Detection of *Toxoplasma gondii* and *Encephalitozoon* spp. in wild boars by serological and molecular methods

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SUMMARY

The study aims to determine the seroprevalence of *Toxoplasma gondii* and of 2 representative microsporidia (*Encephalitozoon cuniculi* and *Encephalitozoon intestinalis*) in wild boars (*Sus scrofa*) and to compare with the molecular detection of parasites by PCR. Sera collected from 91 hunter-killed wild boars from eight different regions of the Slovak republic were assayed for specific antibodies (IgG) using 2 enzyme-linked immunosorbent assays and the presence of parasites were investigated into seropositive animals by standard and real time PCR from blood for *T. gondii* and from stool samples for *Encephalitozoon* spp. Circulating antibodies targeted against *T. gondii, E. cuniculi* and *E. intestinalis* were detected in 18 (19.8%), 4 (4.4%) and 3 animals (3.3%) respectively. The frequency of the anti-*T. gondii* antibodies was significantly higher in young boars (< 1 year old) (37.9%) than in adults (11.3%) ($P < 0.01$) whereas only adult subjects were seropositive for the microsporidia. A DNA fragment (191 bp) from *T. gondii* was detected in only one positive boar in a relatively high number of copies (1.5×10^5) and the presence of *Encephalitozoon* spp. was not confirmed by PCR.
These results indicate that wild boars can be contaminated by *T. gondii* and microsporidia with moderate and low seroprevalences respectively and that they do not represent a great (in quantity) reservoir of these parasites, but the risk of toxoplasmosis transmission to humans remains qualitatively important throughout consumption of meat from an effectively infected boar.

Keywords: Toxoplasmosis, encephalitozoonosis, wild boar, serological survey, PCR assay, seroprevalence.

RÉSUMÉ

Détection de *Toxoplasma gondii* et des *Encephalitozoon* spp. chez les sangliers par des méthodes sérologiques et moléculaires

Les objectifs de cette étude ont été de déterminer les séroprévalences de *Toxoplasma gondii* et de 2 microsporidies représentatives (*Encephalitozoon cuniculi* et *Encephalitozoon intestinalis*) chez le sanglier (*Sus scrofa*) et de comparer les résultats avec ceux de la PCR permettant la détection moléculaire des parasites. Les anticorps spécifiques (IgG) ont été recherchés par deux tests ELISA sur 91 sérums de sangliers tués par des chasseurs dans 8 régions différentes de la République Slovaque et la présence des parasites a été recherchée sur les animaux séropositifs par des techniques de PCR standard et en temps réel à partir du sang total dans le cas du toxoplasme et des fèces dans le cas des sporidies. Les anticorps circulants dirigés contre *T. gondii, E. cuniculi* et *E. intestinalis* ont été respectivement détectés sur 18 (19,8 %), 4 (4,4 %) et 3 (3,3 %) animaux. La fréquence des anticorps anti-*T. gondii* a été significativement plus élevée chez les jeunes (de moins de 1 an) (37,9 %) que chez les adultes (11,3 %) ($P < 0.01$) alors que les anticorps dirigés contre les sporidies n'ont été retrouvés que chez des adultes. L'amplification spécifique par PCR (jusqu'à 1.5×10^5 copies) d'un fragment d'ADN de *T. gondii* (191 pb) n'a pu être obtenue que sur un seul des 18 animaux séropositifs et la présence des microsporidies n'a pas été confirmée par PCR. Ces résultats montrent que les sangliers peuvent être contaminés par *T. gondii* et par les microsporidies avec des fréquences respectivement moyennes et faibles et qu'ils ne constituent pas un réservoir quantitativement important de ces parasites, mais que le risque de transmission de la toxoplasmose à l'homme reste qualitativement important par la consommation de viandes effectivement infectées.

Mots clés : Toxoplasmose, encéphalitozoonose, sanglier, sérologie, PCR, séroprévalence.

