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Small rodents as reservoirs of *Cryptosporidium* spp. and *Giardia* spp. in south-western Poland

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Abstract

Cryptosporidium spp. and *Giardia* spp. have been detected in a range of host species, including rodents. The aim of this study was to determine the distribution of these pathogens and recognition of the reservoir role of rodents in the maintenance of these pathogens in south-western Poland. Additionally, preliminary molecular studies were conducted to elucidate the species and genotypes of *Cryptosporidium* and *Giardia* identified in this study. Stool samples (n=266) from *A. agrarius*, *A. flavicollis* and *M. glareolus*, were subjected for analyses. Values of prevalence were 61.7, 68.3 and 68.1%, respectively, for *Cryptosporidium* spp. and 41.7, 24.4 and 38.4%, respectively, for *Giardia* spp. There was a statistically significant correlation between host species and *Giardia* infection where *A. agrarius* was the species of the highest prevalence. Statistically significant differences were not found for comparisons made for study sites and occurrence of *Giardia* spp. and *Cryptosporidium* spp. Due to preliminary nested PCR results, specific amplifications of *Cryptosporidium* COWP and SSU rRNA genes were obtained for several isolates taken from rodent host species. One isolate recovered from *A. agrarius* (from a semi-aquatic, urban area) was identified as *C. parvum* and revealed 100% similarity with sequences obtained from humans. To the best of the knowledge of the authors, this is the first record of the *C. parvum* zoonotic species from the striped field mouse. Also recorded were the first findings of *C. ubiquitum* from three small rodent species.

Key words

Cryptosporidium, Giardia, rodents, *Apodemus agrarius*

INTRODUCTION

Cryptosporidium and *Giardia* are two genera of parasitic protozoa that can infect the intestinal tracts of humans and a wide range of animals, including livestock, companion animals and wildlife [1]. Of these potential sources of pathogens, wildlife has received the least attention and the risk posed by these populations to public health is not fully understood. Among the range of known hosts, rodents are considered to be a significant risk factor for public health, acting as a reservoir of pathogens in the environment, i.e. contributing to food, water and soil contamination.

Apart from the potential risk of infection, epidemiologic studies regarding these pathogens in wild rodents are not common because of their low economic importance and the difficulty in carrying out surveys. Studies on small rodents have detected *Cryptosporidium* and *Giardia* infections in several species worldwide [2, 3, 4, 5, 6, 7, 8]. In Poland, a few epidemiologic studies have demonstrated the presence of *Cryptosporidium* and *Giardia* in wild living rodents, i.e. from the area of the Mazurian Lake District [9, 10] and Wielkopolska Region [11]. However, only some of them presented molecular characterization of the various *Giardia* and *Cryptosporidium* species and genotypes [8, 12, 13]. Additionally, the cervine genotype, later called *Cryptosporidium ubiquitum*, a new geographically widespread parasite, was molecularly described from wild and domesticated ruminants, rodents, carnivores and primates, including humans. However, it has still not been determined if the transmission is zoonotic or anthroponotic [14].

The role of small rodents as the zoonotic reservoir of *Cryptosporidium* and *Giardia* spp. has not been examined so far in the area of south-western Poland (Lower Silesian Region), especially in the context of different types of the regions, such as urban, suburban and rural, representing also recreation grounds and nature reserves.

The aim of the presented study was determination of the distribution of *Cryptosporidium* spp. and *Giardia* spp., as well as recognition of the role of reservoir rodent hosts in the maintenance of these pathogens in urban and rural areas. Additionally, preliminary molecular studies were conducted to elucidate the precise species or genotypes of protozoa identified in this survey, emphasizing their zoonotic potential. Moreover, the role of *Apodemus agrarius* as reservoir host of pathogens has been recorded and needs to be highlighted.

MATERIALS AND METHODS

Study areas and collection of rodents. Surveys were performed in four localities of south-western Poland, which comprised varied environments: urban, suburban – water distribution area (W1) and irrigation fields (W2) for the Wrocław agglomeration, a rural ornithological reserve – the Milicz Ponds Reserve (M), and recreation grounds surrounding the Ślęża Landscape Park (S).

