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Molecular epidemiology of *Cryptosporidium* spp. in calves in Estonia: high prevalence of *Cryptosporidium parvum* shedding and 10 subtypes identified

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Abstract

We investigated the molecular epidemiology of *Cryptosporidium* spp. in Estonia by testing fecal samples from 486 calves aged <2 months, raised on 53 cattle farms, for the presence of *Cryptosporidium* DNA. The parasites were identified and characterized by sequencing of the 18S rRNA gene and of the 60 kDa glycoprotein (*gp60*) gene. Moreover, using a questionnaire, we surveyed factors that could be relevant for animal-to-human and human-to-animal transmission of *Cryptosporidium* spp. on the farms. *Cryptosporidium* spp. were shed by 23% of the investigated calves and at least one shedding calf was found on 66% of the farms. *Cryptosporidium parvum* was the most common species shed, while *C. bovis* and *C. ryanae* were also detected. More than half of the calves aged 8–14 days shed *C. parvum*. Nine previously described *C. parvum* subtypes (IIaA14G1R1, IIaA16G1R1, IIaA17G1R1, IIaA18G1R1, IIaA19G1R1, IIaA20G1R1, IIaA21G1R1, IIaA22G1R1 and IIaA16G2R1) and an apparently novel subtype IIlA21R2 were found. Calves from farms that reported spreading manure on fields during spring had 10 times higher odds to shed *Cryptosporidium* spp. in their feces than calves from farms that did not. Calves aged 8–14 days had higher odds to shed IIa18G1R1 as well as IIaA16G1R1 than younger calves.

Introduction

Protozoan parasites of the genus *Cryptosporidium* can cause gastro-intestinal disease in several host species, including humans and cattle (Thompson *et al.*, 2016). *Cryptosporidium parvum* and *C. hominis* are considered responsible for most cases of human cryptosporidiosis (Cacciò and Chalmers, 2016). *Cryptosporidium hominis* is known as human-specific species, while *C. parvum* has a wider host spectrum that includes cattle. *Cryptosporidium parvum* has been observed as the dominant *Cryptosporidium* species shed by pre-weaned calves in many countries (Chako *et al.*, 2010), but not in countries nearby Estonia (Silverlås and Blanco-Penedo, 2013; Björkman *et al.*, 2015). Young calves infected with *C. parvum* can shed high numbers of oocysts in their feces (Xiao, 2010; Smith *et al.*, 2014). In addition to animal-to-human transmission of *C. parvum* by direct contact, feces of infected cattle may also contaminate, e.g. water supplies (McLauchlin *et al.*, 2000; Xiao, 2010; Wells *et al.*, 2015) or ready-to-eat vegetables (Åberg *et al.*, 2015).

Cryptosporidium parvum subtype families IIa and IId have been found in both humans and cattle (Xiao, 2010). In Sweden, one of the most common subtype of the *C. parvum* in cattle was IIaA16G1R1 (Silverlås *et al.*, 2010; Björkman *et al.*, 2015), which has been also found in humans (Silverlås *et al.*, 2010; Insulander *et al.*, 2013). In Estonia, the same subtype IIaA16G1R1 has been identified in both cattle and an immunocompetent human with clinical cryptosporidiosis (Lassen *et al.*, 2014). That case and results of a questionnaire study focusing on veterinary students (Dorbek-Kolin *et al.*, 2018) provide evidence for zoonotic transmission of *Cryptosporidium* in Estonia.

In Estonia, cryptosporidiosis in humans is a notifiable but under-reported disease (Lassen *et al.*, 2014; Plutzer *et al.*, 2018). Surveillance data do not provide a good overview of the epidemiology of *Cryptosporidium*, and the need to fill the knowledge gaps with a One Health approach is evident (Plutzer *et al.*, 2018). Previous studies have shown that almost all Estonian cattle farms had cattle that were shedding *Cryptosporidium* spp. (Lassen *et al.*, 2009). However, the zoonotic potential of the *Cryptosporidium* spp. shed and the circulating *C. parvum* subtypes have been unknown.

