

Survey of *Toxoplasma gondii* antibodies in meat juice of wild boar (*Sus scrofa*) in several districts of the Czech Republic

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Abstract

Introduction and objective. The aims of the study were: 1) to detect antibodies against *Toxoplasma gondii* from wild boar meat; 2) establish seroprevalence of toxoplasmosis in the wild boar population; 3) establish risk factors concerned in higher possible seroprevalence; 4) to estimate the usefulness of meat juice for detection of *T. gondii* antibodies in wild boar.

Material and methods. Diaphragm meat juice samples from 656 wild boar (*Sus scrofa*) were collected during the hunting seasons between September 2008 – October 2010 from 9 districts of the Czech Republic. The samples were stratified per age category into 2 groups: piglets (n = 279) and yearlings together with adults (n = 377). The in-house ELISA test was used for the detection of antibodies against *T. gondii* from the meat juice samples.

Results. Antibodies against *T. gondii* were detected by in-house ELISA in 260 of 656 wild boars (40%) with 26% prevalence in piglets (72/279) and 50% prevalence in yearlings and adults (188/377). The district total seroprevalences ranged between 32% – 59%, with a significantly higher prevalence in the district of Havlíčkův Brod (59%). Statistically significant differences (p-value < 0.05) were found between 2 age categories, and between 9 districts, with a significant variability in the district of Havlíčkův Brod. Seroprevalence correlated positively with farm density, but without any statistical significance.

Conclusion. The obtained results indicate that consumption of raw or undercooked meat from wild boars can carry an important risk of toxoplasma infection. *Post mortem* detection of antibodies in meat juice samples using ELISA is a useful alternative to blood serum examination. In addition, a diaphragm sample has been well-proven as a matrix sample for the contemporaneous diagnostics of trichinellosis and toxoplasmosis.

Key words

toxoplasmosis, age categories, meat juice, wild boars, ELISA, risk factors

INTRODUCTION AND OBJECTIVE

Toxoplasmosis is a worldwide distributed zoonosis caused by the coccidian parasite *Toxoplasma gondii*. *Felidae* are the only definitive hosts that excrete million oocysts of *T. gondii* in their faeces and thus contaminate the environment. All warm-blooded vertebrates, including mammals and birds, become infected by ingesting sporulated *T. gondii* oocysts in the environment or by ingesting *T. gondii* tissue cysts within the tissues of intermediate hosts. The wild boar (*Sus scrofa*) is considered a good indicator when monitoring environmental contamination with *T. gondii*.

In recent decades, the population size of wild boars increased dramatically in most European countries. In some regions of the Czech Republic, the constant population of wild boars has been increasing by 11.6% each year [1]. This expansion is associated with environmental and anthropogenic factors, presumably due to the absence of natural selection, lower predation pressure, and due to the availability of food as a consequence of intensive agriculture practicing.

Wild boar meat is usually consumed locally or distributed into the meat food chain. More than 140,000 wild boar

were hunted during the year 2011 in the Czech Republic [1]. Wild boar, currently being one of the leading wildlife species consumed, is a species that carries a particular risk for the transmission of toxoplasmosis to humans. Since toxoplasmosis is considered as an under detected and underreported disease, there is a need for optimising the surveillance and monitoring in wild boars to evaluate disease burden and epidemiological status. Several diagnostic methods are used for serological testing of *T. gondii* infection, carried out mainly on blood serum. However, serological testing of blood serum in wild boars is not practical due to the difficulties with sample collection. Recently, an alternative approach, based on antibody screening performed on meat juice, has been suggested in domestic pigs [2].

The aim of this study was to estimate the usefulness of meat juice for the detection of *T. gondii* antibodies in wild boar, and to establish the seroprevalence and risk factors of toxoplasmosis in the wild boar population.

MATERIALS AND METHOD

Animals and sampling. Diaphragm samples of 656 wild boars were collected by hunters in open areas during the hunting seasons between September 2008 – October 2010. The samples were sent to State Veterinary Institute in

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Jihlava for trichinellosis survey. Five grams of diaphragm were used for detection of trichinellosis by the digestion method. The remaining diaphragms were packed into a plastic bag, immediately frozen at -20°C and stored for up to 60 days. The frozen diaphragm sample was thawed overnight at room temperature, and meat juice samples collected from the plastic bags. Meat juice samples were submitted for serological analysis immediately after thawing.

