Artículo de Investigación Original

KINETOPLAST ULTRASTRUCTURE OF FIVE *Trypanosoma evansi* AND *Trypanosoma equiperdum* VENEZUELAN ISOLATES

E. Sánchez[a], T. M. Perrone[a†], F. Sánchez[b], A. Mijares[a]*

[a] Laboratorio de Fisiología de Parásitos. Centro de Biofísica y Bioquímica. Instituto Venezolano de Investigaciones Científicas. Venezuela.

[b] Unidad de Microscopía Electrónica. Instituto Venezolano de Investigaciones Científicas. Venezuela.

*Corresponding autor. E-mail: amijares@ivic.gob.ve, Phone: +582125041853, Fax: +582125041093

Recibido: Abril 2016. Aprobado: Julio 2016
Publicado: Noviembre 2016.

ABSTRACT

The kinetoplast is a concatenated network of complex mitochondrial DNA comprising of two different structures, known as minicircles and maxicircles. *T. evansi* molecular studies have revealed the complete loss of the maxicircles, while *T. equiperdum* has retained maxicircle fragments. The presence or absence of kinetoplast has been used for comparative studies of trypanosomes from different geographical areas. In this study was analyzed this ultrastructure by transmission electron microscopy of five trypanosoma isolates from two states of Venezuelan plains. *T. evansi* and *T. equiperdum* isolates blood samples previously cryopreserved from natural hosts of three horses, one donkey and one capybara (asymptomatic), were employed to induce experimental infections and expand in rats, then parasites were purified by ion exchange chromatography. These parasite sediments were chemically fixed in suspension. Samples were washed and included in agar, then were exposed to an increasing series of ethanol dehydration and included in Polybed epoxy resin. Sections of 60 nm were placed on copper grids with a collodion-carbon film for positive contrasting. Images were obtained and analyzed by observation in a transmission electron microscope. The photomicrographs shown as expected the integrity of kinetoplast in the five trypanosoma isolates studied, revealed the mitochondrial membrane covering the kinetoplast, which is exhibited in a disk form near the flagelar pocket and adjacent to the base of the flagellum. The kinetoplast's ultrastructures of Venezuelan trypanosoma isolates studied were equivalent to Colombian/ Chinese *T. evansi* and Chinese *T. equiperdum* isolates, but different to akinetoplastic natural ultrastructures of Brazilian *T. evansi* isolates.

Keywords: Kinetoplast, *T. evansi*, *T. equiperdum*, diskinetoplast, electron microscopy.

ULTRAESTRUCTURA DEL CINETOPLASTO DE CINCO AISLADOS VENEZOLANOS DE *Trypanosoma evansi* Y *Trypanosoma equiperdum*

RESUMEN

El cinetoplasto es una red concatenada de ADN mitocondrial compleja, compuesta por dos estructuras diferentes denominadas minicírculos y maxicírculos. Estudios moleculares de *T. evansi* han revelado la completa perdida de los maxicírculos, en cambio *T. equiperdum* ha retenido fragmentos del maxicírculo. La presencia/ ausencia del cinetoplasto ha sido usada para estudios comparativos de tripanosomas provenientes de diferentes áreas geográficas. En este estudio se analizó la ultraestructura del cinetoplasto por microscopía electrónica de transmisión de los cinco aislados venezolanos de tripanosoma. Las muestras sanguíneas previamente criopreservadas de hospedadores naturales de *T. evansi* y *T. equiperdum*: tres caballos, un asno y un chigüire (asintomático), fueron experimentalmente infectadas y expandidas en ratas, luego los parásitos fueron purificados por cromatografía de intercambio iónico. Los sedimentos parasitarios fueron químicamente fijados en suspensión, seguidamente las muestras fueron lavadas, incluidas en agar, deshidratadas en una serie creciente de etanol e incorporadas en una resina epoxyca Polybed. Cortes de 60 nm fueron contrastados positivamente en rejillas con una película de colodión-carbón. Las imágenes fueron obtenidas y analizadas en un microscopio electrónico de transmisión. Las micrografías mostraron la integridad del cinetoplasto en los cinco aislados de tripanosoma estudiados, revelando la membrana mitocondrial recubriendo el cinetoplasto, el cual se observó en forma de disco cerca del bolsillo flagelar y adyacente a la base del flagelo. La ultra-estructura del cinetoplasto de los aislados venezolanos de tripanosoma estudiados fueron equivalentes a los aislados colombianos/ chinos de *T. evansi* y chinos de *T. equiperdum*, pero diferentes a los aislados akinetoplásticos de *T. evansi* brasileros.

