

Detection of *Toxoplasma gondii* by PCR and mouse bioassay in commercial cuts of pork from experimentally infected pigs

[*Detecção do Toxoplasma gondii por PCR e bioensaio em camundongo em cortes comerciais de carnes de suínos infectados experimentalmente*]

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ABSTRACT

The distribution of *T. gondii* in commercial cuts of pork (ham, tenderloin, spareribs and arm picnic) by PCR and bioassay from experimentally infected pigs, was evaluated. Eighteen mixed breed pigs were divided into two groups (G). The G1 animals (n=10) were infected with 4×10^4 oocysts of the *T. gondii* VEG strain and the G2 animals (n=8) were used as control. Pigs of both groups were slaughtered at 59th day after infection, and meat samples were collected for bioassay and PCR. All animals from G1 were positive by at least one or both tests, and all control animals were negative. *T. gondii* was identified in pork by mouse bioassay and PCR in 27/40 (67.5%) and in 9/40 (22.5%) of the evaluated samples, respectively. There were no statistical differences in the distribution of tissue cysts from commercial cuts of pork by bioassay ($P>0.05$). However, statistical differences were observed when mouse bioassay and PCR were compared ($P<0.01$).

Keywords: pork meat, mouse bioassay, PCR, *Toxoplasma gondii*

RESUMO

Avaliou-se a presença de T. gondii em cortes comerciais de carne suína (pernil, lombo, costela e paleta), por meio do bioensaio e PCR, em animais experimentalmente inoculados. Dois grupos (G) foram formados. Os animais do G1 (n=10) foram inoculados com 4×10^4 oocistos da cepa VEG e os do G2 (n=8) permaneceram como grupo-controle, não inoculado. Todos os animais foram abatidos no dia 59 após a infecção, quando foram colhidas as amostras de carne para a realização das provas de bioensaio e da PCR. Todos os suínos do G1 apresentaram-se positivos a pelo menos um dos testes de diagnóstico ou a ambos, e os do grupo-controle permaneceram negativos. Não houve diferenças significativas em relação aos tipos de cortes comerciais e à presença do parasita no bioensaio ($P>0,05$). O bioensaio foi capaz de detectar T. gondii em 27/40 (67,5%) amostras e a PCR em 9/40 (22,5%). O estudo mostrou diferença entre o bioensaio e a PCR ($P<0,01$).

Palavras-chave: carne suína, bioensaio em camundongos, PCR, *Toxoplasma gondii*

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INTRODUCTION

Toxoplasma gondii is an intracellular parasite which can infect cells of human beings and all warm blood animals. It is a world wide protozoan parasite. Humans can become infected by ingesting either oocysts or raw and undercooked meat containing cysts (Garcia et al., 2004). Bradyzoites, located in tissue cysts, are able to survive for long periods within the host; the longevity of tissue cysts in pork can persist for more than two years (Dubey et al., 1998). This is important because pork is one of the most important sources of *T. gondii* infections in humans (Dubey, 1994).

Epidemiological studies in Brazil have showed that the seroprevalence of *T. gondii* in pigs varies from 9.6% to 54.1% (Vidotto et al., 1990; Garcia et al., 1999; Suárez-Aranda et al., 2000). This wide variation could be related to regional/geographical factors and the difference in production systems (Garcia et al., 1999).

The polymerase chain reaction (PCR) is an efficient molecular technique to detect the parasite animal and human tissues. This method is very sensitive, highly specific and rapid (Yai et al., 2003). Mouse bioassay is the principal method used to detect cysts in tissues, but it is arduous and expensive. The objective of this study was to evaluate the distribution of tissue cysts of *T. gondii* in commercial cuts of pork by PCR and mouse bioassay in experimentally infected pigs.

MATERIAL AND METHODS

The *Toxoplasma gondii* VEG and RH strains were used in this experiment. The VEG strain, a type III genotype, was used for pig infection. The RH strain, a type I genotype, was used as the antigen in the indirect fluorescence antibody test (IFAT).

The maintenance and care of experimental animals complied with the National Institute of Health guidelines for the humane use of laboratory animals. Eighteen mixed breed pigs between 6.5-7.5 weeks old, including females and castrated males, were randomly allocated in separate stables. An accommodation period of 6 days pre-experiment was used. Food and water were administered *ad libitum*. All pigs were

serum negative (titer < 64) by the *T. gondii* IFAT. Animals were divided in two groups, G1 (infected group, n=10) and G2 (uninfected control group, n=8). G1 was infected at day 0 with 4×10^4 oocysts of VEG strain by oral route. G1 pigs were treated at day 5 post-infection with an intramuscular dose of sulfadiazida (3mg/Kg) and trimetoprim (15mg/Kg). All pigs were treated because this promotes encystation of parasites and interrupts clinical symptoms (Alexander and Hunter, 1998). At death, muscle samples (ham, tender loin, spareribs and arm picnic) were collected to investigate *T. gondii* by mouse bioassay and PCR.

