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Enterobiasis epidemiology and molecular characterization of *Enterobius vermicularis* in healthy children in north-eastern Poland

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Summary

Enterobiasis is a human intestinal parasitic disease caused by human pinworm, *Enterobius vermicularis*. Despite being the most prevalent nematode infection in Europe and North America, predominantly among in school aged children, the data concerning infection rate and knowledge of genetic variability of pinworms are incomplete. The aim of the study was the estimation of prevalence and molecular typing of *Enterobius vermicularis* among healthy children in north-eastern Poland. In 2013 – 2015, 296 individuals (aged 2 – 18 years) from 12 kindergartens, schools and orphanages were examined by the adhesive cellophane tape method. Data on socio-demographic status were collected using a questionnaire. Molecular analysis was performed using the DNA of adult female pinworms and primers targeting the region of cytochrome oxidase I gene. The overall prevalence of enterobiasis was 10.1 %. *Enterobius vermicularis* infection rates were 3.9 % in children living in families and 32.8 % among the orphans (OR=0.08; 95 % CI: 0.04 – 0.19; p<0.001). There were no associations between distribution of enterobiasis and gender, pets possession and the season of examination. In 43.3 % of the infected children enterobiasis was asymptomatic. Based on a molecular marker three different haplotypes of pinworm were identified. All sequences clustered within type B, together with human *E. vermicularis* isolates from Denmark, Germany, Greece, and Japan. This paper provides complementary data on the occurrence and intraspecific variability of *E. vermicularis* in human population in Europe.

Keywords: *Enterobius vermicularis*; enterobiasis; children; genotyping; *cox1;* Poland

Introduction

The human pinworm, *E. vermicularis* (Nematoda) is a one of the most common human parasitic helminths. It is estimated that about 209 million people worldwide are infected, with children aged 5 – 10 accounting for over 30 % (Cook, 1994; Kucik *et al*., 2004). Enterobiasis is not associated with any particular socioeconomic level, race or culture, although it is facilitated by factors such as poor personal or group hygiene or overcrowding (in preschools, schools, orphanages, family groupings) (Cook, 1994; St Georgiev, 2001; Burkhart & Burkhart, 2005). These conditions favour transmission of pinworm eggs from person to person, directly via anus-to-mouth route, by finger contamination or indirectly by contaminated objects (toys, food) (Cook, 1994; Vermund & Wilson, 2000). Most *E. vermicularis* infections are asymptomatic or cause some indefinite symptoms such as perianal pruritus leading to local epidermis irritation and secondary bacterial infections. Other symptoms include abdominal discomfort, loss of appetite, weight loss, insomnia, restlessness, irritability (Vermund & Wilson, 2000; Burkhart & Burkhart, 2005). Occasionally pinworm infection may

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cause enuresis (Çulha & Duran, 2006; Otu-Bassey *et al.* 2011), urinary tract infections (Zahariou *et al.*, 2007; Ayala Castellanos Mde *et al.*, 2009) or appendicitis (Arca *et al.*, 2004; Ramezani & Dehghani, 2007; Panidis *et al.*, 2011).

Epidemiological studies from different European countries reveal that enterobiasis prevalence showed a declining trend at the end of the 20th century, although it has remained relatively common in children populations (Gauert, 1998; Gale, 2002; Bitkowska *et al.* 2004; Crotti & D'Annibale, 2006; Remm, 2006). Despite that fact, *E. vermicularis* genetic variability and its epidemiology of based on molecular typing have not been fully investigated (Iñiguez *et al,.* 2006; Piperaki *et al.*, 2011; Zelck *et al.,* 2011; Ferrero *et al.*, 2013). Complete data on the DNA sequence are only available in relation to the mitochondrial genome of *E. vermicularis* (Kang *et al.*, 2009). The analysis of the mitochondrial *cox1* gene sequences in pinworm isolates from humans and captive chimpanzees (*Pan troglodytes*) has shown the presence of three different genetic types, designated as type A, B and C (Nakano *et al.*, 2006). Type A has been identified in both species, whereas types B and C only in chimpanzees.

