ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2007, 14, 265-270

DISTRIBUTION OF *CRYPTOSPORIDIUM* AND *GIARDIA* SPP. IN SELECTED SPECIES OF PROTECTED AND GAME MAMMALS FROM NORTH-EASTERN POLAND

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Paziewska A, Bednarska M, Niewęgłowski H, Karbowiak G, Bajer A: Distribution of *Cryptosporidium* and *Giardia* spp. in selected species of protected and game mammals from North-Eastern Poland. *Ann Agric Environ Med* 2007, **14**, 265-270.

Abstract: *Cryptosporidium* spp. and *Giardia* spp. are wide-spread pathogens of humans and many species of mammals. The ways of transmission are very complex and difficult to define. Both parasites occur in similar environments and share a broad host range. However, in Poland there is still little known about the epidemiology of these parasites due to the paucity of data on human cases and only few studies in wildlife. The aim of our study was to determine the distribution of two intestinal protozoa in a few species of protected and game mammals in North-Eastern Poland. Additionally, we wanted to compare prevalence and abundance of these parasites between wild and farm animals, and to determine the species/genotypes of *Cryptosporidium*. Fecal samples collected from protected species (European beaver – 22, grey wolf – 14, European bison – 55, Polish Konik (horse) – 5) and game mammals (red deer – 52, roe deer – 22, boar – 5) were examined by IFA. We also studied a group of samples collected from farm animals: beaver – 30, red deer – 66, Polish konik – 5. *Cryptosporidium* oocysts were identified in 5 of 7 studied animal species (prevalence from 9% in roe deer to 36% in wolves), *Giardia* cysts in 4 of 6 studied species (prevalence from 1.7% in red deer to 7.7% in European beaver). Sequencing analysis of COWP gene fragment revealed that 5 *Cryptosporidium* isolates from wolves were *C. parvum* genotype 2 (zoonotic). The results show the important role of examined species in maintaining the natural sources of *Cryptosporidium* spp. and *Giardia* spp. infections in the environment.

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Key words: *Cryptosporidium*, *Giardia*, reservoir, genotype, wolf, bison, beaver, red deer, roe deer.

INTRODUCTION

Cryptosporidium spp. and *Giardia* spp. are intestinal protozoan parasites which are recognized as prevalent and wide-spread pathogens of humans and many species of mammals. *Cryptosporidium* spp., mainly *C. parvum* is a common cause of gastroenteritis called cryptosporidiosis which manifests as a watery diarrhea in humans. The diarrhea may become profuse and prolonged, and in consequence life-threatening, particularly in immunocompromised (i.e. HIV positive) or immunosuppressed persons [15]. Even in immunocompetent individuals cryptosporidiosis may present as an acute and often persistent enterocolitis. Infections with the other parasite, *Giardia intestinalis* (*G. duodenalis*), are the third most common protozoan infections in humans world-wide. The presence of the parasite may load to a variety of clinical manifestations [32]. Giardiosis is the most prevalent in children and may develop into a chronic disease, resulting in malabsorbtion and stunted growth or body weight loss [23].

Received: 29 January 2007
Accepted: 25 November 2007

The ways of transmission are very complex and difficult to define. Both parasites occur in similar environments and share a broad host range: more than 100 different mammal species [2, 27, 28]. Both cryptosporidiosis and giardiosis are believed to be zoonoses. Both are actually the complexes of different species and genotypes, with a number of pathogenic species/genotypes, some of them being specific to humans, others being zoonotic [6, 8, 38]. The epidemiological surveys proved that the most important sources for human invasions are contaminated drinking and recreational water, food, household animals and infected people [8, 11]. Sources of environment contamination may be very different [37] with a likely important role of a host species reservoir.