The main aim of this study was to characterize *Cryptosporidium* spp. shed by calves in Estonia, with special emphasis on the zoonotic potential. In addition, we surveyed factors

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that could be relevant for transmission of *Cryptosporidium* spp. from animals-to-humans or from humans-to-animals on the farms.

Materials and methods

Study design

Sample size calculation was performed using OpenEpi (Dean *et al.*, 2015): 35 farms was the minimum sample size needed for this study. This calculation was based on a population size of 5572 cattle herds (Estonian Agricultural Register and Information Board, 2018*a*), absolute precision of 10% and an expected proportion of farms with calves shedding *C. parvum* of 10%. The aim was set to sample at least 50 farms. The sampling was proportionally stratified to the 15 Estonian counties according to the number of farms listed in the Estonian Animal Recording Centre (2013) in each county. Expecting that at a given moment, at least 30% of calves would be shedding *Cryptosporidium* spp. oocysts on a farm where *Cryptosporidium* spp. is present (Lassen *et al.*, 2009), it was evaluated that 10 calves per farm would be sufficient to find at least one calf shedding the parasite, if *Cryptosporidium* spp. was present on the farm.

Sampling

The samples were collected by veterinarians from April 2013 to May 2014 and from January to March 2015. Inclusion criteria for farms were: registration in the e-Business Register (Centre of Registers and Information Systems, 2018) and Estonian Agricultural Registers and Information Board (2018*b*), and herd size ⩾50 cattle to ensure a sufficient number of calves for the study. Farms were selected using a random number generator (Microsoft Excel, Microsoft Cooperation). Three farms were randomly chosen as potential replacements for each county and were included in case a farm that was originally selected opted out.

The veterinarians were instructed to collect individual fecal samples from the rectum of up to 10 calves ⩽2 months of age on each farm. Animal-level exclusion criterion was the calf being reported to be >2 months old. Samples were collected in disposable gloves and stored in a transportable cooler during transport to the laboratory. The samples were stored frozen at −18 °C until DNA extraction.

Questionnaire

A questionnaire was designed to collect information on diarrhoea in calves on the farms as well as on factors with potential relevance for animal-to-human and human-to-animal transmission of *Cryptosporidium* spp. on farms (Supplementary Table S1). The questionnaire was filled in by the interviewing veterinarian either on the farm at the time of sampling or by phone interview following the farm visit. The questions were asked in Estonian language.

DNA extraction, polymerase chain reaction and sequencing

We used molecular methods to detect, identify and characterize *Cryptosporidium* spp. from the samples. Genomic DNA was extracted from 200 µg of thawed and homogenized feces using the PSP® Spin Stool DNA Kit (STRATEC Biomedical AG, Birkenfeld, Germany) according to the manufacturer's instructions.

Three microlitres of each DNA sample were submitted to polymerase chain reaction (PCR) amplification targeting the 18S rRNA gene (Xiao *et al.*, 1999). Nuclease-free water and *C. parvum* genomic DNA (kindly provided by the European Union Reference Laboratory for Parasites) were used as negative and positive controls. The nested reaction used 1 µL of the first-round PCR product. The thermal cycling conditions were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min and a final extension of 72 °C for 7 min in both the first and second rounds. The PCR products were run on 2% ethidium bromide-stained agarose gel and visualized under an ultraviolet transilluminator. Products of the expected size (approximately 825 bp) were submitted to sequencing for species identification.

The samples that tested positive were submitted to PCR amplification targeting the 60 kDa glycoprotein (*gp60*) gene for subtype identification (Peng *et al.*, 2001). Three microlitres of DNA sample were used in the first PCR reaction and 1 µL of PCR product in the nested PCR reaction. Nuclease-free water and *C. parvum* genomic DNA were used as negative and positive controls. Thermal conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min and a final extension of 72 °C for 7 min and 10 min in the first and second rounds, respectively. Electrophoresis was performed as described above. Products of approximately 490 bp were selected for subsequent sequencing.