Palabras claves: Cinetoplasto, *T. evansi*, *T. equiperdum*, diskinetoplástico, microscopía electrónica.

INTRODUCTION

Kinetoplastida order are flagellated protozoans, characterized by an extra-nuclear DNA known as kinetoplast (kDNA), with function equivalent to mitochondrial DNA as eukaryotes cell [1]. The kDNA is in a single tubular mitochondrion in trypanosomes, inside of mitochondrial matrix adjacent to the basal body of the flagellum [2]. The kDNA is a concatenated network in a disk form, which it consists of two complex circular structures different; minicircles and maxicircles that occupy 5-25 % of total genome. Minicircles encode guide RNAs for editing mitochondrial transcripts, there are among 5.000-10.000 copies of about 1000 bp [3]. Maxicircles encode the mitochondrial genes necessary for development and differentiation in the insect vector, 50-100 copies that vary in size amongst 20-40 kbp [4-6]. All species of trypanosomes contain partial or complete maxicircles sequences with the exception of *T. evansi* [7], since molecular studies revealed the complete loss of the maxicircles, while *T. equiperdum* has retained maxicircle fragments similar to those present in *T. brucei brucei* [8]. The presence of minicircle structures and fragments maxicircle was defined as diskinetoplast (Dk) strains, although the complete loss of mini and maxicircles was definite as akinetoplast strains (Ak) [9]. *T. brucei brucei* bloodstream can survive with partial or total loss of kDNA, which could occur natural or experimental induction by drugs as ethidium bromide [10], the fragment or lack of kDNA, conditions to trypanosomes at bloodstream stage, since information's kDNA is clearly essential to procyclic stage [11, 12].

Procyclic forms are completely developed and have the mitochondrion fully functional, instead bloodstream forms produce ATP by glycolysis due to that the expression of some mitochondrial functions are inhibited such as the electron transport system [13].

The independence of invertebrate host as reduction of heteroxenous to monoxenous life-cycle stage have dramatic consequences, which is believe paradoxically allowed that trypanosomes leave from Africa areas where predominated the tsetse fly, therefore trypanosomes were expanded to regions and continents adapted to mechanical transmission by new blood-feeding insects [14, 15]. The presence or absence of kDNA (diskinetoplast and akinetoplast, respectively) has been used for comparative studies of trypanosomes from different geographical areas. Fourteen Brazilian *T. evansi* isolates that infected naturally domestic and wild mammals lost the total kinetoplast, therefore were considered as akinetoplast strains as the only Brazilian natural stage or at least the most common [16], whereas Colombian *T. evansi* isolates have been observed with partial loss of kDNA as diskinetoplast strains [7, 17].

The trypanosomosis caused by *T. brucei brucei*, *T. evansi* or *T. equiperdum* constitutes an important limitation for animal protein productivity, and it is widely distributed throughout the world [18, 19]. *T. evansi* causes the disease known as Surra, Derrengadera or "Mal de Cadeiras", while *T. equiperdum* is the etiological agent of Dourine or "Mal du Coit", characterized by venereal transmission and white patches in the genitalia [20]. These three trypanosomes are morphologically indistinguishable that evolved from a common ancestor and acquired important biological differences, including host range, mode of transmission, distribution, clinical symptoms and pathogenicity [21-23]. *T. evansi* and *T. equiperdum* are the only causative agents of equine trypanosomosis in Latin America [24].

Our team previously studied the parasitological and hematologic parameters of Venezuelan *Trypanosoma* spp. isolates, finding statistical significant differences [25]. Recently, coinertia analysis applied to microsatellites, Procyclin PE repearts and maxicircle gene shows that two Venezuelan isolates have the same behavior as *T. equiperdum* STIB841/ OVI strain [26]. In order to corroborate presence or absence of kDNA, in this work was analyzed the ultrastructure of kinetoplast by transmission electron microscopy of five Venezuelan trypanosome isolates.

