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Trypanosoma evansi: A quantitative approach to the understanding of the morphometry-hematology relationship throughout experimental murine infections

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ABSTRACT

The morphometric characteristics of three Venezuelan isolates of *Trypanosoma evansi,* and the basic hematological features as well as host's body weight were registered throughout experimental murine infections. The phenomena, trypomastigote morphometry dynamics, mouse hematological change and mice body variation, are related in statistical terms. In addition, the changeable morphometric characteristics of the hemoflagellate, and the host's mutable hematological attributes, and the variable host's body eight produced three different mathematical models generated by Multiple Linear Regressions that could explain the heterogeneous behavior of this eclectic parasite. Even more, supplementary mathematical models provided by Correspondence Analysis emphasize this reasoning. The results herein presented suggest that the association between trypomastigote morphometry, and host hematology, as well as body eight could be a feasible starting point to understand the heterogeneity of the *T. evansi* isolates.

Key words: *Trypanosoma evansi*; morphometry; hematology

INTRODUCTION

The area of distribution of *Trypanosoma evansi* includes the Palearctic, Ethiopian, Oriental and Neotropical regions, geographical areas where is the etiological agent of "Surra", known as "Derrengadera" in Venezuela, an equine disease with a long recorded history in the Venezuelan savannas (Rangel, 1905; Kubes, 1939).

T. evansi is responsible for an anemic and febrile symptomatology that leads, through a progressive and generalized weakness process, to the host's death (Hoare, 1972; Losos, 1980; Mahmoud and Gray, 1980).

Although the horse (*Equus cabalus*) is the par excellence host in the Neotropical region (Hoare, 1972), *T. evansi* has also been reported in other mammals of the area, such as donkey (*E. asinus*) (Tejera, 1920), capybara (*Hydrochoerys hydrochaeris*) (Mingone, 1910; Tejera, 1920; Gutierrez, 1958; Morales *et al.*, 1976), dog (*Canis familiaris*) (Rangel, 1905), ocelot (*Felis pardalis*) (Shaw, 1977), coati (*Nasua nasua*) (Nunes and Oshiro, 1990), vampire bat (*Desmodus rotundus*) (Hoare, 1965), rice rat (*Oryzomys* sp.) (Nunes, *et al*., 1994) and armadillo (*Dasypus* sp.) (Herrera *et al.*, 2002).

The pathobiology of the disease has not been described in all animals naturally parasitized; also the pathology in naturally infected horses has been slightly described (Seiler *et al.,* 1981; Quiñones-Mateu *et al.*, 1994). Nevertheless *E. asinus* suffers "Derrengadera" in nature its gradual disuse in Venezuela's extensive bovine farming is turning the domestic donkey into a peridomestic, and eventually, a feral species interacting with domesticated and wild horses. Furthermore, the lack of relation to the symptomatology associated with the infection led Morales *et al.* (1976) to suggest that *H. hydrochaeris* could be a suitable reservoir acting as an infection bridge to domesticated and wild horses visiting water bodies usually occupied by the rodents. Therefore, capybara and donkey should fulfill a role in the epidemiology of the disease, and as a logical spin off consequence, the basic biology of the trypanosome's populations dwelling these species should be investigated and compared with those occurring in the horse.

By its part, the morphometric heterogeneity of trypanosomes is a well established attribute (Hoare, 1972). Besides, Gill (1977) and Dávila *et al.* (1998a), among others, have reported some relationship between the parasite's measurable morphology and the host's physical condition. Along these lines, Bookstein (1982, 1986), Bookstein *et al.* (1985), Dalay (1985), Rohlf and Marcus (1993), and Adams and Funk (1997) state that is possible to compute associations between morphometrical characteristics and biochemical, physiological, pathological, genetic and/or environmental features.

In general, morphometric investigations dealing with trypanosomes are uncommon, and usually their biometric methodology focuses on the specific characteristics of the species. In addition, such studies are scanty and diffuse, and mostly time-static, covering in consequence a tiny field of the whole form-function process. Thus investigations monitoring sequential changes in the measurable morphology of the trypanosomes must be carried out and especially those relating morphologic quantifiable characteristics to the host's physiology. For that reason, this work explores with biometric and multivariate tools the association between the measurable morphological changes of three indigenous *T. evansi* isolates derived from different naturally infected hosts (*E. asinus, E. cabalus* and *H. hydrochaeris*) and the basic mouse hematological variations during the complete experimental infection.

MATERIALS AND METHODS

The parasites used in this work derived from a naturally infected *E. asinus* with "Derrengadera" symptomatology ("Terecay" cattle ranch; 7º58' N – 67º28' W, and 68 m above sea level; Guarico State, Venezuela), *H. hydrochaeris* ("El Frio" cattle ranch; 7º35' N – 68º50' W, and 67 m above sea level; Apure State, Venezuela) and *E. cabalus* suffering "Derrengadera" ("Mantecal"; 7º33' N – 69º6' W, and 62 m above sea level; Apure State, Venezuela). Since the qualitative characters of the trypanosomes in all cases were indistinguishable, they were compared on the basis of their measurements. The isolates were species-specific identified on the basis of morphological and morphometrical data (Hoare, 1956, 1972). The isolates were named according to Dávila *et al.* (1998b), as MEQD/VE/99/Trino (*E. asinus* from "Terecay"), MHHY/VE/92/EF-1 (*H. hydrochaeris* from "El Frio") and MEQH/VE/96/Mant (*E. cabalus* from "Mantecal"). All trypanosome samples were maintained in liquid N_2 from isolation to use.