Considering lack of current data on enterobiasis rate and molecular typing of *E. vermicularis* in children in Poland, the aim of this study was to evaluate the prevalence of enterobiasis and investigate the occurrence of *E. vermicularis* genotypes in healthy children and adolescents in the city of Olsztyn, the capital of the Warmia-Masuria province (north-eastern Poland).

Materials and methods

Study populations

The study was conducted from April 2013 to December 2014 in children and adolescents from 5 kindergartens, 3 schools and 4 orphanages located in several districts of the city of Olsztyn, the capital of Warmia-Masuria province (north-eastern Poland), covering an area of 88.33 km^2, with a population of 174,675 (as of 2013). The children enrolled in this study were those whose parents or caregivers collected swabs correctly and delivered them for testing. Consequently, the study population comprised 296 individuals (232 children living in a family and 64 orphans). In the examined population there were 51 % boys and 49 % girls aged 2 – 18 years (mean 7.1). Individuals who underwent antihelminthic therapy within three months preceding the study were excluded from it.

Sample collection

Enterobiasis was examined by Graham's adhesive cellophane tape method (Garcia, 2007). From each child 2 – 3 perianal swabs (each on a separate slide) were obtained. The swabs were collected early in the morning over 3 consecutive days from each child by the parents or caregivers, or self-collected by older children according to the instructions attached to a sampling kit. Until delivered to the laboratory, swabs were handled as infectious material and stored at 4 °C. All perianal swabs were examined under the light microscope at 100× and 400× magnification. Samples containing at least one or more eggs of *E. vermicularis* were regarded as positive. A total of 873 perianal swabs were tested. Additionally, in some egg-positive children, enterobiasis was confirmed by detecting adult worms in stool samples collected after antihelminthic therapy.

Data on socio-demographic status (age, gender, residence, education, siblings) of the patients, symptoms suggestive of enterobiasis, the history of other parasitic infections and their treatment were collected using questionnaire. Participation in the study was voluntary, and the parents or caregivers of the patients gave written informed consent to their children's participation. Participants were guaranteed full confidentiality during data collection and processing.

Molecular analysis

DNA isolation

Molecular investigations were conducted on adult females of *E. vermicularis* obtained from fecal samples or identified in perianal swabs collected from the study population. Human pinworms from feces were washed in tap water and stored in 70 % ethanol at 4 °C. Directly before DNA extraction the human pinworms were washed in a physiological saline solution and mechanically homogenized. Females from positive slides were excised with a piece of tape to extract DNA. A universal kit for DNA isolation from biological traces, Sherlock AX (A&A Biotechnology, Poland) was used according to the manufacturer's instructions with modification according to Piperaki *et al.* (2011) Modification was used in the first step of DNA extraction from females identified on perianal swabs. DNA was eluted in 50 µl of TE buffer.

DNA amplification

For the PCR primers EVM1/EVM2 (Piperaki *et al.*, 2011) targeting the region of *E. vermicularis* mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene (accession no. EU281143) were used. Amplification reaction mixtures (25 µl) consisted of 1 x PCR buffer (A&A Biotechnology), 200 µM dNTPs (Sigma Aldrich, Poland), 0.2 µM of each primers (Sigma Aldrich), 1U *Taq* DNA polymerase (1U/µl) (A&A Biotechnology) and 1µl of sample DNA. Thermal cycling was carried out in iCycler MyiQ (Bio-Rad, Poland) as follows: an initial denaturation step at 94 °C for 5 min was followed by 45 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. Two negative controls were used. The first negative control contained DNA-free water and the second contained a template extracted from a microscopically negative cellophane tape sample. PCR products were separated by electrophoresis on 2 % agarose gel with ethidium bromide and visualized on GelDoc (Bio-Rad). The results were archived using Quantity One software (Bio-Rad). PCR products of positive samples were purified for sequencing using Clean Up purification kit (A&A Biotechnology) according to the protocol of the manufacturer.