MATERIALS AND METHODS

Trypanosoma isolates

The Venezuelan trypanosome isolates were obtained from three horses (TeAp-Mantecal01 *T. evansi*, TeAp-N/D1 and TeGu-N/D1, both *T. equiperdum*) [26], one donkey (TeGu-Terecay323, *T. evansi*). and one capybara (TeAp-El Fríο01, *T. evansi* asymptomatic host) blood samples of natural hosts [27], cryopreserved in liquid nitrogen and conserved in our laboratory, were expanded in rats by experimental infection and purified by DEAE-Cellulose ion exchange chromatography [28].

Electron microscopy

The parasite sediments purified from blood of experimental infected rats were processed in suspension during three hours of chemical fixation with 2.5% glutaraldehyde/ 0.1M cacodylate buffer (pH 7.4, 360 mosm/L, 4 ºC) and 1% OsO_4 solution at 4 ºC. Parasites fixed samples were washes with distillated water at 4 ºC and included in agar, then were exposed to an increasing series of ethanol dehydration (50, 70, 95 and 100%, 4 ºC, 5 min each time) and included in Polybed epoxy resin (Embed 812 1:1, Shell®) first by 4 ºC, 48 hrs, second 60 ºC, 48 hrs. Polymerized blocks resin-parasite samples were cut with ultramicrotome equipped with 2.5 mm diamond knife (Diatome®, Biel-Switzerland). Sections of 60 nm were placed on copper grids 100 mesh covered with a collodion-carbon film and contrasting positive with 4% uranyl acetate and lead citrate [29]. Images were obtained and analyzed by observation of copper with a transmission electron microscope CM10-Fei® at 80kV in the microscopy unit of IVIC.

RESULTS AND DISCUSSION

Morphological characterization of the parasites

Five samples of parasites were analyzed in this study and were visualized by transmission electron microscopy. They are represented by the typical elongated body characteristic of *Trypanosoma* spp. in bloodstream stage, as expected with a kDNA extranuclear adjacent of flagellum based (bfl) and opposite to nucleus, as shown in figure 1A. The photomicrographs revealed expected integrity of kinetoplast as DNA packaging disc-shaped in all trypanosoma isolates studied (Fig. 1:A-F), the figures B, C, D, E exposed the mitochondrial membrane covering the kinetoplast, which is exhibited near the flagellar pocket (fp), (see Figs. 1A and 1F). Additionally figure 1A, exposed the trypanosoma before cytokinesis face observing double nucleolus (n) and kinetoplast (kDNA), reservosomes (r), vesicles (v), nuclear envelope (ne), mitochondrial crest (mc), mitochondrial membrane (mm) and plasma membrane (pm); these structures detected represent the quality of the samples and estimated condition of parasites studied.

Some differences have been observed by our research group, in an initial molecular biological research, a RAPD cladogram analysis revealed that Venezuelan *Trypanosoma* spp. isolates divided in two different cluster, seven isolates clustered together, while two highly virulent horse isolates appeared to be genetically distinct [27]. Secondly in virulence and pathology studies of these isolates, they were divided in three groups by monitoring parasitemia and survival: most, middle and lowest virulences, these results even were overlapping with pathogenicity data [30]. Thirdly microsatellites loci, PE repeats analysis and phylogenetic maxicircle of four gene studies, shown that the seven lowest and middle virulence trypanosomes isolates clustered with *T. evansi* reference strains, whereas the most virulent isolates closely matched to *T. equiperdum* reference strain [26]. In this work, the three *T. evansi* isolates, TeAp-Terecay323, TeAp-Mantecal01 and TeAp-El Frio01 (Fig. 1A, 1B, 1E and 1F, respectively) showed a kDNA as two the *T. equiperdum* isolates, TeAp-N/D1 and TeGu-N/D1 (Fig. 1C and 1D, respectively). In another hand, interestingly, these results revealed difference significant of the kDNA among from Brazilian *T. evansi* isolates and similar to Colombian *T. evansi* and *T. evansi*/ *T. equiperdum* Chinese isolates [6, 16, 21]. Additional microscopic studies in these parasites must be carry out in order to observe possible differences between isolates from

different geographical areas. In the future will be carried out others kinetoplast reconstructions to have more information about this tridimensional structure. A deeper understanding of the tridimensional structure of kinetoplast will be key for examine if the kinetoplast´s structure have a relation with the virulence and pathogenicity of parasites.