However, in Poland there is still little known about the epidemiology of these parasites due to the paucity of data on human cases and only few studies in wildlife [4, 20, 24]. In North-Eastern Poland, due to years of active conservation, including introduction and re-introduction, there are established permanent populations of rare protected species such as the European beaver *Castor fiber*, grey wolf *Canis lupus*, European bison *Bison bonasus* and Polish Konik (horse) *Equus caballus*, in the area of the Białowieska Primeval Forest (BPF) or the Mazurian Landscape Reserve (MLR). These areas are also extensively used for recreational purposes (by sailors, hunters, tourists) with thousands of visitors from Poland and abroad each year. The aim of our study was to determine the distribution

Table 1. Prevalence and abundance of *Cryptosporidium* spp. and *Giardia* spp. among examined groups of animals. N – number of samples (for European beavers the number of animals from which the samples were taken is shown in the brackets)

Group/Species	Year	Season	Wild (w) or Farmed (f)	N	*Cryptosporidium* spp.		*Giardia* spp.	
					Prevalence (% infected)	GM oocysts/ml (95% CL)	Prevalence (% infected)	GM cysts/ml (95% CL)
Protected species:								
European beaver	2003	summer	f	10 (20)				
Castor fiber		autumn	f	10 (20)	10.0	1.9 (0.9 4.1)	10.0	1.9 (0.9 3.8)
	2005	spring	f	10 (20)				
	2003	autumn	w	22	31.8	9.0 (2.0 40.1)	4.5	1.6 (0.6 3.9)
	Total			52 (82)	19.2	3.7 (1.7 8.0)	7.7	1.7 (1.0 3.0)
European bison	2003/04	winter	w	34				
Bison bonasus	2004/05	winter	w	11				
	2005	spring	w	10				
	Total			55	29.1	5.9 (2.8 12.7)	7.5	2.0 (0.9 4.6)
Grey wolf *Canis lupus*	2003	winter	w	14	37.5*	nd	nd	Nd
Polish Konik	2005	spring	w	5				
Equus caballus	2005	spring	f	5				
	Total			10	0	-	0	-
Game species:								
Red deer	2003	spring	w	25				
Cervus elaphus		summer	w	17	26.9	6.3 (2.7 15.0)	1.9	1.1 (0.9 1.4)
		autumn	w	10				
	2003	summer	f	30				
		autumn	f	16	4.5	1.3 (1.1 2.5)	1.5	1.1 (0.9 1.4)
	2005	spring	f	20				
	Total			118	14.4	3.0 (1.9 4.7)	1.7	1.1 (1.0 1.3)
Roe deer	2003	spring	w	19				
Capreolus capreolus		summer	w	1				
		autumn	w	2				
	Total			22	9.1	1.8 (0.8 4.3)	4.5	1.3 (0.8 2.3)
Boar	2003	spring	w	2				
Sus scrofa		summer	w	1				
		autumn	w	2				
	Total			5	0	-	0	-

GM - geometric mean, CL - confidence limits, nd - not done, * results due to nested PCR.

of two intestinal protozoa in few species of protected and game mammals, and to determine the species/genotypes of *Cryptosporidium* infecting these hosts. Additionally, we wanted to compare prevalence and abundance of these parasites between wild and farm groups of animals.

MATERIALS AND METHODS

Sampling. Fecal samples were derived from farm animals (European beaver, Polish Konik, red deer) living in the Research Station for Ecological Agriculture and Preservation of Native Breeds, Polish Academy of Sciences (PAN), in Popielno, MLR, Poland. Time and number of samples are presented in Table 1.

Samples from wild beavers were collected from individuals live-trapped for introduction purposes in the area of Suwałki, NE Poland.

Fecal material from European bison was taken from the colon from individuals killed during selective shootings in winter 2002/03 in BPF. Fecal samples from European wolves were collected during tracing in winter 2003/04 in BPF. Samples from wild red and roe deer and boars were collected during three tracing sessions in the Mazury lake district and once in BPF (Tab. 1).

Laboratory analysis. Detection of *Cryptosporidium* spp. and *Giardia* spp. infections was carried out using immunofluorescent assay (IFA) MerIFluor *Cryptosporidium*/*Giardia* (Meridian Diagnostics, Cincinnati, Ohio, USA) on samples condensed by the Sheather's flotation technique as described previously [4]. Modified Ziehl-Neelsen staining of faecal smears was additionally used for detection of *Cryptosporidium* spp. oocysts [18]. For all *Cryptosporidium*-positive samples and for all wolf samples nested PCR on a fragment of COWP gene were performed [30, 34].

Statistical analysis. Prevalence, abundance of *Cryptosporidium* spp. and *Giardia* spp. and distribution of species richness were analyzed as previously described [4]. Mean parasite species richness for the host species was calculated as an arythmetic mean with standard error.

Table 2. Mean species richness of parasites in examined host species.