Introduction

Toxoplasma gondii (*T. gondii*) is a protozoan parasite of great medical and veterinary importance. Toxoplasmosis is one of the most common parasitic zoonoses in the world afflicting a broad range of both mammals and birds [11]. The aetiological agent is *T. gondii* whose definite hosts are representatives of the family of *Felidae* infected by oocysts from the environment, or by tachyzoites and bradyzoites from intermediary hosts, such as all kinds of vertebrates, including humans. It is a pantropical cosmopolite and facultative heterogenic coccidia. *T. gondii* causes a mild infection in immuno-competent hosts, but in the immuno-compromised hosts, foetus and neonates, toxoplasmosis is severe even leading to death [21].

Encephalitozoon spp. is widespread amongst humans and other mammals. The microsporidium genus *Encephalitozoon* contains three known species that are opportunistic parasites of HIV-infected patients [25]. One of these species, namely *E. cuniculi*, is the most extensively studied mammalian microsporidium because it occurs in a wide range of animals, and spontaneous infections have been documented in various mammals, including non-human primates and human beings [15]. *E. cuniculi* causes infection in wide range of organisms and *E. intestinalis* is the second most prevalent species which causes mostly gastrointestinal infections (local and disseminated)

but it has rarely been reported in animal hosts. Increasing attention has been paid to the study of animal encephalitozoonosis in the field of veterinary and human medicine in the last years [24].

Wild boars are important large game species in the Slovak Republic. Their population has significantly increased during last decade: according to data of the Slovak hunting Association, the number of animals in the spring 1995 was 17 738, while in 2002 it was 26 135 [2]. Wild boars are omnivorous animals that grout in the soil and eat a lot of species of plants and living or death organisms. The most frequent source of infection by *T. gondii* is ingestion of infected rodents and birds and infected cat faeces [14]. Therefore, the *T. gondii* prevalence in wild boars may be a suitable indicator of the environmental contamination. In turn, wild boar meat containing tissue cysts is a potential source of infection for humans, especially hunters and their families who may become infected during evisceration and handling of the game. Consequently, the aim of the study is to determine the prevalence of *T. gondii* and *Encephalitozoon* spp. in wild boars in order to assess the risks for the human health.

Materials and Methods

ANIMALS

The study analyzed 91 sera of wild boars (*Sus scrofa*) hunted in 8 different regions of the Slovak republic. According to the age, wild boars were divided into 2 groups: under 1 year old (n = 29) and adult animals (n = 62). Immediately after death, animals were bled and after coagulation at 22°C for 30 minutes and subsequent centrifugation (2 200g, 10 minutes, 22°C), sera were stored at -20°C until used. On the other hand, EDTA was added to approximately 5 mL of blood and not coagulated blood samples were used for DNA extraction in order to evidence the presence of *T. gondii*. Faeces of wild boars were mixed with tissue-lysing buffer (30% sarcosine, NET 50) and these samples were then disrupted in microwaves (600 W) three times for 20 seconds each before DNA extraction for evidencing *Encephalitozoon* spp.

SEROLOGICAL ANALYSIS

Toxoplasma gondii

An immuno-enzymatic test (ELISA) was carried out for the detection of IgG antibodies targeted against *T. gondii* according to the manufacturer's instructions (Test-Line, Czech Republic). The reaction is based on the principle of an indirect enzymatic reaction. In the first step, specific IgG antibodies present in serum diluted to 1:100 or 1:200 bind to the *T. gondii* antigen coated on the surface of reagent wells and then, the rabbit anti-boar IgG antibodies labelled with peroxidase (Sigma-Aldrich, USA) were applied to the complex formed between the *T. gondii* of antigen and circulating antibodies. After addition of the enzyme substrate, TMB (3,3´,5,5´-tetramethylbenzidine), the absorbance was read at 450 nm using a Dynex spectrophotometer (Dynex Technologies, USA). Positive and negative serum controls previously tested by conventional serological tests were included on each plate. For each sample, the index of positivity (IP) was calculated according to the schema provided by the manufacturer: IP: sample absorbance/average absorbance of cut-off serum (cut-off serum is serum sample which contains antibodies to *T. gondii* in limiting concentration). Samples with IP > 0.8 were considered to be negative, samples with IP between 0.8-1.0 were considered to be dubious and samples with IP > 1.0 were considered to be positive.

