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Abstract

Cystic echinococcosis is a major emerging zoonosis in many Eastern European and Asian countries. Post slaughter examinations of 684 pig livers in Lithuania revealed significantly higher numbers of Echinococcus granulosus infections in animals from family farms (13.2%; 95% CI 10.7-16.2) as compared with those from industrial farms (4.1%; 95% CI 0.8-11.5). The prevalence was also significantly higher in pigs older than 1 year than in younger ones. In addition, in 0.5% of the pigs from the family farms, infertile and calcified E. multilocularis lesions were identified by PCR. Faecal samples from rural dogs (n=240) originating from 177 family farms in 12 villages were investigated for taeniid eggs with two methods. Significantly more dogs excreting taeniid eggs were diagnosed with the flotation/sieving method (n=34) as compared to the modified McMaster method (n=12). Multiplex PCR performed with DNA from taeniid eggs isolated from faeces of 34 dogs revealed 26 infections with Taenia spp., 9 with E. granulosus and 2 with E. multilocularis (4 cases with concurrent Taenia spp. and E. granulosus or E. multilocularis infections). Genotyping of E. granulosus cyst tissues from 7 pigs, 1 head of cattle and from E. granulosus eggs from 8 dog faeces revealed the genotype G6/7 ('pig/camel strain') in all cases. The high infection pressure with Echinococcus spp. in family farms necessitates initiating control programs.
Echinococcosis in pigs and intestinal infection with *Echinococcus* spp. in dogs in southwestern Lithuania

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Abstract

Cystic echinococcosis is a major emerging zoonosis in many Eastern European and Asian countries. Post slaughter examinations of 684 pig livers in Lithuania revealed significantly higher numbers of *Echinococcus granulosus* infections in animals from family farms (13.2%; 95% CI 10.7–16.2) as compared with those from industrial farms (4.1%; 95% CI 0.8–11.5). The prevalence was also significantly higher in pigs older than 1 year than in younger ones. In addition, in 0.5% of the pigs from the family farms, infertile and calcified *E. multilocularis* lesions were identified by PCR. Faecal samples from rural dogs (n=240) originating from 177 family farms in 12 villages were investigated for taeniid eggs with two methods. Significantly more dogs excreting taeniid eggs were diagnosed with the flotation/sieving method (n=34) as compared to the modified McMaster method (n=12). Multiplex PCR performed with DNA from taeniid eggs isolated from faeces of 34 dogs revealed 26 infections with *Taenia* spp., 9 with *E. granulosus* and 2 with *E. multilocularis* (4 cases with concurrent *Taenia* spp. and *E. granulosus* or *E. multilocularis* infections). Genotyping of *E. granulosus* cyst tissues from 7 pigs, 1 head of cattle and from *E. granulosus* eggs from 8 dog faeces revealed the genotype G6/7 (‘pig/camel strain’) in all cases. The high infection pressure with *Echinococcus* spp. in family farms necessitates initiating control programs.

*Keywords: Echinococcus granulosus*, ‘pig strain’, *Echinococcus multilocularis*, Lithuania
1. Introduction

Cystic echinococcosis (CE), caused by *Echinococcus granulosus*, is an important zoonotic infection causing morbidity and mortality in humans and significant economic losses in livestock (Budke et al., 2006). It is an emerging disease in many parts of the world, in particular in countries of the former Soviet Union, in Eastern Europe and in Asia (Romig et al., 2006; Torgerson et al., 2006). In the Central and Eastern European countries such as Poland, Slovakia, Romania, Belarus and Ukraine, the ‘pig strain’ of *E. granulosus* has been described (Eckert and Thompson, 1988; Kedra et al., 1999; Šnábel et al., 2000; Bart et al., 2006). Danilevičius (1964) found 8.6% (2’635/30’641) of pigs infected with *E. granulosus* from six major slaughterhouses during 1961–1962 indicating that the same strain may also occur in Lithuania in a dog/pig cycle.

The prevalence of *Echinococcus* has decreased to 0.8 % (6’010/782’768) in the Lithuanian pig population by 2001 (Anonymous, 2001). However, 36% of pigs are being reared on family farms (Department of Statistics of the Government of the Republic of Lithuania, www.stat.gov.lt), and a considerable number of these pigs are slaughtered at home. As carcasses of such pigs often avoid official post slaughter inspection, the prevalence of CE may substantially be underestimated.