Host species		Mean species richness ± SE
European beaver	farm	0.20 ± 0.22
	wild	0.36 ± 0.17
	total	0.27 ± 0.07
Red deer	farm	0.06 ± 0.04
	wild	0.29 ± 0.06
	total	0.16 ± 0.04
Roe deer		0.14 ± 0.07
European bison		0.27 ± 0.08

Molecular analysis. DNA isolation and purification were carried out using Stool Genomic Mini AX Stool Kit for fecal samples (A & A Biotechnology, Gdynia, Poland). Purified DNAs were stored at -20°C until further use.

Amplification of an N-terminal fragment of the *Cryptosporidium* oocyst wall protein (COWP) gene was performed using a nested PCR protocol [30, 34]. Obtained products were sequenced in ABI-PRISM 377 (Applied Biosystems) in cooperation with the Institute of Biochemistry and Biophysics (IBB PAN, Warsaw). Obtained sequences were compared with sequences deposited in the GenBank data base.

RESULTS

Measures of infracommunity structure. Mean species richness. An almost five times higher mean species richness was found in wild deer in comparison to farm ones, while there was no such difference between wild and farmed beavers. Mean species richness was the lowest among roe deer, and was similar (range 0.16-0.27) for the other studied species (Tab. 2).

Measures of infracommunity structure. Distribution of species richness. For all examined groups of hosts, the majority (>65%) of samples were free of both intestinal parasites (Fig. 1). Infections of one parasite species were more prevalent than co-infection with both parasites. However, among European beaver samples, a higher percentage of

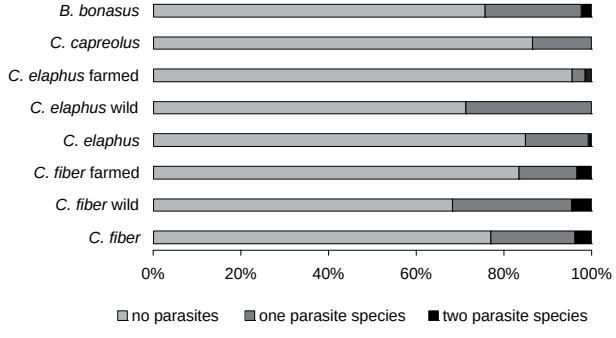

Figure 1. Comparison of distributions of species richness in particular host species.

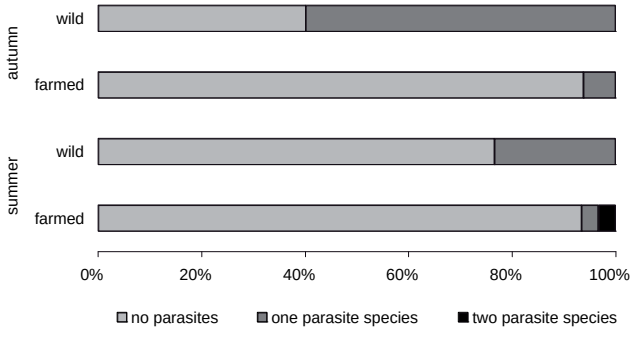

Figure 2. Comparison of distribution of species richness in samples from wild and farmed red deer depending on season.

co-infections was noted in samples from wild individuals (Fig. 1). No co-infection was detected in samples from roe deer. Co-infection with *Cryptosporidium* spp. and *Giardia* spp. was detected in 2.4% of European bison samples and in less than 1% of red deer samples. For the latter host species, statistical analysis revealed the influence of interaction between season of study and the habitat on the prevalence of co-infections (Fig. 2). Majority (60%) of samples collected from wild deer during autumn contained cysts or oocysts, and the prevalence of single infection was in both seasons higher in samples from wild individuals in comparison to farm ones (distribution of species richness x wild/farmed x season, $F_{1,68}$=3.79, 0.1>p>0.05, Fig. 2). However, the only example of co-infection was found in the sample from a farm animal.

***Cryptosporidium* spp.** Summary prevalence and abundance of *Cryptosporidium* spp. are provided in Table 1. *Cryptosporidium* spp. infections were detected in 5 of 7 studied animal species. No oocysts were found in samples from boar and Polish Konik. Overall prevalence of *Cryptosporidium* spp. was high (range 19-38%) in protected species (bison, beaver and wolf). Due to nested PCR results, the highest prevalence of *Cryptosporidium* spp. was found in grey wolves (35.7%). Because of the method, abundance was not calculated for this species. For five wolf isolates of *Cryptosporidium*, the 550 bp products of nested PCR were sequenced. All isolates demonstrated 100% homology with *C. parvum* genotype 2 (bovine) sequence (AF266273).

