Occurrence of *Giardia duodenalis* zoonotic assemblages in red foxes from Romania

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Abstract. The aim of this study was to determine the occurrence and distribution of *Giardia duodenalis* among red foxes (*Vulpes vulpes*) living in Romania. For this purpose, a total of 273 fecal samples from red foxes, shot during hunting seasons or corpses resulted in car accidents between December 2011 and January 2012 were examined using molecular biology methods. Overall, two zoonotic genotypes have been identified, assemblage A and assemblage B with higher prevalence in juvenile male. The results of the present study show that red foxes are reservoirs of *Giardia duodenalis*, this study being the first report on the occurrence of *Giardia duodenalis* zoonotic assemblages in the red fox population from Romania.

Keywords: *Giardia duodenalis*; Red fox; Assemblages.

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Introduction

Red foxes (*Vulpes vulpes*) are the most widespread members of the order of Carnivora. Since they often live in periurban areas, they are a potential reservoir of zoonotic agents transmitted from wildlife to humans or domestic animals. Human infection especially with zoonotic parasites is an emerging health issue, as the human environment is increasingly shared with infected animals, either pets or wild life.

Ecological changes during the past two decades, coupled with successful vaccination of foxes against rabies, have resulted in a significant increase of the fox population and might have accounted for substantial extension of the parasite range (Romig et al., 2006).

Red foxes are a common inhabitant of the region of Romania and are found in a variety of habitats. Despite this wide geographic distribution and published articles regarding parasites distribution, only a small amount of published information is available regarding *Giardia duodenalis* population in this species.

*Giardia intestinalis* is a cosmopolitan pathogen with a very wide host range, including humans,
domestic animals, and wild animal species (Cacciò et al., 2008; Thompson et al., 1993). The high prevalence of *Giardia* spp. was attributed to the fact that this parasite can colonize niche previously occupied by parasites such as *T. canis* and *D. caninum*, and most of the anthelmintics do not interfere in the development of *Giardia* spp. (Bugg et al., 1999).

However, a few studies have been carried out and little information is available on the prevalence, distribution and genetic characterization of *Giardia duodenalis* in red foxes. The aim of this study was to determine the occurrence and distribution of *Giardia duodenalis* among free-living red foxes in Romania, since no studies have been conducted to date in this area.

**Material and methods**

*Animals and samples*

Between December 2011 and January 2015, 217 samples were collected from red foxes originated in 7 counties from Romania. The foxes were originally collected within a National vaccination program against rabies. Samples (gastrointestinal tract) were collected one time from foxes provided of Sanitary Veterinary Institutions following organized hunters and also corpses resulted in car accidents. All the foxes investigated at the laboratory of Parasitology and Parasitical diseases were free of rabies. The corpses were maintained at -20°C until examination. We registered for each fox the following data: age, gender and area.

**PCR reactions**

The amplification of β-giardin gene was performed using a nested-PCR protocol (Cacciò et al., 2002; Lalle et al., 2005). In the first PCR reaction it was obtained a 753bp fragment using the primers pair G7-G759. PCR mix consisted in 10µM of each primers, 12.5µl of MasterMix (Rovalab GMBH, Germany) and 4µl of DNA in a total volume of 25µl. PCR conditions were: initial denaturation for 5 min at 96°C, a set of 40 cycles of annealing (30 sec at 95°C, 30 sec at 50°C and 1 min at 72°C) followed by a final extension of 7 min at 72°C.

In the second PCR reaction it was obtained a 511bp fragment using the primers pair GiarF-GiarR. The PCR mix consisted in 10µM of each primers, 25µl of MasterMix (Rovalab GMBH, Germany), 4µl of DNA in a total volume of 50µl. PCR conditions were: initial denaturation for 5 min at 96°C, a set of 35 cycles of annealing (45 sec at 95°C, 30 sec at 55°C and 45 sec at 72°C) followed by a final extension of 7 min at 72°C (table 1).

PCR products were subsequently sequenced (MACROGEN, Amsterdam, Netherlands).

**Statistical analyses**

Descriptive epidemiology was performed using EpilInfo 2000 software. Frequency, prevalence, 95% confidence interval and the "p" value were established. Age (youth and adults), sex (males and females), origin (county) of foxes provided the basis to analyze the relations between these factors and the presence of zoonotic parasites.

**Results**

The coproparasitological examination was performed and the results were previously published (Onac et al., 2015), the prevalence of *Giardia*’s cysts being 2.8%. The amplification of all 217 samples from foxes revealed 10 samples (4.6%; 95%CI=2.2-8.3) which yielded bands at 511bp (figure 1, table 2). From the positive samples amplified, two samples were from females (2.3%; 95%CI=0.3-8.0) and eight samples were from males (6.2%; 95%CI=2.7-11.9). Regarding age distribution, seven positive samples were from youth foxes (12.3%; 95%CI=5.1-23.7) and three from adults (1.9%; 95%CI=0.4-5.4) (p=0.004). Positive samples stratified by counties were from Bihor (n=4) (7.3%; 95%CI=2.0-17.6), Cluj (n=1) (4.8%; 95%CI=0.1-23.8), Covasna (n=2) (4.1%; 95%CI=0.5-14.0), Hunedoara (n=1) (2.6%; 95%CI=0.1-13.5) and Satu Mare (n=2) (5.4%; 95%CI=0.7-18.2) (table 2).

