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Differential infectivity of two *Trypanosoma cruzi* strains in placental cells and tissue

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ABSTRACT

Congenital Chagas disease, caused by *Trypanosoma cruzi* (*T. cruzi*), has become epidemiologically relevant. The probability of congenital transmission depends on the maternal and developing fetal/newborn immune responses, placental factors and importantly, the virulence of the parasite. It has been proposed, that different genotypes of *T. cruzi* and their associated pathogenicity, virulence and tissue tropism may play an important role in congenital infection. Since there is no laboratory or animal model that recapitulates the complexities of vertical transmission in humans, here we studied parasite infectivity in human placental explants (HPE) as well as in the human trophoblast-derived cell line BeWo of the Y(DTU II) and the VD (TcVI) *T. cruzi* strains; the latter was isolated from a human case of congenital infection.

Our results show that the VD strain is more infective and pathogenic than the Y strain, as demonstrated by qPCR and cell counting as well as by histopathological analysis.

The present study constitutes the first approach to study the relationship between parasite two parasite strains from different genotypes and the infection efficiency in human placenta.

1. Introduction

Congenital Chagas disease, caused by *Trypanosoma cruzi* (*T. cruzi*) is an increasing relevant public health problem in Latin-America and other non-endemic countries (Monge-Maillo and Lopez-Velez, 2017; Pennington et al., 2017). Due to the decline in new cases of infection by insect vectors, congenital transmission has become more important being the main mode of spread of the disease in non-endemic countries (Alvarez et al., 2017) and reaching an estimated 22% of new *T. cruzi* cases in 2015 (WHO, 2015). Even if vector-borne transmission were interrupted today, infected girls and women would continue to transmit the infection to their children, sustaining the cycle across generations in the absence of the vector (Messenger et al., 2015; Schenone et al., 2001).

Congenital Chagas disease is an acute infection with 27–57% asymptomatic cases in children (Carlier et al., 2011; Pennington et al., 2017). Transplacental *T. cruzi* infection has been associated with premature labor, low birth weight, and stillbirths. Older studies report high morbidity and mortality rates, but recent studies refer non-lethal congenital cases (Carlier et al., 2015; Liempi et al., 2016). The probability of congenital transmission depends on the maternal and developing fetal/newborn immune responses, placental factors and importantly, the virulence of the parasite (Fretes et al., 2012; Liempi et al., 2016).

T. cruzi has been classified into seven discrete typing units (DTUs), TcI–TcVI and Tcbat according to biological, biochemical and genetic diversity (Zingales, 2018; Zingales et al., 2012). Each DTU comprises several parasite strains which are related to each other based on shared molecular markers. However, these molecular markers currently used to define the *T. cruzi*-DTUs do not focus on the genes responsible for congenital transmission or pathogenicity of the parasite. Parasites from all DTUs, except TcIV, have been identified in human cases of congenital *T. cruzi* infection (Carlier and Truyens, 2015; Juiz et al., 2017). It has been proposed, that different genotypes of *T. cruzi* and their associated pathogenicity, virulence and tissue tropism may play an important role in congenital infection (Juiz et al., 2017).

During congenital transmission, *T. cruzi* has to cross the placental

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barrier to infect the developing fetus (Carlier et al., 2012; Liempi et al., 2016). This barrier is formed by the trophoblast, a two-layer epithelium which is in direct contact with maternal blood, the fetal connective tissue (villous stroma), the endothelium of fetal vessels and the basal laminae that support the epithelia (Arora et al., 2017; Liempi et al., 2016). Importantly, no laboratory or animal model recapitulate the complexities of vertical transmission in humans. The placenta of the small animals commonly used as experimental models display significant anatomic differences relative to the human placenta (Arora et al., 2017). However, human placental explants (HPE) and the choriocarcinoma-derived cell line BeWo have been successfully used to study *T. cruzi* infection (Fretes and Kemmerling, 2012; Liempi et al., 2015). Here we investigated the differences in parasite infectivity in human placental explants (HPE) as well as in the human trophoblast-derived cell line BeWo of two different *T. cruzi* strains, the Y(DTU II) (Zingales et al., 2012) and VD (TcVI) strains (Juiz et al., 2017; Risso et al., 2004). The latter, also known as Cvd strain (Risso et al., 2004), was isolated from a human case of congenital infection (Juiz et al., 2017; Risso et al., 2004).

Our results show that the VD strain is significantly more infective and pathogenic than the Y strain, as demonstrated by qPCR and cell counting as well as by histopathological analysis.

2. Materials and methods

2.1. Parasites

2.1.1. Parasite strains origin

The Y strain was isolated in Brazil (1953) from a woman and her daughter, both diagnosed with acute Chagas disease. The *T. cruzi* isolated from these patients showed marked mortality among inoculated animals; thus, proving the highly expressive virulence of the strain. Due to this trait, the protozoan in question was the target of special characterization. It received the designation of "Y" strain from the first letter of the young patient's name (Amato Neto, 2010). The strain used in this study has been classified as DTU II (Cura et al., 2015). The VD strain has been isolated from an Argentinean infant with congenital Chagas disease, admitted at the Hospital Ricardo Gutierrez of Buenos Aires. The strain used in this study has been classified as DTU VI (Cura et al., 2015).