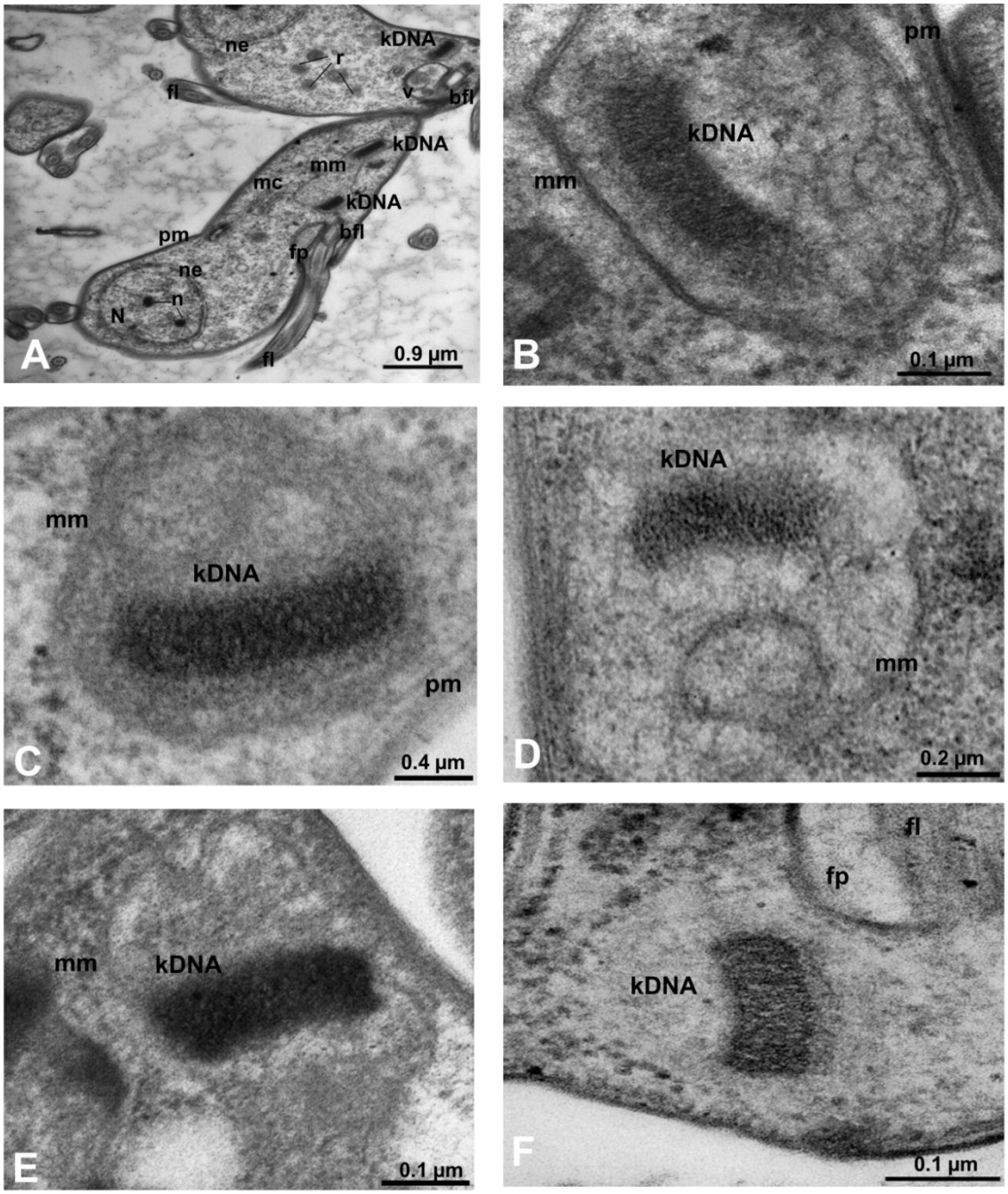

Fig. 1. Five electron micrographs from Venezuelan trypanosome isolates of veterinary interest. (A) and **(B)**: TeGu-Terecay323 donkey isolate (*T. evansi*). (**C**): TeAp-N/D1 horse isolate (*T. equiperdum*). (**D**): TeGu-N/D1 horse isolate (*T. equiperdum*). (**E**): TeAp-Mantecal01 horse isolate (*T. evansi*). (**F**): TeAp-El Frío01 capybara isolate (*T. evansi*). The photomicrographs shown of kinetoplast (**kDNA**) in the five trypanosoma isolates studied, revealed the mitochondrial membrane (**mm**) surrounding the kDNA, which is exhibited in a disk form near the flagelar pocket (**fp**) and adjacent to the base of the flagellum (**bfl**). Others structures observed; **pm**, plasma membrane; **fl**, flagellum; **v**, vesicles; **r**, reservosomes; **n**, nucleolus; **N**, nucleus; **ne**, nuclear envelope; **mc**, mitochondrial crest.