Age of animals was estimated based on body weight and molariform mandibular tooth development. Wild boars were distributed into three age groups: 1) 279 piglets (≤ 12 months old) and 2) adults, 321 yearlings (>12 –24 months old), and 3) 56 older pigs (≥ 24 months).

The sampling was conducted in 9 administrative districts: Břeclav ($n = 66$), Havlíčkův Brod ($n=71$), Hodonín ($n=77$), Jihlava ($n=94$), Jindřichův Hradec ($n=52$), Třebíč ($n=58$), Ústí nad Orlicí ($n=83$), Znojmo ($n=71$) and Žďár nad Sázavou ($n=84$) (Fig. 1). The area of 6 districts (Třebíč, Havlíčkův Brod, Žďár nad Sázavou, Ústí nad Orlicí and Jindřichův Hradec) is consists mainly of highlands with mean altitude ranging from 500–836 m. The climate is humid continental with cold winters. The habitat is characterized by coniferous forest with dominating spruce culture (31.8% of total area), arable land (42.2%) and permanent pastures (11.4%). The landscape of the 3 remaining districts (Znojmo, Hodonín and Břeclav) is formed by lowland with mean altitude varying from 150–340 m. Dry and hot summers are the dominant feature of the local humid, continental climate, with habitat characterized by mixed forest (21.7% of the total area), with oak and pine being the most common species. In total surface, 58% is covered with arable land, while only 4% by permanent pastures.

Serological analysis. Meat juice samples were assayed for the presence of IgG antibodies to *T. gondii* by in-house ELISA test using antigen and other reagents from commercial kit EIA *Toxoplasma* IgG (Test-Line Clinical Diagnostics, Czech Republic). Meat juice dilution was 1:4 due to the lower concentration of antibodies compared with sera standardly diluted 1:20. The procedure in brief: 80 μl of dilution buffer (Test-Line) was added to the wells of microtiter plates, coated with *T. gondii* antigen. Then 20 μl of meat juice was added to each well with dilution buffer, and incubated for 1 hour at 37°C in a wet chamber. The plates were washed 3 times in washing solution (Test-Line) and 100 μl of goat anti-pig IgG-Fc conjugate (diluted 1:40,000, Bethyl Laboratories, Inc., USA) were added to each well. After incubation (30 minutes at 37°C) and second washing (washing solution, Test-Line), 100 μl of TMB Complete substrate – chromogen solution (Test-Line) was added to each well. The reaction was stopped after 10 minutes by adding 100 μl Stop solution (Test-Line). The plates were read at 450 nm (Dynex MRX II) and S/P percentage ($\text{OD sample}/\text{OD positive control} \times 100$) was calculated for each sample. Samples with $\text{S/P} \geq 50\%$ were considered as positive. Each ELISA reaction included 2 blanks, 2 positive and 2 negative controls obtained from experimental infection.

Experimental infection in brief: eight domestic pigs (6 months old, originating from a farm Nové Dvory, Czech Republic) negative for *T. gondii* antibodies tested by commercial ELISA kit (ID Screen® Toxoplasmosis Indirect, ID VET Inc., Montpellier, France) were used for the experiment. Pigs were housed and slaughtered according to the guidelines of the Ethical Commission of the University of Veterinary and Pharmaceutical Sciences in Brno. Two pigs were infected