Following sequencing, three of the samples gave positive results for *Giardia duodenalis* assemblages, respectively one for assemblage B, and another two samples as assemblage A (subassemblage AII).
Table 1. Primers used for the molecular characterization of Giardia isolates

<table>
<thead>
<tr>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>G7 - AAGCCCGAGACCTCACCCGCAGTGC</td>
<td>G759 - GAGGCCGCCCTGGATCTTCGAGAC GAC</td>
</tr>
<tr>
<td>GiarF - GAACGAACGAGATCGAGGTCCG</td>
<td>GiarR - CTCGACGAGCTTGGTT</td>
</tr>
</tbody>
</table>

Figure 1. Samples amplification for β giardin locus
(Lane 1 – Ruller, lanes 2-10, 13 – positive samples, lanes 11, 12, 14, 15 – negative samples)

Table 2. The frequency, prevalence and CI 95% of Giardia duodenalis in nested PCR reaction, targeting β-giardin gene

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Frequency</th>
<th>Prevalence (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>217</td>
<td>10</td>
<td>4.6 (2.2-8.3)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>youth</td>
<td>57</td>
<td>7</td>
<td>12.3(5.1-23.7)</td>
<td>0.004</td>
</tr>
<tr>
<td>adults</td>
<td>160</td>
<td>3</td>
<td>1.9(0.4-5.4)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>females</td>
<td>88</td>
<td>2</td>
<td>2.3 (0.3-8.0)</td>
<td></td>
</tr>
<tr>
<td>males</td>
<td>129</td>
<td>8</td>
<td>6.2(2.7-11.9)</td>
<td>0.3</td>
</tr>
<tr>
<td>County</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>...AB</td>
<td>8</td>
<td>0</td>
<td>0.0 (0.0-36.9)</td>
<td></td>
</tr>
<tr>
<td>...BH</td>
<td>55</td>
<td>4</td>
<td>7.3 (2.0-17.6)</td>
<td></td>
</tr>
<tr>
<td>...CJ</td>
<td>21</td>
<td>1</td>
<td>4.8 (0.1-23.0)</td>
<td></td>
</tr>
<tr>
<td>...CV</td>
<td>49</td>
<td>2</td>
<td>4.1 (0.5-14.0)</td>
<td></td>
</tr>
<tr>
<td>...HD</td>
<td>39</td>
<td>1</td>
<td>2.6 (0.1-13.5)</td>
<td>0.9</td>
</tr>
<tr>
<td>...HG</td>
<td>8</td>
<td>0</td>
<td>0.0 (0.0-36.9)</td>
<td></td>
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<tr>
<td>SM</td>
<td>37</td>
<td>2</td>
<td>5.4 (0.7-18.2)</td>
<td></td>
</tr>
</tbody>
</table>

Discussions

Despite their hunting, the population density and distribution of red foxes has increased throughout Europe as a consequence of intensive oral anti-rabies vaccination programs (Gloor et al., 2001). Environmental pollution with human and domestic-animal fecal material is recognized as a potential pathogen pathway for wildlife infections with zooanthropomorphic protozoan parasites such as Giardia (Appelbee et. al., 2005).

The association between infected beavers and waterborne outbreaks of human giardiasis led the World Health Organization to classify Giardia as a zoonotic parasite (Thompson, 2004).

A few studies have been done for surveillance of Giardia in red foxes. Hamnes et al. (2007)...
detected *Giardia* cysts in 13 (4.8%) fecal samples from the total of 269 Norwegian red foxes, while Beck et al. (2011) found the same prevalence (4.5%) of *Giardia duodenalis* in Croatia. Nucleotide sequence analysis of the gdh, SSU-rDNA and β-giardin genes of *Giardia duodenalis*, demonstrated a high degree of heterogeneity and the genotyping information showed the occurrence of zoonotic Assemblages A and B in red foxes, suggesting a potential role of these animals as a source of infections for humans or other animals (Hamnes et al., 2007; Beck et al., 2011; Hodžić et al., 2014).

Since host-specific genotypes are not frequently seen in wildlife, it is which are the likely contamination sources for assemblage A and B in wild mammals. Assemblages C and D have been found mostly in dogs and other canines (foxes and coyotes) and canine-related animals (seals) (Feng and Xiao, 2011).

In our study, the prevalence of *Giardia* infection was significantly higher in juvenile male foxes than in adult male foxes, with significant differences between age were found. No significant differences in prevalence related to sex or geographical origin of animals were found. Genotyping of *Giardia duodenalis* isolates from three foxes demonstrated a high degree of heterogeneity amongst them, with all isolates belonging to the zoonotic assemblages A and B.

**Conclusions**

In conclusion, the results of the present study show that foxes are reservoirs of *Giardia duodenalis*, but further molecular analysis are necessary to elucidate the routes of transmission of this pathogen. This is the first report on the occurrence of *Giardia duodenalis* zoonotic assemblages in the red fox population from Romania.

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