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Short communication

Comparison of infectivity and virulence of clones of *Trypanosoma evansi* and *Ttrypanosoma equiperdum* Venezuelan strains in mice

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ABSTRACT

Livestock trypanosomoses, caused by three species of the *Trypanozoon* subgenus, *Trypanosoma brucei brucei*, *T. evansi* and *T. equiperdum* are widely distributed and limit animal production throughout the world. The infectivity and virulence of clones derived from *Trypanosoma evansi* and *Trypanosoma equiperdum* Venezuelan strains were compared in an in vivo mouse model. Primary infectivity and virulence determinants such as survival rates, parasitemia levels, PCV, and changes in body weight and survival rates were monitored for up to 32 days. The *T. equiperdum* strain was the most virulent, with 100% mortality in mice, with the highest parasitemia levels (7.0×10^7 Tryps/ml) and loss of physical condition. The *T. evansi* strains induced 100% and 20% fatality in mice. Our results show that the homogeneous parasite populations maintain the virulent phenotype of the original *T. equiperdum* and *T. evansi* stocks. This is the first comparative study of infectivity and virulence determinants among clonal populations of *T. equiperdum* and *T. evansi*.

1. Introduction

Trypanosoma evansi and *Trypanosoma equiperdum*, are closely related, distinct parasites, that have been classified either with the binomial nomenclature (Hoare, 1972; Desquesnes et al., 2013; Carnes et al., 2015; Wen et al. 2016) or the trinomial nomenclature (Claes et al., 2006). *T. evansi* is a blood and extracellular parasite that causes equine trypanosomosis, a disease known as Surra in Africa or Derrengadera in South America. Traditionally, *T. evansi* infections have been observed in domestic and wild animals in acute, sub-acute and chronic forms, depending on the virulence of the strain and the parasite-host interplay. Horses and camels, but also donkeys, mules, llamas, dogs, cats, cattle, and buffalo, are susceptible hosts of economic interest (Desquesnes et al., 2013). Other wild mammals, such as capybaras (*Hydrochoerus hydrochaeris*), act as reservoir hosts (Perrone et al., 2009). The clinical signs of *T. evansi* infection include anemia, recurrent fever, weight loss, emaciation, swelling of the hind limbs and hemostatic abnormalities (Holland et al., 2003; Mekata et al., 2013). *T. equiperdum* causes a venereal disease known as Dourine in horses and donkeys and is morphologically indistinguishable from other *Trypanozoon* species (Verducci et al., 1989; Brun et al., 1998). These host species appear to be the only natural reservoirs for *T. equiperdum*. Dogs, rabbits, rats, and mice are susceptible to experimental infections (Spickler et al., 2009). Dourine has been found in Africa, Asia, Russia, Mexico, Southern and Eastern Europe, and several outbreaks in Italy have been reported in recent years (Dávila and Silva, 2000; Wei et al., 2011; Calistri et al., 2013; Pascucci et al., 2013, Vulpiani et al., 2013). Dourine is a serious, often chronic, venereal disease characterized mainly by swelling of the genitalia, cutaneous plaques, and neurological signs. The symptoms vary with the virulence of the strain, the nutritional status of the host, and stress factors.

Pathogenicity is the aptitude of an organism to produce clinical signs of different severity including mortality and is determined by its infectivity and virulence. For parasites, virulence depends on the capacity to multiply inside a host, while infectivity has been defined as the ability of the microorganism to multiply and be maintained in a given host (Holzmuller et al., 2008a). Based on the median survival time, trypanosome virulence has been classified as extreme, when death occurs within a week, while high, death within a month, moderate, death within several months and low, chronic infection and mild or no clinical signs (Herbert and Parratt, 1979). Comparative virulence studies of *T. evansi* isolates from water buffaloes have been carried out in mice (Verdillo et al., 2012). Other authors have reported clear differences in virulence and/or pathogenicity for *T. congolense* strains or

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isolates in bovines (Bengaly et al., 2002a,b; Masumu et al., 2006) and in mice (Bengaly et al., 2002a), for *T. brucei gambiense* strains in Balb/c mice (Holzmuller et al., 2008a) and *T. congolense* and *T. evansi* in nude rats (Holzmuller et al., 2008b). The goal of the study was to compare the infectivity and virulence, in terms of parasitemia levels, clinical signs and survival rates of mice infected with clones derived from *T. evansi* and *T. equiperdum* strains, that were isolated from three different hosts in Venezuela.