Encephalitozoon spp

Mature spores of microsporidia (*E. cuniculi* and *E. intestinalis*) grown in monolayer cell cultures of rabbit kidney cells (RK 13) in vitro were used as antigens in the serological assays. Permanently infected cells were cultivated in the modified RPMI 1640 medium supplemented with 5% foetal bovine serum and with addition of antibiotics (streptomycin / gentamicin). After centrifugation (4 000 g, 20 minutes, 20°C), spores were isolated from the supernatant, rinsed in PBS, counted in a haemocytometer and finally re-suspended in PBS to the required concentration (10^6 spores/mL). The IgG antibodies against *E. cuniculi* and *E. intestinalis* were detected using a modified ELISA described by Hollister and Canning in 1987 [13]. Briefly, 100 µL of *E. cuniculi* or *E. intestinalis* spores (10^6 spores/mL) in coating buffer (Na_2CO_3 and $NaHCO_3$) were put into each well of ELISA plates and after incubation overnight at 4°C, the plates were dried and fixed with 1:1 mixture of acetone and methanol for 10 min. After blocking the unbound sites with 5% new-born calf serum (NBCS) in PBS for one hour at 37°C, the plates were washed with 0.05% Tween 20 in PBS (T-PBS) three times. Each serum diluted to 1:200 and 1:400 in 2% NBCS in PBS (0.1 mL) was added into the well and after incubation (37°C, 1 hour), plates were washed with T-PBS and incubated with rabbit anti-swine immunoglobulin peroxidase conjugate (Sigma, Germany) for one hour at 37°C. After washing, an enzymatic colour reaction was generated using orthophenylenediamine substrate (OPD). The reaction was stopped with 2M H_2SO_4. Sample absorbance was measured by a spectrophotometer Dynex Elisa Reader (Dynex Technologies, USA) at the wavelength of 490 nm. Sera for which the absorbance was at least 2.1 times higher than the absorbance of a negative control serum were considered positive.

MOLECULAR PCR ANALYSIS

Toxoplasma gondii

DNA was isolated from the leukocyte fraction of the not coagulated blood with the commercial kit QIAamp DNA Mini Kit (QIAGEN, Germany).

Amplification of the isolated DNA was carried out by the standard PCR method from the *T. gondii* gene region TGR1E, repeated in the genome 30-35 times, using the specific primers

TGR1E-1 and TGR1E-2 [8]. PCR was executed in 25 µL reaction volume containing 0.2 µM of each primer (TGR1E-1, TGR1E-2), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 2.5 U of *Taq* DNA polymerase and the reaction was conducted in a thermocycler (Genius, UK) with the following temperature profile: initial denaturation at 94°C for 3 min., 40 cycles of amplification (94°C 1 min., 60°C 1 min., 72°C 1 min.) and final extension at 72°C for 7 min. The PCR products were visualized in 3% agarose gel and stained with ethidium bromide [7]. The final positive PCR product has 191 bp in size.

For quantification of the positive PCR samples, a real time PCR with SYBR Green as detection system was used, using the specific primers TGR1E-1 and TGR1E-2. The standards cloning TA *T. gondii* TGR gene (GenExpress, Germany) diluted to 10^4-10^9 were used for the calibration curve. In each reaction, a melting analysis (comparison of the melting temperature (T_m) of PCR products) was inserted to differentiate specific and not specific PCR products. The reaction volume was 50 µL that contains commercial SYBR GreenER qPCR Supermix (Invitrogen, Brazil) and 0.2 µM primers (TGR1E-1 and TGR1E-2). Real time PCR was executed in the thermocycler Line GeneK with a software Line GeneK Fluorescent Quantitative Detection System (BIOER Technology, China). After incubation at 50°C for 2 min. and initial denaturation at 95°C for 10 min., 40 amplification cycles were performed (95°C for 15 s, 60°C for 1 min.) and the melting analysis was carried out at temperatures ranging from 60°C to 95°C, in which the temperature was gradually increased by 0.5°C and the period of measurement at individual steps was 15 seconds.