Rodents. 266 individuals represented by striped field mouse *Apodemus agrarius* (n=115), yellow-necked mouse *A. flavicollis* (n=82) and bank vole *Myodes glareolus* (n=69), were captured

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in Sherman live traps during 2009–2011. After identification of species and gender, the trapped rodents were euthanized and subjected to parasitological dissection. In the laboratory, individual faecal samples were collected from the colons, and then used for microscopic identification and molecular studies.

All animal procedures were approved by Local Ethical Committee (No 46/2008).

Faecal analysis. Faecal pellets were used to prepare smears from each of specimen in duplicate by mixing a small amount of stools with 0.8% NaCl. For detection of *Giardia* cysts a drop of Lugol's iodine was added to the first set of slides, following examination under ×60 microscope objective magnification. For demonstration of *Cryptosporidium* oocysts, the smears were stained according to a modified Ziehl-Neelsen technique [15], and examined using the oil-immersion objective, i.e. ×100. Oocysts were identified on the basis of their size (4–5 × 3.5–4.5 µm), general morphology and bright pink colour. The immunofluorescent assay MerIFluor Cryptosporidium/Giardia (Meridian Diagnostics, Cincinnati, OH, USA) was used according to the manufacturer's instruction for the confirmation of parasites' presence.

Additionally preliminary molecular studies were conducted on several randomly selected positive samples to elucidate the species and genotypes of examined pathogens.

DNA extraction. DNA was isolated from faecal samples collected in 2010 and 2011 using Gene Matrix Stool DNA Purification Kit (Eurx) according to the manufacturer's instruction. DNAs were stored at -20°C until further use.

Gene amplification and sequencing. Amplification of a 826-bp region of the *Cryptosporidium* SSU rRNA gene [16] and 553-bp of the COWP gene [17] were carried out by nested PCRs.

For the molecular typing of *Giardia* spp., a PCR was performed to amplify a 292-bp region of the *Giardia* SSU rRNA gene [18]. For all PCR reactions, negative and positive controls were performed with sterile water and reference DNA, respectively.

PCR products were subjected to electrophoresis on a 1.0% agarose gel and stained with ethidium bromide. Positive products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced on Applied Biosystems ABI PRISM 3100-Avant Sequencer (Genomed, Warsaw, Poland). In order to elucidate any homologies with previously deposited sequences in the GenBank, a BLAST search (www.ncbi.nih.gov/BLAST/) was carried out. The multiple alignment was performed with the use of CLUSTAL W using MEGA 5.1 package. Phylogenetic analysis of *Cryptosporidium* was inferred using the Maximum Likelihood method. Obtained sequences were deposited in the GenBank under Accession Nos. KC962124 and KC917325.

Statistical analysis. The use of descriptive ecological terms followed Bush et al. [19], and the prevalence of infection for particular host species calculated. The differences between prevalence of infection with regard to the study sites, species and gender of the rodents were tested by the chi-square test (STATISTICA® 8).

RESULTS

Stool samples (n=266) derived from three rodent species were analysed. The overall protozoan prevalence in the studied areas was 75.9% (202/266). Values of prevalence for *Cryptosporidium* spp. ranged from 61.7 – 68.3% and for *Giardia* spp. 24.4 – 41.7%. Co-occurrence of the two studied species in hosts was shown in 64 individuals (31.7%).

The prevalence of parasites was determined according to the study sites and capturing season, as well as species and gender of the hosts. No statistically significant differences were found for comparisons made for study sites and prevalences of *Giardia* spp. (χ^2=6.26; df=3; p=0.09) and *Cryptosporidium* spp. (χ^2=4.07; df=3; p=0.25). The occurrence of both parasites was observed most commonly in the urban and suburban areas of the Wrocław agglomeration (W1 and W2) (Fig. 1a).

