

Prevalence of cystic echinococcosis in pigs from Slovakia, with evaluation of size, fertility and number of hydatid cysts

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Summary

The regional occurrence of cystic echinococcosis in slaughtered pig from Slovakia was studied in the period of 2000 – 2008, along with the quantitative parameters associated with the establishment of cysts (intensity of infection, fertility, size). From 103 pig livers collected from abattoirs in 35 sites Slovakia as suspected for *Echinococcus* infection, 63 were positive for cystic echinococcosis, whereas in 40 livers cysticercosis was diagnosed. Fertile cysts with echinococcal protoscoleces were recorded in 25.4 % of positive pigs, with the 8.9 % fertility rate and the intensity of infection reaching value of 5.9 cysts per liver. The average size of fertile cysts was more than three times larger than diameter of sterile cysts (diameters of 4.67 cm vs. 1.37 cm, respectively). No significant differences in relative amounts of fertile and sterile cysts were found among counties ($P = 0.15$). The annual prevalence of *E. granulosus* in pigs in 2000 – 2008 has ranged from 0.02 % to 0.13 % (average rate 0.08 %), with the decreasing tendency over the period, especially after 2005. The most heavily affected areas with cystic echinococcosis over 2006 – 2008 were indicated in Prešov (eastern Slovakia, 0.68 % prevalence) and Komárno districts (southwestern Slovakia, 0.26 % prevalence). Obtained data are relevant in designing regional control strategies to suppress the occurrence of disease in livestock and risk for humans to be infected.

Keywords: *Echinococcus*; hydatid cysts; prevalence; fertility; intensity of infection; cyst size

Introduction

Cystic echinococcosis (CE) caused by larval stage of *Echinococcus granulosus* tapeworm is a chronic zoonotic infection, mainly transmitted in a cycle between dog as definitive host and livestock as intermediate hosts. Humans are occasional intermediate hosts, and CE accounts for

more than 95 % of the estimated 2 – 3 million human global cases affected by *Echinococcus* parasites (Budke, 2006). The infection in intermediate hosts results in the formation of a hydatid cyst (metacestode larvae). A mature fertile cyst is frequently unilocular and is manifested as anechoic, fluid-filled lesion. More than 90 % cysts occur in liver, lungs or both (McManus *et al.*, 2003). The cyst wall consists of an inner nucleated germinal layer, which gives rise to brood capsules and protoscoleces, and an outer acellular laminated layer adjacent to the host tissue. All cysts start to develop as a typical small unilocular cyst with cyst fluid. In some cases, development progresses no further and the cyst is said to be sterile (nonfertile). In most other cases, however, protoscoleces subsequently bud within brood capsules attached to the germinal layer and are released into cyst fluid. This internal structure can be remained, but collapsed cyst walls, calcification and the presence of additional daughter cysts are often seen. Such heterogeneity in cyst structure has been an issue for clinicians how to treat human CE and is important for monitoring progression of the disease (Altintas *et al.*, 2007; Čulafić *et al.*, 2007). If the viable portions of germinal layer, brood capsule wall or protoscoleces are present, the cyst has the ability to regenerate in some form (Kamenetzky *et al.*, 2000). Some discussions have evolved about the origin of daughter cysts. The former major assumption that these arise by budding from the germinal layer has no histological support and view that cysts develop directly from protoscoleces currently prevails (Rogan *et al.*, 2006). In comparison with investigations in humans, little research has been directed toward the development of *E. granulosus* infection in domesticated animals such as pig. The pig-dog cycle is the most important cycle for perpetuating this parasite in central Europe (Romig *et al.*, 2006). No studies have yet determined the regional-specific prevalence or intensity of infection, nor examined cyst size and fertilities in pig from Slovakia. The pilot study was

therefore undertaken to examine regional occurrence of the tapeworm over the last decade and to assess quantitative parameters associated with the establishment of cysts in pig intermediate host and areas mostly affected by disease.