Sequencing and phylogenetic analysis

Three PCR products (390 bp) from different *E. vermicularis* isolates were successfully sequenced (Macrogen Europe, the Netherlands). DNA sequencing reactions were carried out in both directions. The sequences were aligned and trimmed to the length of 333 bp (primers excluded) according to the previously published *cox1* alignments of *E. vermicularis* isolates (Nakano *et al.*, 2006; Piperaki *et al.*, 2011; Ferrero *et al.*, 2013) available in the GenBank database. Phylogenetic analysis was performed using the neighbour-joining method in a Geneious v.7.0 computer program (Biomatters). The topology of the phylogenetic tree was evaluated using the bootstrap method with 1000 replicates. The *cox1* sequence from *E. anthropopitheci* (AB254450) was included in the tree as the outgroup.

Statistical analysis

The data were analyzed using chi-square test and a significance test of differences between two independent proportions (*t*-test). The association between predictor variables was determined by odds ratio (OR) and their 95 % confidence interval (CI). The significance level ≤0.05 was adopted in all statistical tests. The tests were conducted using the software package SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL).

Results

Enterobiasis prevalence

The prevalence of *E. vermicularis* in the total study population was 10.1 % (30/296) (Table 1). The infection rate was 32.8 % (21/64) in the orphans and significantly lower in the group of children living in families 3.9 % (9/232) (OR=0.08; 95 %CI: 0.04 – 0.19; p<0.001). There were no statistically significant differences in the distribution of enterobiasis between males and females (Table 1). The overall infection rate in the groups was equal (50.0 vs. 50.0 %, p=0.876). In the infected children living in family enterobiasis was diagnosed in 66.6 % (6/9) girls and 33.3 % (3/9) boys. In the group of orphans the opposite trend was observed; infected males accounted for 57.1 % (12/21) and infected females constituted 42.9 % (9/21).

Significant differences were observed in terms of the average age of infected and uninfected groups only in children living in family. In this population the infected children (mean age 8.2 ± 2.64 years) were older than the uninfected (mean age 6.1 ± 2.3 years) (p<0.05). In the group of orphans the opposite age-related trend was observed (Fig. 1). In both examined groups the average age of the infected children was about 8 years old. The highest infection rate was noted in children from 7 to 9 years, 55.5 % (5/9) in children living in family and 28.6 % (6/21) among orphans respectively (Table 1).

No statistically significant relationship was observed between the presence of *E. vermicularis* infection and the number of siblings or roommates living in the same house (p=0.340) (Table 2). However, children or adolescents having siblings or roommates were more likely to be infected as compared to children living in small families (OR=1.69; 95 %CI: 0.57 – 5.06). Only 15.5 % of those examined were only children. None of the children in this group had enterobiasis. There were no statistically significant associations between the distribution of *E. vermicularis* infections and pet possession or the season of enterobiasis examination (Table 2).

In 43.3 % (13/30) of the infected children enterobiasis was asymptomatic. The parents or carers of the other infected children noticed irritability (20.0 %), abdominal pain (16.7 %), anal and perianal itching (10 %) and lack of appetite (10.0 %) (Table 3). These symptoms were also reported in similar proportion in children negative of enterobiasis.

Table 1. Comparison of the enterobiasis prevalence between study group according to the gender and age.

