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DETECTION OF VIABLE *TOXOPLASMA GONDII* IN FREE-RANGE PIGS FROM THE SPECIAL NATURE RESERVE OF ZASAVICA*

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Summary: *Toxoplasma gondii is considered one of the most successful parasites of humans and animals. The ingestion of viable cysts through the consumption of undercooked pork is recognized as a significant route of human infection with T. gondii. The aim of this study was to investigate the presence of viable parasite in tissues of free-range pigs from the Zasavica Special Nature Reserve. All pigs were of the Mangulica breed, raised in a traditional way. The serological screening was performed using a modified agglutination test (MAT). The isolation of viable T. gondii was attempted by a bioassay of pig heart tissue in mice, while the real-time polymerase chain reaction (qPCR) targeted at the 529 bp repetitive element of T. gondii was used to detect parasitic DNA in digested hearts. Specific antibodies were detected in 12 out of 18 pigs examined. The bioassay was performed for five MAT-positive and one MAT-negative pig, and a total of three isolates were obtained. qPCR was performed for all samples, including one MAT-negative sample that was not bioassayed. The presence of T. gondii DNA was confirmed in all hearts with a positive bioassay as well as in one originating from seropositive and one from seronegative pig whose hearts were not bioassayed. The successful isolation of viable cysts, presence of risk factors (such as older age at the time of slaughter) and increased contact with the environment, along with the great appreciation of Serbian consumers towards home-cured Mangulica's meat, make this breed worthy of consideration as a potentially important reservoir of human infection.*

Key words: *Toxoplasma gondii, isolation, free-range pigs, Mangulica*

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan from the phylum *Apicomplexa*. Its worldwide distribution, wide range of hosts (including humans) and ability to parasitize virtually any nucleated cell of birds and mammals make it one of the most successful zoonotic parasites. In contrast to the wide spectrum of intermediate hosts, only members of the family *Felidae* have been recognized as definitive hosts (Dubey, 2008). Immunocompetent humans and the majority of animal species rarely suffer from clinical toxoplasmosis. For immunocompromised persons, however, the infection with *T. gondii* can have rather severe, even lethal, consequences (McLeod et al., 2013; Weiss and Dubey, 2009). In women, the infection during pregnancy can result in abortion or birth of congenitally infected children (Bobić et al., 1998). Main sources of infection in humans are considered to be the ingestion of oocysts through contaminated food and water and the ingestion of tissue cysts, through consumption of undercooked meat of infected animals.

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In many countries, including Serbia, pigs are considered an important source of human infection with *T. gondii* (Cademartori et al., 2014; Dubey, 1986; Klun et al., 2006). Antibodies to *T. gondii* have been found in pigs in all production systems and successful cases of parasite isolation have been reported worldwide (De Sousa et al., 2006; Dubey et al., 2012; Turčeková et al., 2013). Moreover, pigs are considered to be one of the meat-producing animals which most frequently harbour infective cysts of *T. gondii* in their tissues (Tenter et al., 2000). The life-long persistence of tissue cysts, lack of clinical symptoms in the majority of infected pigs (Dubey, 1986), inability of current meat inspection strategies to distinguish between carcasses of infected and uninfected animals (Blagojevic & Antic, 2014) and habit of consuming raw (including cured) meat (Bobić et al., 2012) additionally increase the risk of acquiring *T. gondi* - infection through pork meal. Cases of human toxoplasmosis have already been connected to the consumption of pork products (Choi et al., 1997; Pereira et al., 2010) and viable parasites have been isolated from both fresh meat (Wang et al., 2012) and meat products (Dias et al., 2005; Gomez-Samblas et al., 2015).

Considering a higher risk of infection with *T. gondii* in pigs raised in production systems which imply an increased interaction of animals with the environment (Dubey et al., 2012; Wallander et al., 2016) and lack of data regarding such pigs in Serbia, the aim of this study was to investigate the presence of viable *T. gondii* in tissues of free-range pigs slaughtered for human consumption.