The PCR products were cleaned up and sequenced with Applied Biosystems® 3130xl Genetic Analyzer by a two-directional procedure. Forward and reverse sequences were aligned with BioEdit v7.2.5 software (Hall, 1999) to generate single consensus sequences and correct mismatches. The resulting sequences were compared with nucleotide sequences (Accession numbers KJ941147, HQ005736, AM937006, AB242226) deposited in GenBank using BLASTn (nucleotide Basic Local Alignment Search Tool, Altschul *et al.*, 1990). *Gp60* subtypes were named in agreement with the system proposed by Sulaiman *et al.* (2005) based on the number of serine-coding trinucleotide repeats.

Statistics

Sample size and confidence intervals (CI) (Mid-P exact) were calculated using OpenEpi (Dean *et al.*, 2015). Further statistical analyses were performed using Stata IC 14.2 for Mac software (Stata Corporation, College Station, TX, USA). A calf was considered *Cryptosporidium* spp.-positive if its sample tested positive for *Cryptosporidium* spp. 18S rDNA. A farm was considered positive if at least one calf of the investigated calves tested positive. Multivariable logistic regression models were built for dichotomous animal-level outcomes: calf testing positive for *Cryptosporidium* spp., calf testing positive for *C. parvum*, calf testing positive for *C. parvum* subtype IIa18G1R1 and calf testing positive for *C. parvum* subtype IIaA16G1R1. The farm number was used as a random factor to account for clustering. Variables (Supplementary Table S1) with a P value ⩽0.20 in univariable analysis were first included in the model, followed by a stepwise backward elimination procedure. Biologically meaningful interactions and possible confounding effects were tested. P values <0.05 were considered significant.

Results

Sample

From each of the 53 farms included in the study, 3–14 (median 10) fecal samples were collected, resulting in a total of 522 individual fecal samples. A total of 36 fecal samples were excluded because the same farm had been sampled twice (the samples from second sampling were included), missing labels or insufficient amount of fecal sample available for the analysis. The final sample included in this study comprised individual fecal

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Table 1. Animal-level prevalence of *Cryptosporidium* species and subtypes in fecal samples from calves ($n = 486$), including 454 calves ⩽2 months of age and 32 calves of unknown age, collected from 53 cattle farms in Estonia

Species	Subtype[a]	*n* positive	% positive	95% confidence interval
Cryptosporidium parvum		105	21.60	18.12–25.43
	IIaA18G1R1	34	7.00	4.97–9.53
	IIaA16G1R1	16	3.29	1.96–5.18
	IIaA20G1R1	9	1.85	0.90–3.37
	IIaA14G1R1	8	1.64	0.77–3.10
	IIaA16G2R1	5	1.03	0.38–2.27
	IIaA22G1R1	5	1.03	0.38–2.27
	IIaA21G1R1	5	1.03	0.38–2.27
	IIaA17G1R1	5	1.03	0.38–2.27
	IIlA21R2	5	1.03	0.38–2.27
	IIaA19G1R1	3	0.62	1.16–1.67
Cryptosporidium bovis		4	0.82	0.26–1.97
Cryptosporidium ryanae		1	0.21	0.01–1.01

[a]*Cryptosporidium parvum* subtype data for 95 samples (only species level for 10 *C. parvum*-positive samples).

samples from altogether 486 calves from the 53 farms, 3–14 (median 10) per farm. Information on age was available for 454 calves, and the age of the calves ranged from 1 to 59 days (median 15, mean 18.26). The prevalence estimates were based on the results from 486 calves, i.e. including also the 32 calves with no information of age.