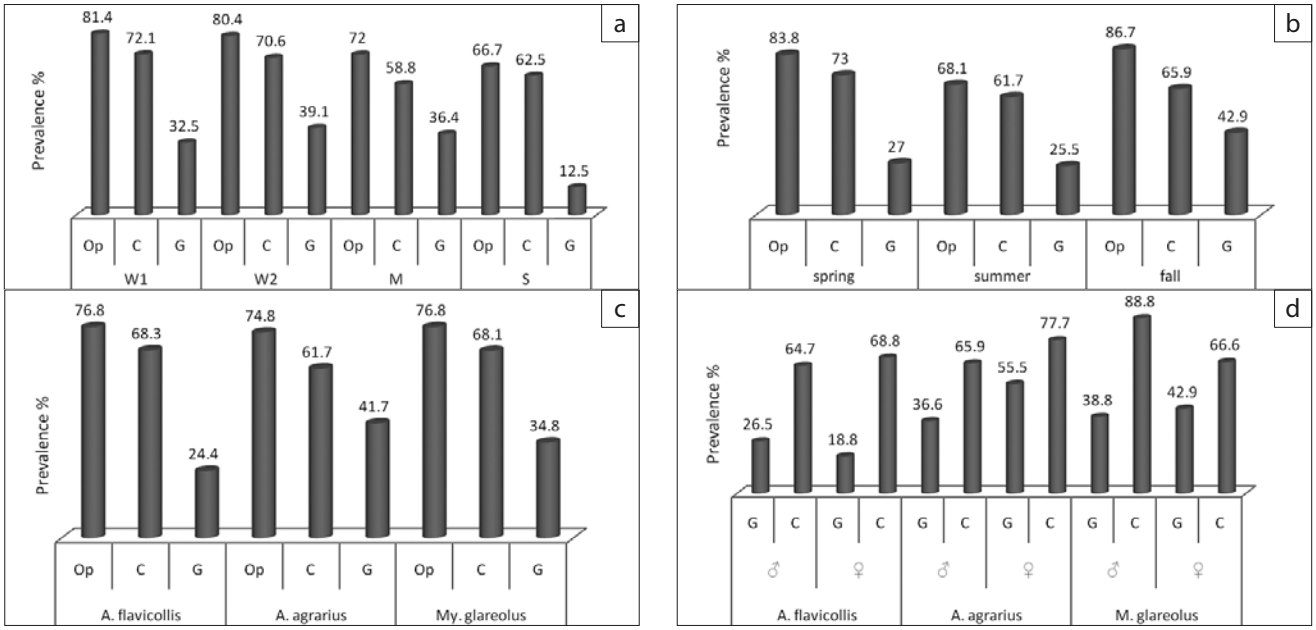

Figure 1. Prevalences of infection with *Cryptosporidium* (C) and *Giardia* spp. (G) obtained with regard to different factors: study sites (a), season (b), host species (c) and gender of the host (d). W1 – water distribution area; W2 – irrigation fields for the Wrocław agglomeration; M – ornithological reserve; S – surroundings of the Ślęża Landscape Park; Op – overall prevalence

With regard to the occurrence of *Giardia* spp. between seasons, it was described as significant (χ^2= 8.53; df=2; p=0.01). The highest prevalence was noted in autumn (42.9%). On the other hand, there were no significant differences in the occurrence of *Cryptosporidium* spp. between the seasons (χ^2=1.52; df=2; p=0.48) (Fig. 1b).

The relationship between *Giardia/Cryptosporidium* occurrence and host species was also tested. There was a statistically significant relationship between host species and *Giardia* infection, with the highest prevalence in *A. agrarius* (41.7%) and the lowest in *A. flavicollis* (24.4%). No statistically significant difference was found for *Cryptosporidium* infection and hosts species (χ^2=1.21; df=2; p=0.55) (Fig. 1c).

The relationship between *Giardia/Cryptosporidium* occurrence and gender of the host species was also examined. There was a statistically significant difference between the gender of *M. glareolus* and *Cryptosporidium* spp. infection, where the parasite occurred more often among males (88.8%) (Fig. 1d).

