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Occurrence and genetic characterization of *Toxoplasma gondii* in naturally infected pigs

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Abstract

The protozoan *Toxoplasma gondii* is an obligate intracellular parasite that infects a wide range of warm-blooded vertebrates. The data about the occurrence of toxoplasmosis in slaughter pigs in the Slovak Republic are still missing. The aim of our study was to estimate the prevalence of toxoplasmosis in pigs from Slovakia during the period of 2006–2010 by ELISA and PCR methods. In sera of 970 slaughter pigs, 2.16% seropositivity to *T. gondii* was detected. In tissue samples of seropositive pigs the presence of *T. gondii* DNA was confirmed. In six monitored Slovak regions the seropositivity varied between 1.11 and 3.48%. The statistically significant differences were recorded between the Košice and Prešov region. The seroprevalence of toxoplasmosis in sows (4.26%) was two times higher than that in slaughter pigs (2.06%) (OR = 2.12; 95% CI = 0.48–9.36). Presence of *Toxoplasma gondii* in tissues of seropositive pig isolates was confirmed by TGR1E and B1 genes and analysis of DNA polymorphism at SAG2 and ROP1 genes revealed the presence of virulent strain of genotype I in 85.7% of infected pigs and an avirulent strain (genotype II) in 14.3% of pigs.

Keywords

Toxoplasma gondii, pigs, seroprevalence, genotypes, Slovak Republic

Introduction

Toxoplasmosis is a common zoonotic disease occurring worldwide that affects a large number of warm blooded vertebrates. Infection can be acquired by ingestion of infectious oocysts from the environment or by consumption of tissue cysts contained in raw or undercooked meat of infected intermediate hosts (Tenter *et al*. 2000). The prevalence of the disease in different countries depends mainly on eating habits of the population. Manipulation and consumption of raw or undercooked meat pose a health risk especially for pregnant women and immunosuppressed patients (Dubey *et al*. 2002, Boyer *et al*. 2005) and in a large-scale study conducted in six European countries it was found to be the reasons of 30–63% cases of *Toxoplasma* infection in pregnant women (Cook *et al*. 2000). Similarly, Wilson and McAuley (1999) reported a high prevalence of toxoplasmosis associated with the consumption of undercooked meat.

In sheep, goats and pigs the prevalence of toxoplasmosis greatly varies. According to Tenter *et al*. (2000), till 2000 the highest prevalence range of toxoplasmosis in the European countries was recorded in sheep (10–92%), lower in goats (20–60%) while pigs were infected less frequently (2–36%).

Studies on genetic structure of *T. gondii* based on multilocus restriction length polymorphism analysis (RFLP) or multilocus enzyme electrophoresis showed the existence of three predominant clonal types (I, II, III) which differ in pathogenicity and virulence in both, animals and humans. *T. gondii* strains can also be characterised by the survival time of mice after being infected with the parasite (Howe and Sibley 1995, Nowakowska *et al*. 2006).

Despite the huge number of literary data on toxoplasmosis, the data concerning its occurrence in the livestock in Slovakia are rather scarce and completely missing in relation to molecular biology. The aim of our study was to determine the seroprevalence of toxoplasmosis in pigs from Slovakia, to diagnose the positive findings by molecular methods and to genetically characterise the causative agent of this serious parasitic zoonosis.

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Materials and Methods

Sampling

Between 2006 and 2010 blood sera and brain and muscle tissues from 970 pigs from 18 districts (6 regions) of Slovakia were collected in slaughterhouses. The majority, 923 of animals, were slaughter pigs at the age of 6 or 7 months and 47 samples were obtained from sows aged between 3 and 4 years. Samples (sera and tissues), if not examined immediately, were stored at –20°C until tested.