The experimental groups, one for each trypanosome isolate, consisted of 30 female NMRI mice weighting 20 g each. The selection of the mouse strain followed the criteria set up by Perrone-Carmona *et al.*

(2006). Animals were assorted in assemblages of ten individuals, and placed in metal boxes with rice husk, and food and water provided *ad libitum*. All rodents were intra-dermal inoculated with 1 trypomastigote/g of body weight, suspended in 100 µl of sterile isotonic saline (0.85% NaCl + 1% glucose). Control groups were identically injected with a hemoflagellate-free sterile isotonic saline. Experiments were repeated three times.

The investigation complies with the norms set out in the Guide for Care and Use of Laboratory Animals, published by the U.S. National Institute of Health (NIH publication Nº85-23, revised 1985).

One day after inoculation, and every other day until parasitemia interruption (mouse death), 100 µl of caudal vein blood was collected with a heparinized microhematocrit tube from nine randomly selected mice (three from each box). Control mice were identically chosen and bled; their blood was also processed.

Collected blood samples were distributed in the following aliquots: 10 µl to make blood smears, 10 µl to determine hemoglobin concentration ([Hb]) according to Van Kampen and Zjlstra (1965), 60 µl to establish packed red cell volume by standard microhematocrit method (PCV), 5 µl to ascertain erythrocytes/blood ml (EC), 5 µl to quantify parasitemia (PAR) according to Brener (1962), and 5 µl to make blood films on glass slides. In addition, mice body weight (MBW) and mice survival time were also registered.

Blood films were fixed with Karnovsky (320 mOsm/l; pH 7.4; 3 min), post-fixed with 70% methanol (3 min), stained with May-Grünwald Giemsa (15 min) and differentiated with acetone dips, if necessary.

Recording of morphometric variables was carried out with the trypomastigotes enclosed in thirty microscopic fields (x1,000) systematically selected from each blood film according to the sampling procedure described by Miles (1978). The images of the trypanosomes (x1,000) were captured with a digital camera attached to the microscope. Digitalized trypomastigote images were used to compute the numeric magnitude of the following morphometric variables: L (total length), BL (body length excluding the free flagellum), W (maximum cell body width through nucleus), KN (space separating kinetoplast and nucleus), PK (distance between kinetoplast and posterior end), PN (space amid nucleus and posterior end), AK (length separating kinetoplast and anterior end), AN (distance between nucleus and anterior end), F (free flagellum length) and TA (total cell area excluding the free flagellum). In addition, the Nuclear Index (NI) (Dias and Freitas, 1943), the Kinetoplast Index (KI) (Keymer, 1967) and the Body Shape Index (BSI) (Bennett *et al.*, 1994) were calculated.

The biometric management of the data included: a) mean contrasting parametric (ANOVA) and non-parametric (Kruskal-Wallis) tests; b) Multiple Linear Regression (MLR), a multivariate procedure which models the relationship between two or more explanatory variables and a response variable by fitting a linear equation to observed data; c) Path Analyses (PA), an extension of the MLR model, used to test the fit of the correlation matrix against two or more causal models which are being compared; and c) Correspondence Analyses (CA), a descriptive and/or exploratory technique designed to analyze simple two-way and multi-way tables able to reduce the number of variables, as well as to detect structure in the relationships among variables. Differences were considered to be statistically significant with values of $p < 0.05$.

RESULTS

The taxonomic status of these trypanosomes was established as *T. evansi* based on morphological and morphometrical data (Hoare, 1956, 1972).

These *T. evansi* isolates exhibited different pre-patent periods varying between 3 days in capybara isolate (MHHY/VE/92/EF-1) and 9 days in donkey isolate (MEQD/VE/99/Trino); the pre-patent period for the horse isolate (MEQH/VE/96/Mant) was 7 days. Although patent parasitemia finished abruptly with the host's death, it showed isolate-dependent dissimilarities. Certainly, rodent (MHHY/VE/92/EF-1) and horse (MEQH/VE/96/Mant) derived isolates displayed a typical sustained increasing parasitemic pattern, ending 23 and 19 days post-inoculation, respectively. For its part, mice inoculated with the donkey derived hemoflagellates (MEQD/VE/99/Trino) revealed a distinctive undulatory-like parasitemic pattern that resembles waves for 39 days. The parasitemic peaks also varied among isolates (Figure 1).