Due to preliminary nested PCR results, specific amplifications of *Cryptosporidium* COWP and SSU rRNA genes were obtained for several isolates taken from rodent hosts. The obtained sequences were compared with those available in the GenBank. Regarding the COWP genes, the sequence of one isolate from *Apodemus agrarius* (urban area W2) (GenBank Accession No. KC917325) was identical (100%) with the sequences of *Cryptosporidium parvum* obtained previously from humans (Fig. 2). Also, five sequences showed 94–96% similarity with *Cryptosporidium ubiquitum* (previously known as cervine, cervid, W4 genotypes or genotype 3). Two sequences of SSU rRNA gene fragment also revealed homology with *Cryptosporidium ubiquitum* (99%) (GenBank Accession No. KC962124) (Fig. 3).

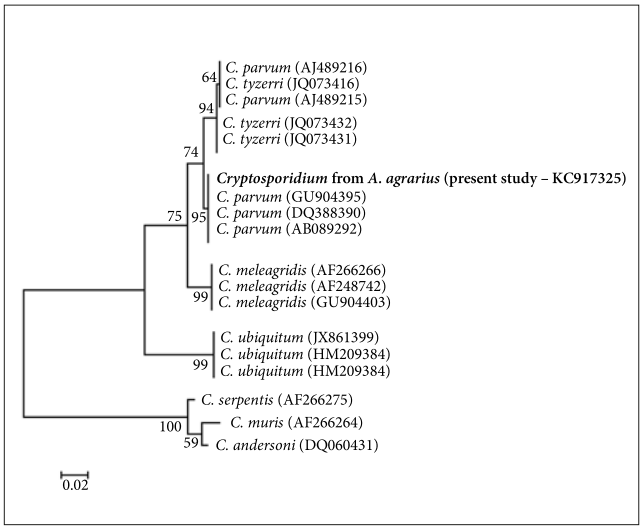

Figure 2. Phylogenetic relationship of *Cryptosporidium* obtained by maximum likelihood method of COWP gene (Tamura 3-parameter model with invariant sites). Sequence obtained in this study is shown in bold, Accession Nos. are shown in parentheses

Molecular typing of *Giardia* SSU rRNA gene revealed a similarity of the examined two isolates with *Giardia microti* (99%) and eight with *G. muris* (92%). The obtained sequences of both *Giardia* spp. were identical.

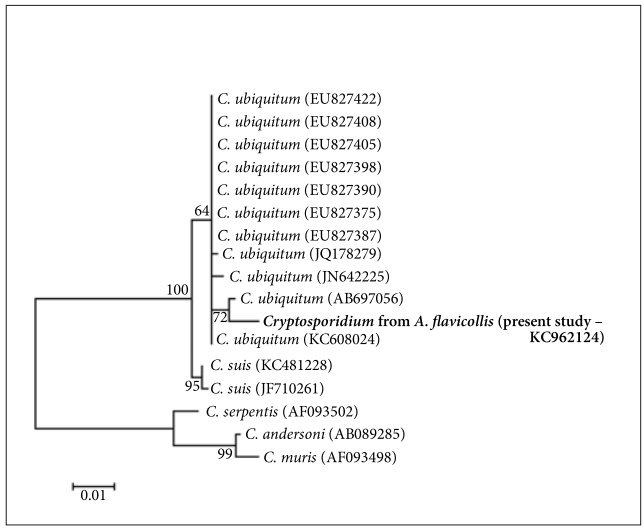

Figure 3. Phylogenetic relationship of *Cryptosporidium* obtained by maximum likelihood method of SSU rRNA (Tamura 3-parameters model with gamma distribution). Sequence obtained in this study is shown in bold, accession numbers are provided in parentheses

DISCUSSION

There have been few epidemiologic studies regarding *Giardia* and *Cryptosporidium* spp. in wild living small rodents because of their low economic importance and the difficulty in conducting studies.