MATERIALS AND METHODS

Sample collection. The sampling was conducted during December of 2015. The paired samples of blood and hearts were collected from pigs slaughtered at a private abattoir in Zasavica II (44º58′N 19º32′E), a village in the Srem District, the Vojvodina province, Serbia. All pigs were of the Mangulica breed, ≥ 18 months old and raised outdoors, allowed to roam. After collection, the samples were transported to the Laboratory for Parasitology of The Faculty of Agriculture, the University of Novi Sad. The blood was centrifuged (2000 rpm for 20 min), the sera were separated, frozen and transported the following day to the National Reference Laboratory for Toxoplasmosis (NRL-Toxo), Centre of Excellence for Food- and Vector-borne Zoonoses, the University of Belgrade Institute for Medical Research, to be examined for *T. gondii* specific antibodies. The hearts were stored at 4°C until the reading of the serological test the following day. The hearts of seropositive pigs were selected for a bioassay in mice. In several cases, digestion had to be done prior to the serological testing and in these cases all hearts were bioassayed, regardless of the serological status of the pig.

Serological examination. The sera of both pigs and mice (from the bioassay) were screened for the presence of *T. gondii*-specific IgG antibodies using the modified agglutination test (MAT), as described by Desmonts and Remington (1980). The formalin-fixed whole RH tachyzoites used as antigens were kindly donated by Dr. Isabelle Villena (Centre National de Référence de la Toxoplasmose, Reims, France). The sera were serially two-fold diluted starting at 1:25 for pigs and 1:20 for mice. All sera reactive at these levels were considered positive and were further titrated.

Isolation of *T. gondii*. The hearts were subjected to the artificial trypsin digestion, and bioassayed according to the slightly modified protocol described by Djurković-Djaković et al. (2005). Briefly, the tissue samples (50±10g) were chopped using an electric blender, mixed with saline solution of trypsin (final concentration 0.25%), penicillin-streptomycin (FM Pharm, Subotica, Serbia) and amoxicillin (Hemofarm, Vršac, Serbia) and incubated at 37◦C, for 1.5h. The suspension was then filtered and washed with saline three times. A fraction (300µl) of the obtained pellet was frozen (-20ºC) for molecular examination and the remaining tissue was mixed with gentamicin (FM Pharm, Subotica, Serbia) and bioassayed. For the purpose of a bioassay, 1 mL of each homogenate was intraperitoneally inoculated into two adult (body mass ≥ 18g) Swiss-Webster mice (Medical Military Academy Animal Research Facility, Belgrade, Serbia). After a minimum of six weeks the mice were euthanized and bled. Squashes were made from frontal portions of brains and microscopically examined for the presence of *T. gondii* cysts. The remaining brain tissue was homogenized with 1 mL of saline and cysts were enumerated at a sensitivity threshold of 10 cysts per brain. The bioassay was considered positive in the case of positive serology, detection of brain cysts, or both.

The bioassay was conducted with the approval of the Experimental Animals Welfare and Ethics Committee at the University of Novi Sad.

DNA extraction and real-time polymerase chain reaction (qPCR). DNA was extracted from 100 µL of heart digests using GeneJet Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the slightly modified "Mammalian Blood Genomic DNA Purification Protocol" provided by the manufacturer. For the purpose of the optimal DNA yield, the elution was performed using 150µl of pre-warmed PCR water, instead of 200µl of the elution buffer.

Table 1. Primers and probes used in qPCR for the detection of *T. gondii* DNA in pig hearts

Description	Name	Sequence
"Forward" primer	HO1	aga gac acc gga atg cga tct
"Reverse" primer	HO2	ccc tct tct cca ctc ttc aat tct
Probe	HOFT	FAM-acg ctt tcc tcg tgg tga tgg cg-TAMRA

The detection of *T.gondii* DNA from heart digests was performed using qPCR, according to the protocol employed by NRL-Toxo. PCR targeted at the 529 bp repetitive element (Gene Bank accession number AF146527). Briefly, the PCR reaction was performed in a final volume of 20µl mixture containing a 10µl of Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA), 0.25 mM of each primer (HO1 and HO2, Table 1), a 0.10 mM of TaqMan HOFT probe (Invitrogen, Life Technologies, Carlsbad, CA, USA), 0.015 U/µl of UNG, 25 mM MgCl2 and nuclease-free water, with the addition of 3µl of extracted DNA. The amplification and detection was performed with the StepOnePlus™ real-time PCR system (Applied Biosystems, California, USA), using the following program: 2 min at 50◦C for the UNG pre-treatment, 5 min at 95◦C for initial denaturation, followed by 45 cycles of 15 s at 95◦C for denaturation and 60s at 60◦C for annealing/extension.