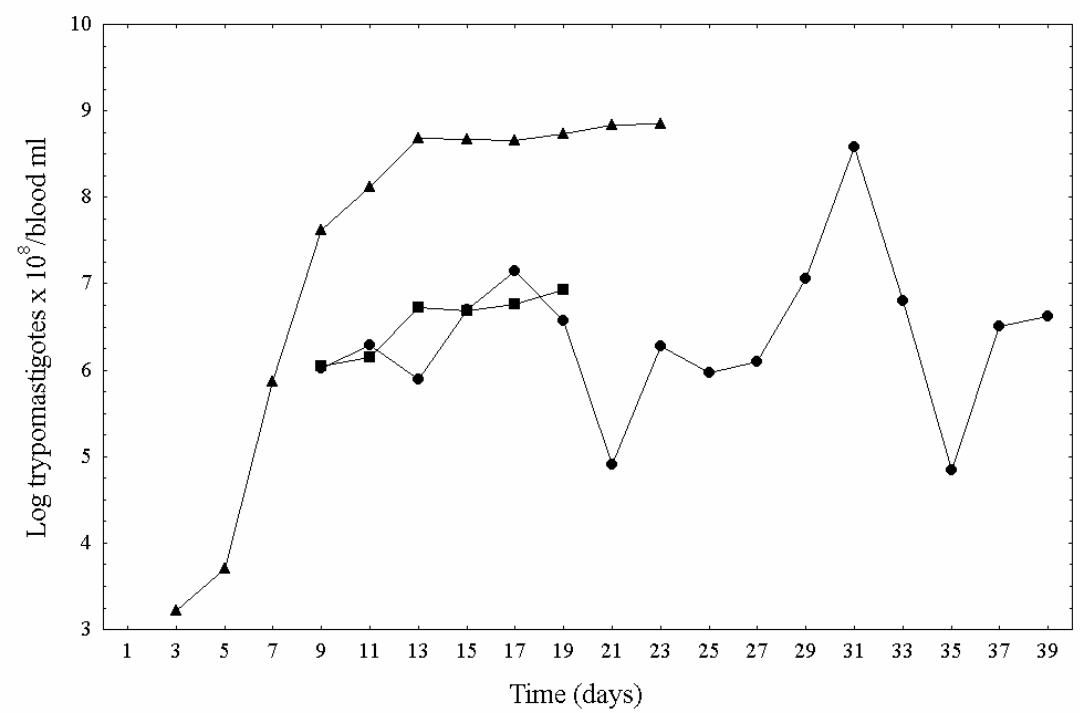

Figure 1. Mouse Parasitemias by three Venezuelan isolates of *Trypanosoma evansi*. Termination of curve indicates mice death. ●: MEQD/VE/99/Trino (donkey isolate); ▲: MHHY/VE/92/EF-1 (capybara isolate); ■: MEQH/VE/96/Mant (horse isolate).

Because of the nature of recorded data, the first approximation to quantitative biology was through mean contrasting tests. Depending on particular conditions, parametric (ANOVA) or non-parametric (Kruskal-Wallis) studies were completed. These analyses facilitate the detection of differences ($p < 0.05$) among the variables through time (Table 1).

Variables	MEQD/VE/99/Trino			MHHY/VE/92/EF-1			MEQH/VE/96/Mant		
			p-level			p-level			p-level
L	F =	2.63	0.05	F =	27.82	0.00 *	F =	5.39	0.00 *
BL	H =	5.0×10^6	0.07	F =	5.46	0.00 *	F =	10.36	0.00 *
W	F =	4.49	0.00 *	H =	1.0×10^6	0.00 *	F =	4.41	0.00 *
KN	H =	2.0×10^6	0.00 *	H =	2.0×10^6	0.00 *	F =	7.88	0.00 *
PK	H =	5.0×10^6	0.07	F =	5.46	0.00 *	F =	10.36	0.00 *
PN	H =	1.0×10^6	0.00 *	H =	2.0×10^6	0.00 *	F =	13.30	0.00 *
KA	F =	1.56	0.20	F =	3.91	0.00 *	F =	8.47	0.00 *
NA	F =	2.91	0.04 *	F =	1.41	0.23	F =	3.01	0.01 *
F	F =	2.50	0.06	F =	22.02	0.00 *	F =	4.00	0.00 *
TA	H =	2.0×10^6	0.00 *	F =	3.64	0.01 *	F =	9.63	0.00 *
NI	H =	2.0×10^6	0.00 *	F =	3.64	0.01 *	F =	9.63	0.00 *
KI	F =	4.11	0.01 *	F =	2.42	0.04 *	F =	3.50	0.00 *
BSI	F =	2.07	0.12	F =	6.33	0.00 *	F =	3.74	0.00 *
[Hb]	F =	9.55	0.00 *	F =	28.74	0.00 *	F =	6.70	0.00 *
PCV	F =	1.77	0.03 *	H =	5.0×10^6	0.00 *	F =	7.09	0.00 *
EC	H =	1.0×10^6	0.13	F =	7.14	0.00 *	F =	14.40	0.00 *
PAR	H =	7.0×10^6	0.00 *	H =	9.0×10^6	0.00 *	H =	6.0×10^6	0.00 *
MBW	F =	0.63	0.88	F =	1.54	0.14	F =	73.67	0.00 *