Materials and methods

In a period of 2000 – 2008, livers with lesions from 103 slaughtered pigs were collected from 35 sites in 7 counties of Slovakia (sites with findings of fertile/sterile echinococcal cysts are shown in Fig. 1). Frozen livers were transported from abattoirs to the laboratory. Cystic formations were diagnosed as being originated due to echinococcosis or cysticercosis. Material was kept at -20°C until microscopical and morphological examinations at the laboratory. Before processing, frozen cysts were rinsed in 70 % ethanol and cut by sterile scissors. Frozen cysts were excised and transferred into beakers, in which the size of cysts was measured at the laboratory temperature. The whole cyst content was passed through a 100 µm sieve to remove wall layers and debris. Resulting hydatid fluid was then centrifuged at 6 000 rpm for 10 min. The sediment was washed in phosphate buffer saline (pH 7.2), and re-centrifuged under same conditions. Supernatant was carefully removed and final sediment and germinal layers were observed under light microscope to determine the cyst fertility. Fertile cyst was considered the cyst with the presence of protoscolexes in the hydatid fluid and/or attached to the germinal layer. In sterile cysts degenerative modifications (cysts with purulent inflammation, caseous necrosis or calcified cysts) were often seen. Morphometric data of echinococcal cysts were evaluated by statistical methods with respect to their geographical origin, number and fertility.

Cyst size related to the geographical origin was evaluated by ANOVA (analyses of variance), supplemented by post hoc analysis by Duncan's test was performed when needed. Relation between fertility and geographical origin was determined by use of Kruskal-Wallis ANOVA with the subsequent HSD (unequal N) test. For evaluation of



Fig. 1. Illustration of sites with findings of cystic echinococcosis (CE) in slaughtered pigs in Slovakia partitioned into counties. CE-positive counties: BA - Bratislava county, TT - Trnava county, NR - Nitra county, BB - Banská Bystrica county, PO - Prešov county, KE - Košice county. Districts mostly affected by CE in 2006 – 2 008, Prešov in Prešov county (prevalence of 0.68 %), Komárno in Nitra county (prevalence of 0.26 %), are designated by hatched areas with diagonal lines. ● - site with recorded fertile cysts; ○ - site with recorded sterile cysts; ◐ - site with both fertile and sterile cyst

relation between cyst size and fertility Student's t-test was used. Spearman's rank correlation coefficient was used for regression analysis of size cysts and their number in the liver. Values for cyst sizes were logarithmically transformed to fit this analysis. All statistical analyses were performed by Statistica 6.1 software (StatSoft, CR, Prague) program. Significant differences were considered when $P < 0.05$.

Results

From 103 pig livers obtained from abattoirs as suspected for CE infection, 63 (i.e. 61.2 %) were positive for cystic echinococcosis (findings are demonstrated in Fig. 2). In remaining 40 (38.8 %) livers cysticercosis was diagnosed (findings illustrated in Fig. 3). Overall, 463 cysts were recorded, 372 of which were due to echinococcus and 91 due to cysticercus. Numbers of examined livers and cysts according to Slovak counties are summarized in Table 1. Only echinococcal cysts were subjected to statistical evaluations. Mixed infections of echinococci and cysticerci were recorded in 4 (3.9 %) of livers.

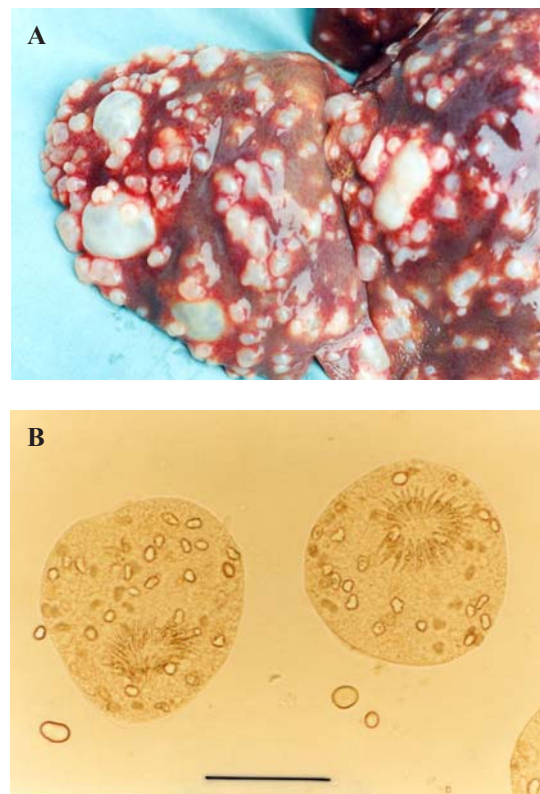


Fig 2. A - *Echinococcus granulosus* cysts in pig liver; B - isolated protoscolexes from cysts. Scale bar - 100 µm.