Serological analyses

Sera of pigs were examined for the presence of IgG antibodies against *T. gondii* by commercial ELISA kit (ELISA *Toxoplasma gondii* serum screening, Institut Pourquier, France) according to the protocol of the manufacturer. Optical densities (OD) of samples were measured spectrophotometrically at 450 nm (Thermo Labsystems Opsys MR, Chantily, Virginia, USA). Percentage rate for each sample was made in equation: S/P% = (OD_{450} sample – OD_{450} negative control)/(OD_{450} positive control – OD_{450} negative control) x 100. Any samples with S/P% ≤ 40% were considered as negative, samples with S/P% between 40% and 50% were characterised as doubtful and samples with S/P% ≥ 50% were considered coming from an animal, which has been infected by *Toxoplasma gondii.*

Isolation of DNA from pig brains or muscles

The samples of brain or muscle tissues from ELISA positive or doubtful pigs were used for molecular analyses. *T. gondii* tachyzoites have been isolated according to Jauregui *et al*. (2001). Brain/muscle tissue (50 g) was homogenised with 5 volumes of saline solution (pH 7.2) and digested with equal volume of warm (37°C) pepsin-HCl (1.4 mg pepsin and 10 mg of NaCl per ml 0.1 N HCl) during 1 hour. The tissue samples were centrifuged for 10 min at 1,800 x g and subsequently neutralised by two washes with 0.1 M Tris buffer (pH 8.0). The sediment was used for DNA isolation.

The acquired sediment was incubated overnight with digestion buffer (pH 8.0) (Jauregui *et al*. 2001) containing 0.5% SDS, 25 mM EDTA, 100 mM NaCl, 20 mM TRIS-HCl and proteinase K with final concentration 10 mg/ml. After digestion, DNA was extracted by phenol-chloroform-isoamyl alcohol in the rate of 25:24:1. The DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol. After centrifugation, DNA pellet was solubilized in TE buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 7.6–8.0) or in redistilled water and stored at – 20°C for next use.

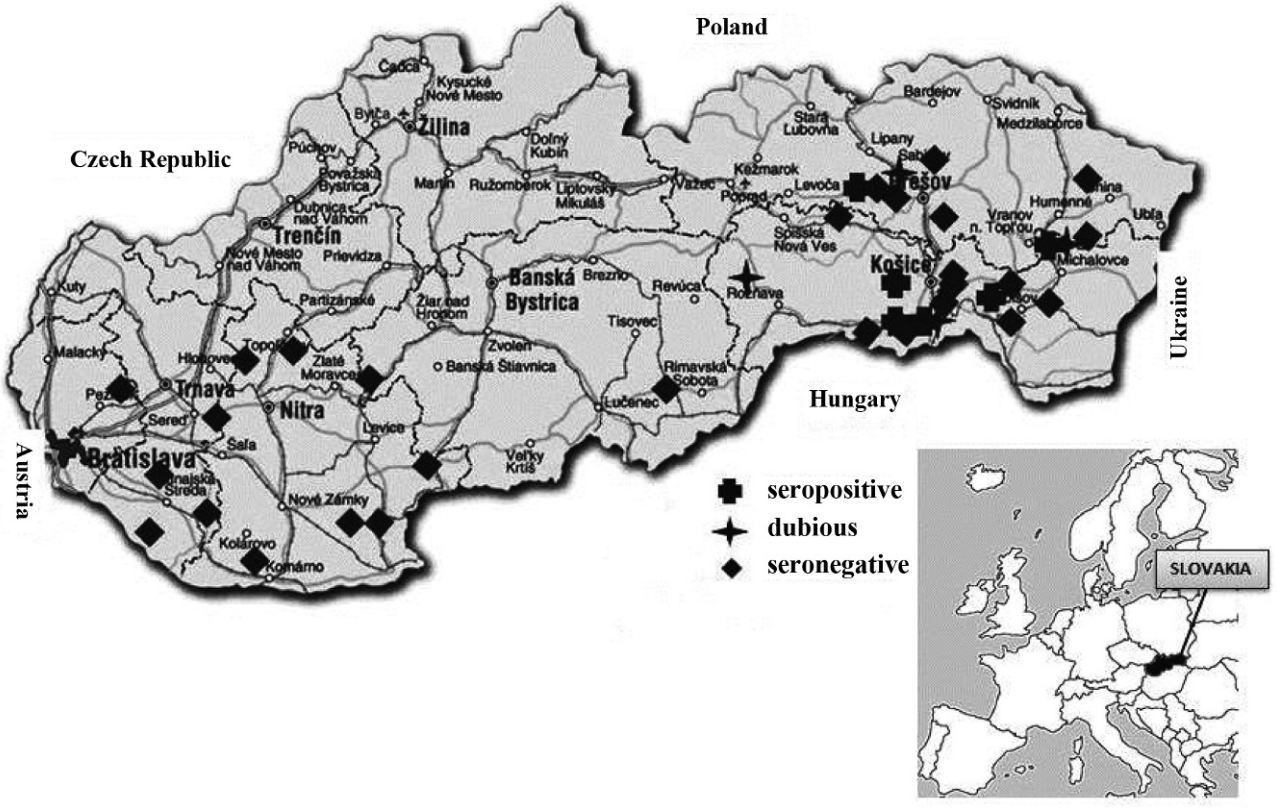

Fig. 1. Occurrence of *Toxoplasma gondii* in pigs from Slovak Republic

Table I. The number of positive cases and representation of *T. gondii* genotypes in pigs from different regions of the Slovak Republic

