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ORIGINAL PAPER

Emergence of novel subtypes of *Cryptosporidium parvum* in calves in Poland

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Abstract The aim of the study was to identify the *Cryptosporidium parvum* subtypes circulating in Polish cattle and their distribution in relation to the age and health status of tested animals. In total, 779 fecal samples were obtained from young cattle originating from 237 farms. *C. parvum* strains were identified at the 18 small-subunit ribosomal RNA (SSU rRNA), COWP, and LIB13 loci and were subsequently analyzed by sequencing at the 60-kDa glycoprotein (GP60) locus for subtype determination. The presence of 71 *C. parvum* strains belonging to IIa, IId, or III subtype families was shown. The strains from the IIa allele family prevailed with IIaA17G1R1, IIaA17G2R1, and IIaA15G2R1 subtypes occurring frequently. Two novel subtypes IIaA10G1R1 and IIIA19R3 were detected for the first time in a bovine host. The highest *C. parvum* prevalence (22.5 %, confidence interval (CI)=2.5 %) was observed among the youngest animals up to 2 weeks of age, followed by the prevalence among those aged 2 to 4 weeks (6.6 %, CI=2.6 %) and then among older cattle (4.9 %, CI=2.1). The occurrence of diarrhea in animals was associated with the presence of the IIaA16G1R1b subtype, while infections caused by IIaA15G2R1 strains were more likely to be asymptomatic. The geographical distribution of subtypes revealed that strains from the IIa subtype family were detected all over the country frequently compared to the IId and III subtypes, the sporadic appearances of which confirmed their endemic occurrence. Subtype analysis revealed the presence of zoonotic strains indicating cattle as a reservoir for human cryptosporidiosis.

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Introduction

Cryptosporidium infections are often reported in cattle raised in different geographical regions under various husbandry systems (de Graaf et al. 1999). Despite observed worldwide prevalence of the parasite in different animal species, infections have usually generated considerable losses in the livestock industry (Olson et al. 2004). Calves acquire infections shortly after birth, and animals below the age of 1 month are the major host of the parasite (Santín and Trout 2008; Rzeżutka and Kaupke 2013). Cattle can be infected by several *Cryptosporidium* species, but only two of these cattle-associated species, *Cryptosporidium parvum* and *Cryptosporidium andersoni*, have been found to cause human *cryptosporidiosis* (Leoni et al. 2006; Liu et al. 2014). It has previously been shown that the bovine host is a major reservoir of zoonotic *C. parvum* and contact with an infected animal or drinking contaminated water can lead to human infection (Hunter and Thompson 2005; Cacciò et al. 2005; Olson et al. 2004). Sequencing of a 60-kDa glycoprotein (GP60) gene is a frequently used subtyping method (Waldron et al. 2009; Plutzer and Karanis 2007; Díaz et al. 2012). This tool also confirmed its usefulness during epidemiological investigation of cryptosporidiosis cases and in surveillance studies of human and animal cryptosporidiosis (Soba and Logar 2008). Based on sequence analysis of the GP60 gene, the existence of 14 (IIa–IIo) *C. parvum* subtype families has been shown in humans and animals (Insulander et al. 2013; Wang et al. 2014). The analysis of a microsatellite region within the GP60 gene of *C. parvum* strains revealed the parasite's heterogeneity, with a large number of subtypes detected within each

subtype family that encompasses zoonotic or human-specific types. Although a lot of studies have been conducted that aimed to subtype *C. parvum* strains detected in humans and animals, our knowledge on subtype occurrence and worldwide distribution in the human and animal host is still not complete (Xiao 2010). So far, little is known about the occurrence and epidemiology of infections in humans caused by zoonotic *C. parvum* subtypes in Poland, although information on possible zoonotic transmission (Majewska et al. 1999; Gait et al. 2008) and identification of zoonotic species present in farm animals exists (Rzeżutka and Kaupke 2013; Kaupke et al. 2014; Rzeżutka et al. 2014; Majewska et al. 2000, 2001, 2004). The aim of the study was to identify the *C. parvum* subtypes circulating in the Polish cattle population, in respect to the presence of zoonotic subtypes. In addition, animal age and animal health as factors in the occurrence and distribution pattern, respectively, of parasite subtypes were investigated.

