# Evaluation of experimental *Toxoplasma gondii* (Nicolle and Manceaux, 1909) infection in pigs by bioassay in mice and polymerase chain reaction

## Avaliação da infecção experimental por *Toxoplasma gondii* (Nicolle e Manceuax, 1909) em suínos pelas provas de bioensaio em camundongos e reação em cadeia pela polimerase

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## Summary

The aim of the present experiment was to standardize a nested polymerase chain reaction (nPCR) for the detection of *Toxoplasma gondii* in tissues of experimentally infected pigs and to compare the performance of nPCR with the standard isolation technique, the bioassay in mice. Comparison between the two methods was done testing eight 4 month-old pigs orally inoculated with 5 x 10⁴ oocysts of *Toxoplasma gondii* (AS-28 strain) and three non-infected pigs at the same age, kept as control. All animals were euthanatized 47 days after infection and samples of brain, heart, tongue and retina were collected from each animal for analysis by nPCR and bioassay in mice. By using the bioassay, *Toxoplasma gondii* was detected in 4 infected pigs, being two in the retina, one in the heart and one in the tongue. *Toxoplasma gondii* DNA was detected in five of the inoculated pigs, being: three in the tongue, two in the brain and heart and one in retina. The detection threshold of the nPCR on mouse brain suspension artificially infected with the RH strain of *Toxoplasma gondii* was 10 tachyzoites/ml. Although both techniques were unable to detect the parasite in all infected pigs, nPCR showed better performance as it was accomplished in a shorter period of time. When used concurrently, both techniques detected the agent in seven infected animals. The only way to increase sensitivity of either method is to increase the amount of tissue to be examined.

## Introduction

Infection with *Toxoplasma gondii* is widespread among warm-blooded animals including birds, wild animals, pets, livestock and humans[1]. Ingestion of tissue cysts from undercooked infected meat and ingestion of food and water contaminated with oocysts from cat feces are the two principal causes of human contamination[2].

Porcine tissues infected with *Toxoplasma gondii* are considered the major source of *Toxoplasma gondii* infection for human beings in the United States[3] where serologic surveys have found infection rate ranging from 4.00% to 69.00%[4]. In Brazil, seroprevalence of *Toxoplasma gondii* infection in pigs ranges from 16.33% to 54.00%[5,6].

*Toxoplasma gondii* has been detected in several tissues of pigs[2,3]. It has been estimated that the number of *Toxoplasma gondii* cysts per gram of tissue from food animals such as pigs, may be less than one tissue cyst per 50g. Therefore, a concentration bioassay technique is often used[7], but this technique is time consuming, potentially hazardous for the operator and very expensive. Molecular methods of diagnosis such as polymerase chain reaction (PCR) offer the advantages of remarkable sensitivity, high specificity and speed[8]. Several PCR-based assays have been developed for the detection of DNA from *Toxoplasma gondii* and the most common target are the B1 repetitive sequence, the P30 (SAG1) gene or rDNA[9]. The B1 PCR is more sensitive than P30, probably because B1 is a repeated DNA sequence with a higher copy number than P30, 35 and 1, respectively[10,11]. There are few reports on the use of PCR for the detection of *Toxoplasma* DNA in animal species[12,13,14,15,16].

The objective of this study was to compare techniques for *Toxoplasma gondii* detection on infected pig tissues by the traditional method of *Toxoplasma gondii* diagnosis, the bioassay in mice, and by nPCR.

## Material and Method

### Tachyzoites

Mature swiss white mice of both sexes from *Toxoplasma gondii* free colonies were used to maintain *Toxoplasma gondii* tachyzoites of the RH strain[17]. Parasites were harvested from the peritoneal cavities of mice that had been infected three days earlier with tachyzoites by intraperitoneal injection (i.p.). *Toxoplasma gondii* RH strain was also used to prepare antigen for the immunofluorescence assay technique (IFAT)[18] as well as to be a source of *Toxoplasma gondii* DNA for the positive control in the nPCR.

### Oocysts

Eight cats approximately 2-month-old and seronegative for *Toxoplasma gondii* by IFAT were used to obtain viable *Toxoplasma gondii* oocysts of AS-28 strain[19], that were used for experimental infection of pigs.

### Experimental infection of swine

Eleven 4-month-old pigs seronegative by IFAT to *Toxoplasma gondii* (titer<64) were allocated into two groups. A suspension containing $5 \times 10^4$ sporulated *Toxoplasma gondii* oocysts in sterile PBS, pH 7.4 was orally administrated to a group of eight animals and the remaining three pigs were used as negative control. Pigs were clinically and serologically (IFAT) monitored during the pos-infectiion (p.i.) period. Serum samples from these animals were analyzed by IFAT using dilutions starting from 1:16 to 1: 4096. A titer $\geq 64$ was considered positive for *Toxoplasma gondii* antibodies[2]. The control and infected groups were killed 47 days p.i. and tissue samples (brain, heart, tongue and retina) were collected for bioassay in mice and nPCR. The organs were examined for macroscopic lesions.