The absence of maxircircle genes in *T. evansi* is the most precise differentiation with *T. equiperdum* and *T. b. brucei* [8]; however, nowadays the literature have many opinions supporting the idea of *T. evansi* and *T.*

equiperdum are "petite mutants" of *T. brucei*, suggesting classify these parasites as subspecies of *T. brucei* [6, 31]. Studies of Venezuelan trypanosoma population described nine isolates by following parasitemia and survival of animals infected experimentally, revealed significant differences in virulence and pathogenicity [30]. Comparison of nine Venezuelan isolates by RAPD profiles, showed two separate clustal, first consisted seven isolates and second with remaining two isolates, TeAp-N/D1 and TeGu-N/D1 [27]. Recent reports of microsatellites, PE repeats and maxicircle genes amplification by end-point PCR exposed that TeAp-N/D1 and TeGu-N/D1 isolates as *T. equiperdum* [26]. In this study were evaluated the ultrastructure of five Venezuelan trypanosome isolates for contribute to knowledge and understanding of the heterogeneity of trypanosoma isolates presents in our country.

The trypanosomes's research has been reporting natural and induced loss kDNA in different trypanosomes species by testing with intercalating dyes. *T. equiperdum* strain was made diskinetoplastic by treatment of infected rats with ethidium bromide and acriflavine, kinetoplast lost was stabilized and reveled by CsCl-dye gradients analysis, this trypanosoma isolate not exhibited virulence changes than wild isolate, this reported suggested that neither the minicircles nor the maxicircles of kDNA are essential to the viability and the pathogenicity of bloodstream trypanosomes [9]. *T. brucei* natural or induced dyskinetoplastic strains are unable carry out the life cycle inside insect vector, attributable to incapacity to make oxidative phosphorylation, frequently maintaining the same virulence as compared with isolates that have intact kDNA, this have done inquiring the revaluation of use the mitochondrial genes as therapeutic targets [10].

Mitochondrial DNA network of kinetoplastid isolates with minicircles and fragments maxicircle is demarcated as diskinetoplast (Dk) strains, while the complete loss of mini and maxicircles is known as akinetoplast strains (Ak) [9]. Molecular and structure studies (PCR, Giemsa, DAPI-stained and transmission electron microscopy) the kinetoplast in bloodstream trypanosomes from domestic and wild animals infected with fourteen Brazilian *T. evansi* strains, revealed the absence of minicircle DNA and total absence of kinetoplast (observed as electron-dense material), even in old, new and successive passages in mice of *T. evansi* isolates stabilized [16]. Additionally in Latin America, molecular reports of Colombian *T. evansi* isolates showed minicircles sequences of kDNA, describing these as diskinetoplast strains [7, 17]. In this study, the kinetoplast structures of five Venezuelan trypanosoma isolates studied were equivalent to kinetoplast structure of Colombian/ Chinese *T. evansi* isolates and Chinese *T. equiperdum* isolates, but different to the natural structure akinetoplastic of Brazilian *T. evansi* isolates [6, 16, 17, 21]. These differences amongst trypanosomes of same continent support the theory about introduction of trypanosomes to South America from Africa by Spanish conquerors that arrived in different moments, causing different start evolution points of adaptation of these trypanosomes in our continent [32-34]. Our results for *T. evansi* (TeAp-El Frio01, TeGu-Terecay323 and TeAp-Mantecal01) and *T. equiperdum* isolates (TeAp-N/D1 and TeGu-N/D1) are agree with Lai et al, [6] for diskinetoplast strains, because the deletion of fragments' maxicircle kDNA, since has no an impact on kinetoplast structure, with conservation its distinctive disk-like characteristic as the Dk strains.