Materials and methods

Origin of the samples

Fecal samples were collected from calves from birth to 2 months of age originating from 237 farms located in all 16 provinces in Poland. In total, 779 cattle fecal samples were obtained during 4 years of monitoring from 2010 to 2014. Sampled farms and the animals on those farms were randomly selected for the studies. They represented different administrative locations across each province. Average herd size differs in the sampled farms, although the minimum number of heads per farm was 20. From each farm, from two to five individual fecal samples of approximately 10–15 g were placed into plastic containers, labeled, and sent to the laboratory. Of these samples, 633 had previously been tested for *Cryptosporidium*, resulting in detection of 34 *C. parvum* strains but without pursuant subtype identification (Rzeżutka and Kaupke 2013). The majority of sampled animals (689 calves) did not show any gastrointestinal disorders, and only 90 calves did, demonstrating diarrheal illness (with loose and watery stools) of an unknown etiology on the day of sampling. The age and health status of sampled cattle are presented in Table 1.

Detection of *C. parvum* in cattle and subtype identification

Samples were analyzed using molecular methods according to a previously described procedure. Briefly, parasite genomic DNA was extracted from 0.1 g of the feces with an alkali wash and a heat lysis method developed by Millar et al. (2001) with further modifications (Rzeżutka and Kaupke 2013). Identification of *C. parvum* was performed at the 18 small-subunit ribosomal RNA (SSU rRNA) (Xiao et al. 1999), COWP (Homan et al. 1999), and LIB13 (Tanriverdi et al. 2003) loci by conventional PCR. The LIB13 PCR was a *C. parvum* and *Cryptosporidium hominis*-specific assay targeting sequence polymorphism of a four-nucleotide deletion on an unknown genomic sequence of *C. parvum*. The positive 18 SSU rRNA (849 bp) and COWP (640 bp) PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis using *Nde*I (Zintl et al. 2007) and *Taq*I (Homan et al. 1999) enzymes. For subtyping, two nested PCR assays that target the GP60 gene locus giving amplicons of approximately 800 or 400 bp were used (Glaberman et al. 2002; Sulaiman et al. 2005). The appropriate positive and negative controls were included during the nucleic acid extraction and PCR analyses. Visualization of PCR amplicons and digested products was performed by electrophoresis in either 1.7 or 2.5 % agarose gels stained with ethidium bromide. The GP60 amplicons were subsequently excised from the agarose gel, purified, sequenced, and compared with the reference sequences of each subtype family submitted to the GenBank database using the NCBI BLASTn program (http://blast.ncbi.nlm.nih.gov). Particular subtypes were identified based on trinucleotide repeats present in analyzed sequences.

Statistical analysis

Moreover, the relationship between cattle age and frequency of *C. parvum* occurrence as well as the dominance of infections caused by *C. parvum* strains from the IIa subtype family over strains belonging to the IId and III families were statistically analyzed using one-way analysis of variance (ANOVA) with Tukey confidence intervals (CIs). Subsequently, the relationship between infections caused by a particular subtype and the presence of diarrhea was determined on the basis of the confidence interval for the mean estimated for all diarrheic animals. A chi-square test (χ^2) was used to determine the frequency of *C. parvum* infections in diarrheic and healthy animals as well as to show the relationship between the animal age and the occurrence of infections caused by various *C. parvum* subtypes. Concluding the statistical work, a two-way ANOVA without interactions was applied to determine how the relationship between the animal age and the presence of each subtype exerts influence on the frequency of infections caused by these strains. All calculations were performed with Statgraphics Centurion v. XV.

