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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

a μDrop Plate DNA quantification system in a Varioskan Flash Multimode Reader (Thermo Scientific, USA). For amplification of human and parasite DNA, two specific primer pairs were used. A 100 bp human GAPDH sequence was amplified using the primers hGDH-F (5′-TGATGCGTGTACAAGCGTTTT-3′) and hGDH-R (5′-ACATGGTATTC ACCACCCCACTAT-3′), which were designed using Primer Express software (version 3.0; Applied Biosystems®). For *T. cruzi* DNA detection, a 182 bp sequence of satellite DNA was amplified using the primers TCZ-F (5′-GCTCTTGCCCACAMGGGTGC-3′) and TCZ-R (5′-CAAGCAGC GGATAGTTCAGG-3′) (Castillo et al., 2012; Cummings and Tarleton, 2003). Each reaction mix contained 200 nM of each primer (forward and reverse), 1 ng of DNA, 12.5 μl of SensiMix® SYBR Green Master Mix (Bioline®, USA) and H_20 for a total volume of 25 μL. The amplification was performed in an ABI Prism 7300 sequence detector (Applied Biosystems®, USA). The cycling program was as follows: an initial incubation at 20 °C for 2 min, a denaturation step at 95 °C for 10 min and 40 amplification cycles at 95 °C (15 s), 60 °C (15 s) and 72 °C (30 s). The final step was a dissociation stage that ranged from 60 to 95 °C (105 s). The relative quantification analysis of the results was expressed as an RQ value determined using the comparative control (ΔΔCt) method (Castillo et al., 2012; Pfaffl, 2001).

2.5.2. Immunofluorescence in BeWo cells

RFP and GFP were detected in BeWo cells cultured onto cover slides and fixed on 70% ice-methanol for 30 min. Following fixation, samples were treated with blocking solution (BSA 1% p/v, saponine 0.1% v/v, calf serum 3% v/v in PBS) for 2 h at 37 °C and incubated overnight at 4 °C with a monoclonal anti-GFP or anti-RFP antibody (Thermo Scientific). Samples were then washed and incubated with a secondary antibody conjugated to Fluoresceine (FITC-antimouse) fluorochrome or Rhodamine (TRITC-antirabbit) (Molecular Probes). Nuclear and kinetoplastid DNA were labeled with 4′,6-diamidino-2-phenylindole (DAPI). Samples were evaluated by fluorescence microscopy observation using 492 nm, 550 nm and 520 nm filters for blue, red and green fluorescence, respectively (Sepulveda et al., 2014). Photographs were processed computationally to determine the overlap of DAPI (blue), rhodamine 405 (red) and fluorescein (green). *T. cruzi* amastigote were recognized by their nuclear size and presence of kinetoplast as well as RFP or GFP immunostaining and quantified using MATLAB® software (Liempi et al., 2015).

2.6. Statistics

All experiments were performed in triplicate. Results are expressed as means ± S.D. The significance of differences was evaluated using Student's *t*-test for paired data.

3. Results

3.1. T. cruzi VD strain is more infective than Y strain during in vitro infection of the trophoblastic cell line BeWo

BeWo cells were incubated in the presence and absence of *T. cruzi* trypomastigotes VD-RFP or Y-GFP lines for 48 h at a cell:parasite ratio of 1:1. Both strains infect the BeWo cells (Fig. 1A), however the percentage of cells infected with VD-RFP is significantly higher (48.8 ± 5.2%) than that of the ones infected with Y-GFP line (37.2 ± 3.7%; $p \leq 0.001$) (Fig. 1B). Moreover, the number of the intracellular amastigote forms per cell is significantly higher ($p \leq 0.001$) in VD-RFP infected cells (2.45 ± 0.05) than in Y-GFP infected ones (1.53 ± 0.1) (Fig. 1C).

In the experiments of co-infection (Fig. 2), cells were challenged at a cell:parasite ratio of 1:0.5 of each parasite line during 48 h (total cell: parasite ratio 1:1). Interestingly, cells infected with one of each parasite line as well as with both of them can be observed (Fig. 2A). The percentage of BeWo cells infected with VD-RFP, Y-GFP or both genotypes were 59.1%, 2.8% and 38.6%, respectively (Fig. 2B).