Regional intensities of infection

In 63 pigs infected with CE, the intensity of infection (number of hydatid cysts per positive animal) has ranged from 1 to 36 cysts per liver, with the average intensity 5.9 (Tab. 2). Significant overall differences ($F = 4.09$, $P < 0.01$) at this parameter were detected between examined

Table 1. Liver examination of slaughtered pigs according to Slovak counties for tapeworm presence

County	Number of livers	Livers with echinococcus	Livers with cysticercus	Number of cysts with	
				echinococcus	cysticercus
BA	1	1	0	2	—
TT	2	2	0	42	—
NR	2	2	0	17	—
TN	—	—	—	—	—
ZA	1	0	1	—	2
BB	2	1	1	24	2
PO	12	7	5	38	15
KE	83	50	33	249	72
Total	103	63	40	372	91

BA - Bratislava; TT - Trnava; NR - Nitra; ZA - Žilina; BB - Banská Bystrica; PO - Prešov; KE - Košice

counties. At the same time, no significant difference was found when counties within groups established by Bratislava, Prešov, Košice, Nitra counties and Trnava, Banská Bystrica counties were compared, whereas these groups differed significantly ($P < 0.05$). Massive infections (more than 10 cysts in liver) were diagnosed in 17.5 % of pigs (11/63).

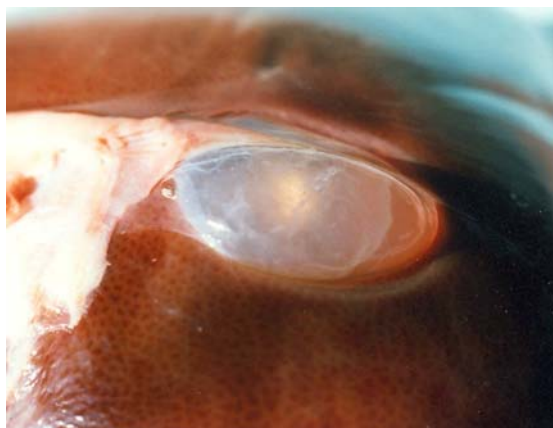


Fig. 3. A - Cysticercus cysts in pig liver; B - cysticercus excised from pig liver

Mean size of cysts

When comparing average diameters of cysts, more striking differences between counties were recorded (Tab. 2, Fig. 4). Lesion sizes from Bratislava county were excluded from this statistical evaluation as only two cysts were obtained. Largest cysts were seen in livers from Banská Bystrica county, the size of which significantly differed from

cysts in Prešov county at confidence level $P < 0.01$ and at level $P < 0.001$ when related to remaining counties. This could have been influenced by the fact that examined cysts from Banská Bystrica county originated from one liver. Smallest diameters of cysts were obtained from Trnava county, but their size was not significantly different from those obtained from Košice, Nitra and Prešov counties.

Distribution of fertile and sterile cysts

No significant differences in relative proportional amounts of fertile and sterile cysts were found among counties ($P = 0.15$). From a total number of 372 cysts 33 (8.9 %) were fertile (Table 2). In Banská Bystrica and Prešov counties the values were lower (0 % and 5.3 %, respectively), but this difference was not yet statistically significant ($P > 0.05$). The closest proportional value of the occurrence of fertile cysts to the overall value was recorded in Košice county (8.7 %). Slightly higher proportions were ascertained in Nitra (11.8 %) and Trnava (16.7 %) counties, without exceeding the probability limit for statistical significance. The proportion value in cysts from Bratislava county ($P < 0.001$) was significantly different, but the cyst number (2) from this county was too small for being considered for statistical evaluation.