In Poland, intestinal protozoa have been investigated and are known to be widely distributed in forest and fallow rodents in the Mazurian Lake District [9, 10]. The results of research by Sinski et al. [20] demonstrated the differences in *Cryptosporidium* spp. prevalence in rodents inhabiting three different habitats (forest, fallow and semi-aquatic). Bajer et al. [21] showed a higher prevalence of *Cryptosporidium* spp. in voles *M. glareolus* and *Microtus arvalis* in forested areas than in mice *A. flavicollis*. The same pattern was observed for *Giardia* infections [9]. Torres et al. [4] stated that the trapping sites and vegetation found in these areas affect the prevalence of the parasites. In the presented research, the study sites did not affect the prevalences of examined pathogens; however, the highest rate of infection was observed in rodents from the semi-aquatic sites (W1 and W2) representing urban and suburban areas within city agglomeration. Additionally, based on the environmental studies, a correlation was found between *Giardia* infection and the year and season of trapping rodents. It was pointed out that *Giardia* spp. occurs more frequently in spring and autumn, which is in agreement with the presented results, when the temperature and humidity affect the survival of protozoan cysts in the environment. Extrinsic factors, such as site and season of trapping rodents, influenced more the prevalence of infection with protozoa than intrinsic factors [9].

Giardia spp. and *Cryptosporidium* spp. were detected in a range of rodent host species from Poland, i.e. from the area of the Mazurian Lake District where the *Cryptosporidium* prevalence was as high as 54–71% for the bank voles (*M. glareolus*), 62–73% for the common voles (*M. arvalis*), and 28% for the yellow-necked mice (*A. flavicollis*). The prevalences of *Giardia* were reported to be higher for the examined species of rodents: 58–94%, 74–96% and 24–48%, respectively [9, 10]. *Giardia* prevalence from the

Wielkopolska Region was significantly lower, i.e. 5.4% for *A. agrarius* and 10.3% for *M. glareolus* [22]. In the presented study, a significant occurrence was noted of *Giardia* spp. in the examined hosts – 41.7% and 34.8%, respectively, and the lowest rate of infection obtained for *A. Flavicollis,* This is in agreement with other studies which stated that this species is the least important reservoir for *Giardia* spp. [9].

Intrinsic factors, i.e. host gender and age seem to have less influence on the rate of infection. Earlier research [3, 10] showed that the prevalence of *Cryptosporidium* did not differ significantly between the genders. However, Torres et al. [4] reported a higher prevalence of infection in female individuals of wild rodents. Bajer [10] noted the varied influence of gender on infection with *Giardia* spp. in *A. flavicollis* from different areas of the Mazurian Lake District. e.g., in males the prevalence was 16.7% and in females 7.6% from Urwitalt village, whereas in analyzing *A. flavicollis* trapped from Lake Talty, infection with *Giardia* spp. was recorded only in females (16%). In *M. arvalis* trapped in the vicinity of Urwitalt, almost the same rate of protozoan infection was reported in females (73.8%) and males (76.6%). In the presented study, in most cases, no relationships were noted between the gender and species of the host, and the prevalence of protozoans. The only significant correlation was recorded for infection of *Cryptosporidium* spp. and gender of *M. glareolus*.

In Poland, there have been few studies using molecular diagnostics of the discussed pathogens in wild-living small rodents. The presented results show a molecular diversity among the isolates recovered from the three host species. One isolate recovered from *A. agrarius* (from a semi-aquatic, urban area) was identified as *C. parvum,* which revealed 100% similarity with sequences obtained from humans (AB089292, DQ388390, GU904395). To the best of the knowledge of the authors, this is the first recorded instance of that zoonotic species from striped field mouse. The identification of zoonotic isolates of *Cryptosporidium* has been previously reported from bank voles (AJ489215), common voles (AJ489216) and yellow-necked mice (AJ489217) as *C. parvum* mouse genotype I from the area of north eastern Poland [12]. The similarity with the current sequence was 99% with an 8-nucleotide difference. Also in Poland, *C. muris* infections were detected in *A. agrarius* and *M. arvalis* the from Wielkopolska Region [11]. Moreover, among the small rodents in Poland, *C. parvum* was detected in wood mouse (*Apodemus sylvaticus*) [5], house mouse [7], white-footed mouse (*Peromyscus leucopus*), deer mouse (*Peromyscus maniculatus*) and red-backed vole (*Clethrionomys gapperi*) [7].