Region	District	N/n/d (%)	Prevalence (95% CI)	PCR positive samples	Genotype		
					I	II	III
Košice	Košice	209/6/1 (2.9)	3.5 (2.4 – 5.0)	6	6	–	–
	Michalovce	258/5/1 (1.9)		5	5	–	–
	Rožňava	30/0/1 (0.0)		–	–	–	–
	Spišská Nová Ves	10/0/0 (0.0)		–	–	–	–
	Trebišov	67/9/0 (0.0)		9	6	3	–
Prešov	Prešov	50/1/1 (2.0)	1.1 (-0.7 – 9.1)	1	1	–	–
	Sabinov	15/0/0 (0.0)		–	–	–	–
	Snina	25/0/0 (0.0)		–	–	–	–
Banská Bystrica	Rimavská Sobota	10/0/0 (0.0)	0.0 (-0.9 – 5.2)	–	–	–	–
	Žarnovica	51/0/ (0.0)		–	–	–	–
Nitra	Komárno	15/0/0 (0.0)	0.0 (-0.9 – 3.6)	–	–	–	–
	Levice	28/0/0 (0.0)		–	–	–	–
	Nitra	15/0/0 (0.0)		–	–	–	–
	Nové Zámky	82/0/0 (0.0)		–	–	–	–
	Topoľčany	15/0/0 (0.0)		–	–	–	–
Trnava	Dunajská Streda	60/0/0 (0.0)	0.0 (-0.9 – 6.2)	–	–	–	–
	Galanta	15/0/0 (0.0)		–	–	–	–
Bratislava	Pezinok	15/0/0 (0.0)	0.0 (0.0 – 22.2)	–	–	–	–
TOTAL	–	**970/21/4**	**2.16**	**21**	**18**	**3**	**0**

N – number of examined samples; n – number of positive samples; d – number of dubious samples; % – percentage of positive samples

PCR analyses

Two genes, most suitable for the diagnostics of toxoplasmosis, were used for the PCR analyses. TGR1E gene was amplified with primers TGR1E-1 5′-ATGGTCCGGCCGGTGTATGATATGCGAT-3′ and TGR1E-2 5′-TCCCTACGTGGTGCCGCATTGCCT-3′ according to Lamoril *et al*. (1996) with one amplification reaction. This gene is of 191 bp in size and is itself repeated in the *T. gondii* genome 30–35 times. The coamplified area of the gene for beta globine of 362 bp in size was used for the PCR control. B1 gene was amplified by hemi-nested PCR method using three primers, B1a 5′-GAGAGGTCCGCCCCCACAAC-3′, B1b 5′-CTGCTGGTGCGAGGGGAGTG-3′ and B1c 5′-CAGGAGTTGGATTTTGTAGA-3′ according to Pujol-Riqué *et al*. (1999), with two amplification reactions. Gene B1, 362 bp in size, is itself repeated in the *T. gondii* genome 35 times. The resulting amplified products were run on 3% agarose gel containing ethidium bromide and visualised with UV lamp at 254 nm.

Genotyping of SAG2 and ROP1 locuses

Genotype of *T. gondii* DNA from brain or muscles of pigs was examined using PCR and subsequent RFLP of SAG2 and ROP1 locuses. For SAG2, samples were analysed separately for the 5’ and 3’ ends of the locus, according Howe *et al*. (1997). The 5’ end of the locus was amplified by nested PCR with the primers SAG2: F4 (5’GCTACCTCGAACAGGAACAC3’) and R4 (5’GCATCAACAGTCTTCGTTGC3’) and following amplification with the internal primers F (5’GAAATGTTTCAGGTTGCTGC3’) and R2 (5’GCAAGAGCGAACTTGAACAC3’). The 3’ end of the locus was amplified using primers F3 (5’TCTGTTCTCCGAAGTGACTCC3’) and R3 (5’TCAAAGCGTGCATTATCGC3’) in the first amplification and the internal primers F2 (5’ATTCTCATGCCTCCGCTTC3’) and R (5’AACGTTTCACGAAGGCACAC3’) in the second reaction. The amplified fragments of the second reaction were used in the RFLP analysis. The 5’end was digested using restriction endonuclease *Sau*3AI (Promega, USA) and 3’end using *Hha*I (Promega, USA).

