Experimental infection with the *Toxoplasma gondii* ME-49 strain in the Brazilian BR-1 mini pig is a suitable animal model for human toxoplasmosis

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Toxoplasma gondii causes toxoplasmosis, a worldwide disease. Experimentation with pigs is necessary for the development of new therapeutic approaches to human diseases. BR-1 mini pigs were intramuscularly infected with *T. gondii* with tachyzoites (RH strain) or orally infected with cysts (ME-49 strain). Haematology and serum biochemistry were analysed anduffy coat cells were inoculated in mice to determine tachyzoite circulation. No alterations were observed in erythrocyte and platelet values; however, band neutrophils increased seven days after infection with ME-49. Serology of the mice inoculated with pig blood leucocytes revealed circulating ME-49 or RH strain tachyzoites in the pigs’ peripheral blood at two and seven or nine days post-infection. The tachyzoites were also directly observed in blood smears from the infected pigs outside and inside leucocytes for longer periods. Alanine-aminotransferase was high at days 21 and 32 in the RH infected pigs. After 90 days, the pigs were euthanised and their tissue samples were processed and inoculated into mice. The mice serology revealed the presence of parasites in the hearts, ileums and mesenteric lymph nodes of the pigs. Additionally, cysts in the mice were only observed after pig heart tissue inoculation. The infected pigs presented similar human outcomes with relatively low pathogenicity and the BR-1 mini pig model infected with ME-49 is suitable to monitor experimental toxoplasmosis.

**Key words:** *Toxoplasma gondii* - pig infection - haematology - parasitaemia - neutrophils
animals were observed daily. The pig infections were con-
cystogenic strain. After infection, the clinical signs of the
and platelets counting (MS4 Melet Schloesing®
processed for automatic white blood cell, red blood cell
cles (3-5 mL) with EDTA from all of the animals were
performed with an ophthalmoscope (ODN 4.4; Eyetec).
Indirect fundoscopy was
a week in the first 14 days, twice a week for an extra 16
days after infection. The pigs were sedated with an intra-
muscular acepromazine (0.22 mg/kg) injection that was
combined with ketamine (20 mg/kg) for blood collection
and homogenised. After quantification, the tachyzoites or
cysts from the mice were used to infect the pigs.

Ethics - This study was carried out in strict accordance
with the Brazilian Law #11794/08. The animal study pro-
tocol was reviewed and approved by the UENF Commit-
tee on the Ethics of Animal Experiments (permit 130).

Infection, blood collection and fundoscopy - Two
groups of four pigs each were infected and a third group
was a non-infected control group. The “ME-49” group
was infected by an oral route with 660 cysts of ME-49
strain and the “RH” group was infected intramuscu-
larly with 1 x 10⁷ RH strain tachyzoites, which is a non-
cystogenic strain. After infection, the clinical signs of the
animals were observed daily. The pig infections were con-
frmed by serological testing with the modified agglutina-
tion test (MAT) (Dubey & Desmonts 1987) before and 30
days after infection. The pigs were sedated with an intra-
muscular acptomazine (0.22 mg/kg) injection that was
combined with ketamine (20 mg/kg) for blood collection
and fundoscopy of all animals. The blood was collected
from the auricular vein with ethylenediamine tetracetic
acid (EDTA) as an anti-coagulant and purified for serum
obtainment. Both procedures were performed three times
a week in the first 14 days, twice a week for an extra 16
days and weekly up to 90 days. Indirect fundoscopy was
performed with an ophthalmoscope (ODN 4.4; Eyetec).

Haematology and serum biochemistry - Blood sam-
ple (3-5 mL) with EDTA from all of the animals were pro-
cessed for automatic white blood cell, red blood cell and platelets counting (MS4 Melet Schloesing®). Haemo-
globin concentrations, haematocrit percentages, the mean
corpulsus volumes and the mean corpuscular haemo-
globin concentrations were also assayed by a cited auto-
mated procedure. In parallel, blood smears were stained
with Giemsa in order to identify and estimate the differen-
tial leucocytes counts and to detect possible morphologi-
al alterations and the presence of parasites. One hundred
leucocytes were differentially counted per animal.