3.2. T. cruzi VD strain is more infective than Y strain in ex vivo infection of HPE

HPE were co-cultured with *T. cruzi* trypomastigotes (1×10^5/ml) from the VD-RFP or Y-GFP lines during 24 h. Samples incubated with the VD-RFP line presented a significant (3.16 ± 0.36; $p \leq 0.0001$) higher DNA parasite load than the explants infected with the Y-GFP line (1.0 ± 0.576) (Fig. 3A). On the other hand, 24 h co-infection of HPE with 0.5×10^5/ml trypomastigotes from each line showed a significantly higher DNA parasite load of VD-RFP

line parasites (2.16 ± 0.888; $p \leq 0.01$) than of the Y-GFP line ones (1.0 ± 0.336) (Fig. 3B).

Moreover, the VD-RFP line induces a more severe histopathological damage in the HPE than the Y-GFP line. The tissue damage can be evidenced by trophoblast destruction and detachment (arrows) as well as by disintegration of the fetal connective tissue of the villous stroma (asterisk). Trypomastigotes from the VD-RFP line induced a severe (+ + +) tissue damage (Fig. 4B) compared to control (Fig. 4A) or to the Y-GFP line infected samples (Fig. 4C) (+). HPE that were co-infected with both strains showed a moderate (+ +) tissue damage (Fig. 4D).

4. Discussion

The interaction between the host and pathogens is the main factor in determining whether an infection is successful. Host-parasite interaction includes invasion of the host through primary barriers (such as the placental barrier), evasion of host defenses, pathogen replication in the host, and immunological capacity of the host to control or eliminate the pathogen (Sen et al., 2016). It has been demonstrated that different *T. cruzi* genotypes and their population

characteristics, such as virulence and tissue tropism, are related to specific clinical manifestations, particularly to the outcome of chagasic cardiomyopathy (Messenger et al., 2015; Rodriguez et al., 2014).

It has been proposed previously by us and others, that the congenital transmission of pathogens is the consequence of complex interactions among the parasite, maternal and fetal/newborn immune responses, as well as placental factors (Carlier and Truyens, 2015; Liempi et al., 2016). All these different factors, involved in the probability of *T. cruzi* transmission, are being investigated but the exact physiopathology is far from being resolved.

It is difficult to study the association between *T. cruzi* genotypes and the risk of congenital

transmission, since only a small sample volume of neonatal blood can be obtained, the access to infected placental tissue samples is complicated (due to the reduced transmission rates), the sensitivity of conventional diagnostic and genotyping methods is low and the follow up of the patients is deficient (Duffy et al., 2013; Juiz et al., 2016).

It has been proposed that the different genotypes of *T. cruzi* may play an important role in congenital infection. However, available data in humans demonstrating an association between particular *T. cruzi* genotypes with congenital infection is not conclusive. There are no differences in the distributions of congenital cases and their respective parasite populations (Burgos et al., 2007; Carlier and Truyens, 2015; Corrales et al., 2009; Messenger et al., 2015; Virreira et al., 2007). Therefore, the genotypes of congenital cases mirror the distribution of lineages observed among the different local chronic adult populations. Moreover, the same genotypes were detected in peripheral blood from mothers and their respective infected newborns (Burgos et al., 2007; Llewellyn et al., 2015; Virreira et al., 2007). Thus, it seems that placental invasion ability depends on each parasite strain rather than on a given genotype.

It is also important to consider that *T. cruzi* also presents an important genetic diversity at the intra-DTU level. Therefore it is reasonable to propose, that there are strains and clones of any lineage