RESULTS

The samples from a total of 18 pigs were collected. *T.gondii*-specific IgG antibodies were detected in 12 pigs, with the following antibody titres: 4 pigs were seropositive at a titre of 1:25, 4 at 1:50, 3 at 1:100 and one pig at a titre of 1:200. Due to technical reasons, bioassays could not be performed for all seropositive pigs (Table 2); however, all of their heart digests were tested by qPCR for *T.gondii* DNA. Also, due to the aforementioned reasons (Materials and Methods), two seronegative pigs were also examined – one both by a bioassay and qPCR and one only by qPCR.

Table 2. Summary of the bioassay and qPCR results for all pigs included in the study according to their serological status

	Examined	Total *T. gondii* positive/tested (%)*	Bioassayed		qPCR only**	
			Tested	Isolated (%)	Tested	Positive (%)
Seropositive	12	3/12 (25)	5	2 (40)	7	1 (14.3)
Seronegative-	6	1/2 (50)	1	1 (100)	1	0 (0)
Total	18	4/14 (28.6)	6	3 (50)	8	1 (12.5)

* by either bioassay or qPCR

** These samples were examined only by PCR. No bioassay was performed, due to technical reasons.

All hearts that were positive according to the mouse bioassay were also positive according to qPCR (Table 3). None of the positive mice died prior to the termination of the bioassay. On microscopic examination, the cyst burden in their brains ranged from 20 to 270 cysts per mL of brain homogenate.

Table 3. Pigs with *T. gondii* DNA detected and/or viable parasites isolated

Pig ID	Antibody titre	Results of qPCR	Bioassay results	
			Mice with cysts/ Mice inoculated	MAT titre
17 BM	< 1:25	positive	2/2	> 1:40
1 BM	1:50		1/2	> 1:40
12 BM			ND	ND
6 BM	1:100		2/2	≥ 1:40

ND – not done

DISCUSSION

During the past 10 years, one nation-wide (Klun et al., 2006) and two local seroepizootiological studies in pigs (Klun et al., 2011; Kuruca et al., 2014) have been conducted in Serbia, revealing seroprevalence of 29%, 9% and 4.5% respectively. Viable parasites have been isolated from the blood of pigs with parasitaemia and tissues of other meat animal species, such as sheep (Klun et al., 2011; Marković et al., 2014). Earlier work showed isolation of *T. gondii* from the hearts of 8 out of 620 examined pigs from the farms in the Belgrade area. However, authors provided no data on the serological status of these animals (Simitch et al., 1967). In our study, *T. gondii*-specific antibodies were detected in 12 out of 18 free-range pigs and viable cysts were demonstrated in two out of five bioassayed hearts. Cysts were also observed in mice inoculated with material from MAT-negative pig. Isolation from tissues of seronegative pigs has been previously reported by other authors. In a study conducted by Hejlíček and Literák (1993) *T. gondii* was demonstrated in 18 pigs whose dye test results were negative. Dubey et al. (2002) isolated *T. gondii* from two pigs in which specific antibodies could not be demonstrated with either the MAT, dye test or Western blot. Out of 170 isolates from sows in Iowa, 29 originated from seronegative pigs (titer < 20) (Dubey et al., 1995). The isolation of *T. gondii* from seronegative pigs indicates that either these pigs were recently infected and had not yet developed specific antibodies, or that the antibody titres had declined to undetectable levels. Furthermore, antibody response in naturally infected pigs is generally considered to be low (Dubey, 2000) which is also in accordance with low antibody levels detected in the majority of animals in our study. A low percentage of *T. gondii*-infected pigs is likely to go undetected by antibody screening, even when the MAT is used, which is regarded as the most sensitive and specific test used to detect *T. gondii* infection in naturally infected pigs (Dubey et al., 1995). None of the isolates obtained in our study was pathogenic for the mice.

Among the animals whose hearts were not bioassayed, two samples (one from a MAT-positive and one from a MAT-negative pig), were positive for *T. gondii* DNA. Since bioassay is the only method capable of assessing the viability of the parasites, in its absence it is difficult to speculate on the infectivity of meat originating from these two pigs. However, the presence of DNA is indicative of the presence of *T. gondii* infection and it is reasonable to assume that some of the parasites might have remained viable in other (unexamined) tissues of the animals.