Table 1. Critical points for ANOVA and Kruskal-Wallis analyses. F, Fisher's F; H, Kruskal-Wallis estimator; *: significative differences. L: total length; BL: body length excluding the free flagellum; W: maximum cell body width through nucleus; KN: space separating kinetoplast and nucleus; PK: distance between kinetoplast and posterior end; PN: space amid nucleus and posterior end; AK: length separating kinetoplast and anterior end; AN: distance between nucleus and anterior end; F: free flagellum length; TA: total cell area excluding free flagellum; NI: nuclear index; KI: kinetoplast index; BSI: body shape index; Hb: hemoglobin concentration; PCV: packed red cell volume by standard microhematocrit method; EC: erythrocytes/blood ml; PAR: parasitemia; and MBW: mice body weight.

The pattern of morphologic change exhibited by these isolates revealed consistent differences in the way morphometric variables change in the course of time. Figure 2, focuses on BL comparisons for the isolates under study; all other morphometric variables (L, W, KN, PK, PN, AK, AN, F and TA) revealed

similar differential outlines. The undulatory-like parasitemic pattern resembling waves previously described with the MEQD/VE/99/Trino (donkey isolate) is also exhibited in its trypomastigote's body length change (Figure 2).

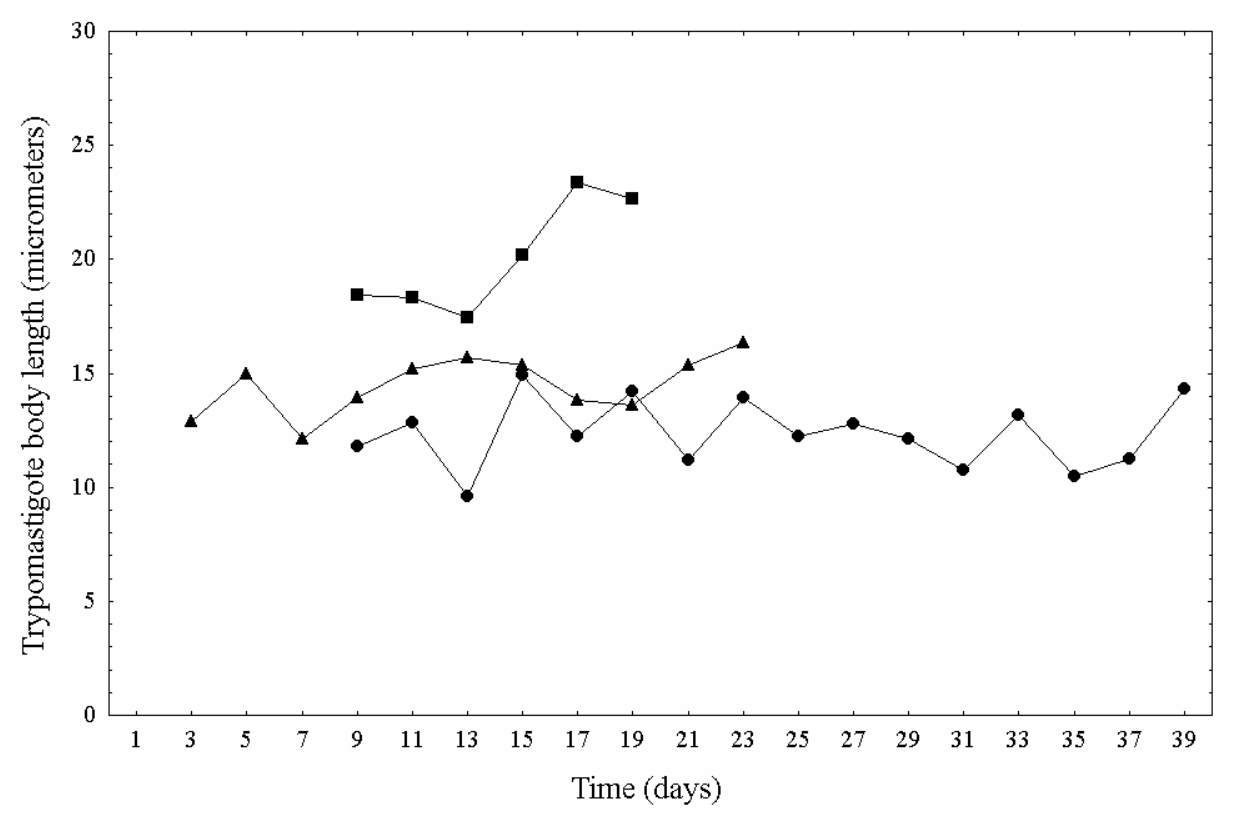

Figure 2. Variation in the body length of the trypomastigotes in the blood of female *Mus musculus* experimentally infected with three Venezuelan isolates of *Trypanosoma evansi*. Termination of curve indicates mice death. ●: MEQD/VE/99/Trino (donkey isolate); ▲: MHHY/VE/92/EF-1(capybara isolate); ■: MEQH/VE/96/Mant (horse isolate).