Serum was collected from 5 mL of blood right after
coaagulation, stored in individual vials and frozen (-70°C)
until biochemistry determination of the following was
conducted: urea, creatinine, alanine aminotransferase
(ALT), aspartate aminotransferase and alkaline phos-
phatase. The serum biochemistry was assayed in a semi
automatic spectrophotometer (BTS 310 BioSystems®).

Parasitaemic determination - During the first 14 days,
parasitaemia was determined by mouse bioassays. Blood
collected with EDTA (5 mL) was centrifuged (500 g, 10
min), the buffy coat was collected and washed once with
PBS and the cell pellet was resuspended in 1.5 mL of PBS.
This suspension was inoculated intraperitoneally (0.5 mL)
in three adult mice. After 40 days, the serum of the mice
that survived was submitted to the MAT with a cut-off titre
of 1:25 (RH and ME-49 groups) and the brains were ob-
served for the presence of cysts (the ME-49 group only).

Necropsy and histopathology - After 90 days post-
infection (dpi), all of the pigs were euthanised, necrop-
sied and the tissues was processed for pathology exami-
nation. Tissues were fixed in 10% buffered formalin,
routinely processed in alcohol and xylol, embedded in
paraffin and 5 μm sections were stained with haematox-
ylin and eosin. Eleven organs were analysed, including
the heart, lung, liver, spleen, kidney, ileum, brain, me-
senteric lymph node, skeletal muscle, tongue and retina.

Renal stereology - The kidney volumes of all four pigs
in each group (infected with the ME-49 or RH strains
and non-infected control), cleared of adipose tissue, was
measured by the water displacement method. The right
kidneys were transversely sliced in 2 cm sections that
were fixed in 4% formaldehyde for later cortical-medullar
ratio determination by the point-counting method (de
Souza et al. 2011) and the absolute cortical volume was
calculated by multiplying the cortical-medullary ratio by
the renal volume. The left kidneys were sectioned frontally
and then randomly picked cortical area fragments were
fixed in 10% formaldehyde and routinely processed for
paraffin embedding. From each animal, 26 histological
fields that were obtained from different cortex sections
were acquired with a digital camera that was coupled to a
microscope. An M42 test-system was used for glomeru-
lar volume density estimation by the point-counting tech-
nique. The volume weighted mean glomerular volume
was estimated with the point-sampled intercept method
(de Souza et al. 2012) and 50 glomeruli per animal were
analysed. The total glomeruli number per animal estimation
was calculated by multiplying the cortical volume by
the glomerular volume density and then divided by the
volume weighted mean glomerular volume.

Tissue bioassay in mice - Eleven organs from each
pig were analysed with the bioassay method and the fol-
lowing were collected and processed: approximately 100
g of heart, lung, liver, spleen, kidney, brain, muscle and
tongue tissue, 10 cm ileum fragments, an entire eye and
the mesenteric lymph nodes. Tissues were digested in
pepsin and their homogenates were inoculated in mice
according to the Dubey (1998) protocol, with minor mod-
ifications, as follows. Each tissue was ground separately
with saline and the homogenates were incubated for 60
min in a shaker at 37°C with 200 mL of pre-warmed
acidic pepsin solution (0.52%, pH 1.2). The product was
filtered through two layers of gauze and centrifuged at
1,200 g for 10 min. The supernatant was poured off,
the sediment was resuspended in 20 mL of PBS and it
was then transferred to a 50 mL centrifuge tube with a
conical bottom and neutralised with 15 mL of sodium
bicarbonate (1.2%, pH 8.3). After it was mixed, the solu-
tion was centrifuged at 1,200 g for 10 min and the sedi-
ment was resuspended with 10 mL of PBS that contained
1,000 units of penicillin and 100 μg/mL of streptomycin.
Blender jars, cutting boards and other materials were sterilised with soap and hot water (100°C) between tissue processing. One millilitre of tissue homogenate was inoculated intraperitoneally for each of three adult mice and a control group was inoculated with the same volume of PBS. Serology and brain cyst searches were performed for parasitaemia determination.

Statistical analysis - The numerical data were analysed statically and compared using the Wilcoxon test and the one-way-ANOVA followed by Dunnett’s test. p < 0.05 was considered significant.

RESULTS

Clinical data - Two pigs out of four per group became apathetic and lethargic between 2-9 dpi for the RH group and 4-11 dpi for the ME-49 group. One RH group pig, which had clinical acute phase signs of the disease, presented increased and abnormal aggressiveness during the chronic phase. No ophthalmic lesion was found in the pigs from both infected groups.