		Children in family (n=232)		Orphans (n=64)		Total (n=296)	
		No. of positive cases (%)	*p*-value*	No. of positive cases (%)	*p*-value*	No. of positive cases (%)	*p*-value*
Gender	Male	3 (33.3)	0.272	12 (57.1)	0.532	15 (50.0)	0.876
	Female	6 (66.7)		9 (42.9)		15 (50.0)	
Age	1.3	0 (0.0)	0.054	2 (9.5)	0.225	2 (6.7)	0.118
	4.6	2 (22.2)		5 (23.8)		7 (23.3)	
	7.9	5 (55.6)		6 (28.6)		11 (36.7)	
	10.12	1 (11.1)		3 (14.3)		4 (13.3)	
	13-15	1 (11.1)		2 (9.3)		3 (10.0)	
	>16	-		3 (14.3)		3 (10.0)	
Total		9 (3.9)[a]		21 (32.8)[b]		30 (10.1)	
	p-value*		<0.001				

*chi-square test; [a,b] – different letters mean significant differences

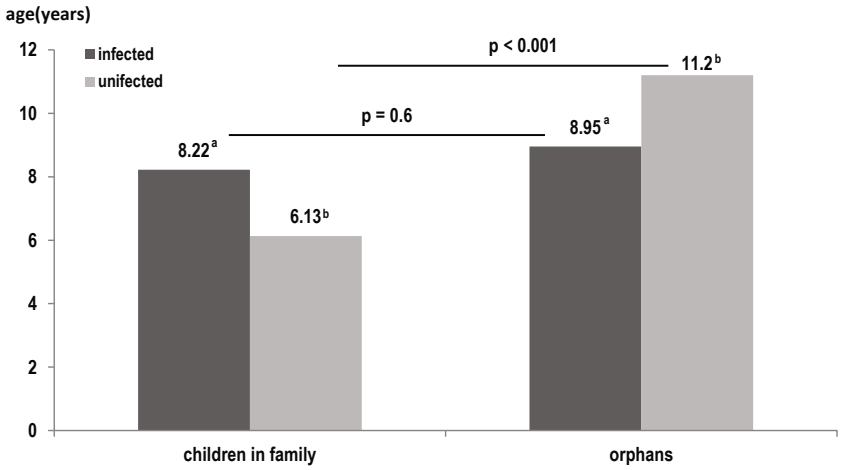

Fig. 1. Average age of infected and uninfected children in study groups.
[a,b] – different letters mean significant differences, t-test (p<0.05)

Enterobius vermicularis genotyping

All three analyzed isolates of *E. vermicularis* marked with symbols PL29, PL36, PL50 represented different haplotypes. The sequences were deposited in GenBank with accession no. KX527600-KX527602. Nucleotide variations among the sequences were found in two positions (2 SNP) and located at the third position of codons. They were transitions. This type of substitutions did not change the amino acid sequences (Fig. 2). PL36 haplotype (KX527601) was novel. The sequence of the PL29 (KX527600) haplotype was identical to a (AB221468) sequence previously isolated from a chimpanzee in Japan and (JQ411487) sequence from a pinworm isolated from a human in Denmark. The sequence of the PL50 isolate (KX527602) was identical to the sequence obtained from the chimpanzee (AB2211469) and to that isolated from a Danish patient (JQ411483). All sequences from Poland clustered within the type B (Nakano *et al.*, 2006), together with human *E. vermicularis* isolates from Denmark, Germany, Greece, and Japan (Fig. 3).

Fig. 2. The nucleotide sequences fragment of mitochondrial cytochrome c oxidase (*cox1*) gene of Polish *E. vermicularis* haplotypes with marked changes in the nucleotide sequence compared to sequence of *cox1* gene (accession no. EU281143) [22]. Dots denotes identical nucleotide to top sequence.

Discussion

In developed countries located in the temperate areas, such as Europe and North America, due to high standards of hygiene, health

Table 2. Association of risk factors with *E. vermicularis* infections in examined population.