The calculated indexes (NI, KI and BSI) indicate a fattening up tendency in the flagellates, making stouter trypomastigotes when the parasitemia reaches higher population densities. Concomitantly, the kinetoplast moves toward the nucleus (data not shown).

Figure 3, shows changes in the haemoglobin concentration ([Hb]) throughout the infection. The donkey isolate (MEQD/VE/99/Trino) reveals the undulating tendency already mentioned in the parasitemia and the trypomastigote body length. The other hematologic variables (PCV and EC), as well as MBW, followed similar tendencies to those presented in the figure.

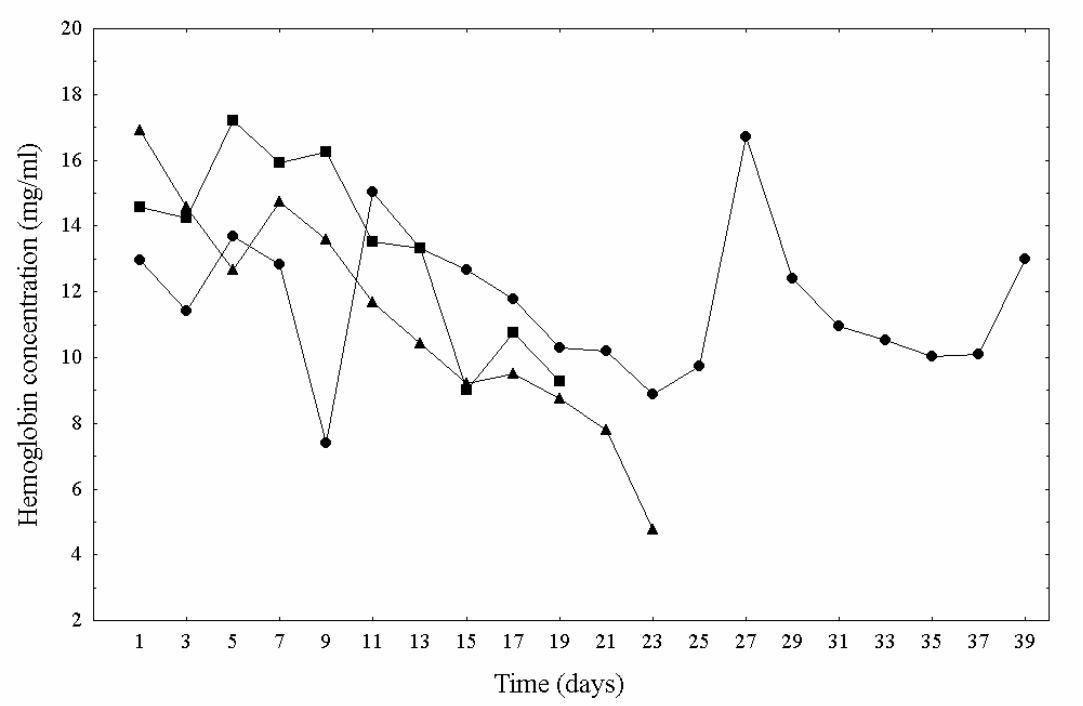

Figure 3. Variation in the plasma hemoglobin concentration of female *Mus musculus* experimentally infected with three Venezuelan isolates of *Trypanosoma evansi*. Termination of curve indicates mice death. ●: MEQD/VE/99/Trino (donkey isolate); ▲: MHHY/VE/92/EF-1 (capybara isolate); ■: MEQH/VE/96/Mant (horse isolate).

The interactions among the collected data (morphological, parasitemic, mice weight and hematologic) were analyzed quantitatively by means of MLR analyses. The MLR relates every kind of variable under experimentation, assumed as independent variables (morphological, haematological and MBW) to the dependent variable, the population density of the parasites (parasitemia). The derived models provide a numerical interpretation of the effect of all variables under investigation upon parasitemia. Table 2, shows the equation of the obtained models.

ISOLATE	EQUATION	R^2
MEQD/VE/99/Trino	PAR = 0.60W + 1.93Hb - 1.10BL	0.999
MHHY/VE/92/EF-1	PAR = -0.92Hb - 0.38MBW - 0.65BL	0.586
MEQH/VE/96/Mant	PAR = -0.51W -1.12PCV - 0.51MBW+ 0.31EC	0.985

Table 2. Equations derived from Multiple Lineal Regression analyses using parasitemia as the dependent variable, and all remaining variables as independent ones; R^2: multiple correlation coefficient. PAR: parasitemia (population density); W: maximum cell body width through nucleus; Hb: hemoglobin concentration; MBW: mice body weight; BL: body length excluding the free flagellum; PCV: packed red cell volume by standard microhematocrit method; and EC: erythrocytes/blood ml.

In order to scrutinize point-by-point the particular interactions among the variables remaining in the MLR models, PA was carried out. PA complements MLR through a clear and detailed illustration of the dependence processes. PA results are presented in Figure 4.