Evolutionary origin of Americans equine trypanosomes has been reexamined in last five years. First scheme proposed the homogenization of minicircles in the *T. b. brucei* bloodstream form, consequent loss of the ability to differentiate within the insect vector and developing the sexually transmitted between horses as *T. b. equiperdum*, after of many generations with the consequent gradual loss of selective pressure to preserve the maxicircles finally gave rise to *T. b. evansi* [15].
Second model suggested that ancestral trypanosoma lost

maxicircle genes in three [35] or four independent times until produce stable diskinetoplastic forms [31], this lack was recompensed by different mutations on the ATP synthase γ-subunit [35]. Third approach advocated that *T. b. evansi* and *T. b. equiperdum* experimented individual evolutionary progressions from a *T. b. brucei* ancestor [23]. Latest phylogenetic description of Venezuelan *Trypanosoma* spp. isolates supports the hypothesis of different evolutionary lineages with the first molecular report of *T. equiperdum* in Latin America from two new strains, TeAp-N/D1 and TeGu-N/D1 [26].

A report of morphometric studies of *Trypanosoma danilewskyi* by animals infection showed that the distance of the kinetoplast from the posterior end, length of free flagellum, nuclear length, and area of nucleus were similar among samples from the same host but differed significantly among samples from different host species [36]. Another comparable report was *in vitro* attenuation of *Cryptobia salmositica* by culture in minimum essential medium, revealed attenuated forms morphologically similar to virulent bloodstream forms. However, these forms were more slender with a shorter anterior flagellum, nucleus and kDNA smaller than virulent form. Interestingly, the attenuated form returned to its normality and multiplicity when inoculated into new fish host [37]. In this study of five Venezuelan trypanosome isolates analyzed by ultrastructure of transmission electron microscopy, revealed some differences respect to Brazilian trypanosome isolates. An additional evaluation to delve by other techniques, as the three-dimensional structure of kDNA, to know possible changes of kDNA size that could have relation with virulence and pathogenicity, among Venezuelan trypanosome isolates from different host species is indispensable. Amongst horses, donkeys and capybaras, the horses and donkeys are the most susceptible hosts affected by *T. evansi* and *T. equiperdum* [38], and capybara had been reported as a reservoir of *T. evansi* in America, which live in the same environment as most susceptible hosts [39, 40].

CONCLUSION

The kDNA ultrastructure differences between Venezuelan and Brazilian trypanosome isolates support the theory of introduction of trypanosomes to South America from Africa in different moments, causing different evolution start points for adaptation of these trypanosomes in our continent. The electron microscopy is not sufficient to establish differences among isolates of the close species, however is necessary to evaluate of the three-dimensional kinetoplast, as its dimensions, it could establishing the possible relationship between the kinetoplast's sizes; the virulence and pathogenicity of the isolates are required.

ACKNOWLEDGEMENTS

Instituto Venezolano de Investigaciones Científicas (IVIC) No 1365. Unidad de Microscopía Electrónica del IVIC: MSc. B.S. Jorge Rivas (Fotographer). Unidad de Microscopía Electrónica de la Universidad Simón Rodríguez (USR): PhD. Jesús Palminio Rodríguez.

REFERENCES

[1] Borst P., Hoeijmakers J.H. (1979) "Kinetoplast DNA" *Plasmid* 2 (1): 20-40.

[2] Heywood P., Weinman D. (1978) "Mitosis in the hemoflagellate Trypanosoma cyclops" *J Protozool* 25 (3 Pt 2): 287-92.

[3] Yurchenko V., Kolesnikov A.A. (2001) "Minicircular kinetoplast DNA from Trypanosomatidae" *Mol Biol* 35 (1): 3-13.

[4] Borst P., Fase-Fowler F. (1979) "The maxi-circle of Trypanosoma brucei kinetoplast DNA" *Biochim Biophys Acta* 565 (1): 1-12.

[5] Clayton C.E., Michels P. (1996) "Metabolic compartmentation in African trypanosomes" *Parasitol Today* 12 (12): 465-71.

[6] Lai D.H., Hashimi H., Lun Z.R., Ayala F.J., Lukes J. (2008) "Adaptations of Trypanosoma brucei to gradual loss of kinetoplast DNA: Trypanosoma equiperdum and Trypanosoma evansi are petite mutants of T. brucei" *Proc Natl Acad Sci U S A* 105 (6): 1999-2004.