Overall prevalence of *Cryptosporidium* spp. in European beaver was 19.2%. For wild beavers prevalence was three times higher than for farm ones, but this difference was not statistically significant. The same trend was demonstrated in abundance, which was 4.5 times higher in wild beavers, but again the difference was not significant. However, the overall mean number of excreted oocysts was low (Tab. 1) and no positive results of nested PCR were obtained.

Both the prevalence and abundance of *Cryptosporidium* spp. were high in European bison (Tab. 1). Despite the high geometric mean number (GM) of excreted oocysts in bison samples, again, both the DNA extraction and amplification were unsuccessful for these samples.

Overall prevalence of *Cryptosporidium* spp. was lower in samples collected from game animals (9% in roe deer, 14% in red deer). However, the prevalence exceeded 27% in samples collected from wild red deer, in comparison to 4.5% in farm one (χ^2_1= 10.62, p=0.0011). A similar effect of the place of living was observed in abundance. Geometric mean no. of oocysts was 5 times higher in samples collected from wild deer in comparison to farm ones ($F_{1,117}$=6.88, 0.025>p>0.01) (Tab. 1). The season did not affect the prevalence and abundance of *Cryptosporidium* infections. Once again, the concentration of oocysts was too low for successful DNA extraction and amplification.

The prevalence and abundance of *Cryptosporidium* spp. were slightly lower in roe deer than in red deer (Tab. 1), and no further molecular analysis of these isolates was conducted.

***Giardia* spp.** The summary prevalence and abundance of *Giardia* spp. are displayed in Table 1. Again, no cysts were found in samples from boar and Polish Konik. Grey wolves' samples were not diagnosed for this parasite. Overall prevalence of *Giardia* spp. was higher in protected species than in game animals (Tab. 1). The highest prevalence (7.7%) and high abundance were detected in European beaver samples. Both were higher in farm animals than in wild ones (10%, 1.9 cysts/ml and 4.5%, 1.6 cysts/ml, respectively).

In European bison, the prevalence of *Giardia* spp. was also high (7.5%), and the geometric mean number of excreted cysts was the highest of all examined species (2 cysts/ml).

Overall prevalence of *Giardia* spp. was again lower in samples collected from game animals (4.5% in roe deer, 1.7% in red deer). Also, the abundance of this parasite was not high in red deer (GM = 1.3 cysts/ml). The lowest percentage of infected samples and the lowest number of excreted cysts were found in material collected from red deer (Tab. 1). The prevalence was slightly higher for wild animals, though the difference was not significant.

DISCUSSION

In our study, we have presented the comparison of distribution of intestinal protozoa among protected and game species, and among wild and farm individuals. Prevalence and abundance of both parasites were higher in protected animals (beavers, bison and wolves) than in game species-roe and red deer.

Cryptosporidium spp. infections were three times more common and more intense in wild beavers in comparison to farm ones, and up to five times more common and more intense in wild red deer in comparison to farm individuals. However, *Giardia* spp. infections were more prevalent in farm beavers in comparison to wild ones, and no differences were found in the distribution of this parasite between wild and farm red deer.

No parasites were detected in Polish Konik and boar samples, which may reflect rather the small number of studied samples (Tab. 1) than the real distribution of these protozoa.

The highest prevalence of *Cryptosporidium* spp. was detected due to nested PCR in wolf samples, which confirms our earlier results [20]. Identified genotype – *C. parvum* bovine – is one of the most relevant in epidemiology of human cryptosporidiosis [25, 26]. High distribution of *C. parvum* in wolves together with known high mobility of these hosts and increasing number of wolves in Poland constitute the important reservoir of this parasite and potential human health risk factor. To our knowledge, this is the first study confirming the *C. parvum* infection in grey wolf.