2. Materials and methods

Two *T. evansi* strains (TeAp-ElFrio01, from capybara and TeGu-Terecay323, from donkey) and one *T. equiperdum* strain (TeAp-N/D1, from horse) were used in this study (Perrone et al., 2009; Sánchez et al., 2015a,b). To obtain homogenous trypanosome populations, NMRI mice were immunosuppressed with cyclophosphamide (Endoxan, 300 mg/kg) 48 h prior to infection with 1 trypomastigote/mouse (Smith et al., 1982). When parasitemia reached 1×10^6 tryp/ml, mice were bled to obtain the parasites and two similar passages were performed.

For experimental infections, forty male, 12 weeks old NMRI inbred mice, were inoculated subcutaneously with 1 trypomastigote/mouse according to the ethical guidelines of the "*Código Nacional de Ética para La Vida* and the *Manual de Ética para la Producción y Uso de Animales de Laboratorio* (MPPEUCT-Universidad Central de Venezuela-Facultad de Ciencias, 2011)". Parasitemia level, hematocrit %, and body weight were recorded every two days and compared to the control group. Mice deaths were registered daily. All experiments were performed in triplicate and the means and standard errors were determined. The arithmetic means of the infected and the control group were calculated over the course of the experiment, and analyzed by the non-parametric, one-way Analysis of Variance (ANOVA); $P < 0.05$ was considered significant.

3. Results

Mice infected with the *T. equiperdum* clone showed rapidly increasing parasitemia up to day 8, while the groups infected with the two *T. evansi* clones showed lower and fluctuating parasitemias (Fig. 1a). According to the prepatent periods for each of the strains, the TeAp-N/D1 clone can be considered as extremely virulent (2 days), TeAp-ElFrio01 as moderately virulent (5 days) and TeGu-Terecay323 is a low virulence clone (8 days).

The survival of mice curves presented in Fig. 1b show that the highest % survival was observed for the *T. evansi* clone TeGu-Terecay323 (80%). In contrast, the TeAp-ElFrio01 and *T. equiperdum* TeAp-N/D1 clones rendered 100% mortality. Several infectivity and virulence parameters were evaluated for each strain throughout the mice experimental infections (Table1).

The development of anemia is shown in Fig. 1c. In the mice infected with TeAp-ElFrio01, the average hematocrit decreased 15% with respect to the values at the beginning of the infection. In contrast, in the infection with TeGu-Terecay323, the mean hematocrit decreased by 3%. In mice infected with the TeAp-N/D1, the mean hematocrit decreased rapidly and significantly (22%) until day 8 post-infection, when mice died. No anaemia was observed in the control group.

Body weight loss was rapid in mice infected with the *T. equiperdum* clone and correlated with its extreme virulence. A decreasing trend in body weight was observed in the group of mice infected with the moderately virulent *T. evansi.* For the low virulent *T. evansi* clone, no changes in body weight were observed, and this trend correlated with the maintenance of hematocrit values

4. Discussion

In order to evaluate the infectivity and virulence of clonal populations of *T. equiperdum* and *T. evansi*, we focused this study on a parasitological and clinical observation of experimental infection in a susceptible mice model. The rapid death of the group infected by the *T. equiperdum* clone can be attributed to the rapid propagation and high parasitemia levels that translate into energy requirements that exceed the capacity of the host. Both *T. evansi* and *T. equiperdum* are mainly extracellular parasites, the success of the infection is based on the "crosstalk" with the host immune cells through the release of molecules that play a key role on the course and immunopathology of the disease. *T. brucei gambiense* has been shown to promote the release of cytokines such as tumor necrosis factor (TNF), gamma interferon (IFN-γ) and interleukins by macrophages (Holzmuller et al., 2008a). In mice infected with T. brucei, these molecules appear to have toxic effects on the endocrine system of the host (Bosschaerts et al., 2010). Furthermore, IFN-γ produced by $CD8^+$ T cells acts as a growth factor in *T. congolense* (Holzmuller et al., 2008a,b). Likewise, *T. brucei* secretome-triggered BMDCs inhibit LPS-stimulated production of TNF-α and IL-6 proinflammatory cytokines, as well as IL-10. These cytokines are key mediators of Th1 and Th2 responses and are directly involved in the induction of an acquired immune response. The decrease of these cytokines favors parasite growth in the host (Garzón et al., 2013).