The fact that all of the pigs included in the study were of the Mangulica breed is of interest, for several reasons. Mangulica is an indigenous, primitive, fat-type pig breed. It takes a minimum of 18 months for Mangulica to reach its market-weight (Živković and Perunović, 2012). This means that, at the time of slaughter, pigs of this breed are usually twice as old as modern pig breeds. Studies have shown that, in pigs, risk of acquiring *T. gondii* infection increases proportionally with the age of animals. As a consequence, the highest seroprevalence has been demonstrated in sows (Edelhofer, 1994; Klun et al., 2006) reaching, in some cases, 100% of animals tested (Venturini et al., 2004). Furthermore, Mangulica pigs are usually traditionally raised, meaning that they are allowed to roam and graze throughout the greater part of the year. Increased contact with the environment increases the chances of pigs to get into contact with the infection sources, leading to a greater risk of acquiring toxoplasmosis (García-Bocanegra et al., 2010; Wallander et al., 2016). As a consequence, a high prevalence of infection can be observed in organically (90.9%) (Dubey et al., 2012) and extensively (47.4%) (Gamble et al., 1999) raised pigs. Finally, mature pork is most often used for production of cured meat delicatessen (Klun et al., 2006) and procedures of salting and curing have not always been efficient in killing *T. gondii* cysts, especially in case of home-made products (Gomez-Samblas et al., 2015; Tenter, 2009). This makes traditionally raised Mangulica a potentially important reservoir of human infection, which necessitates further research.

CONCLUSION

A high prevalence of *T. gondii*-specific antibodies was detected in free-ranging pigs of the Mangulica breed. Viable cysts were demonstrated in both seropositive and seronegative pigs. The late maturation, tradition of outdoor breeding and great appreciation of Serbian consumers towards home-cured Mangulica's meat make this breed worthy of consideration as a potentially important reservoir of human infection.

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DETEKCIJA VIJABILNOG PARAZITA *TOXOPLASMA GONDII* KOD SLOBODNO DRŽANIH SVINJA IZ SPECIJALNOG REZERVATA PRIRODE ZASAVICA*

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Izvod: *Toxoplasma gondii* predstavlja jednog od najuspešnijih parazita ljudi i životinja. Ingestija vijabilnih cista, putem konzumacije termički nedovoljno obrađenog mesa, smatra se jednim od dominantnih puteva humane infekcije ovim parazitom. Cilj ovog istraživanja bio je da se ispita prisustvo vijabilnih parazita u tkivima slobodno držanih svinja u Specijalnom rezervatu prirode "Zasavica". Sve ispitane svinje su pripadale rasi mangulica i gajene su na tradicionalan način. Serološka ispitivanja su sprovedena upotrebom testa modifikovane aglutinacije (MAT). Za izolaciju vijabilnih parazita iz tkiva (srca) svinja korišćen je biološki ogled na miševima, dok je reakcija lančane polimeraze u stvarnom vremenu (qPCR), kojom se detektuje 529 bp repetitivni element genoma *T. gondii* upotrebljena za detekciju parazitske DNK. Specifična antitela su pronađena kod 12 od 18 ispitanih svinja. Biološki ogled je postavljen za 5 MAT-pozitivnih i jednu MAT-negativnu svinju, iz čega su dobijena ukupno tri izolata *T. gondii*. qPCR je urađen za sve uzorke, uključujući i jedan MAT-negativan uzorak, za koji nije postavljen biološki ogled. DNK *T. gondii* je dokazana u digestima srca svih svinja iz kojih je parazit uspešno izolovan, kao i kod jedne seropozitivne i jedne seronegativne svinje za čija srca nije postavljen biološki ogled. Izolacija vijabilnih cista, prisustvo faktora rizika kao što je kasnostasnost rase, povećan kontakt sa spoljašnjom sredinom kao i činjenica da su domaći suhomesnati proizvodi od mesa mangulice visoko cenjeni od strane srpskih potrošača, čine ovu rasu svinja potencijalno značajnim rezervoarom *T. gondii* za ljude.

Ključne reči: *Toxoplasma gondii, izolacija, slobodno držane svinje, mangulica*

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