Questionnaire

Of the 53 farms, 49 (92.5%) answered the questionnaire. Supplementary Table S1 shows the distribution of the answers. The majority of farms had more than 150 cattle (79.2%). One (2.1%) farm had bought cattle from abroad during the previous 5 years. Almost a quarter (24.4%) of the farms were located close to natural waterbodies. Altogether, 10 (18.9%) of the farms reported that *Cryptosporidium* spp. had been diagnosed in calves during the previous 5 years.

Prevalence of Cryptosporidium spp.

Cryptosporidium spp. DNA was amplified and sequenced from 110 (22.63%, 95% CI 19.08–26.51) of the 486 fecal samples. Of the 110 *Cryptosporidium* spp.-positive fecal samples, 105 (95.45%, 95% CI 90.22–98.32) were *C. parvum*-positive, four (3.64%, 95% CI 1.17–8.53) were *C. bovis*-positive and one (0.91%, 95% CI 0.05–4.40) was *C. ryanae*-positive. Of the 105 *C. parvum*-positive fecal samples, 95 (90.48%, 95% CI 83.68–95.06) were successfully sequenced and typed by *gp60* analysis.

At least one of the investigated calves was *Cryptosporidium* spp.-positive on 35 (66.0%, 95% CI 52.6–77.8) of the 53 farms (Table 2, Fig. 1). On 33 farms (62.3%, 95% CI 48.7–74.5), at least one of the investigated calves was *C. parvum*-positive. *Cryptosporidium bovis* was detected in fecal samples from two farms (3.8%, 95% CI 0.6–11.9), and on one of these farms, *C. parvum* was also detected. *Cryptosporidium ryanae* was detected as the only *Cryptosporidium* species on one farm.

Cryptosporidium parvum DNA was almost exclusively found in fecal samples of calves ⩽28 days old, with the exception of one calf that was 36 days old, and seven calves of unknown age (Fig. 2). A total of 64 (52.03%, 95% CI 43.21–60.76) of the 123 calves aged between 8 and 14 days shed *C. parvum*. *Cryptosporidium bovis* and *C. ryanae* were detected in feces of calves that were >14 days old.

Cryptosporidium parvum subtypes

A total of 10 different subtypes were identified (Table 1 and 2). The majority (9/10) of the subtypes were in the IIa subtype family, while one subtype was identified as novel IIlA21R2 (Accession numbers MH509210–MH509219). The most common subtype was IIaA18G1R1, which was found in 35.79% (34/95) of the *C. parvum*-positive samples and on 15 (45.5%) of the 33 farms where *C. parvum* was found. The second most common subtype was IIaA16G1R1, which was identified in 16 (16.84%) of the *C. parvum*-positive samples and on four (12.1%) of the *C. parvum*-positive farms. A single *C. parvum* subtype per farm was found on all except one farm. Three *C. parvum* subtypes (IIaA18G1R1, IIaA20G1R1 and IIaA21G1R1) were identified on a farm located in southern part of the country (Fig. 1).

Subtypes within the IIa family differed by the number of TCA repeats. The exception was the subtype IIaA16G2R1, which had an additional TCG triplet. The novel IIlA21R2 had no TCG triplets, an ACATCA sequence that repeated twice and several single nucleotide polymorphisms when compared with the other IIa subtypes.

Models

Based on the final multivariable model, calves from farms that spread manure on the fields during spring had 10.1 (CI 1.18–86.27) times higher odds to have *Cryptosporidium* spp. DNA in the feces than calves from farms that did not. Calves that were from farms that reported mortality during the first month of life in calves with severe diarrhoea, which had received veterinary treatment, had 6.2 times higher odds (CI 2.46–15.66) to shed *Cryptosporidium* spp. (Supplementary Table S2) and 7.4 times higher odds (2.60–21.10) to shed *C. parvum* (Supplementary Table S3). The odds of a calf aged 8–14 days being *Cryptosporidium* spp. or *C. parvum*-positive were 10.1 (CI 4.53–22.36) and 10.4 (CI 4.58–23.74) times higher than the odds of a calf aged up to 7 days, respectively (Supplementary Tables S2 and S3). Based on the final models, the odds of a calf being