During the first 10 days p.i., pigs were clinically examined and diarrhea, coughing, dyspnea, appetite, rectal temperature and general condition were recorded once a day.

**Bioassay in mice**

Swiss mice weighing 20-25 g were used in the bioassay. Tissue samples of cardiac muscle and tongue from the pigs were submitted to artificial digestion[20] and 1 ml of each tissue homogenate was intraperitoneally inoculated into five mice per each tissue sample. Brain and retina of the pigs were not digested. Fragments of the brain weighting approximately 20g and the entire retina were ground, suspended in phosphate buffer solution (PBS) pH 7,2 0,01M, filtered through gauze, and injected into mice in the same way as the digested material.

Mice were daily inspected for signs of febrile response that might indicate acute toxoplasmosis. Mice which showed signs of illness were culled immediately and a sample of peritoneal exudate removed and inspected for tachyzoites by microscopic examination. Blood samples were collected after 6-8 weeks from surviving mice and the sera were tested for specific *Toxoplasma gondii* antibodies by IFAT. The brain of all inoculated mouse was fixed in 10.00% neutral buffered formalin. After trimming, the tissue samples were embedded in paraffin, 5mm sectioned and stained with hematoxylin and eosin (H.E.) and examin *Toxoplasma gondii* cysts by direct microscopy.

Mouse inoculation was considered positive if either tachyzoites or cysts were seen or if mice developed antibody to *Toxoplasma gondii*[21]. Mouse sera demonstrating a titer $\leq 16$ were considered negative[22].

**nPCR**

The DNA of parasites was detected by nPCR based on primers directed to the sequence of repetitive gene B1[11]. The primers Toxo 1 (5' AGC GTC TCT CTT CAA GCA GCG TA 3') and Toxo 2 (5' TCC GCA GCG ACT TCT ATC TCT GT 3') were used for the primary reaction whilst primers Toxo 3 (5' TGG GAA TGA AAG AGA CGC TAA TGT G 3') and Toxo 4 (5' TTA AAG CGT TCG TGG TCA ACT ATC G 3') flanking 155-bp of the B1 gene were used for the nested reaction.

Fragments of brain, cardiac muscle, tongue and retina were stored at –20°C prior the extraction of DNA for nPCR analysis. Approximately 16g of each sample were homogenized in ultra pure water in homogenater (Stomacher 400 ® Seward) to a final concentration of 20.00% (w/v) and divided into three aliquots of 400ml. To each aliquots it was added 400μl of TE (20 mMTris HCl, 2mM EDTA, pH 8.0) following by centrifugation at 12000 x g for 5 min. The supernatant was discarded and pellets were resuspended in 500 μl of TE and heated in a dry-bath (99.8°C $\pm$ 2°C) for 16 min. The extracts were then purified by standard phenol/chloroform extraction, followed by precipitation with sodium acetate and ethanol[23].

Samples (5μl) of specimen preparations were added to a final volume of 50μl of PCR mixture consisting of 50mM KCl, 10mM Tris-HCl (pH 8.3), 50mM $MgCl_2$, 200μM each of dATP, dTTP, dCTP, dGTP, 10 pmol of each primer (Toxo 1 and Toxo 2), 5.00% glycerol and 2.5 U of Taq DNA polymerase.

The mixture was amplified in thermocycler PTC-200 (MJ-Research, Watertown, Massachusetts, USA) for 25 cycles. After an initial denaturation for 3 min at 94°C, the following cycle was used: denaturation at 94°C for 45 s, annealing at 55°C for 1 min and extension at 72°C for 30 s. A final extension step was continued for a further 10 min. Both negative (TE or mouse brain suspension,

20.00% w/v, from *Toxoplasma gondii* seronegative mice) and positive controls (brain tissue suspension from mice spiked with $10^7$ tachyzoites of the RH strain of *Toxoplasma gondii*) were included.

For nPCR, 50 µl of the second PCR mixture were added to all volume from the first amplification assay. The second PCR mixture consisted of milli-Q water, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200µM each deoxynucleotide triphosphate, 50 pmol of each primer (Toxo3 and Toxo4), 5.00% glycerol pure, and 5 U of Taq DNA polymerase (Life Tech, Gibco, BRL, MD, USA). Samples were amplified for further 35 cycles. Each cycle was performed in the same way as the first round. For monitoring cross-contamination, at each set of 12 test samples it was included one negative control (PCR mixture with water as sample). Ten microlitres of reaction mixture were electrophoresed on agarose 2.00% gel stained with ethidium bromide and compared to a 100-bp DNA ladder (Life Tech, Gibco BRL, MD, USA).