Results

Molecular identification: PCR-RFLP, GP60 sequencing, and sequence analysis

Cryptosporidium detection performed at the 18 SSU rRNA, COWP, and GP60 loci revealed the presence of *C. parvum* in 76 samples analyzed; however, a successful subtype identification was obtained for 71 strains belonging to the IIa, IId, or

Table 1 The age, health status, and occurrence of *C. parvum* subtypes in sampled cattle

Animal age	Number of samples (positive/tested)	Number of cattle (diarrheic/non-diarrheic)	Occurrence of *C. parvum* subtypes			
			Non-diarrheic cattle (*n*=689)	GenBank accession no.	Diarrheic cattle (*n*=90)	GenBank accession no.
>1 day–2 weeks	43/191	38/153	IIaA10G1R1 (*n*=2)	KP997142	IIaA15G2R1 (*n*=1)	KP997149
			IIaA14G1R1 (*n*=2)	KP997144	IIaA16G1R1b (*n*=6)	
			IIaA15G2R1 (*n*=2)	KP997143	IIaA17G1R1 (*n*=11)	KP997151
			IIaA17G1R1 (*n*=3)		IIaA17G2R1 (*n*=4)	
			IIaA17G2R1 (*n*=4)	KP997146	IIdA24G1 (*n*=1)	KP997148
			IIaA19G1R1 (*n*=1)	KP997145	NIS (*n*=2)	
			IIdA22G1b (*n*=1)	KP997137		
			IIdA24G1c (*n*=1)	KP997140		
			NIS (*n*=1)			
>2–4 weeks	16/242	30/212	IIaA15G2R1 (*n*=6)		IIaA17G2R1 (*n*=4)	KP997152
			IIaA17G1R1 (*n*=3)	KP997139	IIlA19R3 (*n*=1)	KP997147
			IIdA23G1 (*n*=2)	KP997136		
>4–8 weeks	17/346	22/324	IIaA15G2R1 (*n*=5)		IIaA16G1R1b (*n*=2)	KP997150
			IIaA16G3R1 (*n*=1)	KP997138	IIaA17G1R1 (*n*=3)	
			IIaA17G1R1 (*n*=1)		NIS (*n*=1)	
			IIaA17G2R1 (*n*=2)			
			IIaA18G1R1c (*n*=1)	KP997141		
			NIS (*n*=1)			

NIS not identified subtype

III subtype families. The IIa subtype family prevailed (84.2 %, CI=3.6 %) compared to IId (9.5 %, CI=4.2 %) and III (6.0 %, CI=7.2 %) subtypes. The IIa subtype family was represented by the following subtypes: IIaA17G1R1 (*n*=21), IIaA17G2R1 (*n*=14), IIaA15G2R1 (*n*=14), IIaA16G1R1b (*n*=8), IIaA10G1R1 (*n*=2), IIaA14G1R1 (*n*=2), IIaA16G3R1 (*n*=2), IIaA18G1R1c (*n*=1), and IIaA19G1R1 (*n*=1). The IId subtype family was found in five samples, specifically IIdA23G1 (*n*=2), IIdA24G1c (*n*=2), and IIdA22G1b (*n*=1), and one sample contained III with the IIIA19R3 identity. For the first time, two novel subtypes, IIaA10G1R1 and IIIA19R3, were detected in a bovine host. None of the tested samples contained more than one *C. parvum* GP60 subtype family. PCR amplification with GP60 primers was unsuccessful only for five *C. parvum* strains, probably either because of the low abundance of *Cryptosporidium* DNA present or because of heterogeneity within the primer's sequences to the DNA sequence of the subtype present. Sequences representing each subtype within the tested group of animals were deposited in GenBank (Table 1).

Distribution of *C. parvum* subtypes in relation to health status and animal age

C. parvum infections were significantly more frequently identified (χ^2=25.24, P<0.001) in diarrheic cattle (40 %) than in healthy animals (5.8 %). The frequency of infections between different age groups of calves (birth–2 weeks, >2–4 weeks, and >4–8 weeks) differed significantly (P=0.022, α=0.05). Indeed, the highest *C. parvum* prevalence (22.5 %, CI=2.5 %) was observed in animals at the age of up to 2 weeks. It was significantly lower for animals at the age between 2 and 4 weeks (6.6 %, CI=2.6 %) and older (4.9 %, CI=2.1 %) (Fig. 1).