Three previous helminthological investigations of necropsied dogs in Lithuania reported prevalences of *E. granulosus* of 6.0% (5/83), 7.8% (8/102) and 21.1% (4/19) (Musteikaitė et al., 1961; Danilevičius, 1964; Kazlauskas and Prūsaitė, 1976). However, more recent data on the prevalence of *Echinococcus* in dogs is not available. Therefore, this pilot study was initiated to investigate the epidemiology of *E. granulosus* with special reference to family farms.
2. Materials and Methods

2.1. Collection and examination of intermediate host samples

The study area was the southwestern part of Lithuania where most pigs in villages are reared for home consumption (with mostly home slaughter) and/or selling locally (with slaughtering usually in local slaughterhouses). During post slaughter examination, pigs’ organs were examined for CE. To evaluate the prevalence of CE, pig livers with visible lesions were collected once a week at two local slaughterhouses between October 2005 and January 2006. This sampling method was based on the experience that CE mostly was found in the liver and very rarely in other organs (unpublished data). The date of slaughter, the total number of slaughtered pigs per day at the slaughterhouse, the pig identification number, age (obtained from the owners) and family (small number of pigs and mainly home slaughtering) or industrial (numerous pigs and abattoir slaughter) farm from which the pigs originated were recorded. The liver samples originated from family farms (n=612) and from industrial farms (n=73). The livers with lesions and/or cysts were frozen at –20 °C and later examined at the Department of Infectious Diseases, Lithuanian Veterinary Academy, where they were cut into 2 cm slices, and all visible cysts or necrotic lesions were excised, opened and morphologically examined. Unidentifiable necrotic lesions as well as the germinal membrane and protocolices of typical *E. granulosus* cysts were collected and frozen at –20 °C for further molecular analyses.

2.2. Collection and examination of dog faecal samples

Twelve villages from areas where *E. granulosus* positive pig samples originated were chosen for a further investigation of dogs. In total, 177 family farms were visited on which 336 dogs were kept. Fresh or a few days old faecal samples were collected from the ground from 240 individual dogs either within the area accessed by chained dogs or, in case of
unchained dogs, from defecation places pinpointed by the owners. Prior to examination, faecal samples were frozen at –80 °C for 5 days for safety reasons and then stored at –20 °C.

To determine the number of helminth eggs in the faeces of dogs, all samples (n=240) were examined using the McMaster method according to Roepstorff and Nansen (1998) with some modifications. Four grams of faeces were resuspended in 56 ml of water and left for 30 minutes, carefully mixed, filtered through gauze, the filtrate stirred and a subsample of 10 ml poured into a centrifuge tube. After centrifugation for 7 min at 1200 rpm, the supernatant was discarded. The tube was filled with commercially available sucrose ('Panevėžio cukrus') flotation solution (density 1.4) up to 4 ml. The content of the tube was thoroughly mixed with a Pasteur pipette and aliquots were carefully transferred into the chambers (150 µl) of the McMaster slide. All parasite eggs under the two separate grids were counted. The number of the different helminth eggs per gram of faeces was obtained by multiplying the total number of eggs in both chambers by 20.

A further 4 grams of the well mixed faecal samples (n=240) were investigated by a combination of flotation in zinc chloride solution and sequential sieving through nylon nets followed by taeniid eggs isolation and identification using an inverted microscope (egg F/Si method) (Mathis et al., 1996).

2.3. Questionnaire

A questionnaire was administered to all farmers (n=177, from 12 villages) asking for information on identification, age, gender, name and brief physical description of the dogs, their feeding habits: offal (raw organs or meat) or kitchen slops (boiled meat, porridge, vegetables, soups etc.); anthelmintic treatment (yes/no, if treated how often) and how they were restrained (chained or unchained). The type and breed of the dogs were not recorded as most rural dogs are guard dogs of mixed breed.
2.4. Molecular genetic analysis

For the genetic identification of *Echinococcus* to species/strain level, PCR was performed with DNA from typical hydatid cysts from pigs (n=7), morphologically unidentifiable lesions from pigs (n=3), nonfertile cysts from cattle (n=3) and from taeniid eggs isolated from dog faecal samples (n=34) (Mathis et al. 1996).

DNA isolations from cysts and lesions were performed using a commercial kit (Qiamp DNA mini kit, Qiagen, Hilden, Germany) according to the manufacturer’s instruction. PCR with these samples was done according to Bowles et al. (1992). DNA from unidentifiable lesions was additionally tested by a PCR using *E. multilocularis*-specific primers (Stieger et al., 2002). Negative controls (distilled water) were included in all PCR assays and the uracil DNA glycosylase system was used to prevent carry over contamination.