The threshold of detection nPCR was evaluated using nPCR negative mice brain homogenates spiked with tachyzoites. Briefly, 20.00% suspension of mice brain in water was spiked with free tachyzoites in order to obtain from $10^7$ to $10^{-1}$ parasites/ml. Each dilution was then tested by nPCR.

## Results

### Experimental infection of swine

Clinical signs relating to toxoplasmosis were not observed in any pig except for a moderate elevation of the rectal temperature (> 39.9°C) noted in four pigs. In those animals the rise in temperature was first noted around day 4 and was pronounced until day 7 p.i. After this period no temperature changes were observed.

All animals manifested specific seroconversion after oral infection. Eight days after inoculation, all pigs developed a rise in *Toxoplasma gondii* titers ranging from 64 to 4096 which last until the euthanasia. The uninoculated pigs remained seronegative.

### Bioassay in mice

The bioassay gave positive results in four of the eight inoculated pigs as showed in Table 1. By histopathology

**Figure 1**
Threshold of detection of nPCR.
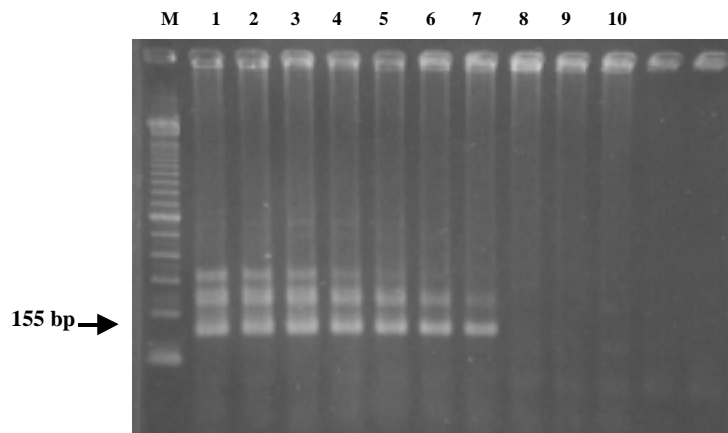
(H.E.), no tissue cyst was observed in the samples of brain from inoculated mice.

### nPCR

The threshold of detection experiment showed that nPCR was able to detect as few as 10 tachyzoites/ml in the spiked sample (Figure 1). As shown in Table 1, nPCR gave positive results on tissues samples from five of the eight inoculated pigs, being from tongue (three pigs), brain tissue (two pigs), heart (one pig) and retina (one pig).

## Discussion

*Toxoplasma gondii* rarely causes clinical disease in pigs[24,25] irrespective of using high or low infective dosis. The same picture was also assumed in the present study, where only four of the eight infected animals showed mild rise in temperature. However, all the infected pigs seroconverted by IFAT with titers ranging from 64 to 4096, confirming the infection with *Toxoplasma gondii*. No macroscopic injuries were observed in the examined pig tissues.

By biossay, four of the eight inoculated pigs were positive at least in one tissue, with *Toxoplasma gondii* been isolated from the retina of two pigs, heart of one pig, tongue of one pig and no isolation was obtained from the brain samples. Verma and Bowles[26] examining brain of 12 pigs and Viotti et al.[27] examining brain, retina, muscle, tongue and lung of six pigs, recovering *Toxoplasma gondii* from 50.00% of them.

The percentage of positivity confirmed by the bioassay was lower than that reported by Dubey et al.[24] that, from 35 inoculated pigs (> 1000 oocysts) isolated *Toxoplasma gondii* from the tongue of 24 animals (68.50%), from the brain in 27 (77.10%) and from the heart in 25 (71.40%).

Otherwise, Gajadhar et al.[28]

analyzing samples of diaphragm and heart from 2800 market-pigs from Canada, isolated no *Toxoplasma gondii* by bioassay from any sample, even though 240 of them were seropositive.

The low level of isolation by the bioassay in mice could be due to the stage of development of the parasite. In this trial, the pigs were killed 47 days after the experimental inoculation and, at that age, the cysts are small and might contain a low number of bradyzoites.

According to Dubey[29] in the bioassay, mice are inoculated with 25.00% of the digested material and one *Toxoplasma gondii* digested cyst release about 10 bradyzoites. Based on the aforementioned assumptions, if the total amount of digested material contains a single cyst, then the amount of inocula is likely to contain about only two bradyzoites.

The explanation for the failure of the bioassay in the detection of *Toxoplasma gondii* could be extended for the performance of the nPCR. In the infected pigs, the DNA of *Toxoplasma gondii* was detected in five of them (62.50%) with the tongue showing the higher percentage of detection (37.50%). In two of the three pigs in which *Toxoplasma gondii* was detected in the tongue, all the three examined aliquots were positive. However in the other examined organs this uniformity was not observed and the agent was only detected in one or two aliquots from the same kind of tissue.