To verify the results of SAG2 locus genotyping, the analysis of ROP1 gene encoding a rhoptry protein was performed according to Howe and Sibley (1994). After the PCR amplification with primers ROP1I 5’CGT GAC ATA TAC TGC ACT GAC 3’ and 5’CAT CTG CAA ACT CGA TCA C 3’ the ROP1 locus was analysed with endonucleases *Ddel* (Promega, USA) and *Hha*I (Promega, USA).

Table II. Prevalence of antibodies to *Toxoplasma gondii* in different age categories of pigs

Age category	Examined pigs	ELISA		Prevalence (%)	OR 95%	CI
		Positive	Dubious			
Slaughter pigs (6–7 months)	923	19	4	2.06	0.47	0.9–3.8
Sows (3–4 years)	47	2	0	4.26	2.12	-1.0–18.3
Total	970	21	4	2.16	–	–

OR – odds ratio; CI – confidence interval.

Toxoplasma gondii genotype was determined according to the location of restriction fragments of the 3' and 5' ends after the visualisation in 1.2% agarose gel.

Statistical analysis

The statistical analyses were performed using Fisher's exact test (GraphPad Prism_5) with corresponding odds ratio (OR). The seroprevalence values were given with 95% confidence intervals (CIs). The differences were considered statistically significant when $P<0.05$.

Results

Totally 970 pigs sera were examined during the monitoring of toxoplasmosis between 2006 and 2010; 923 sera from slaughter pigs and 47 sera from sows. Pigs came from 36 farms located in six Slovak regions (18 districts) (Fig. 1). Antibodies to *T. gondii* were detected in 21 animals (2.16%) and four sera were interpreted as dubious. Seropositivity to *T. gondii* varied in different regions. The highest prevalence was observed in Košice (3.5%) and Prešov (1.1%) region, while in pigs from central (Banská Bystrica region) and western regions (Nitra, Trnava and Bratislava region) seropositivity was not detected (Table I). The difference between seropositivity in Košice and Prešov region was not significant, but differences in the prevalence of antibodies in individual districts of Eastern Slovakia were of statistical significance ($P = 0.0001$). Statistically significant was also the difference in positivity between Eastern Slovakia and the rest of the country ($P = 0.0005$).

The age did not influence the positivity of animals significantly, even though the sows were infected two times more frequently (4.3%; OR = 2.12; 95% CI = 0.48–9.36) than slaughter pigs (2.1%; OR = 0.47; 95% CI = 0.11–2.09) (Table II).

Brains and muscles from positive and dubious animals were used for molecular analyses. Analysis of TGR1E and B1 genes confirmed *T. gondii* in all seropositive animals, but all dubious ones were found to be negative. RFLP analysis of SAG2 locus showed the presence of genotype I in 18 positive samples (85.7%) and 3 (14.3%) animals were infected with genotype II. Genotype II was detected only in slaughter pigs from a farm in Trebišov district, where also the presence of genotype I was confirmed (Table I). RFLP analysis of ROP1 locus confirmed the results of SAG2 analysis.