2.1.2. Epimastigote cultures

Both Y and VD strains were routinely maintained in axenic culture at 28 °C in liver infusion (LIT) media supplemented with 10% fetal calf serum, 20 μg/mL haemin, 100 μg/ml of streptomycin and 100 U/ml of penicillin (Ponce et al., 2017).

2.1.3. Transfection of parasites with GFP (green fluorescence protein) and RFP (red fluorescence protein)

Epimastigotes were harvested at exponential phase after 48 h of culture by centrifugation at 3000 × *g* for 10 min at room temperature. Following pellet washing in PBS, 1×10^8 parasites were suspended in 350 μl of electroporation buffer (PBS 1x, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$) and mixed with 10–15 μg of the transfection vectors pTREXrfp (VD strain) or pTREXgfp DNA (Y strain). Parasite suspensions were electroporated in 0.2 cm gap cuvettes with a discharge of 400 V 500 μF, yielding time constants varying between 3.5 and 5 ms. Parasites were then diluted in 5 ml of LIT medium and incubated at 28 °C for 48 h to allow recovery before the addition of 100 μg/ml of G418 (Sigma). Following 7–10 days of incubation, mock-transfected parasites completely stopped dividing, and resistant parasites were incubated in the presence of G418 for another two weeks before cloning them by serial dilution in 96-well plates. Only stable cell lines were used in this work. Transfection efficiency was assessed by fluorescence microscopy (Ponce et al., 2017).

2.1.4. Trypomastigotes

Metacyclic trypomastigotes were obtained from TAU3 AAG medium as previously described (Contreras et al., 1985). Vero cells (ATCC® CCL-81) grown in RPMI medium supplemented with 5% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin) at 37 °C in a humid atmosphere at 5%CO_2 were infected with metacyclic trypomastigotes or trypomastigotes from a previous culture. Trypomastigotes then invaded the cells and replicated intracellularly as amastigotes, after 48–72 h, amastigotes transformed back to trypomastigotes and lysed host cells. The infective trypomastigotes were separated from cellular debris by low-speed centrifugation (500 × *g*). From the supernatant, the parasites were isolated by centrifugation at 3500 × *g*, suspended in RPMI media (without FBS, 1% antibiotics) (RPMI 1640, Biological Industries Ltd.) and quantified in a Neubauer Chamber (Castillo et al., 2013; Liempi et al., 2014).

2.2. Infection of BeWo cells with T. cruzi trypomastigotes

BeWo cells (ATCC CCL-98) were grown in DMEM-F12 K medium supplemented with 10% FBS, L-glutamine and antibiotics (penicillin-streptomycin) (Drewlo et al., 2008). Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2, with replacement of the culture medium every 24 h. BeWo cells were detached by trypsinization, sedimented and resuspended in 10% FBS-containing medium. Next, 2×10^5 cells were seeded in 6-well plates. The cells were allowed to adhere to the bottom for 3 h and then challenged with the parasite at a BeWo cell:parasite ratio of 1:1. The cells were analyzed at 48 h post-infection (Liempi et al., 2015).

2.3. HPE culture and infection with T. cruzi trypomastigotes

Human term placentas were obtained from uncomplicated pregnancies from vaginal or cesarean deliveries. Informed consent for the experimental use of the placenta was given by each patient as stipulated by the Code of Ethics of the Faculty of Medicine of the University of Chile. The exclusion criteria for the patients were the following: major fetal abnormalities, placental tumor, intrauterine infection, obstetric pathology, or any other maternal disease. The organs were collected in a cold, sterile saline-buffered solution (PBS) and processed no more than 30 min after delivery. The maternal and fetal surfaces were discarded, and villous tissue was obtained from the central part of the cotyledons. The isolated chorionic villi were washed with PBS to remove blood, dissected into approximately 0.5-cm^3 fragments and co-cultured with *T. cruzi* trypomastigotes (1×10^5/ml) for 24 h in RPMI culture medium supplemented with inactivated FBS and antibiotics (Duaso et al., 2010).

2.4. Histopathology

HPE were fixed in 4% formaldehyde 0.1 M phosphate buffer (pH 7.3) for 12 h at room temperature and processed by standard histologic methods for the inclusion in paraffin blocks. Haematoxylin-Eosin (HE) staining was performed with Haematoxylin solution Gill N°2 and Eosin Y solution (Sigma-Aldrich®). Slides were Mounted with Histofluid Mounting Media (Marienfield®), and images were captured in a Motic BA310 microscope equipped with a 5.0 M P Moticam Camera. Then, ten fields were selected randomly, the tissue damage was analyzed, and scored as follows: +, low; + +, moderate; + + +, severe (Duaso et al., 2010).

2.5. Parasite detection

2.5.1. DNA amplification by real-time PCR (qPCR)

Genomic DNA was extracted from HPE and BeWo cells with a Wizard Genomic DNA Purification Kit (Promega®, USA) according to the manufacturer's instructions. The resulting DNA was quantified with