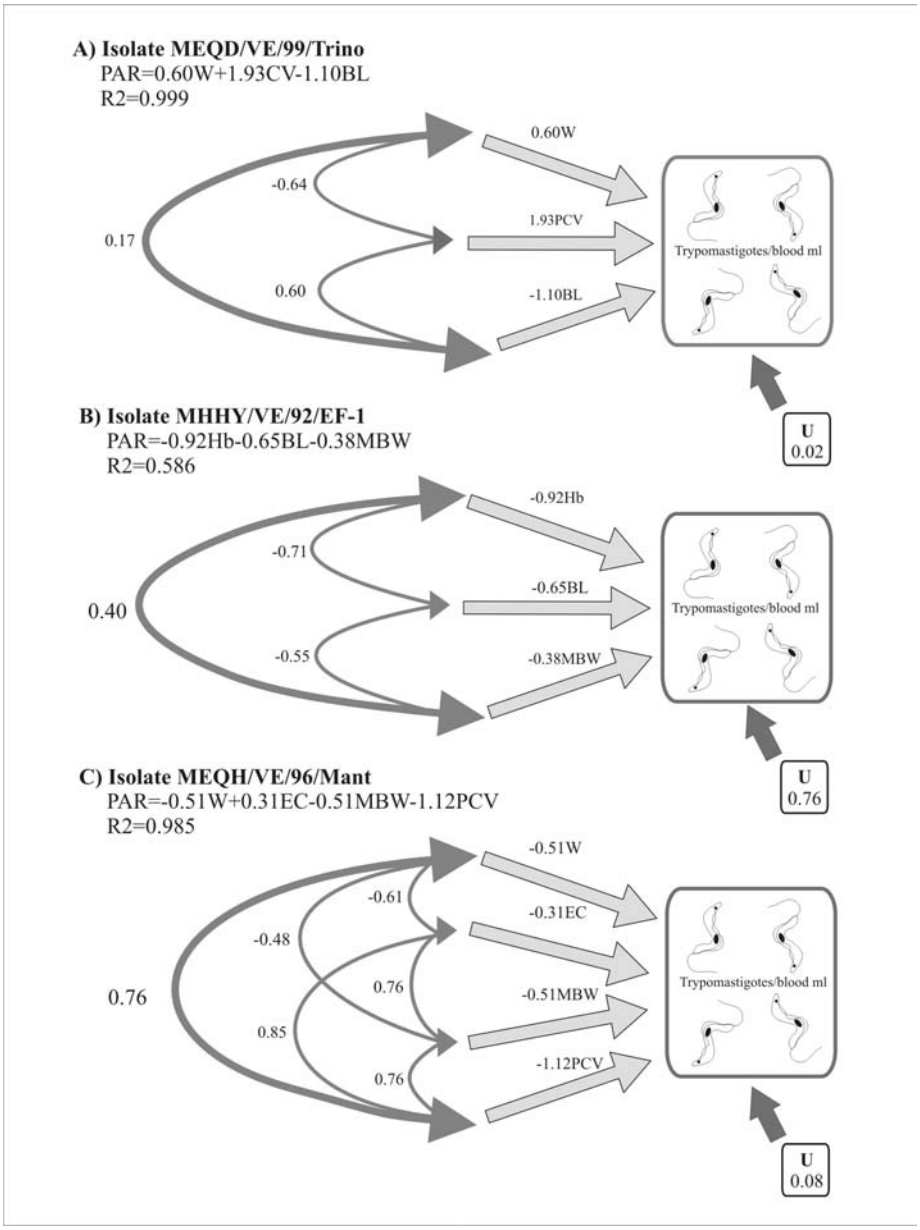

Figure 4. Path Analysis models showing details of the Multiple Liner regression models, coefficients and the unexplained variance of the models. Coefficients underline the weight of the variables on the population density (parasitemia).

The PA (Figure 4) graphically emphasizes the results summarized by MLR equations (Table 2). The PA makes clear the diversity of the investigated *T. evansi* isolates: A heterogeneous behaviour put in evidence through changes in variables of different nature.

Finally, data were considered by means of CA, a statistical technique used to study the dependence relationship among categories that allow analyzing the structure of the association describing, besides, proximities able to define cause categories causing the association. The analysis maintained in the mathematical model the haematological variables ([Hb], PCV, and EC), MBW and the parasitemia. The model collected 98% of the total system variance in two components that discriminate the isolates. The MEQD/VE/99/Trino (donkey isolate) and MHHY/VE/92/EF-1 (capybara isolate) groups share a small portion of the reduced variables space. On the contrary, MEQH/VE/96/Mant (horse isolate) appears as a totally discrete entity (Figure 5).