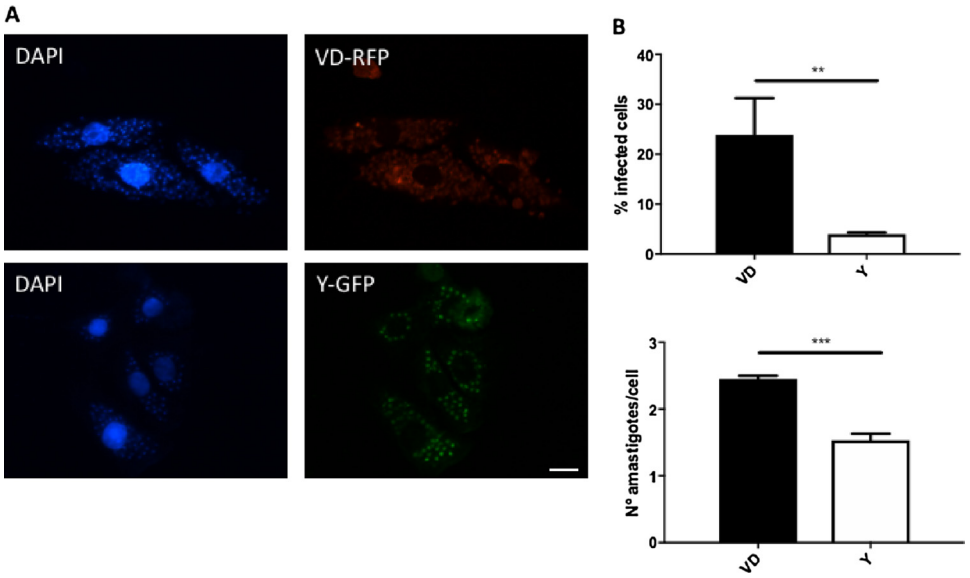

Fig. 1. *T. cruzi* VD strain is more infective than Y strain during *in vitro* infection of the trophoblastic cell line BeWo.
Cells were incubated in the presence and absence of *T. cruzi* trypomastigotes VD-RFP or Y-GFP lines for 48 h at a cell:parasite ratio of 1:1. (A) Representative images of cells infected with the VD strain (upper panels) or the Y strain (lower panels) are shown, scale bar: 5 um. (B) Data shows the percentage of infected cells and (C) the average of amastigotes per cell infected with which each strain. Data represents means ± SD and were analyzed by Student *t*-test $^{**}p < 0.01$; $^{***}p < 0.001$.

better adapted for transplacental infection (Messenger et al., 2015).

There are several evidences that certain *T. cruzi* genotypes present a tropism toward the human placenta. For instance, in a study where infected human placental tissue was analyzed, additional minicircle signatures not observed in matched maternal blood samples were detected (Bisio et al., 2011). Others have described parallel discordant minicircle profiles between paired maternal–neonate blood specimens, implying either the generation of novel mutations by rapid parasite multiplication during acute neonatal infection or selective transmission of parasite subpopulations (Virreira et al., 2007).

Different placental tropism of *T. cruzi* strains has been described in mice (Andrade, 1982; Cencig et al., 2013; Juiz et al., 2017; Solana et al., 2002). Thus, a study comparing two different strains (K98 and RA, belonging to TcI and TcVI, respectively) evidenced differences in inflammatory response in the genital tract, the outcome of pregnancy and transmission of congenital infection (Solana et al., 2002). Another study, comparing TcI, TcII and TcVI genotypes demonstrated a low transmission rate for all the studied strains, but TcVI was more infective (Cencig et al., 2013). Additionally, other study (Juiz et al., 2017) showed that VD (TcVI) was more infective in murine placenta than K98, a clone of the non-lethal myotropic CA-I strain; as it was demonstrated by comparing parasitic burden in maternal blood, placental and fetal tissues as well as by parasite viability, determined by *T. cruzi* 18S RNA-based expression. Moreover, the K98 and VD strains elicited a significant different gene expression profile in murine placentas, as determined by means of functional genomics and biological network analyses (Juiz et al., 2017). Indeed, VD was associated with a higher degree of up-regulation in murine genes related to innate immunity and response to interferon-gamma (Juiz et al., 2017).