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Table 2. Farm-level prevalence of *Cryptosporidium* species and subtypes on cattle farms ($n = 53$) in Estonia

Species[a]	Subtype[b]	*n* positive	% positive	95% confidence interval
Cryptosporidium parvum		33	62.3	48.72–74.50
	IIaA18G1R1	15	28.3	17.44–41.48
	IIaA16G1R1	4	7.5	2.44–17.21
	IIaA20G1R1	4	7.5	2.44–17.21
	IIaA14G1R1	2	3.8	0.64–11.91
	IIaA22G1R1	2	3.8	0.64–11.91
	IIaA21G1R1	2	3.8	0.64–11.91
	IIaA16G2R1	1	1.9	0.09–8.95
	IIaA17G1R1	1	1.9	0.09–8.95
	IIIA21R2	1	1.9	0.09–8.95
	IIaA19G1R1	1	1.9	0.09–8.95
Cryptosporidium bovis		2	3.8	0.64–11.91
Cryptosporidium ryanae		1	1.9	0.09–8.95

A farm was considered positive if at least one of the 3–14 calves investigated from the farm tested positive.
[a]One farm had both *C. bovis* and *C. parvum*.
[b]*Cryptosporidium parvum* subtype data for 31 farms (only species level for two *C. parvum*-positive farms). A single *C. parvum* genotype per farm was found on all except one farm. Three *C. parvum* subtypes (IIaA18G1R1, IIaA20G1R1 and IIaA21G1R1) were found on that one farm.