[7] Borst P., Fase-Fowler F., Gibson W.C. (1987) "Kinetoplast DNA of Trypanosoma evansi" *Mol Biochem Parasitol* 23 (1): 31-8.

[8] Li F.J., Gasser R.B., Lai D.H., Claes F., Zhu X.Q., Lun Z.R. (2007) "PCR approach for the detection of Trypanosoma brucei and T. equiperdum and their differentiation from T. evansi based on maxicircle kinetoplast DNA" *Mol Cell Probes* 21 (1): 1-7.

[9] Riou G.F., Belnat P., Benard J. (1980) "Complete loss of kinetoplast DNA sequences induced by ethidium bromide or by acriflavine in Trypanosoma equiperdum" *J Biol Chem* 255 (11): 5141-4.

[10] Schnaufer A., Domingo G.J., Stuart K. (2002) "Natural and induced dyskinetoplastic trypanosomatids: how to live without mitochondrial DNA" *Int J Parasitol* 32 (9): 1071-84.

[11] Durieux P.O., Schutz P., Brun R., Kohler P. (1991) "Alterations in Krebs cycle enzyme activities and carbohydrate catabolism in two strains of Trypanosoma brucei during in vitro differentiation of their bloodstream to procyclic stages" *Mol Biochem Parasitol* 45 (1): 19-27.

[12] Cristodero M., Seebeck T., Schneider A. (2010) "Mitochondrial translation is essential in bloodstream forms of Trypanosoma brucei" *Mol Microbiol* 78 (3): 757-69.

[13] Jensen R.E., Simpson L., Englund P.T. (2008) "What happens when Trypanosoma brucei leaves Africa" *Trends Parasitol* 24 (10): 428-31.

[14] Lun Z.R., Desser S.S. (1995) "Is the Broad Range of Hosts and Geographical-Distribution of Trypanosoma-Evansi Attributable to the Loss of Maxicircle Kinetoplast DNA" *Parasitol Today* 11 (4): 131-133.

[15] Lun Z.R., Lai D.H., Li F.J., Lukes J., Ayala F.J. (2010) "Trypanosoma brucei: two steps to spread out from Africa" *Trends Parasitol* 26 (9): 424-7.

[16] Ventura R.M., Takata C.S., Silva R.A., Nunes V.L., Takeda G.F., Teixeira M.M. (2000) "Molecular and morphological studies of Brazilian Trypanosoma evansi stocks: the total absence of kDNA in trypanosomes from both laboratory stocks and naturally infected domestic and wild mammals" *J Parasitol* 86 (6): 1289-98.

[17] Songa E.B., Paindavoine P., Wittouck E., Viseshakul N., Muldermans S., Steinert M., Hamers R. (1990) "Evidence for kinetoplast and nuclear DNA homogeneity in Trypanosoma evansi isolates" *Mol Biochem Parasitol* 43 (2): 167-79.

[18] Luckins A.G., Dwinger, R.H.,. (2004) "Non-tsetse-transmitted Animal Trypanosomiasis" *The Trypanosomiases*, I. Maudlin, Holmes, P. H., Miles, M.A., ed., Oxfordshire: CABI Publishing, pp. 269-281.

[19] Desquesnes M., Holzmuller P., Lai D.H., Dargantes A., Lun Z.R., Jittaplapong S. (2013) "Trypanosoma evansi and Surra: A Review and Perspectives on Origin, History, Distribution, Taxonomy, Morphology, Hosts, and Pathogenic Effects" *BioMed Research International 2013:1-22.*

[20] Hoare C.A. (1972) "*The Trypanosomes of mammals. A zoological monographs*", Oxford, Blackwell Scientific Publications.

[21] Brun R., Hecker H., Lun Z.R. (1998) "Trypanosoma evansi and T. equiperdum: distribution, biology, treatment and phylogenetic relationship (a review)" *Vet Parasitol* 79 (2): 95-107.

[22] Claes F., Buscher P., Touratier L., Goddeeris B.M. (2005) "Trypanosoma equiperdum: master of disguise or historical mistake?" *Trends Parasitol* 21 (7): 316-21.

[23] Wei Y., Wen Y-Z., Desquesnes, M., Lun, Z-R. (2011) "Molecular Epidemiology of Trypanosoma evansi and T. equiperdum and Atypical Human Infection by Animal Trypanosomes" *The Molecular Epidemiology of Trypanosomes and Leishmania.*: Geoff Hide pp. 1-14.