C. ubiquitum has been reported in the faeces of humans and wild and domesticated ruminants [23]. *C. ubiquitum* has also been found in faeces from a variety of wild rodents, including squirrels, chipmunks, woodchucks, beavers, deer mice and raccoons [6, 7, 14]. Additionally, molecular analysis has detected *C. ubiquitum* in the large Japanese field mouse (*Apodemus speciosus*) [23]. The similarity of that sequence (AB697056) with the presented study was 99%. *C. ubiquitum* were identified in stool isolates derived from all three examined species of rodents, and to the best of the authors' knowledge, this is the first finding of that pathogen in yellow-necked mouse, striped field mouse and bank vole.

C. ubiquitum has been found in humans, especially in children and immunosuppressed patients, mainly in developed and industrialized countries, i.e. the Unites States, United Kingdom, New Zealand, Canada and Slovenia [14]. A recently released publication of Cieloszyk et al. [24] reports on *C. ubiquitum* (JN642225) in an immunocompetent child from Spain, supporting the zoonotic transmission of the parasite. It is suggested that *C. ubiquitum* could emerge as an important human pathogen, but to date in Poland it has been identified only occasionally in humans with no record on this parasite. The lack of host specificity of *C. ubiquitum* and habitat-sharing of its hosts serving as pathogen reservoirs, probably contribute to its widespread distribution, being potentially hazardous for animal and human health alike [14].

Molecular studies have shown that *Giardia* zoonotic species and genotypes are present in rodent populations in Poland, i.e. *G. intestinalis* Assemblage A [10]. In the presented study, pathogens specific for rodents, i.e. *G. muris* and *G. microti,* were detected.

Due to the limited geographical extension, the striped field mouse has been the subject of very few studies. *A. agrarius*, the most numerous species of the study, prefers moist habitats and lives near streams, in forests, agricultural land and meadows. It tends to over-winter near human settlements, in haystacks, storehouses and dwellings [25]. In recent decades, its marked spread into new areas has been observed in many central European countries. Regarding the high density of *A. agrarius* with detected infections of both pathogens, it is supposed that this species of rodent may have an important role in the circulation of some potentially zoonotic *Cryptosporidium* and *Giardia* spp. in south-west Poland.

Due to preliminary type of study, the number of sequences obtained was limited, and additional precise molecular information on the parasite species/genotypes of zoonotic importance among wild rodents is necessary to address this issue further.

REFERENCES

1. Xiao L, Fayer R. Molecular characterization of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. Int J Parasitol. 2008; 38: 1239–1255.
2. Pacha RE, Clark GW, Williams EA, Carter AM, Scheffelmaier JJ, Debusschere P. Small rodents and other mammals associated with mountain meadows as reservoirs of *Giardia* spp. and *Campylobacter* spp. Appl Environ Microbiol. 1987; 53: 1574–1579.
3. Chalmers RM, Sturdee AP, Bull SA, Miller A, Wright SE. The prevalence of *Cryptosporidium parvum* and *C. muris* in *Mus domesticus, Apodemus sylvaticus* and *Clethrionomys glareolus* in an agricultural system. Parasitol Res. 1997; 83: 478–482.
4. Torres J, Gracenea M, Gomez MS, Arriza-Balaga A, Gonzalez-Moreno A. The occurrence of *Cryptosporidium parvum* and *C. muris* in wild rodents and insectivores in Spain. Vet Parasitol. 2000; 92: 253–260.
5. Hajdusek O, Ditrich O, Slapeta J. Molecular identification of *Cryptosporidium spp.* in animal and human hosts from the Czech Republic. Vet Parasitol. 2004; 122: 183–192.
6. Feng Y, Alderisio K, Yang W, Blancero LA, KuhneWG, Nadareski CA, Reid M, Lihua Xiao L. *Cryptosporidium* genotypes in wildlife from a New York watershed. Appl Environ Microb. 2007; 6475–6483.
7. Ziegler PE, Wade SE, Schaaf SL, ChangYF, Mohammed HO. *Cryptosporidium* spp. from small mammals in the New York City watershed. J Wild Dis. 2007; 43: 586–596.
8. Lv C, Zhang L, Wang R, Jian F, Zhang S, Ning C, Wang H, Feng C, Wang X, Ren X, Qi M, Xiao L. *Cryptosporidium* spp. in wild, laboratory, and pet rodents in China: prevalence and molecular characterization. Appl Environ Microbiol. 2009; 75: 7692–7699.
9. Bajer A, Bednarska M, Pawełczyk A, Behnke JM, Gilbert FS, Siński E. Prevalence and abundance of *Cryptosporidium parvum* and *Giardia* spp. in wild rural rodents from Mazury Lake District region of Poland. Parasitology 2002; 125: 21–34.