Similar results were also observed in samples of cerebro-spinal fluid from patients with toxoplasmic encephalitis, where the authors have found 62.00% of positivity by nPCR[30]. Esteban-Redondo and Innes[31], using a nPCR directed to B1 gene, detected *Toxoplasma* DNA in heart, brain and esqueletical muscle of eight from 12 experimentally infected sheep.

Despite of the fact that no *Toxoplasma gondii* was isolated from brain samples by bioassay, nPCR detected the parasite in the brain of two pigs. Similarly

Owen, Clarkson and Trees[32], dealing with cotyledons of seropositive lambs, detected three positive samples by PCR which were negative by bioassay. In the present work, when both nPCR and bioassay were positive for the same pig, *Toxoplasma gondii* was not detected in the same tissues. This discrepancy should be due to the rather sparse and focal distribution of the parasites in the tissues.

The size of the examined sample is an important parameter for the detection of the parasite. In this experiment only 16g of tissue was examined by nPCR, and negative result of any sample do not necessary mean that the whole organ is free from the agent. The low number of tissue cysts in infected pigs, less than 1 cyst per 50g of tissue, and the very sparse and focal distribution of parasites in the tissues[33] are important characteristics that should be in mind when results of *Toxoplasma gondii* are discussed.

The use of nPCR and bioassay as associated diagnostic techniques increased the sensibility of detection from 62.50% (nPCR) and 50.00% (bioassay) to 87.50%. Both techniques showed a relatively poor ability to identify parasites in the tissue, which means that many samples will have no parasites. The performance of the nPCR is not a significant improvement over bioassay. If there are no parasites in the tissue sample they will appear negative even if the animal harbours parasites and will be equally negative by both techniques. If combining the two techniques improves sensitivity, it is true to accept that double the number of tissue had been examined by each technique would increase the sensitivity of each test.

In conclusion, the only way to increase sensitivity is to find a method to increase the amount of tissue which can be examined. In fact, if the PCR does not significantly increase sensitivity or specificity it is, otherwise cheaper and faster than mouse inoculation test. The nPCR performed here takes about 48 hours while bioassay could last up to 6 weeks.

Bioassay detects only viable parasites. Conversely, PCR may detect DNA of parasites even if the tissue is in a decomposed state or contaminated which is a fact that becomes favorable to PCR, due to the conditions of sending and aconditioning biological material. In addition, for diagnostic purposes, PCR dispenses with the need for testing animals.

The threshold of detection of 10 taquyzoites in the suspension of mice brain, showed by nPCR was similar to the results reported in suspension of human leukocytes[11]; in aqueous humor, cerebral spinal fluid and sera[13] and in samples of placenta[32].

In the present study some alterations in the protocol as the homogenization of the tissues with the stomacher, keeping away the sample from contamination, the extraction of DNA with simple boiling, and the use of only one tube in nPCR, associated with the safety and speed of the technique must be emphasized.

## Acknowledgements

## Resumo

O objetivo desse trabalho foi o de padronizar uma técnica de reação em cadeia pela polimerase, nested PCR, para detectar *Toxoplasma gondii* em tecidos de suínos infectados experimentalmente e comparar os resultados obtidos com a técnica padrão de isolamento, o bioensaio em camundongos Para testar a técnica

**Palavras-chave**
*Toxoplasma gondii*.
Suíno.
NestedPCR.
Bioensaio.
Diagnóstico.

Evaluation of experimental *Toxoplasma gondii* (Nicolle and Manceaux, 1909[23]) infection in pigs by bioassay in mice and polymerase chain reaction

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padronizada, foram inoculados, oralmente, oito suínos com quatro meses de idade, com $5 \times 10^4$ oocistos de *Toxoplasma gondii* (cepa AS-28) e três suínos com a mesma idade, não infectados, foram mantidos como controles. Todos os animais foram sacrificados 47 dias após a infecção e colheu-se amostras de cérebro, coração, língua e retina de cada animal para as provas de bioensaio e PCR. Pela prova de bioensaio foi isolado o parasita quatro dos animais inoculados, sendo dois na retina, um no coração e um na língua. O DNA de *Toxoplasma gondii* foi detectado em cinco dos suínos inoculados, sendo: em três dos animais na língua, em dois no cérebro e no coração e na retina de um dos animais. O limiar de detecção da nPCR foi de 10 taquizoítas/mL de suspensão de cérebro de camundongos artificialmente contaminada. Nenhuma das técnicas detectou o agente em todos os animais inoculados, entretanto a nPCR, apresentou maior capacidade de detecção e menor tempo para a obtenção do diagnóstico. A utilização das técnicas em associação foi capaz de detectar o agente em sete dos animais inoculados. A única forma de melhorar a sensibilidade do método seria aumentando a quantidade de tecido a ser examinado.

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