The haematological and serum biochemistry parameters - No alterations in the global number of leucocytes, erythrocytes or platelet values were found among the pig groups, but a significant increase in band neutrophils was seen at 7 dpi in the ME-49 group (Fig. 1A). Moreover, we observed a tendency for monocytosis at 7 dpi in the ME-49 group pigs (Fig. 1B). These changes were not seen in the RH and control group pigs.

A bioassay in the mice that were administered swine blood leucocytes - A bioassay test that was performed with the harvested blood leucocytes showed parasitaemia only at 9 dpi in the RH group pigs and at 2 and 7 dpi in the ME-49 group pigs (Table I). However, tachyzoites

![Fig. 1: neutrophils and monocytes numbers during the infection of BR-1 pigs with Toxoplasma gondii of the ME-49 or RH strains. Band neutrophils (A) and monocytes (B) were differentiated counted in each of the four animals per group to determine possible changes caused by the infection; data are presented as means and standard deviation per group. An increase in the number of band neutrophils and a tendency for monocytosis at seven days post-infection in pigs of the ME-49 group (gray) were observed when compared with the RH (white) and control (black) groups. Asterisk means significantly different (p < 0.05) from the respective non-infected group value.](image1)

![Fig. 2: alanine aminotransferase (ALT) activity during the infection of BR-1 pigs with Toxoplasma gondii of the ME-49 or RH strains. ALT was evaluated in each of the four animals per group to determine possible changes caused by the infection; data are presented as means and standard deviation per group. A tendency at six days post-infection (dpi) and a significant increase at 21 and 32 dpi was observed compared with the controls at those days (Fig. 2). All other serum biochemistry assays were not different during the infection period.](image2)

| Pig ID | ME-49  | RH  |
|-------|--------|-----|
| 4     | -      | -   |
| 5     | -      | -   |
| 11    | -      | -   |
| 12    | -      | -   |
| 3     | -      | -   |
| 1     | -      | -   |
| 2     | -      | -   |
| 10    | -      | -   |
| 14    | -      | -   |

Table I: Bioassay in mice inoculated with blood leukocytes of pigs experimentally infected with RH or ME-49 Toxoplasma gondii strains

| Pig ID | ME-49  | RH  |
|-------|--------|-----|
| 2     | +\*    | -   |
| 4     | -      | -   |
| 7     | -      | +   |
| 9     | -      | -   |
| 10    | -      | -   |
| 12    | -      | -   |
| 14    | -      | -   |

\*: days post-infection; \*: seropositive by the modified agglutination test (cut-off titre 1:25).
were observed in the peripheral blood of the pigs before and after these periods. The RH group pigs showed tachyzoites inside neutrophils at early (Fig. 3A, B) and late (Fig. 3C) dpi and they were also observed outside leucocytes in the late infections (Fig. 3D). Additionally, ME-49 strain tachyzoites were also seen in neutrophils at early (Fig. 3E) and late dpi (Fig. 3F) and in monocytes in the early infections (Fig. 3G). As expected, no parasites were found in the blood of the control group animals.

**Tissue bioassay in the mice** - Serology of the mice that received tissues from the ME-49 group pigs that were infected for 90 days indicated the presence of *T. gondii* in the heart, mesenteric lymph nodes and ileum (Table II). However, cysts were only visualised in the brains of the mice that were inoculated with the pig hearts (Table II). Inoculation of tissues from the RH group did not serum convert the mice because this strain does not form cysts in pig tissues (Dubey et al. 1994).

**Histological analyses** - No histopathology alteration or cysts were seen in the pig tissues examined. Additionally, the stereological analysis of the swine kidneys showed no significant differences among the groups. These numerical results are presented in Table III.

