;171(2):64–73.
[10] Reifur L, Garcia-Silva MR, Poubel SB, Alves LR, Arauco P, Buiar DK, et al. Distinct subcellular localization of tRNA-derived fragments in the infective metacyclic forms of *Trypanosoma cruzi*. Mem Inst Oswaldo Cruz 2012;107(6):816–9.
[11] Zheng LL, Wen YZ, Yang JH, Liao JY, Shao P, Xu H, et al. Comparative transcriptome analysis of small noncoding RNAs in different stages of *Trypanosoma brucei*. RNA 2013;19(7):863–75.
[12] Ullu E, Tschudi C, Chakraborty T. RNA interference in protozoan parasites. Cell Microbiol 2004;6(6):509–19.
[13] Kawamura Y, Saito K, Kin T, Ono Y, Asai K, Sunohara T, et al. *Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature 2008;453(7196):793–7.
[14] Haussecker D, Huang Y, Lau A, Parameswaran P, Fire AZ, Kay MA. Human tRNA-derived small RNAs in the global regulation of RNA silencing. RNA 2010;16(4):673–95.
[15] De Gaudenzi JG, Noe G, Campo VA, Frasch AC, Cassola A. Gene expression regulation in trypanosomatids. Essays Biochem 2011;51:31–46.
[16] Elbarbary RA, Takaku H, Uchiumi N, Tamiya H, Abe M, Takahashi M, et al. Modulation of gene expression by human cytosolic tRNase Z(L) through 5′-half-tRNA. PLoS One 2009;4(6):e5908.
[17] Nakashima A, Takaku H, Shibata HS, Negishi Y, Takagi M, Tamura M, et al. Gene silencing by the tRNA maturase tRNase ZL under the direction of small-guide RNA. Gene Ther 2007;14(1):78–85.
[18] Wen YZ, Zheng LL, Liao JY, Wang MH, Wei Y, Guo XM, et al. Pseudogene-derived small interference RNAs regulate gene expression in African *Trypanosoma brucei*. Proc Natl Acad Sci USA 2011;108(20):8345–50.
[19] Chen X, Liang H, Zhang J, Zen K, Zhang CY. Horizontal transfer of microRNAs: molecular mechanisms and clinical applications. Protein Cell 2012;3(1):28–37.
[20] Regev-Rudzki N, Wilson DW, Carvalho TG, Sisquella X, Coleman BM, Rug M, et al. Cell–cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell 2013;153(5):1120–33.
[21] Garcia-Silva MR, Cura das Neves RF, Cabrera-Cabrera F, Sanguinetti J, Medeiros LC, Robello C, et al. Extracellular vesicles shed by *Trypanosoma cruzi* are linked to small RNA pathways, life cycle regulation, and susceptibility to infection of mammalian cells. Parasitol Res **113** (1), 2013, 285-304.
[22] Hecht MM, Nitz N, Araujo PF, Sousa AO, Rosa Ade C, Gomes DA, et al. Inheritance of DNA transferred from American trypanosomes to human hosts. PLoS One 2010;5(2):e9181.