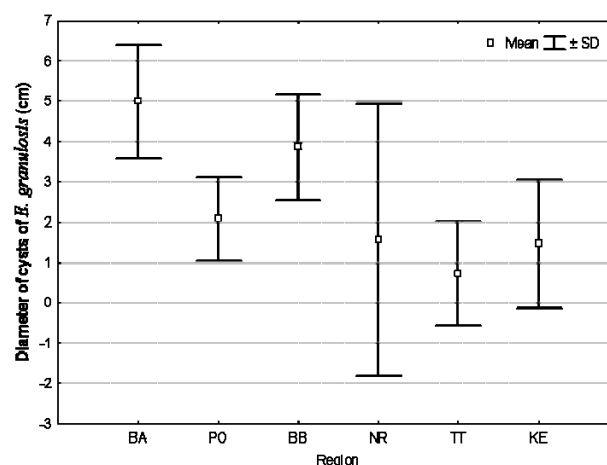


Fig. 4. Graph depicting distribution of cyst sizes according to counties

Size of sterile and fertile cysts

Size of echinococcal cysts in relation to their fertility was evaluated. Highly significant difference in cyst diameters

Table 2. Numbers, sizes and fertility of echinococcal cysts

County	Cyst numbers	Cyst sizes (cm)	Number of sterile cysts	Number of fertile cysts	Proportion of fertile cysts (%)
	Mean ± S.D.				
BA	2,0 ± 0,0	5,00 ± 1,41	0	2	100
TT	21 ± 15*	0,73 ± 1,30	35	7	16.7
NR	8,5 ± 9,2	1,56 ± 3,37	15	2	11.8
BB	24 ± 0,0*	3,87 ± 1,31	24	0	0
PO	5,4 ± 7,0	2,08 ± 1,04	36	2	5.3
KE	5,0 ± 5,6	1,46 ± 1,59	229	20	8.7
Total	5,9 ± 7,2	1,79 ± 3,01	339	33	8.9

BA - Bratislava; TT - Trnava; NR - Nitra; ZA - Žilina; BB - Banská Bystrica; PO - Prešov; KE - Košice

*counties with significantly higher cyst numbers relative to remaining counties ($P < 0.05$)

was observed between fertile and sterile cysts ($t = -12,037$, $df = 370$; $P < 0.001$); the average diameter measured in fertile cysts was three times larger than average diameter in sterile cysts, with mean values 4.67 cm and 1.37 cm, respectively (Fig. 5).

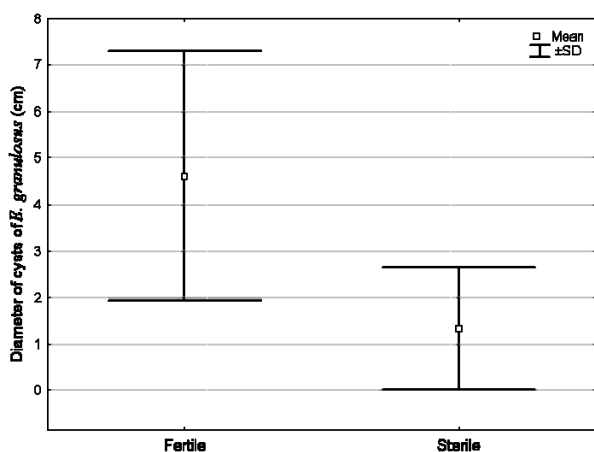


Fig. 5. Graph depicting cyst size in relation to fertility/sterility

Regression analysis of number and size of cysts

In evaluation of relation of number of cyst size (regardless of fertility/sterility) and their number in liver the tendency for decreased size with increased number of cysts in liver was apparent. With increasing number of cysts the trend for cyst diminution has declined. As shown in Fig. 6, when size was reciprocally transferred, positive linear relation of

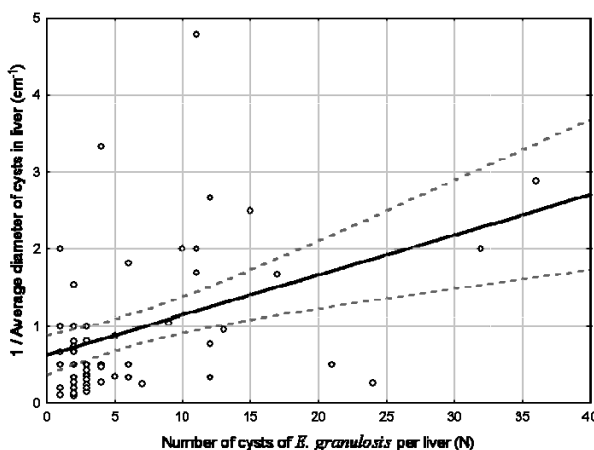


Fig. 6. Graph depicting relation between number and size of cysts based on reciprocal transformation of values of cyst diameters

transformed data with respect to the increase in cyst number in liver was evident, resulting in highly significant correlation coefficient ($P < 0.001$).