Calves were mostly infected with four out of 14 subtypes detected (IIaA17G1R1, IIaA17G2R1, IIaA15G2R1, and IIaA16G1R1b). The part of diarrheic animals in all cattle infected by these subtypes was 57.74±37.63 %. The designated confidence interval for the mean (20.11–95.37) shows that the infections caused by IIaA15G2R1 were more likely to be asymptomatic (7.1<20.11 %), while the presence of IIaA16G1R1b subtypes was always associated with diarrhea (100>95.37 %) (Fig. 2). Of note, these animals were kept on two farms situated in different areas in Wielkopolska

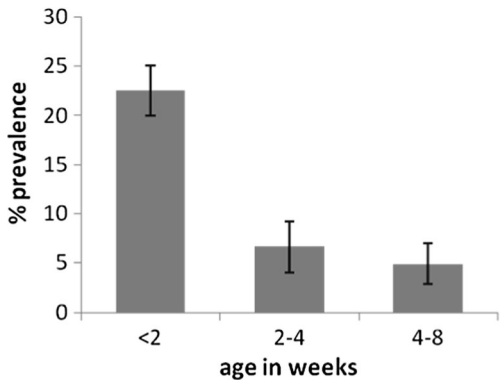

Fig. 1 The relationship between animal age and frequency of *C. parvum* infections

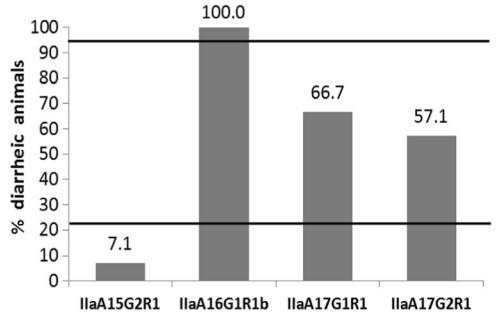

Fig. 2 The relationship between *C. parvum* subtype and presence of diarrhea. The lines indicate the lower and upper limit of the confidence interval. Values outside the confidence interval indicated *Cryptosporidium* subtypes which differ significantly from the mean

Province. For IIaA17G1R1 and IIaA17G2R1 subtypes, there was no relationship observed between the presence and absence of diarrhea during the course of infection. Nevertheless, it should be noticed that animals infected by these subtypes are more likely to develop diarrhea (values of 66.7 and 57.1 % are within the confidence interval for the mean).

There was no correlation observed between the cattle age and the presence of a particular subtype (P=0.869, α>0.05). Also, there was no statistically significant relationship present between the animal age (P=0.452, α>0.05), the presence of specific subtype (P=0.213, α>0.05), and the frequency of infections caused by them. Nevertheless, particular subtypes were only shown in animals at a certain age. For example, IIaA10G1R1, IIaA14G1R1, IIaA19G1R1, IIdA22G1b, and IIdA24G1c subtypes were detected in the youngest animals (below 2 weeks of age), while IIdA23G1 and IIIA19R3 subtypes occurred in cattle at the age between 2 and 4 weeks, being followed by the IIaA18G1R1c subtype found solely in cattle older than 4 weeks (Fig. 3).

Geographical distribution of subtypes

The infected animals were detected on 44 (18.6 %) of the 237 monitored farms. Among the three commonly detected IIa subtypes, IIaA17G1R1 was found in six out of 16 Polish administrative provinces. In comparison to subtypes from the IId and III allele families, strains from the IIa subtype family were frequently detected all over the country (Fig. 4). They were present in 39 (16.4 %) of the investigated farms. The IId subtypes were found sporadically in four farms localized in Lublin, Pomerania, and West Pomerania provinces. The animal harboring IIIA19R3 was raised in Lublin Province.