**DISCUSSION**

Clinical signs of toxoplasmosis were observed during the acute phase of the disease in two pigs of each group infected with the RH or the ME-49 strains. These results differ from oocysts infection (AS-28 strain) in which no major clinical alterations were observed in infected animals, except a moderate elevation of rectal temperature (Yai et al. 2003). Moreover, our clinical results are different from those reported by Wingstrand et al. (1997), in which pigs were infected with oocysts (SSI-119 strain) and cysts (SSI-119 and R92 strains). They observed severe clinical signs between 3-5 dpi, such as inappetence, transient fever and diarrhoea; however, the authors used eight-week-old pigs, which are more susceptible to *T. gondii* infection. It was shown that 21-day-old pigs that were infected with the RH strain presented eye discharge and temperature elevation between 3-5 dpi (Bugni et al. 2008). In contrast, Moura et al. (2007) observed neither

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**TABLE II**

| Pig ID | Spleen | Heart | Brain | Mesenteric lymph node | Retina | Ileum | Skeletal musculature | Lung | Liver | Tongue | Kidney |
|--------|--------|-------|-------|-----------------------|-------|-------|----------------------|------|-------|--------|--------|
| 4      | -      | +b,a  | -     | -                     | -     | -     | -                    | -    | -     | -      | -      |
| 5      | -      | -     | -     | -                     | -     | -     | -                    | -    | -     | -      | -      |
| 11     | -      | -     | -     | -                     | -     | -     | -                    | -    | -     | -      | -      |
| 12     | -      | +a    | -     | +a                    | +a    | +a    | +a                   | +a   | +a    | +a     | +a     |

*a:* seropositive by the modified agglutination test (cut-off titre 1:25); *b:* positive for cyst visualisation in mice brain tissue.

**TABLE III**

Kidney stereological data expressed as mean and standard deviation of all four pigs per group experimentally infected with *Toxoplasma gondii* or inoculated with phosphate buffered saline (control)

| Parameter                  | Control         | RH              | ME-49 (control) | p     |
|----------------------------|-----------------|-----------------|-----------------|-------|
| Kidney volume (cm³)        | 51.8 ± 4.2      | 70.5 ± 8.0      | 65.0 ± 4.5      | 0.09  |
| Cortical-medullary ratio (%)| 16.4 ± 0.6      | 14.5 ± 1.2      | 13.4 ± 0.6      | 0.12  |
| Cortical volume (cm³)      | 8.6 ± 1.3       | 10.0 ± 1.3      | 8.8 ± 1.3       | 0.73  |
| Vv [Glom] (%)              | 4.6 ± 0.4       | 3.5 ± 0.4       | 4.4 ± 0.3       | 0.15  |
| VWG[V] (10⁴ µm³)           | 102.8 ± 4.0     | 104.3 ± 1.7     | 92.0 ± 7.5      | 0.22  |
| N [Glom] (x 10⁷)           | 4.0 ± 0.9       | 3.5 ± 0.8       | 4.4 ± 8.5       | 0.81  |

*a:* glomerular volume density; *b:* volume weighted mean glomerular volume; *c:* numbers of glomeruli per kidney.
clinical nor haematological alterations in male adult pigs that were infected with oocysts (P strain) or tachyzoites (RH strain). Thus, the different clinical manifestations that were observed between these studies, including our observations, may be due to the breed, sex and age of the pigs and also the quantity, form and parasite strain that was used for the infections. It was reported that a pig that presented with neurological signs four days after oral infection with *T. gondii* oocysts died (Wingstrand et al. 1997). In our work, one of the RH group pigs presented aggressiveness during the chronic phase of the disease, which may probably be an indication of neurological alterations caused by the infection.

Significant haematological changes were only detected in the ME-49 group that presented an increase in band neutrophils. This clearly indicates that an immune response based on this cell was triggered after the infection. Because band neutrophils were only seen in the pigs that were orally infected (ME-49 strain), this response may probably be due to ileitis that increases recruitment of these cells in mice (Bliss et al. 2001) and in considerable amounts in the ileum itself (Dunay et al. 2008). Neutrophil infiltration during the oral toxoplasmosis acute phase in mice has been described and its depletion is lethal (Bliss et al. 2001); however, evidence was shown that neutrophils are not crucial for *T. gondii* control (Dunay et al. 2010). Nevertheless, these cells are important because they chemotactically attract dendritic cells that are essential to promote a T-helper 1 response (Benoua et al. 2003). On the other hand, a neutropenia tendency by 3 dpi was observed in *T. gondii* infected pigs; however, this study was carried out with very young pigs, as discussed above (Bugni et al. 2008), but with the same dose, parasite form and strain (RH) used in this work. Neutropenia was observed during toxoplasmosis due to neutrophil migration to infected tissues (Dubey et al. 1994). The observed increase in band neutrophil counts was not sufficient to induce leucocytosis. It is known that only extensive tissue damage causes a significant increase of blood neutrophils that generates leucocytosis (Thrall et al. 2004). Thus, the ME-49 infection in the pigs may have caused enough tissue damage to increase band neutrophils in the blood, but it was not sufficient enough to induce leucocytosis.