Both parasites were also detected for the first time in European bison, which is still a very rare species in Europe. Poland remains the only country in which the population of European bison exceeds 800 animals [21, 36]. Although genotyping of *Cryptosporidium* in samples from bison was unsuccessful, the dimensions of oocysts (Ø 4-5.5 µm) suggested *C. parvum* infection. Prevalence close to 30% is similar to that found in 1-2 year old cattle [12]. Because the European bison is still a very rare species, living and breeding in closely related groups in well protected areas, the importance of this host species as a reservoir of parasites is much lower, but infections may constitute a health risk factor for bison calves. In cattle, *Cryptosporidium* and *Giardia* infections are responsible for severe diarrhea and weight losses in calves [1, 14].

For years, semi-aquatic rodents, involving beavers, are believed to be an important source of water contamination with protozoan cysts/oocysts [22]. However, there are only a few reports on *Cryptosporidium* and *Giardia* infections in beavers conducted in USA and Canada [10, 13, 29]. European beaver is still a very rare species and its distribution in Europe is restricted to East and North regions (Poland, Sweden, Finland, Norway, Ukraine, Russia and other countries of Eastern Europe) and France and Germany [17]. However, in Poland, beaver populations are constantly increasing and the state of this species may change to game species in the next few years. Our study was conducted on samples collected from a beaver farm and captured wild beavers, both serving as sources of individuals for re- and introduction purposes. Intestinal parasites were detected in both sets of samples, suggesting the possibility of environment contamination with beavers' translocation. Overall prevalence of *Cryptosporidium* and *Giardia* infections in beavers (close to 19% and 8%, respectively) in this study is much higher than in our previous study performed on similar material (4.5% for *Cryptosporidium*, 0% for *Giardia*, [3]) suggesting the increasing role of this species as a reservoir host. Other studies in nuisance beaver *Castor canadiensis* reported on *Giardia* prevalence in the range of 9-33% [9, 10, 13, 29]. However, to date, other species of semi-aquatic rodents, i.e. muskrat *Ondatra zibethicus* or nutria *Myocastor coypus*, are believed to constitute more important reservoir of both pathogens with much higher prevalence (up to 100% for *Cryptosporidium* [30] and 37-96% for *Giardia* [3, 9, 13,19]).

In our study an overall prevalence of *Cryptosporidium* spp. was higher in wild red deer than in roe deer (27% and 9%, respectively), which is similar to other researchers' results (red deer: 20% in [7]; roe deer: 6% in [16]). Lower distribution was determined for *Giardia* in both species of deer (2 and 4.5%) which is in agreement with other studies. *Giardia* cysts were detected only in 1.7% to 7.5% of samples of red deer [7, 16, 29] and in 16% of roe deer [16]. Reviewing other data on different species of Cervidae, overall prevalence of both intestinal protozoa very rarely exceeds 10% [7, 16, 31, 33, 35] but the world-wide distribution of these game species supports their role as a reservoir host for these human pathogens. Although in our study the genotyping of *Cryptosporidium* isolates from red deer was unsuccessful, the other study determined that species involved in Cervidae infections is often zoonotic *C. parvum* [5].

We observed a higher level of *Cryptosporidium* infection in wild animals than in farm ones. This may be a result of regular prophylactic treatment and controlled quality of water and food maintained for captive deer and beavers. On the other hand, the high density of animals in the area of the farm could facilitate parasite transmission, and would be a factor which enables possible mass infections.

Our study constitute the first attempt at determining the distribution of two intestinal protozoa in a few species of rare, protected species, resulting in the first record of *C. parvum* (bovine genotype) in grey wolf, the first finding of *Cryptosporidium* spp. and *Giardia* spp. in European bison, and the first record of *Giardia* spp. in European beaver. Two common Cervidae species were confirmed as the reservoir hosts for these parasites in Poland.

Acknowledgements

The study was supported by the Ministry of Science and Higher Education (former KBN), Grant No. 2PO4C09827. We thank Jan Goździewski, MSc, Polish Hunting Association, Suwałki, for his assistance in the collection of beaver samples; Agnieszka Kloch, MSc, Department of Zoopsychology and Animal Ethology, Institute of Environmental Sciences, Faculty of Biology and Earth Sciences, Jagiellonian University of Krakow, for providing wolves' samples and Prof. Zygmunt Giżejewski, Research Station for Ecological Agriculture and Preservation of Native Breeds, Polish Academy of Sciences, Popielno, for his help with collecting samples from farm red deer.

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