Risk factors	Infected n (%)		Uninfected n (%)		OR (95% CI) *p*-Value
Siblings/ housemate					
● yes	30	(100)	220	(82.7)	
≤3	9	(30.0)	177	(66.5)	1.69 (0.57 – 5.06)
>4	21	(70.0)	43	(19.5)	*0.340*
● no	0	(0)	46	(17.3)	
Haushold pets					
● yes	11	(36.7)	130	(48.9)	0.61 (0.28 – 1.32)
● no	19	(63.3)	136	(51.1)	*0.204*
Season					
● spring/summer	11	(36.7)	120	(45.1)	0.70 (0.32 – 1.54)
● autumn/winter	19	(63.3)	146	(54.9)	*0.377*

OR: odds ratio; CI: confidence interval;

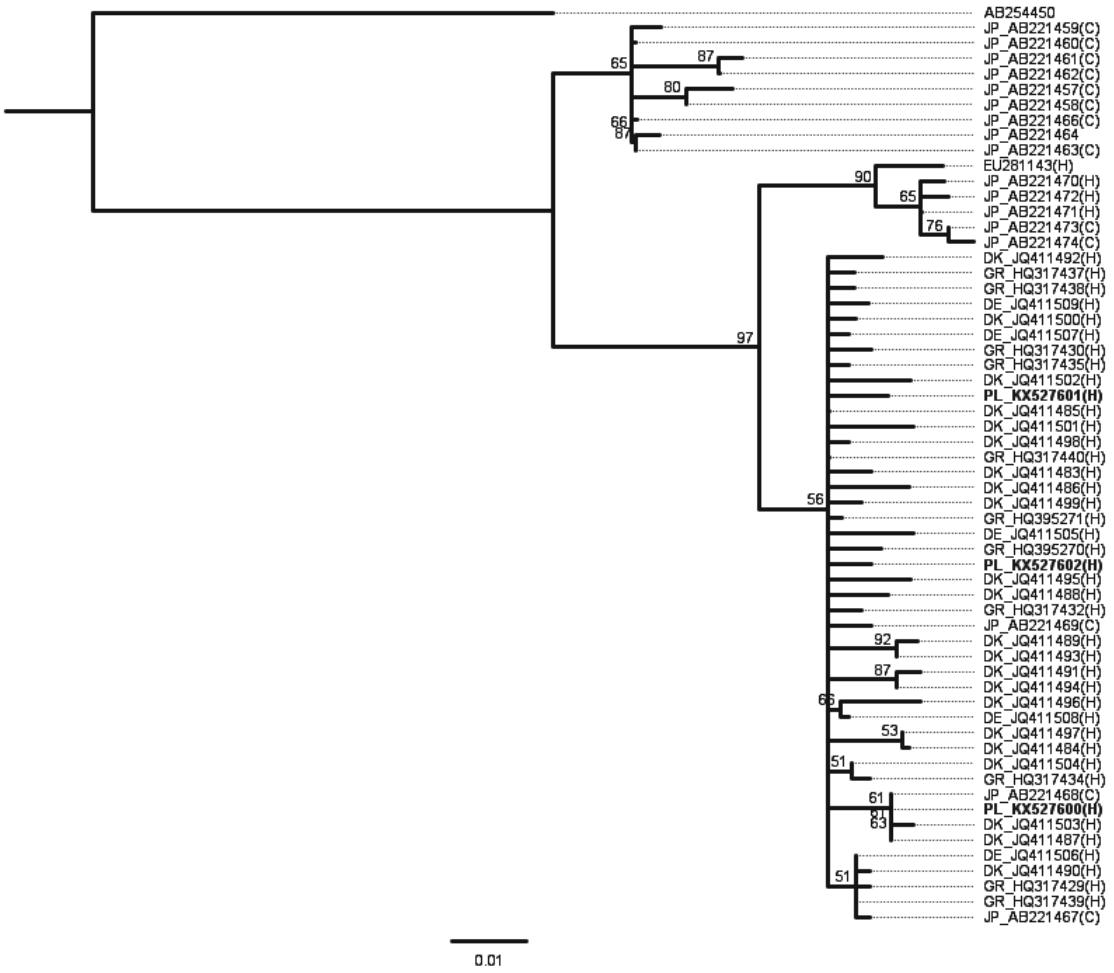

Fig. 3 Phylogenetic tree constructed using neighbor joining method. GenBank accession numbers of the sequences obtained in the present study are indicated with bold letters. Danish (DK), German (DE), Greek (GR) and Japanese (JP) haplotypes based on 333 bp of the *cox1* gene were included in the tree. Accession number AB254450 (*E. antropopitheci*) was included as outgroup. Numbers on the branches indicate bootstrap values. Only values over 50 % are included. (H) indicates human samples and (C) indicates samples derived from chimpanzees.