In the *T. equiperdum* mice infection, the sharp hematocrit decrease correlated with high parasitemias and accelerated death. In *T. evansi* infections, anemia was observed only in TeAp-ElFrio01. The hematocrit decrease is the most common feature in infections by Salivarian trypanosomes and is used as one of the main disease indicators. It has been described in several hosts of different trypanosomes, including sheep, dogs, coatis and camels (Audu et al., 1999; Reid and Husein, 2001; Herrera et al., 2002; Masumu et al., 2006). The mechanisms that underlie anemia have not been fully elucidated and the pathogenesis caused by these trypanosomatids is extremely complex, multifactorial and appears to include the host response. Several theories have been proposed to explain the anemia that accompanies animal trypanosomosis caused by *T. evansi*. According to Juyal (2002), anemia is a consequence of the host immunological response. They propose that erythrocytes acquire trypanosome antigens, which triggers an immunological reaction and recognition by complement. de Menezes et al. (2004) propose three alternative hypothesis, hemolysis due to the mechanical action of the parasites on the red blood cells, the increase of endocytosis by spleen cells or intravascular clotting due to microvascular lesions and hemolysis. Dargantes et al. (2005) propose that the anemia observed in goats infected by *T. evansi* is caused by the massive destruction of red blood cells by the mononuclear phagocytic system.

Together with the anemia, observation of body weight, rough hair coat and behavior are important parameters to be considered in trypanosome infections. Weight loss in murine models infected with *T. evansi* is due to appetite loss, which in turn results in lipid and copper deficiencies (Desquesnes et al., 2013). Lipids are needed for natural mice hair coat, while lusterless hair coat has been observed in mice infected with either highly virulent or moderately virulent strains (Verdillo et al., 2012). In agreement with previous reports of experimental mice and rat *T. equiperdum* infections, that showed anemia, and progressive loss of condition, mice infected with the TeAp-N/D1 cloned strain did not show local oedema of the genitalia, the mammary glands or oedematous cutaneous eruptions, typical of Dourine (Scacchia et al., 2011; Hagos et al., 2010). The absence of these clinical signs of Dourine may be due to the high mortality and virulence of this strain which results in a very short life for the infected mice.

Biological and pathological features observed upon infection with the *T. equiperdum* and *T. evansi* strains were maintained throughout the stages of cloning (1, 2 and 3, data not shown) and were consistent with the infectivity and virulence characteristics of the heterogeneous strains from which they originated (Holzmuller et al., 2008b). These findings suggest that the infectious phenotype appears to be stable in a cloned population; while plasticity of trypanosome infectivity probably occurs at the population level due to parasite adaptation during transmission cycles over the years.