To our knowledge, the present study constitutes the first approach to directly study the difference of two *T. cruzi* strains from different genotypes and the efficiency of infection in human placenta. Our results clearly show that parasite strain VD is much more infective than the Y strain (Figs. 1–4). The Y strain was isolated from a mother and her seven-month old daughter, both with acute Chagas disease and becoming most probably infected by vectorial transmission at their rural residence in the state of Sao Paulo, Brazil. The strain was well characterized and showed high virulence. The baby was not treated and when she was followed-up 25 years later, she presented asymptomatic chronic infection (Amato Neto, 2010). In contrast, the VD strain was isolated from a human case of congenital infection (Juiz et al., 2017; Risso et al., 2004), which supposes a higher capacity to surpass the placental anatomical barrier. The mechanisms of the strain-associated virulence and infectivity in human placenta remains to be elucidated. Different virulence factors of the parasite, such as *Trans*-sialidase (Burgos et al., 2013), mucins and mucin-associated surface proteins as

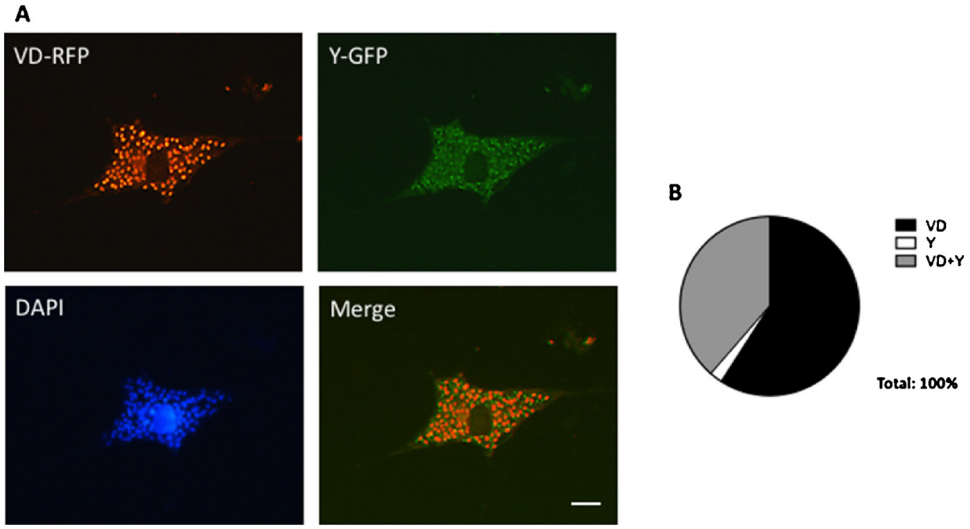

Fig. 2. *T. cruzi* VD strain is more infective than Y strain during *in vitro* co-infection of the trophoblastic cell line BeWo.
Cells were incubated in the presence and absence of *T. cruzi* trypomastigotes of both VD-RFP or Y-GFP lines for 48 h at a total cell:parasite ratio of 1:1. The cell: parasite ratio of each strain was 1:0.5. (A) Representative images of a cell infected with both, VD and Y strains, are shown, scale bar: 5 µm. (B) Data shows the percentage of infected cells with only each strain and both.

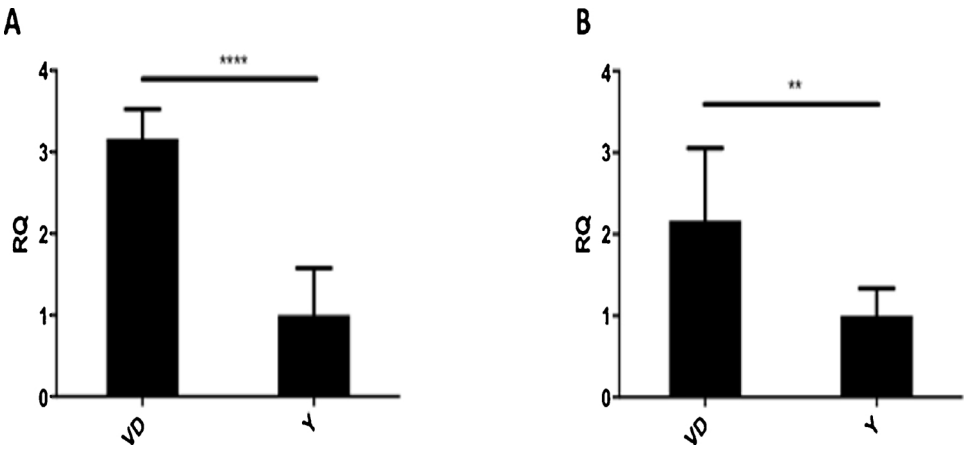

Fig. 3. *T. cruzi* VD strain is more infective than Y strain during *ex vivo* infection of human placental chorionic villi explants.
HPE were incubated in absence or presence of 10^5 tripomastigotes VD-RFP or Y-GFP lines (A) or in presence of 0.5×10^5 parasites of each line (B) for 24 h. Data are a comparison of parasite DNA in 1 ng of total DNA isolated from infected HPE. Real-time quantification by qPCR was performed using ΔΔCt method. Data represents means ± SD and were analyzed by Student *t*-test ** $p < 0.01$, **** $p < 0.0001$.