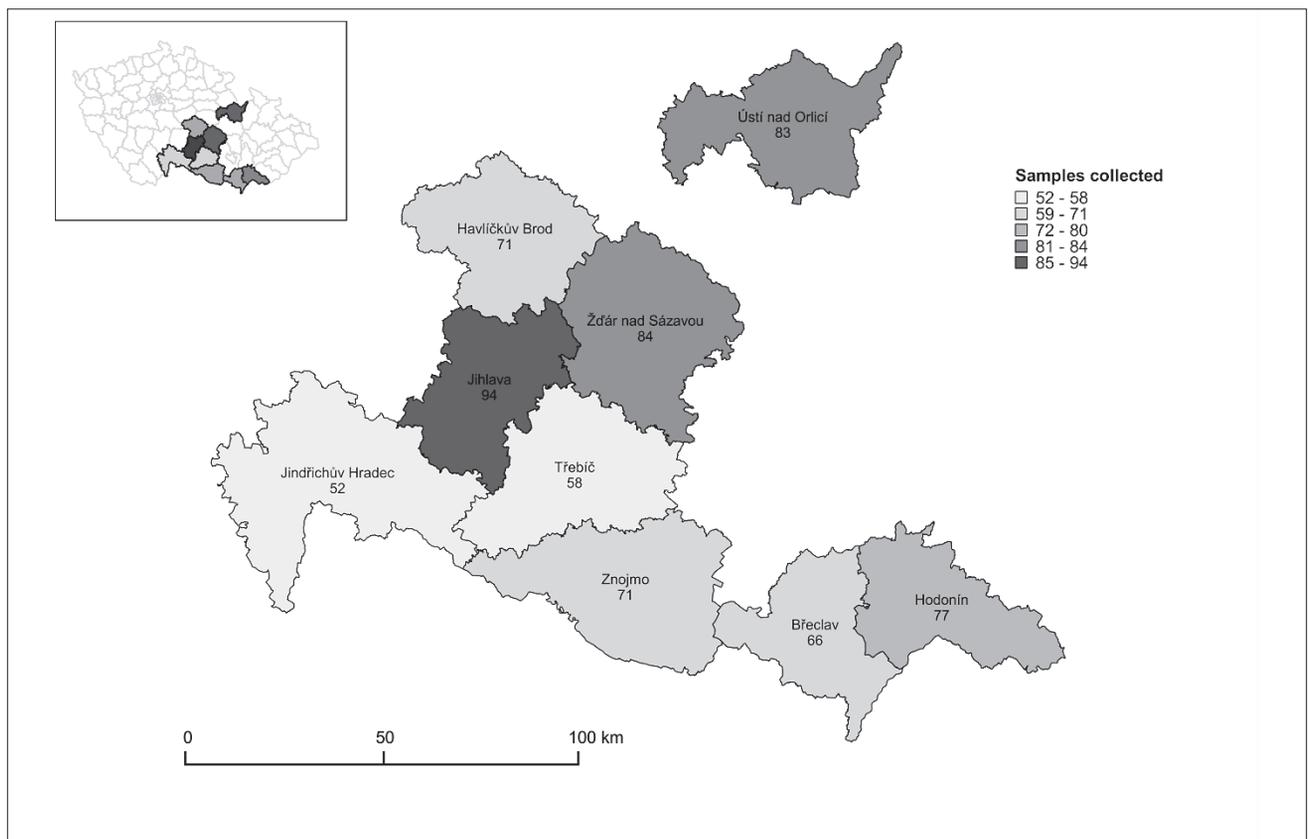


Figure A.1. Sampling coverage by districts of the Czech Republic

by oral administration of 20,000 *T. gondii* oocysts (tiger isolate, genotype II) and 6 pigs were kept non-infected as a control group. All animals were slaughtered 3 months post-infection and samples of serum, meat juice, brain and muscles of the hind limbs were collected. Serum and meat juice from the experimentally infected animals were strongly (S/P 100%) positive for *T. gondii* antibodies in commercial ELISA, and were used as a positive control for in-house ELISA. Brain and muscle samples of experimental pigs were positive for *T. gondii* in PCR and mice bioassay. Samples (serum, meat juice, brain and muscle) of 6 control animals were negative for *T. gondii*. Serum and meat juice from the negative animals were used as a negative control for in-house ELISA.

The results obtained by in-house ELISA were compared with the results obtained by a commercially available ELISA kit (ID Screen® Toxoplasmosis Indirect, ID VET Inc., Montpellier, France) when 100 randomly selected samples of meat juice were tested according to the manufacturer's instructions. The level of agreement between these 2 ELISA tests was determined by using the kappa statistic.