Encephalitozoon spp

DNA was extracted from spores of wild boar faeces using the commercial isolation kit QIAamp DNA Stool Kit (QIAGEN, Germany) according to the manufacturer's instruction.

Specific primer pairs (ECUNF and ECUNR for *E. cuniculi* and SINTF and SINTR for *E. intestinalis*, 0.2 µM of each primer) [23] were used for the amplification of fragments (550 bp and 545 bp respectively) of the small subunit rRNA gene. The PCR reaction was prepared and performed by a standardized protocol, including 35 cycles, at 95°C for 1 min, 48°C or 55°C (for *E. cuniculi* and *E. intestinalis*, respectively) for 1 min. and 72°C for 1 min in a thermocycler (Techne Genius). PCR products were analyzed using electrophoresis in 1.5% ethidium bromide-stained agarose gel examined under ultraviolet light.

STATISTICAL ANALYSIS

The significance of the differences in prevalence of toxoplasmosis and encephalitozoonosis according to the age of animals was evaluated by Fisher Exact Test or a chi 2 test and was considered as positive when *P* value was less than 0.05.

Results

On 91 wild boars examined, 18 (19.8%) exhibited a positive serological reaction for *T. gondii* and the frequency of *T. gondii* contamination was significantly greater in young animals (less than 1 year old) (37.9%) than in adults (11.3%) ($\chi 2$ test: $P < 0.01$) (Table I). The overall *Encephalitozoon* seroprevalence was 7.7% and the proportions of animals infected by *E. cuniculi* and by *E. intestinalis* were similar (4/91 and 3/91 respectively). No antibody against *E. cuniculi* or *E. intestinalis* was found in animals younger than one year (Table I).

Using the standard PCR, the *T. gondii* DNA was identified only in one blood sample (figure 1) and by the real time PCR, the number of copies of the *T. gondii* DNA was 1.05 x 10^5 copies. No DNA was found in stool samples of wild boars seropositive to *E. cuniculi* or *E. intestinalis*.

Discussion

Wild boar is an autochthonous species of leafy exploitation of the Slovak republic. Due to the agricultural exploitation of landscape, disseminating of maize production, intensive forest management and low mortality of piglet during snow and absence of big carnivores, the wild boar population started to increase from the second half of the 20th century. The raising number of wild boars causes economic losses and may represent a source of dissemination of different diseases. Tissue cysts of *T. gondii* in meat of different game species are potential sources of human infection [12].

The overall *T. gondii* seroprevalence in wild boars was 19.8% in the present study and was significantly higher in

Wild boars	***T. gondii***	***Encephalitozoon***		
	SP (N/n)	**Overall SP (N/n)**	***E. cuniculi* SP (N/n)**	***E. intestinalis SP (N/n)***
Total	**19.8** (18/91)	**7.7** (7/91)	**4.4** (4/91)	**3.3** (3/91)
< 1 year old	37.9 (11/29**)	0.0 (0/29)	0.0 (0/29)	0.0 (0/29)
Adult	11.3 (7/62)	11.3 (7/62)	6.5 (4/62)	4.8 (3/62)

N: number of positive samples; n: number of examined samples; SP: seroprevalence (%)
*** P < 0.01.*

TABLE I: Seroprevalence of *T. gondii* and *Encephalitozoon* (*E. cuniculi* and *E. intestinalis*) in wild boars (n = 91) in the Slovak Republic.