Discussion

Genome sequencing and multilocus analysis of highly polymorphic sequences have significantly increased subtyping resolution and knowledge of the genetic structure of *Cryptosporidium* (Xiao 2010). In this study, subtyping of *C. parvum* strains was performed based on the DNA sequence polymorphism analysis of the GP60 gene. *Cryptosporidium* infections have been reported in cattle worldwide with a varied prevalence regardless of the animal age ranging from 3.5 % (Epe et al. 2004) to 60.2 % (Mišic and Abe 2007). In the studies presented, *C. parvum* prevalence was estimated at 9.7 % only for animals up to the age of 2 months. This prevalence is not different from that reported by

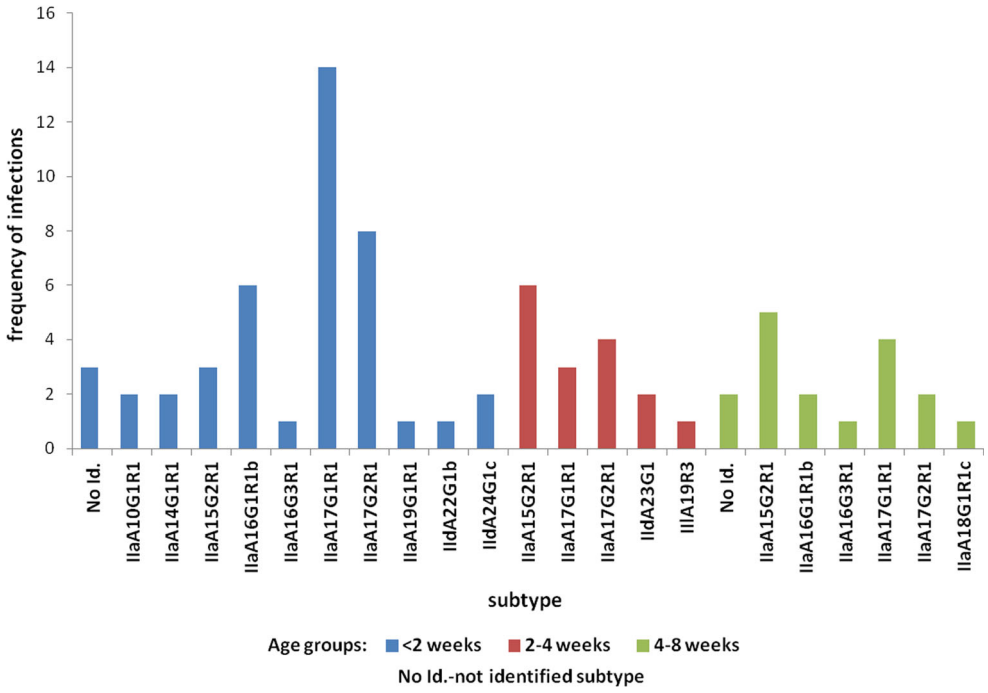

Fig. 3 The frequency of infections caused by each *C. parvum* subtype in relation to animal age

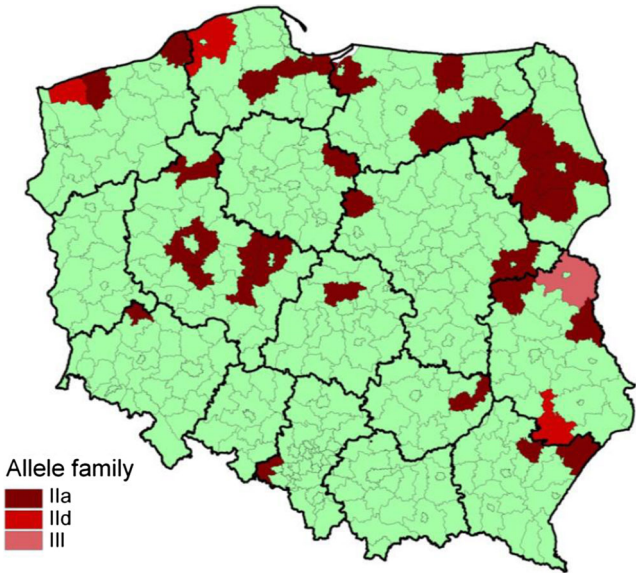

Fig. 4 Map of Poland showing the distribution of *C. parvum* strains from IIa, IId, and III allele families