The presence of antibodies against *T. gondii* in serum samples of pigs and mice were measured by IFAT (Camargo, 1973) considering as positive pigs with titer ≥ 64 and mice with titer ≥ 16 .

Muscle samples (50g of ham, tenderloin, spareribs and arm picnic) from each pig were used to evaluate the presence of *T. gondii* cysts as previously described (Dubey, 1998). Briefly, each sample was homogenized in a blender for 30 seconds in 250ml of saline solution (0.14M NaCl). After homogenization, 250 ml of pepsin solution was added and incubated at 37°C for 1hr. The homogenate was filtered through two layers gauze and centrifuged at 1180xg for 10min. The supernatant was discarded and the sediment was resuspended in 20ml PBS (pH 7.2) and 15ml 1.2% sodium bicarbonate (pH 8.3) was added and centrifuged at 1180xg for 10min. The supernatant was discarded and the sediment was resuspended in 5ml of antibiotic saline solution (1,000U penicillin and 100 μ l of streptomycin/ml of saline solution) and inoculated subcutaneously into 3 mice (1ml/mouse). Impression smears of lung from the mice that died were fixed in methanol, stained with Giemsa, and examined microscopically. Blood samples were obtained from mice that survived 60 days after inoculation. Brain of mice that survived were examined microscopically for *T. gondii* tissue cysts by squashing a portion of brain between a cover slip and a glass slide. Serum from each mouse was diluted at 1:16 and 1:64 and examined for anti-*T. gondii* antibodies using IFAT. A result was considered positive either by the detection of tissue cysts from brain samples or when a IFAT titer ≥ 16 was observed.

Muscle samples were digested as described above. The final sediment was resuspended in 3ml of TNE buffer (200mM NaCl, 20mM Tris, 50mM EDTA, pH8.0), and DNA was extracted as previously described (Silva and Langoni, 2001). Briefly, 3.6µl of each sample was transferred to a microtube and diluted in 246.4µl of TNE and 250µl of TNE extraction buffer (200mM NaCl, 20mM Tris, 50mM EDTA, 0.6mg K proteinase and 2% SDS). The samples were then incubated at 56°C for 90min, with frequent mixing (15min) by inversion. The extracts were purified by standard phenol/chloroform (Theil et al., 1981). Final volume (20µl) was resuspended in 80µl of ultra pure sterile water, and it stored at - 20°C until PCR analysis.

DNA amplification of *T. gondii* was performed using the method described by Homan et al. (2000).

Primers

TOX4 (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3') and TOX5 (5'-CGCTGCAGACACAGTGCATCTGGATT-3') were used, and these flanked a 529bp fragment of *T. gondii* DNA. PCR reaction was performed in a mixture containing 5µl of DNA extracted plus 20µl (final volume of 25µl) of mixture of 0.5mM of each primer, 100mM dNTP (Invitrogen), 60mM Tris±HCl (pH9.0), 15mM (NH₄)₂SO₄, 2mM MgCl₂, 0.5U Taq DNA polymerase (Gibco-BRL). Amplification of DNA from parasites were performed over 35 cycles in Eppendorf Mastercycler Gradienty, using the following cycling conditions: 7min at 94°C for denaturation in cycle one, followed by 33 cycles on 60s at 94°C for denaturation, 60s at 55°C for annealing and 60s at 72°C for extension, cycle 35 was followed by a final extension of 10min at 72°C. Aliquots of each

PCR were electrophoresed on 1% agarose gel and ethidium bromede staining (Pereira et al., 1983).

Tachyzoites of RH strain were obtained from peritoneal fluid of infected Swiss Webster mice. The material was passed thrice through a 26 gauge needle for purification and washed twice with 10mM phosphate buffered saline (PBS, pH7.5). After washing, tachyzoites were prepared at a concentration of 1.13x10⁶, 1.13x10⁵, 1.13x10⁴, 1.13x10³, 113 and 11,3/ml. To evaluate sensitivity of the 529bp primers, DNA extraction and PCR of the cell suspensions were performed as described above.

Differences among test proportions were compared by chi-square (χ^2) test with Yates correction, using EpiInfo 6.01 statistical package. A P value of <0.05 was considered as significant.

RESULTS

The results of mouse bioassay and PCR are summarized on Table 1. Infected pigs had muscle samples recorded as positive by either or both PCR and mouse bioassay in every case, and there were no positive samples in G2 (control group). Mouse bioassay detected 27/40 (67.5%) positive samples; being seven from tenderloin, nine from ham, five from spareribs and six from arm picnic. With PCR 9/40 (22.5%) of evaluated samples were positive: three from tenderloin, four from ham, one from spareribs, and one from arm picnic. No statistical differences (P>0.05) were observed in the detection of *T. gondii* from commercial cuts of pork by bioassay. However, when the test were compared, differences were observed (P<0.01).

Table 1. Outcome of *Toxoplasma gondii* tissue cysts in commercial cuts of pork by mouse bioassay and polymerase chain reaction (PCR) obtained from infected pigs.