Discussion

In the present study, regional patterns derived from numbers, fertility and size of *E. granulosus* larvocysts in pig livers from Slovakia were studied. The proportion of animals with fertile cysts is an important indicator as only viable cysts assure the completion of life cycle and an active role in *E. granulosus* transmission. Fertile cysts were found in 25.4 % (16/63) of CE positive pigs in our report. Overall fertility rate in examined cysts was 8.9 % (33/372), which is lower value compared to situation in Lithuania where pigs harboured 23.4 % (11/47) of fertile cysts (Bružinskaitė *et al.*, 2009). This would be likely accounted for by higher proportion of young pigs (< 1 year) included in the survey within available records from Slovakia (92.2 %); we do not presume differences in infectivity of G7 genotypes circulating in these countries. Older pigs are more effective parasite transmitters due to both the higher chance to be infected with parasite and the drop in immunity owing to aging which allows the cysts to develop protoscoleces. Varcasia *et al.* (2008) found high fertility rate in Peloponnesus (Greece), where 50 % (14/28) of goats were infected with CE (G7 genotype), thus confirming suitability of this intermediate host for G7 transmission. Unlike this, for highly infective *E. granulosus* sensu stricto (G1 genotype) for humans, a high proportion of sterile cysts in pig is characteristic (Eckert *et al.*, 1993). This general feature did not conform to findings of Varcasia *et al.* (2006) from Sardinia, where a remarkable 69.2 % (18/26) fertility rate was recorded in pigs infected with G1 (highly predominant variant in the region). Pig host thereby potentially plays an important role in CE transmission in this island, in addition to sheep. The average cyst diameter of *Echinococcus* cysts, herein measured in 63 infected pig livers, was 1.79 cm. The average diameter of fertile cysts was more than three times larger than diameter of sterile cysts (4.67 cm vs. 1.37 cm). The same pattern was also found in a study of Dueger and Gilman (2001) on ovine echinococcosis in Peru, in which for 1-cm increase in size the odds of having protoscoleces increased by 5.5-fold (mean diameter of fertile cysts was 5.48 cm). Based on survey on 100 necropsied yaks Yang *et*

al. (2009) derived that frequencies of fertile cysts increased in lesions with a diameter > 2.1 cm. Pawlowski (1997) considered that cysts larger than 5 cm usually induce symptoms in humans. Many previous studies illustrated that large cysts, 10 – 20 cm in diameter, are generally fertile (Rausch, 1993). In our survey largest cysts were obtained from sites in Komárno district (Nitra county, southwestern Slovakia), with diameters of 10 and 11 cm (found in single liver), and in Trebišov district (Košice county, eastern Slovakia) 9.5 cm lesion was recorded, all hydatids were fertile.

The average intensity of infection in pigs was 5.9 cysts per liver in the present survey (G7 genotype involved, Šnábel *et al.*, 2008). In recent studies measuring this parameter of parasite burden, a high intensity of infection was found especially in sheep in Peloponnesus (Greece), reaching a value of 16.3 (measured in 64 animals, 53.1 % fertility; G1 involved), and cattle in Sardinia (Italy) - 12.5 (in 14 animals, although only 2.6 % of animals harbored fertile cysts; G1 involved) (Varcasia *et al.*, 2006, 2008). The eight cysts per yak and 5.8 cysts in sheep/goats were found in western Sichuan, China (Yang *et al.*, 2009), 8.1 in cattle and 4.3 in buffalo in Campania, southern Italy (Capuano *et al.*, 2006; Rinaldi *et al.*, 2008), 7 in sheep in Sardinia (Varcasia *et al.*, 2006), and 5.8 in goats in Greece (Varcasia *et al.*, 2008).