DNA extraction from taeniid eggs isolated from faeces was performed as described (Stefanić et al., 2004), and multiplex PCR according to Trachsel et al. (2007) for the simultaneous detection of *E. granulosus* (all strains), *E. multilocularis* and *Taenia* spp. was performed. Amplicons of *E. granulosus* (117 bp) were extracted from agarose gels using NucleoSpin Extract II (Macherey-Nagel, Germany), cloned using the topo TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced by a private company (Microsynth, Balgach, Switzerland).

2.5. Statistical analysis

Data was entered onto an Excel spreadsheet and then imported into R 2.4.0 (The R Foundation for Statistical Computing, CRAN.R-project.org) or GraphPad Prism version 4.00 for Windows® (GraphPad Software, San Diego California USA, www.graphpad.com) for analysis.

A multilevel modelling approach was used which included analysing dog and village level variables. The R function glm and lmer (from the lme4 package) were used with a
binomial (logit) link function. Variables were eliminated in a backward selection process with variables having a $p>0.15$ eliminated at each step. Fixed effect model was compared to mixed models (fixed effects and farm or village level effects) by Akaike Information Criteria (AIC) (Akaike, 1974) and the model with the lowest AIC was selected. The isolation of taeniid eggs and *Toxocara* eggs were examined as dependent variables. Non-linear effects of age were also examined by using general additive modelling (GAM function). Prevalences were reported with the exact binominal 95% confidence intervals for proportions (CI). Fisher’s Exact Test was used to compare the differences in dog infection with taeniid eggs established by two methods (egg F/Si and McMaster), and the infections in pigs reared in industrial and family farms. Variables with a $p$ value of $<0.05$ were considered significant.

3. Results

3.1. Examination of pigs and *Echinococcus* identification

Total 13.2% (81/612; 95% CI 10.7–16.2) of pig livers originating from family farms were infected with metacestodes of *E. granulosus*. From 47 of 81 hydatid cysts investigated for the presence of protoscolices 11 (23%) were fertile. Larval stages of *T. hydatigena* were macroscopically identified in the liver subserosa in 2.5% (15/612; 95% CI 1.4–4.0) of pigs. In two pig livers, both parasites were detected. In 3 (0.5%; 95% CI 0.1–1.4) liver samples, where morphological identification of multiple necrotic and calcified lesions was not possible, PCR confirmed the presence of *E. multilocularis*.

The age of the pigs was available for 549 of the 612 examined animals from family farms and varied from 4 to 26 months. Of 480 pigs less than 1 year of age, 61 were infected with hydatid cysts (12.7%; 95% CI 9.9–16.0) that 3 of 37 were fertile, whereas 19 of the 70 pigs (27%; 95% CI 17.2–39.1) aged 1 year or older, were infected and 6 of 10 cysts were
fertile. Thus, older pigs were more likely to be infected than younger ones (p<0.01, Fisher’s Exact Test). Only one pig out of 63 pigs whose age was not recorded was infected (1.6%; 95% CI 0.04–8.5).

Only 3 of the 73 pigs from industrial farms were infected with hydatid cysts (4.1%; 95% CI 0.8–11.5). This was significantly lower than the proportion of infection on the family farms (p=0.02, Fisher’s Exact Test). Neither infections with *T. hydatigena* nor *E. multilocularis* were recorded in the pigs from industrial farms.

3.2. Examination of dogs

The faeces of 240 dogs from family farms were examined by both the modified McMaster and the egg flotation/sieving (egg F/Si) methods. Eggs from all the taeniid-positive samples detected by F/Si were examined by multiplex PCR to determine the taeniid genera. The results, including those of molecular analysis on taeniid eggs isolated from faeces, are summarised in Table 1. Examination of dog faeces by the McMaster method revealed that the most prevalent helminth in dogs was *Toxocara* spp. (10.8%). Taeniid eggs were detected in 5.0% of the dogs by this method. However, using the egg F/Si method, a significantly higher percentage (14.2%, p<0.05) of dogs were identified as shedding taeniid eggs, including all McMaster positive ones. Molecular analysis of taeniid eggs isolated from faeces revealed *Taenia* spp. in 10.8%, *E. granulosus* in 3.8% and *E. multilocularis* in 0.8% of samples. Three of these infected dogs had co-infections with *E. granulosus* and *Taenia* spp. and one with *E. multilocularis* and *Taenia* spp. One of the 34 samples positive for taeniids by the egg F/Si method was negative by multiplex PCR. Notably, when using the McMaster method, in none of the patent infections with *Echinococcus* spp. taeniid eggs were detected, with the exception of two co-infections with *Taenia* spp. (Fig. 1).
The highest numbers of eggs (EPG) originated from taeniids, *Toxocara* spp. and *Toxascaris leonina* (Table 1).