Neutrophil changes were not seen in the RH group pigs, suggesting a lower immune response in comparison with the ME-49 group. Additionally, the infection venue may induce a completely distinct local response with different reflections in the systemic response, which is seen as leucocyte fluctuation in the peripheral blood.

The bioassay test done with blood leucocytes showed that the parasitaemia occurred only in the early days after infection of the pigs and the ME-49 strain presented a broader range. Nevertheless, tachyzoites were seen in blood smears in the early and late days of the infection. Thus, these strains probably gained blood circulation right after the infection and persisted in the pig circulation after considerable time without clinical signs or physiological alterations that were detected by routine haematological methods. Despite the low parasitaemia with the microscopic evaluation, *T. gondii* was more frequently observed in neutrophils, suggesting that this leucocyte can be important for parasitic dissemination in the pigs as demonstrated in mice (Norose et al. 2008, Coombe et al. 2013). The biological tests indicated that the ME-49 strain tachyzoites reached the blood faster, in higher amounts and for a longer period of time after infection by the oral route than that observed via intramuscular inoculation with the RH strain tachyzoites. This indicates that the former may be a better experimental model due to its faster dissemination. The data obtained with the RH group pigs were similar to a study that used the same strain that described positive biological assays between 3-47 dpi (Moura et al. 2007). It is commonly known that *T. gondii* can only be seen in the peripheral blood during the acute phase. In fact, most of the studies concerning *T. gondii* isolation in humans occurred in immunocompromised (Hofflin & Remington 1985) or immunocompetent patients who presented ocular toxoplasmosis (Amendoa & Coutinho 1982). However, a recent study has broken this paradigm, because it showed that *T. gondii* could circulate in the blood of immunocompetent chronically infected humans (Silveira et al. 2011). Thus, the observed tachyzoites in the chronically infected pigs reported here was similar to what was reported in humans, indicating that the pig model may be a good model to understand chronic toxoplasmosis in humans.

The higher serum ALT levels in the RH group pigs may be a probable consequence of direct hepatic injury due to parasitic replication. The liver is an important organ for multiplication or defence against the RH strain, mainly until 14 dpi (Dubey et al. 1994). Lack of ALT changes in the ME-49 group pigs may suggest that this infection was well managed by the liver.

The ileum, mesenteric lymph nodes and heart were the only ME-49 infected pig tissues that induced serum conversion in the mice; however, with the bioassay, cysts were only observed in the brains of mice that were inoculated with heart tissue, suggesting a higher concentration of parasites in the heart tissue. These results are similar to those obtained in pigs that were infected with 10 or less VEG strain oocysts, which showed many cysts in the tongue, brain and heart (Dubey et al. 1996).

No morphological alteration was detected in the examined infected pig tissues, which was similar to results reported by Wingstrand et al. (1997). Kidney transplant may cause toxoplasmosis (Gharavi et al. 2011), indicating that this organ may harbour this parasite and, consequently, present morphological changes. By using mice, we recently observed nephron loss occurrences with a consequent kidney volume decrease, as well light glomerular and tubular lesions (D Benchimol de Souza et al., unpublished observations). However, no such changes were observed in pigs in the present study. Lack of pathological changes in the tissues of the infected pigs reported here might be due to the period of time between the infections and analyses. Changes would probably be detected if the tissues were examined soon after the infection. Additionally, kidney morphological alterations and other haematological parameters, such as the monocytosis tendency seen at 7 dpi after infection with the ME-49 strain, would be better evaluated and understood.
if higher pig numbers were used for these experiments. The BR-1 mini pigs that were infected with the ME-49 strain used in this work resulted in a suitable model for studying toxoplasmosis because these animals carried the infection, but survived and presented chronic toxoplasmosis, which is similar to the immunocompetence observed in humans. Additionally, Toxoplasma gondii was able to infect these pigs with prolonged parasitemia without clinical signs.

ACKNOWLEDGEMENTS
To Andrea Carvalho César, for proof reading the manuscript, and to Dr JP Dubey, that ceded the antigen for the MAT.

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