The typical transmission pattern of the “pig strain” (G7 genotype) is maintained between pig and dog. In a region of central-eastern Europe, a contiguous area of G7 was established that encompasses at least territories of Poland, Slovakia and Ukraine (Kedra *et al.*, 1999; Šnábel *et al.*, 2000; Turčeková *et al.*, 2003). The G7 was also reported from isolated foci of Spain (Daniel-Mwambete *et al.*, 2004), Italy, Sardinia (Varcasia *et al.*, 2006), Greece (Varcasia *et al.*, 2008), Romania (Bart *et al.*, 2006), Lithuania (Bružinskaitė *et al.*, 2009), Turkey (Šnábel *et al.*, 2009), Armenia (Gevorgyan *et al.*, 2006), Argentina (Kamenetzky *et al.* 2002) and Mexico (Cruz-Reyes *et al.*, 2007). Besides domestic pigs, wild boar was confirmed as transmitting pig strain in Ukraine, Spain and Germany (Kedra *et al.*, 2000; Gonzales *et al.*, 2002; Dinkel *et al.*, 2006), and parasite was also found in European beaver in Poland (Tkach *et al.*, 2002) and cattle in Slovakia (Turčeková *et al.*, 2003). In Europe, human infections with G7 (and its variant G9) were confirmed in Poland (Scott *et al.*, 1997) and Slovakia (Turčeková *et al.*, 2003). There is circumstantial evidence suggested that humans are poorly susceptible or refractory to infections with pig strain due to little evidence of human CE in endemic foci of G7 (Pawlowski, 1985; Thompson *et al.*, 2008). Infectivity of G7 for humans appears to be lower compared with closely related G6 genotype (camel strain), which e.g. in Argentina contributes for up to 37 – 44 % of human CE cases, despite the presence of highly infective sheep strains (G1, G2 genotypes) in the country (Rosenzvit *et al.*, 1999; Guarnera *et al.*, 2004). The close relationships among camel (G6), pig (G7) and G8/G10 (cervid) strains were demonstrated by recent genetic studies (Lavikainen *et al.*, 2006; Nakao *et al.*,

2007; Moks *et al.*, 2008). If the species categorization for this cluster is taxonomically verified, in terms of time priority it is named *E. canadensis* according to the former designation of cervid strain (Webster and Cameron, 1961). However, some genetic structuring between G6/7 and G8/G10 pairs is apparent from both mitochondrial and nuclear evidences (Nakao *et al.*, 2007; Saarma *et al.*, 2009), and it has to be yet resolved whether these groups of genotypic pairs merit an additional species/subspecies status.

Within herein examined echinococcal samples from Slovak pigs, genetic structuring of selected isolates was studied in fragments of mitochondrial genes (*cox1*, *atp6*, *nad1*) along with those from neighbouring countries (Ukraine, Poland, Hungary) (Šnábel *et al.*, 2008). In *cox1* fragment, the two G7 lines were detected, differing in two nucleotide sites. Major line was composed of 20 isolates from Slovakia, 5 isolates from Ukraine and 1 from Poland (assigned to as G7A variant). The G7B variant with two transitive mutations was detected in three samples from north-eastern Ukraine (Sumy region) and one isolate from southeastern Slovakia (Veľký Horeš, Trebišov district). In *atp6* gene, three polymorphic sites were detected within samples from Slovakia. The most common polymorphic site (238) with G/T substitution was recorded in the above isolate from Trebišov district and two locations (Dunajská Streda and Komárno districts) in southwestern Slovakia. All examined samples from central-eastern European region were identified as bearing the G7 genotype structure, except for Hungarian isolate (Békés county) from patient attributed to the G1. Endemic foci of G1 in Ukraine and southern European countries cannot exclude the spread of this genotype to Slovakia in further period, through mobile definitive hosts as jackal and wolf and/or livestock market.

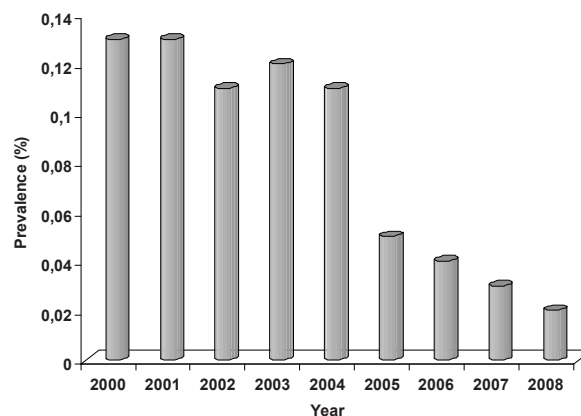


Fig. 7. Graph with annual prevalence rates of cystic echinococcosis in slaughtered pigs in Slovakia over 2000 – 2008