care and education and availability of effective antihelminthic drugs, the prevalence of gastrointestinal nematodes has been significantly reduced. However, the most common gastrointestinal parasitic disease is enterobiasis, predominantly among school-aged children (Stepek *et al.,* 2006; Alum *et al.,* 2010). Although the parasitological study in children populations indicated that the prevalence has declined during the last decades, enterobiasis still remain common. Depending on the country the *E. vermicularis* infections rate ranges from 7.3 % (Greece) to 28 % (Sweden) in examined children populations (Patsantara *et al.*, 2015, Değerli *et al.*, 2009, Kang *et al.*, 2006, Bøås *et al.*, 2012, Herrström *et al.*, 2001, Crotti & D'Annibale, 2006; Remm, 2006). In Poland, nationwide parasitological studies (periodically repeated from 1988 to 2003) confirmed the decreasing trend in the prevalence of *E. vermicularis* among 7-year-old children (from 21 % to 12.2 %) (Bitkowska *et al.*, 2004). The highest number of infected individuals was found in the Warmia-Masuria province (north-eastern Poland). In this area, in the years 2003 – 2006, enterobiasis was diagnosed in 9.5 % in children under 7 years of age (preschoolers), 36.7 % orphans and in 30.6 % of 7-year-old children (Bitkowska *et al.*, 2004; Kubiak *et al.*, 2015). The results of our parasitological test among children from north-eastern Poland, repeated after 10 years, confirm the consistently high levels of *E. vermicularis* infection (10.1 % of study group). Also, the proportions of infected and not infected children in particular study groups have remained similar over the years, with the highest prevalence of enterobiasis in children from orphanages (32.8 %) and significantly lower in the group of children living in families (3.9 %). Despite the fact that the risk of enterobiasis increases in the older nursery and school children (over the age of 5 years) (Song *et al.,* 2003; Remm 2006; Li *et al.,* 2015), some researchers have not found a relationship between the age of children and the prevalence of *E. vermicularis* infection. In our study, similarly to the study results from Argentina (Pezzani *et al.*, 2004), Estonia (Remm & Remm, 2008) and Norway (Bøås *et al.*, 2012), the gender of children was unrelated with the enterobiasis prevalence, although several reports have shown boys to be more frequently affected than girls (Remm, 2006;

Table 3. Association of symptoms with *E. vermicularis* infections in examined population.

Symptoms	Infected n (%)	Uninfected n (%)	OR (95% CI) *p*-Value
Anal ich			
● yes	3 (10.0) (90.0)	33 (12.4)	0.79 (0.23 – 2.73)
● no	27	233 (87.6)	*0.702*
Abdominal pain			
● yes	5 (16.7) (83.3)	52 (19.5)	0.82 (0.30 – 2.25)
● no	25	214 (80.5)	*0.704*
Lack of appetite			
● yes	3 (10.0) (90.0)	38 (14.3)	0.67(0.19 – 2.31)
● no	27	228 (85.7)	*0.519*
Weight loss			
● yes	0 (0.0) (100)	2 (0.8)	0.99 (0.98 – 1,00)
● no	30	264 (99.2)	*0.634*
Irritability			
● yes	6 (20.0) (80.0)	74 (27.8)	0.65 (0.26 – 1.65)
● no	24	196 (72.2)	*0.361*
Urinary tract infections			
● yes	0 (0.0) (100)	6 (2.3)	0.98 (0.96 – 1.00)
● no	30	260 (97.7)	*0,406*
Lack of symptoms			
● yes	13 (43.3) (56.7)	146 (54.9)	1.59 (0.74 – 3.40)
● no	17	120 (45.1)	*0.229*