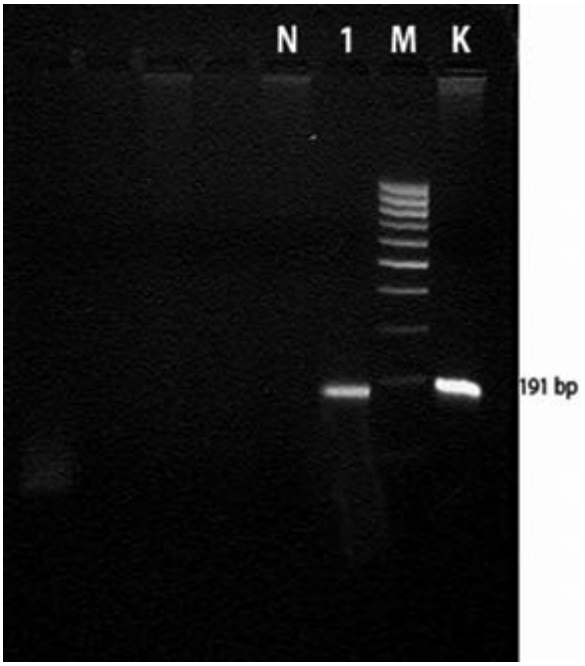

FIGURE 1: PCR fragment of T. gondii DNA (191 bp) in a 3% agarose gel. K: positive control, M: marker of size (100bp plus DNA ladder, Fermentas, Germany); lane 1: positive wild boar sample, N: negative control.

young animals (37.9%). This corresponds to the fact that toxoplasmosis is most commonly seen in young animals, especially in neonates and in immuno-compromised animals. Toxoplasmosis in young animals causes severe damage such as intra-uterine growth restriction, icterus, hepato-splenomegaly, myocarditis, pneumonitis, and various rashes. Neurologic involvement, often prominent, includes chorio-retinitis, hydrocephalus, intracranial calcifications, microcephaly, and seizures [12]. In Europe, anti-*T. gondii* antibodies were found in 8.1% (26/320) or 38.5% (5/13) of wild boars from Slovakia [2, 6], 26.2% (148/565) from the Czech Republic [5], 16.6% (2/12) from Bulgaria [3] and 21.1% (11/52) from Eastern Poland [20].

The occurrence of circulating antibodies against two major representatives of *Encephalitozoon* spp. was also investigated in wild boars in the current study. No data about the occurrence of anti-*E. cuniculi* or *E. intestinalis* antibodies in wild boars in Europe was available, but the *E. cuniculi* seroprevalence was previously determined in other wild animal species (wild rabbits, foxes, European brown hare and koalas). In 1997 THOMAS *et al*. [22] reported the presence of serum anti-*E. cuniculi* antibodies in 22 wild rabbits out of the examined 81 sera examined (seroprevalence: 27.2%). In 2003, AKERSTED *et al*. [1] found no seropositive response among 230 Arctic foxes from Greenland. In agreement with that, only 4 boars gave a positive response to *E. cuniculi* and the seroprevalence determined in the present study was low (4.4%). *E. intestinalis* is microsporidia specie specific to humans. It could be occasionally found in animals but its prevalence in wild boars is not available. This presence was rarely investigated in wild animals. However, *E. intestinalis* has been evidenced by PCR in the small intestine of 2 koalas [17] and in kidneys of 2 European brown hare (*Lepus europaeus*) [10]. In the present study, the *E. intestinalis* seroprevalence in wild boars was also low (3.3%).