### 3.3 Molecular analysis of *E. granulosus*

Sequence analysis of amplicons obtained from cyst tissue from 7 pigs and 1 head of cattle as well as from eggs of 8 dogs revealed in all cases a 100% identity with the sequences attributed to the ‘G6/7’ strain (accession numbers AY462128, AY462126, L49456).

### 3.4 Risk factors

The questionnaire revealed that two dogs on average (range: 1–7 dog) were kept on each yard. The majority (82.4%; 178/216) of the dogs never received anthelmintic treatment and 42.1% (32/76) of the treated dogs were treated irregularly, often only as puppies. One third of the dogs (31.0%; 67/216) were fed with offal and 6.0% (4/67) of them were infected with *E. granulosus*. Additionally, 2.7% (4/149) of dogs fed with kitchen slops (69.0%; 149/216) were infected with *E. granulosus*.

According to the information provided by the dog owners, 92.5% (260/281) of the dogs in villages were kept chained. Nine (3.5%) of them were infected with *E. granulosus* and one (0.4%) with *E. multilocularis*. No *Echinococcus* spp. eggs were detected in the faeces of dogs from village farms without pig production (25 of 177) while 16% of these dogs were excreting *Taenia* spp. eggs. However, no significant risk factor for infections with *E. granulosus* or *Taenia* spp. could be identified.

### 4. Discussion

This investigation confirms the presence of the ‘pig strain’ (G6/7) of *E. granulosus* in the Baltic States extending the proven distribution of this strain in other East European
countries (Poland, Ukraine, Slovakia and Romania) where pigs are commonly reared on family farms (Romig et al., 2006). In contrast to other *E. granulosus* endemic areas, where several genotypes can coexist and can be identified in the domestic dog as the definitive host (Ziadinov et al., 2008), the identification of the *E. granulosus* genotype G6/7 in all *E. granulosus* egg isolates from dogs and from all isolates of pig and cattle origin indicate that the pig strain predominates in Lithuania. This hypothesis is further supported by the fact that *E. granulosus* cysts had only occasionally been recorded in cattle (0.8%) and sheep (2.7%) in a previous study (Danilevičius, 1964). This is consistent with our experience that cattle are rarely infected and mostly with sterile cysts only (unpublished data). Therefore, domestic ruminants are probably accidental hosts of the *E. granulosus* ‘pig strain’ and are of no epidemiological significance in Lithuania. However, our data do not strictly exclude the presence of other strains in Lithuania.

The present study revealed a high prevalence of *E. granulosus* metacestodes in pigs from southwestern Lithuania (13.2%). This is in agreement with a previous study from the same area in Lithuania (8.4%) (Danilevičius, 1964) and results from Poland where up to 13.4% of pigs were found to be infected with hydatid cysts (Deryło et al., 2001). However, our findings are different compared to other East European countries i.e. Slovakia (0.13-0.29%) (Kedra et al., 1999) and Romania (4%) (Bart et al., 2006).

In the present study we compared the prevalence of CE in pigs reared in family and industrial farms. We found significantly higher numbers of infected pigs in family farms when compared to those of industrial farms. However, in a previous study from the same area, there were no differences of prevalence recorded between pigs from family and industrial farms (8.4%, 21/250 and 8.5%, 20/235 respectively) (Danilevičius, 1964). This may be because in the past dogs were often kept close to industrial farms. However, such practices occur to a much lower extent these days due to enforcement of veterinary regulations.
This study revealed a prevalence of *T. hydatigena* larval stages in pig livers of 2.5%. However, the prevalence may be underestimated as the abdomen cavity, where cysts are often located, was not examined.

The findings of *Echinococcus* spp. and *T. hydatigena* metacestode stages in pigs from family farms raise the question of the source of infection. Rural dogs, which are often kept to guard the personal property, may play an important role in the transmission of *E. granulosus* especially with infrequent anthelmintic treatment. However, no risk factors for dogs infected with taeniids could be identified. Most surprisingly, dogs from properties without pig husbandry often were infected. This suggests that there may be various sources for *E. granulosus* infections on family farms. A previous study of helminths of 19 dogs of unknown origin in Lithuania had revealed high prevalences for *E. granulosus* (21.6%), *T. hydatigena* (36.8%), *T. pisiformis* (5.2%) and *D. caninum* (26.3%) (Kazlauskas and Prūsaitė, 1976). In the present study, the species of *Taenia* were not specified.