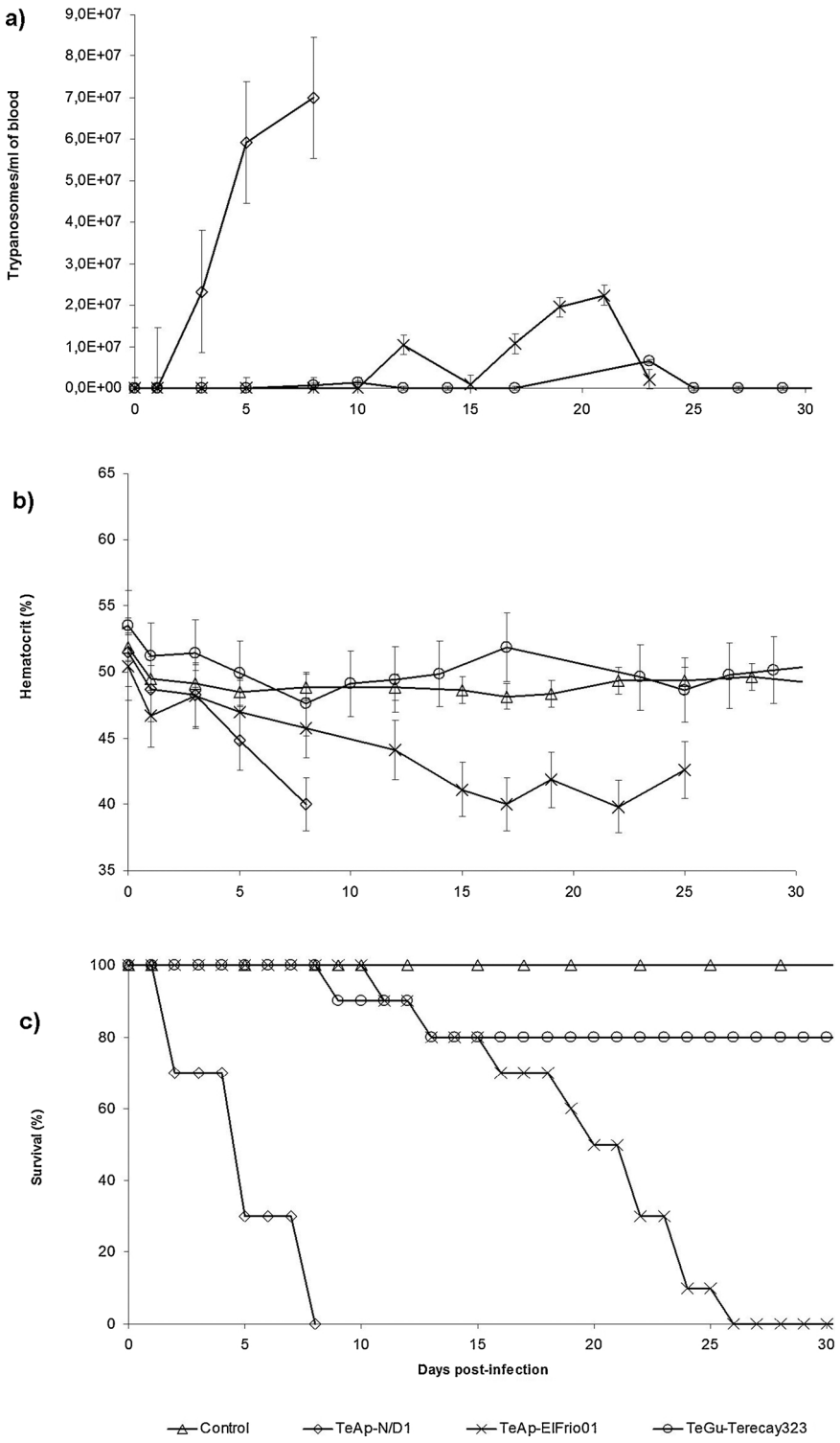

Fig. 1. a) Average parasitemia in experimental mice infections with*Trypanosoma evansi* clones (TeAp-ElFrio01 and TeGu-Terecay323) and a *Trypanosoma equiperdum* clone (TeAp-N/D1). b) Survival curves of the experimental mice. c) Changes in hematocrit values in mice experimentally infected with clones. NMRI mice were inoculated subcutaneously with 1 trypomastigote of either *T. evansi* or *T. equiperdum* cloned strains. A group of non-infected mice was used as control. Mice survival rates were registered daily.

5. Conclusion

Clones derived from the recently characterized Latin American *T. equiperdum* strain TeAp-ND1 maintained the highly virulent and pathological features of the strain, but clinical signs of Dourine were not shown through the experimental infection since mice died within a short period, due to the high infectivity of the strain. Some of the clinical signs in the mice experimentally infected with the *T. equiperdum* clone, including anemia, progressive weight loss, and body condition, were similar to those observed with one of the *T. evansi* clones (i. e. TeAp-ElFrio01). On the contrary, the clone from the TeGu-Terecay323 *T. evansi* strain caused asymptomatic disease, no significant alteration of hematocrit values and very low parasitemias. The differences in virulence and infectivity between the *T. evansi* and *T. equiperdum* clones are consistent with the genetic heterogeneity of these species or subspecies, as reported by Wei et al., 2011; Verdillo et al., 2012;

Table 1
Biological data for the mice infections using the *T. evansi* and *T. equiperdum* clones. % Infection = number of mice with at least 1 trypanosome detected during the follow-up/number of inoculated mice ×100; % Mortality = number of dead mice/number of inoculated mice ×100; Prepatent period = average period prior to the first appearance of parasites in the blood; Maximum parasitemia reached (×10^6 trypanosomes/ml).

Strain	N°.mice inoculated	Prepatent period (days)	Infection (%)	Mortality (%)	Period of mortality (Days)	Maximum parasitaemia (× 10^6 trypanosomes/ml blood)
TeAp-N/D1	10	2	100	100	8	89.8
TeAp-El Fríο01	10	5	100	100	25	59.9
TeGu-Terecay323	10	8	40	20	31	6.48

Desquesnes et al., 2013; Carnes et al., 2015; Sánchez et al., 2015a,b.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contributions

NP propagated the Trypanosoma evansi and Trypanosoma equiperdum strains and obtained the clones used in this study. Analyzed the clinical -hematological parameters throughout the course of the mice infections and contributed to the manuscript draft. TP initiated the molecular characterization of the strains, directed the cloning and the clinical evaluation of the mice infections. PMA conceived the cloning and characterization of the strains. AM participated in the experimental design of the cloning of the strains and contributed to the manuscript draft. PH participated in the experimental design and analysis of all the results and contributed to the manuscript draft. MG participated in the experimental design and contributed to the manuscript draft. All authors read and approved the final manuscript.

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