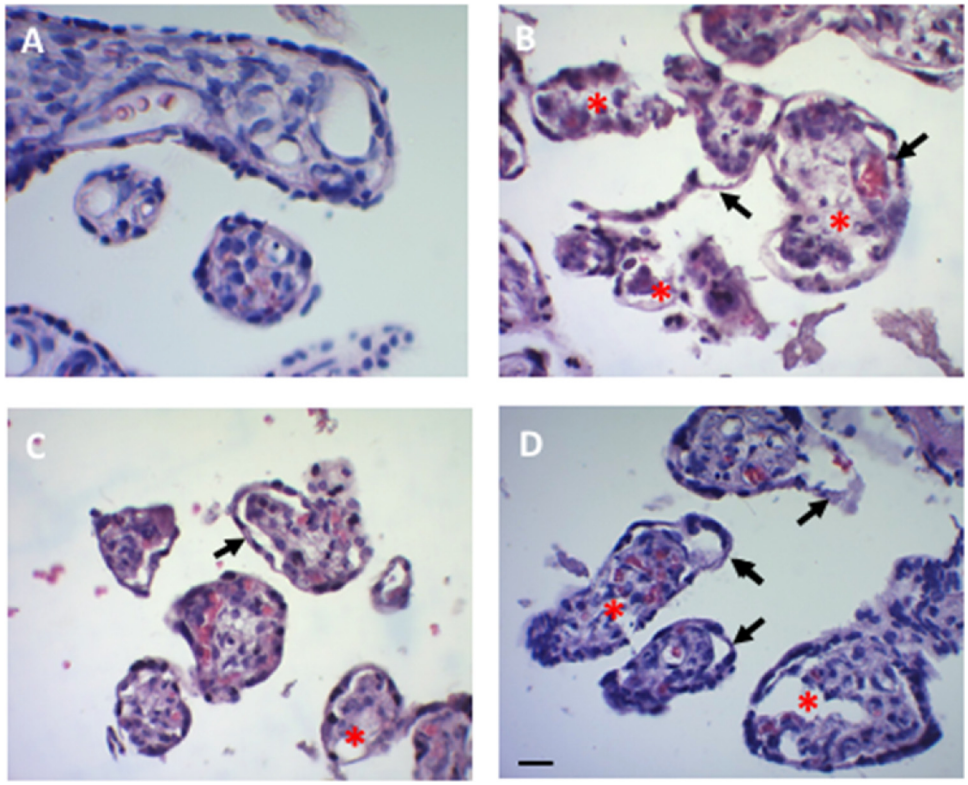

Fig. 4. *T. cruzi* VD induces a more severe tissue damage than Y strain during *ex vivo* infection of human placental chorionic villi explants.
HPE were incubated in absence (A) or presence of 10^5 tripomastigotes VD-RFP (B), Y-GFP lines (C) or in presence of 0.5×10^5 parasites of each line (D) for 24 h. Tissue were processed for routine histological methods and stained with Hematoxylin-Eosin, scale bar: 20 µm.

well as exovesicles (De Pablos et al., 2016), between others might be involved. On the other hand, host factors such as sequence polymorphisms and level of expression in placentary expressed genes as well as in genes related to the immune-response may be also involved in the probability of congenital transmission (Juiz et al., 2016). These are open questions needing urgent answers to better understand the mechanisms involved in *T. cruzi* congenital infection.

5. Conclusion

We conclude that the VD (DTU VI) strain present a higher infectivity in the human placenta than the Y (DTU II) one, pointing to the importance of parasite strains in human congenital infections.

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