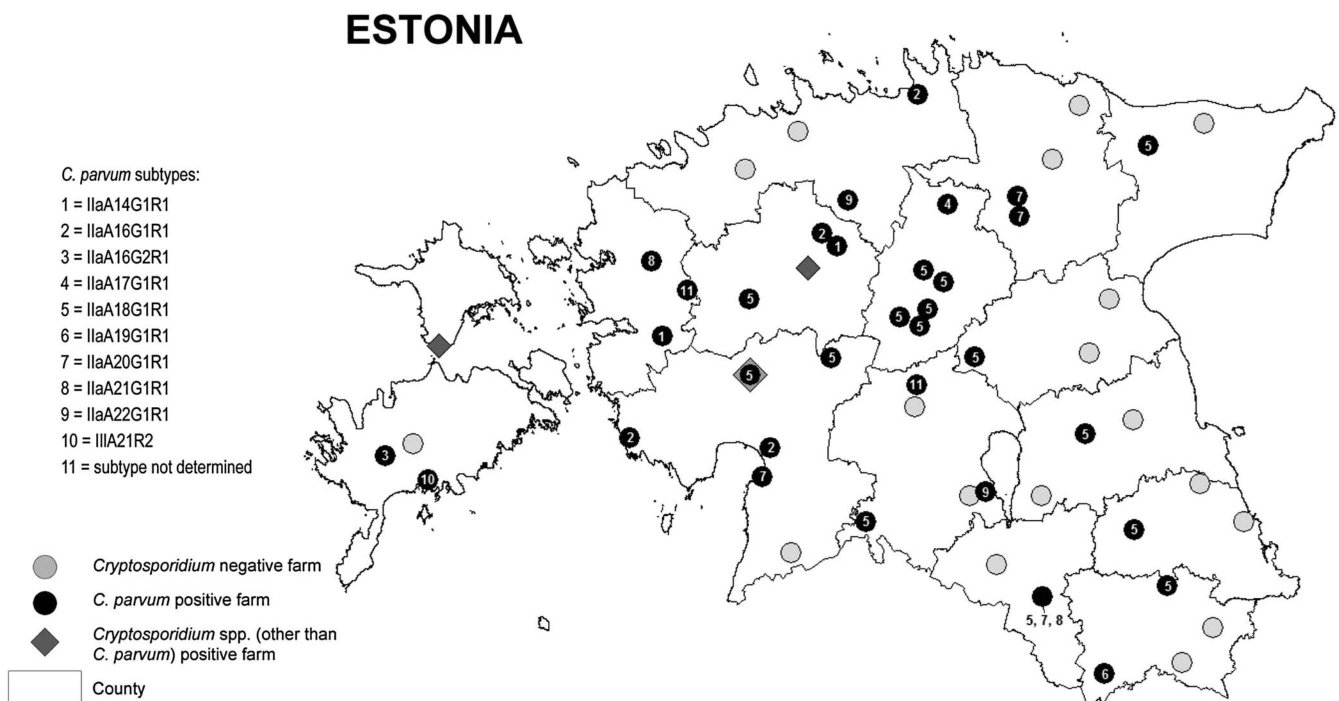

Fig. 1. Map of Estonia showing 18 farms negative for *Cryptosporidium* spp. (grey circles), three farms positive for *Cryptosporidium* spp. other than *C. parvum* (grey diamonds), and 33 farms positive for *C. parvum* (black circles). We tested fecal samples from three to 14 (median 10) calves per farm, and a farm was considered positive if fecal sample of at least one of the sampled calves tested positive.

C. parvum-positive for subtypes IIa18G1R1 and IIaA16G1R1 were 4.00 ($P = 0.024$) and 25.47 ($P = 0.018$) times higher in a calf aged 8–14 days than in a calf aged up to 7 days.

Discussion

The results of this study showed that *Cryptosporidium* spp. were commonly shed by calves in Estonia. This result, which is based on molecular detection, is in line with the previous sample-level and farm-level microscopy-based prevalence estimates (Lassen *et al.*, 2009).

In this study, *C. parvum* was the predominant *Cryptosporidium* species detected. This is in contrast to reports from calves of comparable age groups from nearby countries Sweden (Silverlås *et al.*, 2010; Björkman *et al.*, 2015) and Finland (Seppä-Lassila *et al.*, 2015), where the dominant species found were *C. bovis*, and *C. bovis* and *C. ryanae*, respectively. The results of this study resemble the results from Belgium, Slovenia and the Czech Republic, where *C. parvum* accounted for most of the *Cryptosporidium* findings from calves (Geurden *et al.*, 2007; Soba and Logar, 2008; Kváč *et al.*, 2011).

Ten different *C. parvum* subtypes were identified in the fecal samples in this study, indicating a high genetic diversity within

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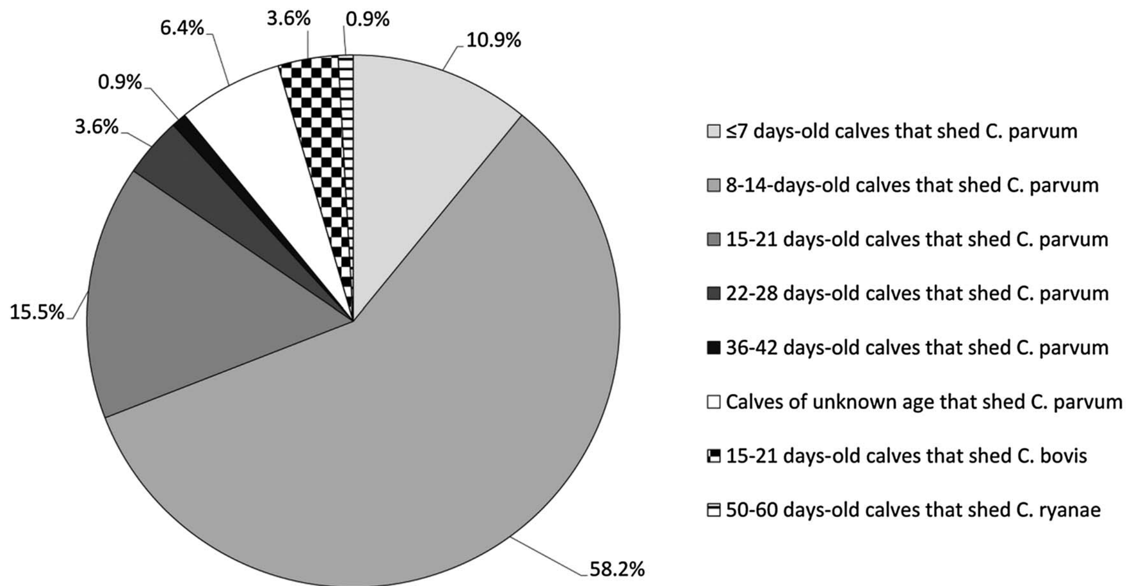