other authors in cattle being at the same age (Brook et al. 2009; Björkman et al. 2015). Nevertheless, only 18.6 % of farms were positive for this parasite species, indicating its low occurrence on the farm level. The differences observed between farms could be associated with the age of animals sampled, as the prevalence is age related and is higher in younger animals and varied geographical occurrence and distribution across the sampled regions; therefore, studies limited only to restricted area will not give a good estimation for parasite prevalence. Other factors that have an influence on prevalence are the herd size, type of breed, farm management, or husbandry system.

It was shown that the majority (91.5 %) of identified *C. parvum* strains in cattle belonged to the IIa zoonotic allele family. There were also infections detected which were caused by IId (7.04 %) and, rare for Europe, III (1.4 %) subtype families. It was statistically confirmed that the IIa subtypes prevailed in cattle regardless of animal age or geographical location of the farm. Subtype analysis revealed that the IIaA17G1R1 isolate was mainly responsible for the infection, as it was present in 29.6 % of *C. parvum*-positive samples. This dominating subtype in Polish cattle has previously been found in sheep (Plutzer and Karanis 2007; Wielinga et al. 2008; Thompson et al. 2007; Stantič-Pavlinic et al. 2003) and in cattle with prevalence among tested animals ranging from 53.3 to 98.4 % (Geurden et al. 2007; Brook et al. 2009; Duranti et al. 2009; Broglia et al. 2008; Wielinga et al. 2007; Alves et al. 2003, 2006; Imre et al. 2009, 2010; Soba and Logar 2008; Quilez et al. 2008; Rieux et al. 2013). Apart from the dominating IIaA17G1R1 isolates, IIaA15G2R1 and IIaA17G2R1 subtypes also frequently occurred. It is worth noting that eight (A14G1R1, A15G2R1, A16G1R1b, A16G3R1, A17G1R1, A17G2R1, A18G1R1c, and A19G1R1) out of nine IIa subtypes identified in this study have previously been described as cattle pathogens (Thompson et al. 2007; Brook et al. 2009; Mišic and Abe 2007; Silverlås et al. 2013). Furthermore, concurrent infections caused by more than one subtype were not reported in any animal. It was shown that the frequency of *C. parvum* infections in cattle is age related, with the highest occurrence of this parasite in animals at the age of 1 day to 2 weeks. For frequently appearing subtypes (IIaA15G2R1, IIaA16G1R1b, IIaA17G1R1, and IIaA17G2R1), the relationship between the occurrence of subtypes and its higher pathogenicity as manifested by cattle diarrhea was investigated. In fact, this association was only shown for the IIaA16G1R1b subtype as diarrhea was attributed to all infections caused by this strain. Profuse diarrhea was also seen in one calf harboring the IIlA19R3 subtype. Despite treatment, this animal died from a diarrheal disease, and subsequent laboratory investigation excluded bacteria as a primary cause of disease (personal communication). However, when the course of the infection was asymptomatic, it was associated with the presence of IIaA15G2R1. The relationship between the presence of any specific *C. hominis* or *C. parvum* subtype and the occurrence of clinical symptoms was also investigated in humans by Cama et al. (2007) and in calves by Geurden et al. (2007). However, in the case of *C. parvum* subtypes, the existence of such a congruity was not confirmed. In contrast to our findings, the IIaA15G2R1 strain was found not only in diarrheic but also in non-diarrheic dairy calves in France (Rieux et al. 2013). Nevertheless, the relationship between clinical signs and subtype occurrence was not analyzed statistically.