On the other hand, the standard PCR has detected the specific DNA fragment (191 bp) of T. gondii from not coagulated blood in only one wild boar whereas the serum anti-*T. gondii* antibodies have been evidenced in 18 animals. Moreover, a relatively high number of copies of the Toxoplasma genome were found by the real time PCR ($1.5.10^5$ copies). In France, RICHOMME *et al*. [19] tested 148 sera and tissues of wild boars for Toxoplasma infection from two French regions, one continental and one insular. Antibodies to *T. gondii* were found in 26 (17.6%) of 148 wild boars using the modified agglutination test (MAT) with 1:24 as the positivity threshold but the seroprevalence was higher (45.9%) when considering a threshold of 1:6. Hearts from individuals with a positive agglutination (starting dilution 1:6) (n = 60) were bioassayed in mice for isolation of viable *T. gondii*, and 21 *T. gondii* isolates were obtained. In other wildlife from France conducted by AUBERT *et al*. [4], *T. gondii* antibodies were found in 14 of 19 (73.7%) red foxes, with titres between 1:25 and 1:6400 and parasite isolation was successful in 9/13 seropositive animals (69.2%). Thirty-six of the 60 roe deer (60%) showed antibodies with titres between 1:6 and 1:6400 and the toxoplasma isolation was successful in 36.4% animals whose antibody titres were comprised between 1:25 and 1:6400. In addition, the anti-*T. gondii* antibodies were found in 4 of 24 red deer (17%) with titres ranged from 1:6 to 1:25 and a viable parasite was isolated from the heart of only one red deer (antibody titre: 1:6). No parasite was isolated from fallow deer and only one animal was seropositive. In the same way, the toxoplasmosis seroprevalence in mouflons was 22.5% (7/31) but the parasite was isolated from only one sample. In brown hares (*L. europaeus*), a common species of wild mammals in Europe extensively hunted, the authors detected serum anti-*T. gondii* antibodies in 9% of the hare population but they failed to isolate viable parasites. Consequently, results of the serological tests did not reflect the course of the present infection. The presence of specific serum antibodies is detected in potential hosts who come in contact with the pathogen. The serological prevalence in an animal population only evidences a possible relationship between population and morbidity. As the investigated serum antibodies were immunoglobulins G which are lately produced during the infection and represent one of mechanisms of memory immunity, their occurrence in serum reveals that the animal has been in contact with the parasite and that the parasite is not necessary always present in the organism. Consequently, serological results traduce the passage of the parasite into the animal population whereas molecular results detect its persistence into the population and discrepancy between these data may evidence the disappearance of the parasite into the wild boar population, i.e. that wild boars probably do not constitute a reservoir of the parasites. The advantage of serological tests is that specific antibodies can be detected two week before histological evidence of parasite into lesions and 4 weeks before the molecular identification of parasites in infected tissues. Gene amplification methods (PCR, LCR, NASBA, etc.) are now used widely in the diagnosis of infectious diseases. Key advantages are their relative speed, the potential to detect very low numbers of pathogens (or, more precisely, specific nucleic acid sequences from pathogens) and the ability to discriminate accurately at the species or

sub-species level. In the case of non-persistent pathogens that are cleared from the body, a positive PCR finding is usually significant. The diagnosis of toxoplasmosis by PCR, however, is complicated by the fact that the parasite persists (principally in heart brain and skeletal muscle in the form of quiescent tissue cysts) for many years after active infection has ceased. Thus, the presence of *T. gondii* in such tissues does not necessarily equate to active toxoplasmosis. Therefore is possible to find discrepancy between the serological results and results of molecular methods [16].

The presence of the 2 *Encephalitozoon* species from the faeces of contaminated wild boars was not confirmed by PCR. Negative results with PCR would be caused by the presence of inhibitors into samples. Various compounds including phenols, glycogen, fats, cellulose, some constituents of bacterial cells, non-target nucleic acids and heavy metals can inhibit the PCR reaction [26]. The presence of PCR inhibitors also depend on the food composition [18]. As wild boars are omnivorous animals that grout the soil and eat a lot of species of plants and living or death organisms, they probably ingested numerous and various PCR inhibitors like cellulose in the same way. The PCR inhibitors stemming from dietary components are frequent in stool samples [9] and lead to false negative PCR results.

As a conclusion, using specific serological ELISA tests, the possibility of contamination of the wild boars by *T. gondii* and *E. cuniculi* or intestinalis has been demonstrated although the observed seroprevalences remained mild (19.8% for the toxoplasmosis) to low (4.4% for *E. cuniculi* and 3.3% for *E. intestinalis*). However, only one parasite (*T. gondii*) was detected by PCR into the wild boar population investigated in the present study. Consequently, it may conclude that wild boars can be contaminated by these parasites (*T. gondii, E. cuniculi* and *E. intestinalis*) but the wild boar population cannot be considered as a main reservoir for these parasite species, although consumption of insufficiently cooked meat from wild boars effectively carrying the parasites constitutes a real risk for toxoplasmosis transmission.

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