Discussion

Toxoplasmosis belongs to very frequent human parasitic infections with estimated prevalence values ranging between 30 to 50% (Tenter *et al*. 2000; Flegr and Havlíček (1999). In recent study in Slovakia, seropositivity to *T. gondii* was confirmed in 42.1% women with habitual abortions (Pavlinová *et al*. 2011). Pigs are considered to be the most probable source of human infection (Gamble *et al*. 1999, Tenter *et al*. 2000). Manipulation with slaughter pigs and pork has probably contributed to infection of as many as 53% of meat industry workers from Slovakia (Studeničová *et al*. 2003). In Europe, the seropositivity of slaughter pigs to *Toxoplasma gondii* varies between 5% in Austria and Netherland, to 26.4% in Poland and 28.9% in Serbia (Dubey 2009). While on a large-scale farm in the Czech Republic the seroprevalence of toxoplasmosis was only 0.5%, in the southern Europe it has reached 15.6% (de Sousa *et al*. 2006). Examination of pigs from 18 Slovak districts revealed 2.16% mean seropositivity to *T. gondii.* The differences in antibody seroprevalences between individual districts, with highest seropositivity in districts of eastern Slovakia were detected. Similarly, Turčeková *et al*. (2006) found 2.5% seropositivity to *T. gondii* in slaughter pigs from the Košice and Prešov region. Nguyen *et al*. (2012) also reported the disparities in prevalence of antibodies to *T. gondii* among different geographical regions.

In the study, the difference in the seropositivity of age groups was recorded. Anti-*T. gondii* antibodies were more frequent in sows (4.3%) than in six or seven-month old animals (2.1%). Similarly, lower prevalence of antibodies to *T. gondii* in young slaughter pigs than in animals over 8 or 24 months recorded Klun *et al*. (2006) and Villari *et al*. (2009). That may be due to a longer contact of older animals with potentially infected environment (Villari *et al*. 2009). Surprisingly, the meat of older animals is usually added to sausages, frankfurters and salamis (Dubey 2000), in a preparation process of

which undercooked or row pork meat is often used. It has been estimated that such production process can lead to consumption of a single pig by 200–400 consumers (Fehlhaber *et al*. 2003).

As the virulence of different strains varies, the determination of *T. gondii* genotypes is of great importance. Three predominant clonal types (I, II, and III) are usually described (Dardé 2008). Genotype I is relatively rare and comprises only about 10% isolates from Europe and USA. It is considered to be highly virulent in mice with a great capacity to cross tissue barriers in vitro and in vivo. Genotype II is more frequent in Europe and USA (80% isolates). Strains of genotype II are relatively avirulent in mice, establishing chronic infections characterized by tissue cysts that are highly infectious by the oral route. It usually affects immunocompromised persons, and in farm animals causes chronic form of the disease (Sibley and Boothroyd 1992, Sibley 2003, Dardé 2008). In our study genotype I, detected in 85.7% of pig isolates, predominated and genotype II was confirmed only in 14.3% animals. In animals, mixed infection was not detected and occurrence of both genotypes was recorded only on a farm in Trebišov district. In Slovakia, the predominance of genotype I was also recorded in red foxes, wild boars and rodents. In cattle, the proportion of genotype I and II was the same, while in goats only genotype II was confirmed (Spišák 2010; Spišák *et al*. 2010; Turčeková et al. in press). Our results are similar to data of Aspinall *et al*. (2002) who found genotype I in 85.0% of *T. gondii* positive samples of meat products. Similar method of genotype determination used de Sousa *et al*. (2006) in Portugal, who detected genotype II in 11 isolates and genotype III in 4 isolates. In Switzerland, results of genotyping of samples from cat oocysts and sheep and pig-meat samples suggest the predominance of clonal Type II *T. gondii* alleles, but authors also discuss the possibility of clonal types I and II or atypical clonal types (Berger-Schoch *et al*. 2011). The occurrence of genotype I in both, domestic and wild animals suggests its circulation in sylvatic and synanthropic cycle in Slovakia. Similarly, Richomme *et al*. (2009) stated that strains present in wildlife probably do not differ from strains from the domestic environment.

The most often sources of *T. gondii* infection for pigs presents the food and water contaminated by sporulated oocysts from faeces of infected cats, but pigs can become infected also after the consumption of infected rodents and birds, milk of infected sows, after congenital transmission or cannibalism (Lehmann *et al*. 2003, Kijlstra *et al*. 2008). Improvement of animal hygiene together with the control of the access of cats to farm buildings are considered the main reasons of seroprevalence decrease (Vostálová *et al*. 2000). Therefore the seropositivity to *T. gondii* can be the indicator of hygiene and infection risk on the farm.

We can conclude that although pig toxoplasmosis is not frequent in the Slovak Republic, concerning the high numbers of pigs slaughtered per year, it is not negligible for professionally exposed workers (staff of slaughter house and meat industry) as well as for public. The human health risk enhances the dominance of highly virulent genotype I in pigs from the territory of Eastern Slovakia.

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