In this study, four different subtypes comprising IIdA22G1b, IIdA23G1, IIdA24G1c, and IIlA19R3 from the less common IId and III genetic families were detected. Some of these subtypes have previously been found in cattle in Spain and Sweden (Quilez et al. 2008; Silverlås et al. 2010) with the high natural prevalence of IId strains reported in cattle housed outside Europe (Amer et al. 2010; Muhid et al. 2011; Zhang et al. 2013). In contrast to IIa and IId subtypes, isolates from the III genetic lineage have also occasionally been reported in Europe (Soba and Logar 2008; Wielinga et al. 2008). In this study, there were no unusual subtypes detected except one IIaA10G1R1, identified in two asymptomatic animals kept on the same farm. This is the first report describing its presence in cattle. However, based on the existing data, it is difficult to determine its significance in the epidemiology of bovine cryptosporidiosis. There was no correlation observed between strain distribution and region of the country, except in the case of isolates from the IId and III allele families present solely in north and east Poland. When a subtype distribution is compared with this observed in neighboring countries, the dominating subtype (IIaA17G1R1) in Poland has not been found in cattle neither in Germany nor in the Czech Republic. Surprisingly, the IIaA15G2R1 subtype prevailing in Germany has also wide distribution in the west and central part of Poland.

Genetic diversity of detected strains within the same farm was not observed, suggesting endemicity of a single subtype

on a farm. As seen in recent studies, this finding cannot be considered as a rule (Abeywardena et al. 2012); however, similar, low variation among subtypes detected in animals housed on the same farm has been described in Slovenia, Serbia, Montenegro, and France (Mišic and Abe 2007; Soba and Logar 2008; Rieux et al. 2013). Certainly, greater genetic variability within the analyzed population of *C. parvum* strains might be found if multilocus analyses targeting mini- and microsatellite loci were analyzed. The association between the animal age, the occurrence of particular subtype, and the frequency of infections was not confirmed statistically. The lack of interaction in the statistical model resulted from non-orthogonality of data, i.e., the presence of different subtypes and their presence in varying numbers in each group of tested animals.

It is known that cryptosporidiosis in humans is not mainly caused by *C. hominis*, but rather that other *Cryptosporidium* species present in livestock and free-living animals represent more important human pathogens and a common cause of diarrhea (Ryan and Hijjawi 2015). Although *C. parvum* subtypes from IIa and IId allele families are considered to be zoonotic, not all currently identified members of these families have been detected in humans. This data may partly support assumptions that animals could be an exclusive reservoir for some subtypes (Abe et al. 2006; Wielinga et al. 2007; Soba and Logar 2008; Hijjawi et al. 2010; O'Brien et al. 2008; Waldron et al. 2009; Budu-Amoako et al. 2012). Nevertheless, the zoonotic nature of some *C. parvum* strains detected in this study has previously been confirmed (Soba and Logar 2008; Hijjawi et al. 2010; Chalmers et al. 2011; Lassen et al. 2014) with the IIaA15G2R1 subtype often being detected in both humans and animals (Chalmers et al. 2011; Drumo et al. 2012). In fact, cases of human cryptosporidiosis in Poland are only sporadically identified and typically without subsequent information on disease-causing subtypes. This lack of data significantly hinders an epidemiological investigation aiming at recognition of the infection source. Although the studies were conducted on randomly selected animals originated from farms at different locations, the number of cattle tested from each province did not reflect the size of population being kept in this region. It could be taken as a major limitation in the interpretation of results on the occurrence and prevalence of *C. parvum* subtypes in Polish cattle. In addition, an attempt was undertaken to investigate the relationship between infections caused by particular subtypes and the presence of diarrhea in infected animals. Although some relationships were shown and confirmed statistically, the low number of strains representing rarely detected subtypes did not allow to fully investigate their clinical significance. *Cryptosporidium* diarrhea in calves is often associated with *C. parvum* infections compared to other parasite species frequently detected in this type of host. Because the etiology of diarrhea in calves was not thoroughly investigated for the possible co-infections with bacterial and viral pathogens, therefore, its strict association solely to *Cryptosporidium* infection might tend to overestimate some findings.

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