OR: odds ratio; CI: confidence interval;

Kim *et al*., 2010). No significant seasonal variation of *E. vermicularis* infections was identified, which was congruent with the results of a study of healthy Norwegian children population (Bøås *et al*., 2012). It seems that staying in big peer groups on a constant basis plays an important role. Overcrowding, even in the home (more people per room, more people sleeping in one bed) is an important element related to the transmission of infections. A study conducted by Cazorla *et al.* (2006) showed that in families with three or more people occupying one room, and in families where members share a bed, the percentage of infected individuals was higher, by 36 % and 43 %, respectively. A greater number of children in the family or keeping a pet are also indicated by Remm and Remm (2008) and Artan *et al.* (2008) as factors increasing the risk of enterobiasis. In our study, in children living in families, each patient infected with *E. vermicularis* had siblings. In this group the greatest number of the children infected were aged 7 to 9. In the group of orphanage children enterobiasis was diagnosed also in younger children, under the age of 7 years, and in adolescents. Remm and Remm (2008) indicated that staying in mixed-age groups with an age range of 4 years turned out to be a significant risk factor for the younger members of the group. Although the prevalence is usually low among children aged 1 – 4, in mixed-age groups they are likely to be infected by the older companions. In such settings, as indicated by Kim *et al.* (2015) maintaining good standards of personal hygiene and monitoring children's health on a regular basis are crucial in limiting the spread of infection. In the group of children living in families an important factor which decrease *E. vermicularis* infection rate is parents' level of education and knowledge of enterobiasis, and also instilling good hygiene practices, teaching children how to maintain personal hygiene and keep their surroundings clean (Artan *et al*., 2008, Kim *et al*., 2010; Kang *et al*., 2012).

Epidemiological studies are typically based on the description of the frequency, and distribution of disease and then attempt to associate these patterns with the frequency and distribution of independent variables or risk factors, which allows to prepare targeted control programs. However, the application of molecular and analytical tools from the fields of population genetics and systematics enable the reconstruction of evolutionary relationships between parasites over a wide range of temporal and spatial scales, improving our ability to identify parasites, to gain insight into their geographic dispersal, diversity and their host range and helping to understand the role they play in disease causation (Lymbery & Thompson, 2012).

Molecular characteristics of *E. vermicularis* isolates described in this work are the starting point for defining the genetic diversity of human pinworm occurring in the child population in Poland. All of the isolates studied were different in terms of the sequences within the cytochrome c oxidase subunit 1 (*cox1*) gene; two of them, however, were identical with those earlier identified in the *E. ver-*

micularis populations from Denmark (Ferrero *et al.*, 2013) and Japan (Kang *et al,.* 2009). On the other hand, just like in Danish and Greek populations, a new haplotype, unregistered in GenBank, was detected. The *E. vermicularis* haplotypes revealed in hereby studies in Poland, similarly to the haplotypes from humans in Greece, Denmark and Germany (Piperaki *et al.*, 2011; Ferrero *et al.*, 2013) clustered within type B, together with the Japanese haplotypes from captive chimpanzees and humans. Type B is the only genetic type of this parasite which has so far been identified in Europe. For further conclusions about genetic diversity of the Polish *E. vermicularis* population more comprehensive molecular analyses are required.

In conclusion, *E. vermicularis* infections are still common among preschool and school children in north-eastern Poland. Children living in overcrowded and mixed age groups such as orphanages, are at greater risk of enterobiasis. Consequently, helminthological screening tests of school children, the promotion of health education, raising awareness among parents and educational staff at institutions about *E. vermicularis* infections and their prevention in children still remain necessary. To the best of our knowledge, our study presents first results of *E. vermicularis* genotypes from the human population in Poland. However, a larger number of *E. vermicularis* isolates from Poland are needed for further extensive molecular analysis and in order to shed more light on the genetic variability of the Polish population of this helminth and the geographical distribution of its genetic types.

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