Epidemiological analysis. The prevalence estimates were computed using R 3.0.0 [3] with an add-on package epiR [4]. The sample descriptive data (sample identification, date of collection, district area of collection and age category) together with the ELISA results formed the analysis dataset (n=656). The prevalence over the whole study period was estimated on a per-district basis (n=9) as the proportions of positive samples, together with binomial exact 95% confidence intervals, under assumptions of representative sampling, both in terms of the population densities and the proportional representation of the 2 age categories. The total overall prevalence and the overall prevalence in the 2 age groups were estimated under an additional assumption of proportional representation of districts included in the study. The overall prevalence in the piglets (≤ 12 months) and the yearlings and adults (> 12 months) categories were compared by means of the two-group binomial proportion test. The cartograms of sampling coverage and estimated prevalence on per-district and age category basis were produced in the QGIS 2.0.1-Dufour software [5].

Statistical analysis. Seroprevalence was statistically analyzed, considering the variables of age, geographical areas (districts) and farm density (Tab. 1). The data analysis was performed by Chi-Square test for independence using

STATISTICA Cz 12 [6]. The null hypothesis that *T. gondii* seroprevalence does not depend on age, origin (district) and farm density per square kilometer was tested. The differences were considered statistically significant when p-value of chi-square test < 0.05 . Odds ratio (OR), 95% confidence intervals (CI) for the odds ratio and partial correlation coefficient $r(X,Y)$ were computed to quantify the association between selected variables and serological *T. gondii* status.

RESULTS

Of the 656 examined wild boars, 260 (40%, 95% – C.I. 36–43%) were positive for antibodies against *T. gondii* with statistical difference ($p=0.000$) between piglets (26%, 95% C.I. 21–31%) and adults (50%, 95% – C.I. 45–55%) (Tab. 1). The null hypothesis that *T. gondii* seroprevalence in piglets is identical with the seroprevalence in adults was rejected on the significance level 0.05, whereas the test statistic value of Chi-Square test for independence was 38.798, $p=0.0000$ and odds ratio 0.350 with 95 % confidence interval for the odds ratio (0.250; 0.489).

A statistically significant difference was also found between districts ($p=0.0218$), with prevalence ranging from 32% – 59% (Tab. 1). The highest prevalence was found in the Havlíčkův Brod district (59%, 95% C.I. 47–71%) compared to 32% – 47% prevalence in the other 8 districts. The null hypothesis that *T. gondii* seroprevalence does not depend on geographical region (district) was also rejected on the significance level 0.05, whereas the test statistic value of Chi-Square test for independence was 17.928 and $p=0.0218$. The seroprevalence was significantly higher in Havlíčkův Brod district with a test statistic value of Chi-Square test for independence 12.681, $p=0.0004$, odds ratio = 2.438 with 95 % confidence interval for the odds ratio (1.476; 4.028)

The seroprevalence of *T. gondii* antibodies correlated positively with farm density per square kilometer, but without statistical significance ($r=0.275$; $p=0.475$).

The agreement between two ELISA tests, used for serological investigation of 100 randomly selected meat juice samples, was evaluated as excellent (kappa=0.92)

Table 1. Total prevalence of antibodies to *Toxoplasma gondii* in wild boars from the Czech Republic by sampling sites and age category

District	Area (km ²)	Density of farms/km ²	Total		Piglets (≤ 12 months)		Adults (> 12 months)		p-value of chi-square test	odds ratio
			N	Positive (%)	N	Positive (%)	N	Positive (%)		
Břeclav	1038.25	0.49	66	21 (32%)	22	3 (14%)	44	18 (41%)	0.17	0.685
Havlíčkův Brod	1264.95	1.57	71	42 (59%)	28	11 (39%)	43	31 (72%)	0.00	2.438
Hodonín	1099.13	0.61	77	26 (34%)	31	6 (19%)	46	20 (43%)	0.26	0.752
Jihlava	1199.32	1.15	94	44 (47%)	41	9 (22%)	53	35 (66%)	0.12	1.410
Jindřichův Hradec	1943.69	0.84	52	19 (37%)	25	7 (28%)	27	12 (44%)	0.63	0.867
Třebíč	1463.07	1.26	58	21 (36%)	29	8 (28%)	29	13 (45%)	0.58	0.853
Ústí nad Orlicí	1258.31	2.10	83	32 (39%)	38	12 (32%)	45	20 (44%)	0.83	0.949
Znojmo	1590.50	0.76	71	24 (34%)	26	7 (27%)	45	17 (38%)	0.29	0.755
Žďár nad Sázavou	1578.51	1.84	84	31 (37%)	39	9 (23%)	45	22 (49%)	0.58	0.876
Total			656	260 (40%)	279	72 (26%)	377	188 (50%)		