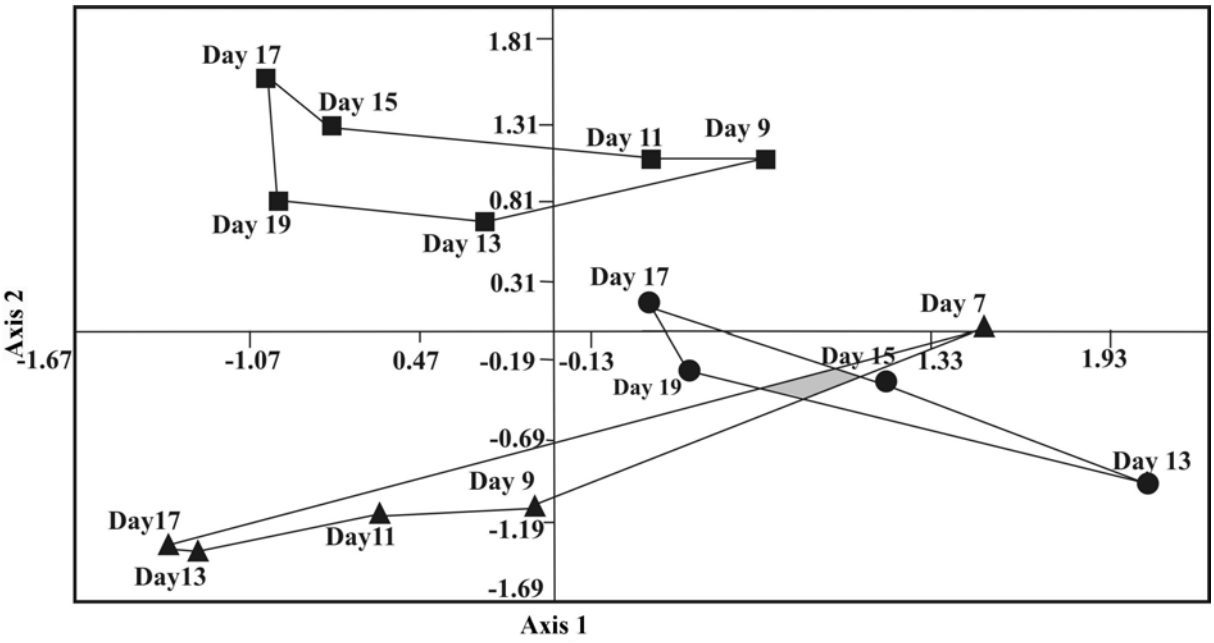

Figure 5. Graphic representation of the Correspondence Analysis. The analysis evidences the morphofunctional relationship among the isolates. The polygon delimited by ● corresponds to MEQD/VE/99/Trino isolate (from donkey); the one bordered by ▲ represents MHHY/VE/92/EF-1 isolate (from capybara); and the one enclosed with ■ stands for MEQH/VE/96/Mant isolate (from horse). Shady area implies superimposition.

The numerical behaviour of all variables (*T. evansi* isolates), shows three numerical entities, one of them completely separated and the remaining two exhibiting some degree of superimposition meaning share of properties. Notice the small dimensions of the superimposed site (shady area).

DISCUSSION

The literature regarding *T. evansi* is copious and gives an account of etiology, epizootiology, epidemiology, diagnosis, clinical signs, pathogenesis and pathology, immunology, as well as chemotherapy and control. Even though they are relevant areas of the *T. evansi* biology, the connection between morphology and host-parasite relationship are aspects that have not been strongly investigated in present time. Particularly, quantitative morphology and its relation to the host's physiological conditions have been superficially studied.

Differences in the parasitemia produced by Surra-causing trypanosomes have been described since the mid-twentieth century. According to Hoare (1943), these hemoflagellates comprise strains differing in their effect upon diverse hosts in the same geographical area being attributed to heterogeneity. Our parasitemia results showed evident dissimilarity (Figure 1), ascribable to the mentioned heterogeneity.

Trypanosoma pleomorphism has also been investigated in depth. Although *T. evansi* is considered a monomorphic trypanosome (Hoare, 1956, 1972; Gill, 1977; among others), there are exceptions (Losos, 1980). Morphometric differences of *T. evansi* have already been observed by other investigators (Ray, 1945; Hoare, 1956, 1972; John *et al.*, 1992; Ramirez *et al*., 1997; Dávila *et al.*, 1998a; among others), but most focus on measures made from samples collected on the parasitemia's peak, and the comparison of trypomastigote dimensionality of different hosts and/or dissimilar geographical areas.

Indeed, the shape modification implies dimensional variation in time. Figure 2, shows obvious differences in trypomastigote's BL among isolates, and in addition, within isolates. According to Stephen (1986), the morphology of trypanosomes may vary in the same host from day to day. We interpret such change through time in morphometric variation terms, probably leading to the expression of different pleomorphic states. In such a way, Gill (1977) and Dávila *et al.* (1998a), reported a certain kind of relationship between the parasite pleomorphism and the host general condition. Nevertheless, lack of correlation between fever and parasitemia was reported in sheep (Katunguka-Rwakishava *et al*., 1992), capybara (Franke *et al.*, 1994), buffalo (Kandavel and Nedunchelliyan, 1994), donkey (Soodan *et al*., 1996), and coati (Herrera *et al*., 2001).