10. Bajer A. Between-year variation and spatial dynamics of *Cryptosporidium* spp. and *Giardia* spp. infections in naturally infected rodent populations. Parasitology 2008; 135: 1629–1649.
11. Solarczyk P, Majewska AC. Molecular identification of *Giardia* spp. and *Cryptosporidium* spp. in humans and animals. Wiad Parazytol. 2007; 53: 110.
12. Bajer A, Caccio S, Bednarska M, Behnke JM, Pieniazek J, Sinski E. Preliminary molecular characterization of *Cryptosporidium parvum* isolates of wildlife rodents from Poland. J Parasitol. 2003; 89: 1053–1055.
13. Cacciò SM, Beck R, Almeida A, Bajer A, Pozio A. Identification of *Giardia* species and *Giardia duodenalis* assemblages by sequence analysis of the 5.8S rDNA gene and internal transcribed spacers. Parasitology 2010; 137: 919–925.
14. Fayer R, Santin M, Macarisin D. *Cryptosporidium ubiquitum* n. sp. in animals and humans. Vet Parasitol. 2010; 172: 23–32.
15. Henriksen SA, Pohlenz JFL. Staining of cryptosporidia by a modified Ziehl – Neelsen technique. Acta Vet Scand. 1981; 22: 594–596.
16. Xiao L, Escalante L, Yang C, Sulaiman I, Escalante AA, Montali RJ, Fayer R, Lal AA. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl Environ Microb. 1999; 1578–1583.
17. Pedraza-Dıaz S, Amar C, Nichols GL, McLauchlin J. Nested polymerase chain reaction for amplification of the *Cryptosporidium* oocyst wall protein gene. Emerg Infect Dis. 2001; 7: 49–56.
18. Hopkins RM, Meloni BP, Groth DM, Wetherall JD, Reynoldson JA, Thompson RC. Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. J Parasitol. 1997; 83: 44–51.
19. Bush AO, Lafferty KD, Lotz JM, Shostak SW. Parasitology meets ecology on its own terms: Margolis etal. revisited. J Parasitol. 1997; 83: 575–583.
20. Siński E, Bednarska M, Bajer A. The role of wild rodents in ecology of cryptosporidiosis in Poland. Folia Parasit. 1998; 45: 173–174.
21. Bajer A, Bednarska M, Siński E. Środowiskowe uwarunkowania zarażeń *Cryptosporidium parvum* w populacjach drobnych gryzoni. Wiad Parazytol. 2001; 47: 747–753 (in Polish).
22. Majewska AC, Werner A, Słodkowicz A, Piłacińska B. Prevalence of intestinal protozoa parasites in wild rodents and insectivores captured in Wielkopolska region. Wiad Parazytol. 2001; 47 (supl.2): 29.
23. Murakoshi F, Fukuda Y, Matsubara R, Kato Y, Sato R, Sasaki T, Tada C, Nakai Y. Detection and genotyping of *Cryptosporidium* spp. in large Japanese field mice, *Apodemus speciosus*. http://dx.doi.org/10.1016/j.vetpar (access: 2013.02.011).
24. Cieloszyk J, Goni P, García A, Remacha MA, Sánchez E, Clavel A. Two cases of zoonotic cryptosporidiosis in Spain by the unusual species *Cryptosporidium ubiquitum* and *Cryptosporidium felis*. http://dx.doi.org/10.1016/j.eimc (access: 2012.04.11).
25. Stanko M. Bionomics and ecology of *Apodemus agrarius* (Pall.) (Rodentia: Muridae) on Vychodoslovenska Nizina lowland. II. Population structure and density. Biologia, Bratislava 1994; 49: 797–805.