DISCUSSION

The total *T. gondii* seroprevalence (40%) found in wild boar in this study was comparable with the results obtained by modified agglutination test (MAT) in some European countries: 36% – 38% in Spain [7, 8] or 55% in France [9]. In Finland, 33% seroprevalence was detected in farmed wild boars using the commercial direct agglutination test [10]. In the Netherlands [11] and Switzerland [2], a lower prevalence 24% and 7% was detected in wild boars by ELISA, respectively. Compared to the presented results, a lower seroprevalence was also detected in neighbouring countries: 21% in Germany [12] and 19% in Austria [13] by indirect immunofluorescence antibody test (IFAT), 21% in Poland by MAT [14] or 19% in Germany [12] and 8% in Slovakia [15] by ELISA. In the Czech Republic, an increasing trend of *T. gondii* positivity has been recorded since it had been 15% [16] and 26% [17] in previous studies. Data obtained in these studies can vary due to the different sampling strategies, different methods and cut-off used.

Indirect ELISA is considered a suitable method for the detection of antibodies since it correlates well with MAT [18] and was found as the most sensitive test for the analysis of animal sera and meat juice [19]. Meat juice as a sample matrix of various wild and domestic animals was assayed by ELISA, e.g. in Sweden [20], New Caledonia [21] and Brazil [22], but only one study from Switzerland was focused directly on wild boars [2].

In the presented study, different *T. gondii* prevalence was found in the studied districts. There is a hypothesis that wild boar acquire infection during digging in soil contaminated by *T. gondii* oocysts, or by accidental ingestion of infected rodents, carcasses or visceral organs of domestic animals [23]. Seroprevalence of *T. gondii* antibodies in the current study correlated positively with farm density per square kilometer without, however, statistical significance. Nevertheless, the farm density may present possible risk factor of *T. gondii* infection due to higher concentration of domestic cats, rodents and other infectious material coming from carcasses of domestic animals [9].

The survival of infectious *T. gondii* oocyst depends on environmental conditions, such as temperature and rainfall. Higher seroprevalence was found in wild animals originating from areas of higher altitude, rainfall and lower temperatures [7]. This fact can elucidate the cause of lower seroprevalence found in a study in the districts of Znojmo, Břeclav and Hodonín in southern Moravia region, compared with the seroprevalence in the remaining districts.

In the presented study, a statistically higher prevalence was found in adults compared to piglets. This can indicate the peroral origin of infection [24]. Age prevalence relation has been observed up to 10 months of age, but remains stable thereafter, whereas no significant variation over the sampling years or between sampling sites has been shown which can indicate a constant infection pressure from the environment [11].

About 50% of all human toxoplasmosis cases are related to foodborne infection, associated with consumption of raw or undercooked meat or other edible parts of animals [25]. Tissue cysts of *T. gondii* remain alive for the lifetime of the animal and there is no way to distinguish infected from uninfected carcasses during slaughter inspection; therefore, all edible parts seropositive for *T. gondii* should be considered

infectious. Wild boar becomes the most frequently consumed game species in the Czech Republic due to their increasing population during the last decades; they therefore carry a particular risk of infection for humans.

CONCLUSIONS

The results of this study provide baseline information on the occurrence of toxoplasmosis in wild boar in the regions of the Czech Republic, and refer to an important human health and hygienic risk associated with the consumption of raw and undercooked meat from these animal species. Statistically significant differences were found between 2 age categories and districts of origin. Indirect ELISA test was found to be promising for future post-harvest surveillance and monitoring of *T. gondii* in wild boar meat or meat products. Meat juice was approved as a reasonable sample for antibody detection by ELISA in wild boar meat or meat products. The diaphragm is a prospective matrix for parallel serological diagnostic of toxoplasmosis and diagnostics of trichinellosis by the digestion method.

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