By the way, Menezes *et al*. (2004), point out that survival time and parasitemia should not be the only parameters to be taken into account for biological characterization and comparison of *T. evansi* populations.

The preceding paragraphs emphasize the necessity for research able to relate host and *T. evansi* from a holistic approach. In this respect, the results reported in this paper reveal undescribed aspects of the *T. evansi* murine experimental infection.

Parasitologic and hematologic characteristics of the *T. evansi* infection have already been reported by Losos, 1980; Mahmoud and Gray, 1980; Jenkins and Facer, 1985; Silva *et al.,* 1995; Onah *et al.,* 1996; De La Rue *et al.,* 1997; Aquino *et al.,* 2002; and Menezes *et al.,* 2004. However, none of these articles take into account the quantitative relationship between the parasite's continuous morphometric change throughout the entire parasitemia and its close association to some concomitant basic haematological variations of the host.

Thus, our first approach to quantitative biology (ANOVA and Kruskal-Wallis) denotes changes in the magnitude of the considered variables, independently of its nature (morphometric, hematological, parasitological or the MBW) (Table 1). Furthermore, the dimensionality of the trypomastigotes can be statistically related to some haematological changes in the infected mouse, and such differences show up a great biological heterogeneity. This fact could explain the variety of parasitological behavior observed among donkey (MEQD/VE/99/Trino), capybara (MHHY/VE/92/EF-1) and horse (MEQH/VE/96/Mant) isolates.

We have demonstrated significant statistical correlation between measurable morphologic characteristics of the flagellate (morphometry) and variations in some basic haematological characteristics of the host, not only in a precise moment of the parasitemia, but throughout the whole infection.

The aforementioned association would suggest that the pathological and the parasitological processes could be related to the measurable trypomastigotes transformation occurring throughout the parasite's ontogeny. Such corollary is in concordance with Menezes *et al.* (2004), since these authors indicate that the disease due to *T. evansi* infection is probably multifactorial.

Furthermore, the described measurable changes seem to be isolate-dependent in view of the fact that they could express different ontogeny developmental patterns in the donkey (MEQD/VE/99/Trino), capybara (MHHY/VE/92/EF-1) and horse (MEQH/VE/96/Mant) (Table 2). This hypothesis reveals a close relationship between particular isolate-dependent patterns of morphometric change and the host's resources being exploited by the parasite, which is also in concordance with Menezes *et al*. (2004), since *T. evansi* was able to colonize several niches.

This variety could also be used as a practical tool in the study of the morphofunctional segregation of *T. evansi* isolates. In this respect, the analysis of the interactions among variables (Figure 4) emphasizes, in detail, the above-mentioned heterogeneity. PA coefficients are standardized regression coefficients (β) showing the direct effect of an independent variable on a dependent variable in the path model. Moreover, these integers are partial regression coefficients which measure the extent of effect of one variable on another in the model. The variety of coefficients shown in Figure 4, underlines the heterogeneity of the form-function complex in terms of the morphometrical change observed in the parasite, its ability to grow and to make differential use of the resources found in the experimental host. The model proved its robustness with the MEQD/VE/99/Trino (donkey) and the MEQH/VE/96/Mant (horse) isolates since U (unexplained variance) is 0.02 and 0.08, respectively. On the other hand, the model showed its weakness with the MHHY/VE/92/EF-1 (capybara) isolate in view of the fact that U = 0.76.

The use of CA, a multivariate technique able to reduce the number of original variables (n = 18) and capable of detect structure in the relationships between variables, provides a new perspective to the study of the *T. evansi*-*M. musculus* experimental interaction. The method discriminates isolates in time, providing in our case three separate polygons representing the *T. evansi* isolates included in the work. The robustness of the technique is guaranteed since it collects 98% of the total variance of the system. The superimposition between polygons indicates common characteristics between the involved groups (*T. evansi* isolates), and the disjointing between polygons is a sign of discrimination. The more distant the polygons are, the more different the isolates are, and *vice versa*. CA results show a clear discreteness of the MEQH/VE/96/Mant isolate (horse), and a small superimposition (shady area) between MEQD/VE/99/Trino (donkey) and MHHY/VE/92/EF-1 (capybara) isolates, implying donkey and capybara isolates share a small fraction of their qualities.

The technique also emphasizes a clear difference with respect to the characteristics of the MEQH/VE/96/Mant (horse isolate), since the colonization and subsequent niche (host) exploitation (parasitization) could induce adaptive changes expressed as distinctive morphofunctional isolate-dependent attributes.

The direct association between the parasite dimensionality and the expression of its function through changeable host haematological features could be the starting point to understand the manifest heterogeneity of the pathology and symptomatology of the sickness, since particular disease characteristics should derive from specific morphofunctional qualities of particular isolates.

Besides the manifest virulence of Venezuelan *T. evansi* isolates to female *M. musculus,* this work put in evidence the hemoflagellate heterogeneity, not only from a morphometric point of view, but in host-parasite relationship terms, and reinforce the use of multivariate tools in the study of such relationships.

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