Fig. 2. Distribution of the 110 *Cryptosporidium* spp. shedding calves by age group and the *Cryptosporidium* species identified.

C. parvum in Estonia. The most frequently found subtype in this study, IIaA18G1R1, has been reported in cattle in Hungary (Plutzer and Karanis, 2007), the Czech Republic (Kváč *et al.*, 2011), the Netherlands (Wielinga *et al.*, 2008) and Serbia (Misic and Abe, 2007). IIaA16G1R1, the second most commonly found subtype in this study has been reported in the Czech Republic (Ondrácková *et al.*, 2009; Kváč *et al.*, 2011), Hungary (Plutzer and Karanis, 2007), Romania (Imre *et al.*, 2011; Vieira *et al.*, 2015), Slovenia (Soba and Logar, 2008) and Sweden (Björkman *et al.*, 2015). IIaA17G1R1 has been reported in Poland (Kaupke and Rzeżutka, 2015) and the UK (Smith *et al.*, 2014); IIaA16G2R1 in Belgium (Geurden *et al.*, 2007) and Spain (Quilez *et al.*, 2008); IIaA14G1R1 in Poland (Kaupke and Rzeżutka, 2015) and Spain (Ramo *et al.*, 2014); IIaA20G1R1 in Serbia (Misic and Abe, 2007); and IIaA21G1R1 and IIaA22G1R1 in Sweden (Silverlås *et al.*, 2010) and Germany (Broglia *et al.*, 2008). At least six of the subtypes identified in this study from calves, including the two most common ones, have also been found in humans (Soba and Logar, 2008; Chalmers *et al.*, 2011; Lassen *et al.*, 2014; Lobo *et al.*, 2014), highlighting the zoonotic potential of *C. parvum* shed by cattle. Subtype IIaA16G1R1, which has been connected to zoonotic transmission from a calf to a human in Estonia (Lassen *et al.*, 2014), was the second most common *C. parvum* subtype and was identified in feces of 3.3% of the calves and on 7.5% of the investigated farms.

The subtype allele family IIl (also indicated as IIj, Soba and Logar, 2008) has been reported from calves in Serbia (Misic and Abe, 2007), Poland (Kaupke and Rzeżutka, 2015) and Lithuania (Wielinga *et al.*, 2008), and in calves and humans from Slovenia (Soba and Logar, 2008). To our knowledge, the IIlA21R2 identified in this study is a new subtype.

Cryptosporidium parvum is commonly regarded as a zoonotic pathogen (Cacciò and Chalmers, 2016). However, not all *C. parvum* infections in humans result from zoonotic transmission. Molecular subtyping is a useful tool for determining whether human infections originate from animals. The results of studies like ours might be useful for back-tracing potential sources of *Cryptosporidium* infections and for evaluating the likelihood of the involvement of local cattle in outbreaks. Nevertheless, the definitive discrimination in such cases must resort to a multilocus approach (Chalmers *et al.*, 2016; Chalmers and Cacciò, 2016).