Pig #	IFAT ¹	Tenderloin ⁴		Ham ⁴		Spareribs ⁴		Arm picnic ⁴	
		Bioassay ^{2,5}	PCR ^{3,5}	Bioassay ⁵	PCR ⁵	Bioassay ⁵	PCR ⁵	Bioassay ⁵	PCR ⁵
		0(1)/3	+	3/3	+	0/3	-	0/3	-
2	256	2/3	-	2(1)/3	-	2/3	+	3/3	-
3	256	1(1)/3	+	1(2)/3	-	3/3	-	3/3	+
4	256	0/3	-	1(2)/3	+	0(1)/3	-	0(2)/3	-
5	1024	0/3	-	0(1)/3	+	0/3	-	0(1)/3	-
6	64	0(2)/3	-	0(2)/3	+	0(3)/3	-	0(3)/3	-
7	256	0/3	+	0/3	-	0/3	-	0/3	-
8	256	0(1)/3	-	0(2)/3	-	0/3	-	0/3	-
9	256	2(1)/3	-	0(3)/3	-	0(2)/3	-	0/3	-
10	256	0(1)/3	-	0(1)/3	-	0/3	-	0(1)/3	-

¹Antibody titers of each pig, at slaughter day, in indirect fluorescence assay (IFAT) ²Results are expressed as number of mice positive for *T. gondii* of three mice inoculated with pepsin digest of pig tissue. Numbers in parenthesis indicate the number of mice with antibody titers ≥ 16 (IFAT), but in which cysts were not seen in their brain; tissue cysts were seen in the other mice. ³PCR was performed from pork. ⁴P>0.05; ⁵P<0.01

Detection of *Toxoplasma gondii* by PCR...

The sensitivity of PCR is shown in Fig. 1. PCR used in conditions of this experiment was able to

detect the DNA of the parasite at a concentration of 10^3 tachyzoites/ml.

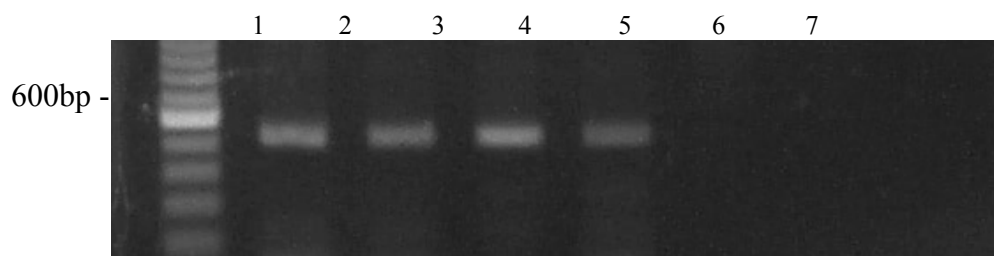


Fig. 1. Electrophoresis, on 1% agarose gel, of polymerase chain reaction (PCR) of tachyzoite dilutions showing sensitivity of 529bp PCR. Lanes 1 to 6 represent dilutions: 1.13×10^6 ; 1.13×10^5 ; 1.13×10^4 ; 1.13×10^3 ; 113; 11.3 of tachyzoites, respectively. Lane 7: negative control.

DISCUSSION

Comparatively, bioassay detects viable parasites and PCR can detect DNA of parasites, even if the tissue is in a decomposed state (Yai et al., 2003). However, PCR may not express the risk of human infection (Aspinall et al., 2002).

Among the PCR targets used to detect *T. gondii* from blood, fluids and tissue samples, the B1 gene is the most widely used and includes a B1 nested-PCR. The used PCR amplified a 529bp fragment that is repeated 200-300 times in *T. gondii* genome, and it was described as being more sensitive than B1 PCR (Homan et al., 2000). However, more DNA targets should be tested to ensure progress in the molecular diagnosis of toxoplasmosis (Chabbert et al., 2004).

Distribution of tissue cysts in commercial cuts has been described in pigs (Dubey, 1988). In the present study there were no differences in the detection of *T. gondii* among muscle samples. However, differences in distribution of tissue cysts are observed in other experimental animals (Dubey, 1997).

Mouse bioassay was more effective for the identification of parasite than PCR. During PCR analysis, DNA of the parasite was only detected in 22.5% of the samples examined. This may be due to the fact that PCR was able to produce a linear relationship between the relative amounts of PCR product and the number of tachyzoites in

order of 10^3 tachyzoites. Similar results 21.8% (7/32) were obtained by nested PCR when tissue samples of pigs challenged with oocysts were evaluated (Yai et al., 2003). Commercial meat products from the UK were evaluated for *T. gondii* by SAG2 amplification and the authors (Aspinall et al., 2002) found 27/71(38%) positive samples.

During this study it was not possible to demonstrate the association between the distribution of *T. gondii* with commercial cuts of pork, while mouse bioassay was more sensitive than PCR.

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