Egg isolation using the egg F/Si method (Mathis et al., 1996) was more sensitive for the detection of *Echinococcus* eggs in animals with low infections than the McMaster method. The majority of infections with *E. granulosus* and *E. multilocularis* were not detected by the McMaster but only by the egg F/Si method. The present study supports recent findings (Al-Sabi et al., 2007) that the McMaster method is not highly sensitive for the detection of low numbers of taeniid eggs. Similarly, the prevalence of *Trichuris* eggs which was determined with the McMaster method may also be underestimated. The egg F/Si method was recently also shown to have a higher sensitivity for the diagnosis of *Echinococcus* spp. in dogs (78% for *E. granulosus* and 50% for *E. multilocularis*) when compared to arecoline purgation (Ziadinov et al., 2008).

Infections of domestic pigs with metacestodes of *E. multilocularis* have been described in Japan and Central Europe (Deplazes et al., 2001). In the study from Switzerland, alveolar echinococcosis was also detected in pigs reared outdoors in areas endemic for *E.*
*multilocularis* (Sydler et al., 1998). In Eastern Europe, infections of pigs and dogs from villages with *E. multilocularis* are reported for the first time in the present study. A high prevalence of *E. multilocularis* in red foxes was reported from various districts of Lithuania (Bružinskaitė et al., 2007). The presence of AE in pigs reared indoors with limited access to contaminated environment together with *E. multilocularis* infections in chained dogs emphasizes the fact that there is a high infection pressure in the rural environment, also posing a high risk for humans. Indeed, it was recently revealed that human AE is an emerging problem in Lithuania (Bružinskaitė et al., 2007).

From the present pilot study we can conclude that the ‘pig strain’ of *E. granulosus* is highly endemic in the southwestern part of Lithuania and that transmission is more likely in family farms. However, for a better understanding of the epidemiology of *E. granulosus* in the other parts of the country, further studies should be initiated in Lithuania.
Acknowledgements

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References


Table 1

Helminth eggs detected in faecal samples of 240 dogs from family farms in Southwestern Lithuania.

<table>
<thead>
<tr>
<th>Parasite species</th>
<th>Prevalence</th>
<th>95% CI</th>
<th>Intensity</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>McMaster method</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Toxocara</em> spp.</td>
<td>10.8</td>
<td>7.2–15.5</td>
<td>343</td>
<td>20–2080</td>
</tr>
<tr>
<td><em>Toxascaris leonina</em></td>
<td>2.1</td>
<td>0.7–4.8</td>
<td>960</td>
<td>20–2420</td>
</tr>
<tr>
<td>Hookworms</td>
<td>3.3</td>
<td>1.4–6.5</td>
<td>198</td>
<td>20–980</td>
</tr>
<tr>
<td><em>Capillaria</em> spp.</td>
<td>3.3</td>
<td>1.4–6.5</td>
<td>45</td>
<td>20–120</td>
</tr>
<tr>
<td>Taeniidae</td>
<td>5.0</td>
<td>2.6–8.6</td>
<td>1689</td>
<td>10–10120</td>
</tr>
<tr>
<td><strong>Egg flotation/sieving method</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taeniidae</td>
<td>14.2</td>
<td>10.8–19.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Multiplex PCR</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Taenia</em> spp.</td>
<td>10.8</td>
<td>7.2–15.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>E. granulosus</em></td>
<td>3.8</td>
<td>1.7–7.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>E. multilocularis</em></td>
<td>0.8</td>
<td>0.1–3.0</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> Roepstorff and Nansen, 1998

<sup>b</sup> Mathis et al., 1996 (all samples positive for taeniids in the McMaster method included)

<sup>c</sup> PCR with samples containing taeniid eggs (Trachsel et al., 2007). Three samples with co-infections *E. granulosus*/*Taenia* spp. and one with *E. multilocularis*/*Taenia* spp.
Fig. 1. Detection of taeniid eggs by the modified McMaster method (the solid line indicates the lower detection limit) (Roepstorff and Nansen, 1998) and by the flotation/sieving method (F/Si) (Mathis et al., 1996). All samples with egg counts below 20 and the 3 *E. granulosus*-positive samples with more than 20 eggs were detected with the F/Si method and the total number of eggs was quantified by counting the eggs directly under the inverted microscope. Open symbols represent mixed infections with *Taenia* spp.