Either IIaA16G1R1 (Iqbal *et al.*, 2015) or IIaA16G2R1 (Ranjbar *et al.*, 2016), both reported to cause human cryptosporidiosis (Lassen *et al.*, 2014; Hijjawi *et al.*, 2017), was identified in 4.3% of the fecal samples. These subtypes have also been found in river water in Romania (Imre *et al.*, 2017) and sewage in Portugal (Lobo *et al.*, 2009). More studies are needed on the role of calves in shedding *C. parvum* in their feces and into the environment. In this study, a calf from a farm that reportedly spread cattle manure on the fields during spring had higher odds of shedding *Cryptosporidium* spp. as well as *C. parvum*. In the UK, a peak in human cryptosporidiosis cases caused by *C. parvum* during springtime was suspected to be linked with livestock (McLauchlin *et al.*, 2000). In the largest human cryptosporidiosis outbreak, which occurred in Milwaukee in 1993, run-off from cattle farms was suspected as the potential cause of the water contamination that resulted in human *C. parvum* infections (Mac Kenzie *et al.*, 1994). Several of the subtypes identified in the current study have been reported in wildlife (Krawczyk *et al.*, 2015) and fish (Certad *et al.*, 2015), which adds a sylvatic aspect to the epidemiology. *Cryptosporidium* spp. infection epidemiology is One Health epidemiology where humans, animals and the environment need to be considered.

High rates of *Cryptosporidium* spp. infection have been reported in calves of 1–3 weeks of age (Abeywardena *et al.*, 2015). In all our models, being 8–14 days old was a risk factor for the calf to shed *Cryptosporidium*. Young animals are usually more susceptible to *Cryptosporidium* spp., and may act as amplifiers and infection sources to other animals (Geurden *et al.*, 2010). In the current study, being 8–14 days old was a risk factor for shedding *C. parvum* subtype IIaA16G1R1 (OR 20.6), as well as for shedding IIaA18G1R1 (OR 4.0), indicating that this specific age group is a risk group for zoonotic subtypes. This information can be used to design measures that may improve animal health and reduce the occupational risks to humans: considering feces of this age group of calves as likely infective and handling them accordingly could be advisable.

Cryptosporidium parvum can cause high morbidity in calves, and the typical profuse diarrhoea can result in high mortality (Abeywardena *et al.*, 2015). Outbreaks with a high mortality in calves due to *C. parvum* have also been described in Estonia (Lassen and Talvik, 2009; Niine *et al.*, 2017). In the current study, calves from farms reporting mortality of calves with severe

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diarrhoea that had received veterinary treatment had higher odds to shed *Cryptosporidium* spp. as well as *C. parvum* in feces.

The design of this study succeeded in obtaining a well-representative sample from cattle farms all over the country (Fig. 1). We chose not to concentrate the fecal samples before extracting the DNA. Consequently, calves shedding only a few oocysts may have been missed. The results are thus mainly representing calves shedding moderate-to-high numbers of oocysts, and the prevalence estimates should be considered conservative. It should be noted that PCR methods targeting the 18S rRNA and direct sequencing are likely to detect only the most abundant species and genotype in the specimen and underestimate the occurrence of mixed infections (Hadfield *et al.*, 2011; Mercado *et al.*, 2015).

The *gp60* sequence analysis we used is a common approach employed to characterize *C. parvum* (Xiao, 2010). The findings of this study indicate that subtypes of *C. parvum* that have also been found in humans were the rule, not the exception, in calves raised in Estonia. It would be important to characterize *Cryptosporidium* spp. from humans in the country as well, to evaluate the proportion attributable to zoonotic transmission.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182018001348.

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Conflict of interest. None.

Ethical standards. The study was approved by the Ethical Committee of Ministry of Agriculture (currently, Ministry of Rural Affairs, permit nr. 7.2-11/46). The participating farms were recruited during farm visits for unrelated reasons, or contacted by phone by a veterinarian. Participation was voluntary and oral informed consent was given.

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