Average annual prevalence of CE in slaughtered pigs in Slovakia in 2000 – 2008 was 0.08 % (Štefančíková & Dubinský, 2000 – 2008). As shown in Fig. 7 summarized according to reports from regional veterinary services, the prevalence of *E. granulosus* ranged from 0.02 to 0.13, with the decreased tendency over the period, especially since

2005. Compared with the situation in 1990's with the average rate of 0.18 % (Svobodová *et al.*, 2006), more than two-fold reduction of the disease occurrence in pig was recorded. Based on reports from surveillances of zoonoses in Slovakia (Štefančíková & Dubinský, 2000 – 2008) we have derived that areas attributed to districts of Prešov (eastern Slovakia) and Komárno (southwestern Slovakia) have been the most important endemic areas of CE in pig over the last 3 years, with average prevalences of 0.68 % and 0.26 %, respectively. Respective areas are designated by hatched lines in Fig. 1. Within neighbouring countries, in Hungary the prevalence of CE in pigs decreased from 0.20 % in 1994 to 0.11 % in 2004 (Svobodová *et al.*, 2006), i.e. to a similar amount as observed in Slovakia in the corresponding period. Czech Republic has documented only 16 pigs with CE in 2004 and no other livestock was infected (Šmatlová & Papežiková, 2005). Higher overall prevalence of CE in pig is being registered in Poland (4.32 % in 2002) compared with Slovakia, with eastern voivodships of the country more encountered by disease (Svobodová *et al.*, 2006).

Annually, 0 – 7 human clinical cases were confirmed in Slovakia through 2000 – 2008 (except for 2000 with 31 registered cases), accounting for the average incidence rate (per 100 000 population) of 0.103 (Kinčeková *et al.*, 2008). The incidence of CE has fluctuated from 0 to 0.58%. The average rate has been similar compared with Poland (0.096) measured through the same period (Czarkowski *et al.*, 2008). In Hungary, according to available data from 2000 – 2004, the incidence was slightly lower (0.084) (Svobodová *et al.*, 2006). In the Czech Republic only imported cases of human CE were recorded in last years (Doležal *et al.*, 2008). Decrease of CE occurrence in pig and ruminants over the last decade in the above four countries correlates with a decline of incidence in humans compared with 1990's. Ukraine is with 44.370 registered animal CE cases per year among most heavily infected countries (Šmatlová & Papežiková, 2005). The prevalence of parasite varies substantially in different geographical zones; highest occurrence in humans in southern steppe zone of Ukraine is confined to sheep-raising areas (Malczewski *et al.*, 2002). 32 % of sheep, 20 % of cattle and 9 % of pigs were infected in this region in late 1980's (Shablovskaya *et al.*, 1989).

The contribution of infections in cattle and sheep to transmission of CE appears to be marginal in Slovakia. In both ruminants only sterile cysts were mostly recorded in our observations (Turčeková *et al.*, 2003). Over the period of 2000 – 2008, the prevalence of CE in slaughtered cattle ranged from 0.002 % (2006, 2007) to 0.04 % (in 2008), with the average annual rate 0.01 %. In sheep the average annual prevalence was 0.79 %. These values were likely partly overestimated due to occasional categorization of *Taenia*-type cysts as *Echinococcus*-type in slaughters, given that intention was to provide solely echinococcal cysts for the current survey. Larval stages of *Taenia* spp. (cysticercus) were presented in livers by fluid-filled cyst containing a single invaginated protoscolex. Prevalence of

cysticercosis in pig in Slovakia in 2000 – 2008 ranged from 0.002 to 0.006 % (Štefančíková & Dubinský, 2000 – 2008). Numbers of *Taenia* infections in Slovakia substantially decreased after 1981 following prohibition of preparing improperly cooked meat in restaurants and further decline was registered after 1989 owing to socio-economic changes implying marked decrease of cattle rearing (Štefančíková & Dubinský, 1995).

Further studies on *E. granulosus* establishment in Slovakia will be focused on epidemiological and genetic studies combined with factors as age-prevalence, genetic variant/prevalence, age-intensity and logistic modeling that might more precisely elucidate local epidemiological factors and ecological patterns of transmission necessary to implement an effective regional control strategy.

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