[24] Desquesnes M. (2004) "Trypanosomes and Diagnosis" *Livestock Trypanosomoses and their*

Vectors in Latin America, Paris, France: Office International des Epizooties, pp. 15-21, 65-83.

[25] Holzmuller P., Grebaut P., Peltier J.B., Brizard J.P., Perrone T., Gonzatti M., Bengaly Z., Rossignol M., Aso P.M., Vincendeau P., Cuny G., Boulange A., Frutos R. (2008) "Secretome of animal trypanosomes" *Ann N Y Acad Sci* 1149: 337-42.

[26] Sanchez E., Perrone T., Recchimuzzi G., Cardozo I., Biteau N., Aso P.M., Mijares A., Baltz T., Berthier D., Balzano-Nogueira L., Gonzatti M.I. (2015) "Molecular characterization and classification of Trypanosoma spp. Venezuelan isolates based on microsatellite markers and kinetoplast maxicircle genes" *Parasit Vectors* 8 (1): 536.

[27] Perrone T.M., Gonzatti M.I., Villamizar G., Escalante A., Aso P.M. (2009) "Molecular profiles of Venezuelan isolates of Trypanosoma sp. by random amplified polymorphic DNA method" *Vet Parasitol* 161 (3-4): 194-200.

[28] Lanham S.M., Godfrey D.G. (1970) "Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose" *Exp Parasitol* 28 (3): 521-34.

[29] Reynolds E.S. (1963) "The use of lead citrate at high pH as an electron-opaque stain in electron microscopy" *J Cell Biol* 17: 208-12.

[30] Perrone T.M. (2003) "Tipificación De Aislados Venezolanos de Trypanosoma evansi", Biology, Universidad Simón Bolívar, Sartenejas. Estado Miranda.

[31] Carnes J., Anupama A., Balmer O., Jackson A., Lewis M., Brown R., Cestari I., Desquesnes M., Gendrin C., Hertz-Fowler C., Imamura H., Ivens A., Koreny L., Lai D.H., MacLeod A., McDermott S.M., Merritt C., Monnerat S., Moon W., Myler P., Phan I., Ramasamy G., Sivam D., Lun Z.R., Lukes J., Stuart K., Schnaufer A. (2015) "Genome and phylogenetic analyses of trypanosoma evansi reveal extensive similarity to T. brucei and multiple independent origins for dyskinetoplasty" *PLoS Negl Trop Dis* 9 (1): e3404.

[32] Hoare C.A. (1972) "*The trypanosomes of mammals: a zoological monograph*",Oxford,, Blackwell Scientific Publications.

[33] Canelón J. L. M.R.D. (2003) "Posible Origen del Trypanosoma evansi en Venezuela" *Veterinaria Tropical* 28: 155-167.

[34] Silva-Iturriza A., Nassar J.M., Garcia-Rawlins A.M., Rosales R., Mijares A. (2013) "Trypanosoma evansi kDNA minicircle found in the Venezuelan nectar-feeding bat Leptonycteris curasoae (Glossophaginae), supports the hypothesis of multiple origins of that parasite in South America" *Parasitol Int* 62 (2): 95-9.

[35] Schnaufer A. (2010) "Evolution of dyskinetoplastic trypanosomes: how, and how often?" *Trends Parasitol* 26 (12): 557-8.

[36] Woo P.T., Black G.A. (1984) "Trypanosoma danilewskyi: host specificity and host's effect on morphometrics" *J Parasitol* 70 (5): 788-93.

[37] Woo P.T., Li S. (1990) "In vitro attenuation of Cryptobia salmositica and its use as a live vaccine against cryptobiosis in Oncorhynchus mykiss" *J Parasitol* 76 (5): 752-5.

[38] Maudlin I., Holmes P.H., Miles M.A. (2004) "*The trypanosomiases*",Wallingford, UK ; Cambridge, MA, USA, CABI Pub.

[39] Clarkson M.J. (1976) "Trypanosomiasis of domesticated animals of South America" *Trans R Soc Trop Med Hyg* 70 (2): 125-6.

[40] Reverón I. (1992) "Tripanosomiasis en chigüires (Hydrochaerus hydrochaeris) y su diagnóstico mediante el ensayo ELISA", Biología, Universidad Simón Bolívar, Caracas.