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Prevalence and molecular characterization of *Cryptosporidium* spp. in dairy cattle in South Bohemia, the Czech Republic

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Annotation

The prevalence and molecular characterization of *Cryptosporidium* spp. in slaughtered cattle 6 months and older was performed. Three species of *Cryptosporidium* were identified. A subtype of *C. parvum* was obtained.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své magisterské práce, a to v nezkrácené podobě fakultou elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

Prohlašuji, že jsem tuto práci vypracovala samostatně s použitím uvedené literatury.

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Ondráčková Zuzana

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Poděkování

Chtěla bych poděkovat všem, kteří mi byli nápomocni při práci. Největší dík patří mému školiteli. Dále děkuji všem pracovníkům laboratoře lékařské a veterinární parazitologie za veškerou pomoc, cenné rady a přátelskou atmosféru. Poděkování patří také těm, kteří mi poskytli materiál nebo mi pomohli při jeho sběru.

Na závěr bych ráda poděkovala rodičům a Petrovi za podporu při studiu.

Content:

Abstract

A total of 995 samples from slaughtered dairy cattle (6 months and older) were collected during two consecutive years (from 2007 to 2008), stained by aniline-carbol-methyl violet and examined microscopically. DNA was extracted from *Cryptosporidium* positive samples and from 200 randomly selected samples. Nested PCR was performed to amplify the partial SSU rRNA and GP60 genes of *Cryptosporidium* that were subsequently digested by *Ssp*I, *Vsp*I and *Mbo*II restriction enzymes to determine the *Cryptosporidium* species and genotype present. The highest prevalence of infection (18.2%) was in the animals in age group of 12-18 months, but no statistical differences among age categories were detected. The sequence analyses of PCR-positive specimens identified 41 samples as *C. andersoni* (4.1%), 2 samples as *C. bovis* (0.2%), and 1 sample as *C. parvum* (0.1%). *Cryptosporidium bovis* was detected only in a group of cattle older than 18 months and *C. parvum* in heifer which was older than 14 months. Seasonal dependency of *Cryptosporidium* spp. prevalence was not proven to any significant degree. Infection intensity was generally low. Sequence analysis of the glycoprotein (GP60) gene showed that detected *C. parvum* belonged to the IIa subtype. This is the first published report about subtyping of the *Cryptosporidium* GP60 gene from cattle in the Czech Republic.

1. Introduction

Cryptosporidium species are unicellular parasites that inhabit the mucosal epithelium of the respiratory and gastro-intestinal tracts of a variety of vertebrate hosts including humans (O'Donoghue, 1995).

Cryptosporidium infections have been reported in livestock worldwide, regardless of the husbandry system. Moreover, cattle could be infected with at least 10 various *Cryptosporidium* species or genotypes, and have been considered the major reservoir of *Cryptosporidium* for human infections (Xiao and Feng, 2008). Beside sporadic infections with *C. suis* (found in calves in USA and Zambia), *C. suis*-like (in Denmark), *C. hominis* (in Scottland, India, United States and Korea), *C. felis* (in Poland) and *Cryptosporidium* pig genotype II (in Denmark) (Bornay-Llinares et al., 1999; Tanriverdi et al., 2003; Smith et al., 2005; Fayer et al., 2006; Feng et al., 2007; Geurden et al., 2006; Park et al., 2006; Langkjær et al., 2007) and experimentally verified *C. canis* calf infection (Fayer et al., 2001), cattle are commonly infected with four *Cryptosporidium* species worldwide: *C. parvum*, *C. andersoni* (formerly known as *C. muris*-like), *C. bovis* (formerly known as bovine genotype B), and *C. ryanae* (formerly known as *Cryptosporidium* deer-like genotype) (Meuten et al., 1974; Anderson, 1987; Santín et al., 2004).

Except *C. andersoni*, which could infect all age categories of their hosts (Kváč et al., 2008; Kváč et al., 2009a), the other three species are age-specific. *Cryptosporidium parvum* is dominant in pre-weaned calves (< 2 months); *C. bovis* and *C. ryanae* are considered to be predominant in post-weaned calves (2-11 months) (Santín et al., 2004; Fayer et al., 2006). In contrast to *C. parvum* infections, characterized by profuse watery diarrhea (Fayer et al., 1997), *C. andersoni* and other *Cryptosporidium* species infections have been associated with few or no clinical signs. Moreover, there are no reports of subclinical pathology in most cases (Anderson, 1998, Kváč and Vítovec, 2003).

Although the first case of bovine cryptosporidiosis in the Czech Republic was described in 1981 (Pavlásek, 1981), the identification of *Cryptosporidium* in most of the subsequent studies was based on oocyst morphology only (Pavlásek, 1995; Kváč and Vítovec, 2003; Kváč et al., 2006).

Therefore the present study was undertaken to evaluate the occurrence of *Cryptosporidium* species and to identify the genotypes using molecular methods in dairy cattle 6 months and older, slaughtered over an area encompassing the South Bohemia region in the Czech Republic.

2. Materials and methods

2.1. Sample collection and examination

A total 995 fecal samples from slaughtered cattle (6 months and older) from 79 different farms were collected from the rectum immediately after being slaughtered. These samples were obtained at four slaughterhouses in the Czech Republic during the period of January 2007 to January 2009. Each sample was individually placed in a plastic dish without fixation. Samples were stored in the dark at 4°C and analyzed within 24 h using the aniline-carbol-methyl violet staining method (Miláček and Vítovec, 1985). Entire smears were microscopically examined by light microscopy at 1,000 × magnification. The number of shed oocysts was counted using semiquantitative method. Infections were tabulated as follows: sporadic infections up to 10 oocysts/slide, weak infections up to 200 oocysts/slide, medium infections up to 10 oocysts/30 fields, and strong infection more then 10 oocysts/30 fields. All of the above counts were carried out at magnification of 1,000 ×.

2.2. Detection of *Cryptosporidium* spp. and subtyping of *C. parvum*

Genomic DNA was isolated from all *Cryptosporidium* positive samples and from 200 randomly selected samples as described previously (Sak et al., 2008) using PSP Spin Stool DNA Kit (Invitek). Acquired DNA extracts were stored at -20°C. A fragment of the *Cryptosporidium* small subunit ribosomal RNA (SSU rRNA) gene, approximately 830 bp in length, was amplified by nested polymerase chain reaction (PCR) according to Jiang et al. (2005). Genotyping of *C. parvum* was performed by sequence analysis of the GP60 gene. A fragment of this gene (800 to 850 bp long) was amplified by nested PCR according to Alves et al. (2003). Both SSU and GP60 PCR mixture contained 1× PCR buffer (Top-Bio, Praha, Czech Republic), 3 mM $MgCl_2$ (Top-Bio), 200 µM dNTP (Top-Bio), 1 U *Taq* / 25 µl (Top-Bio), 1 µl BSA (10 mg/ml, Sigma) / 25 µl and 200 nM of each forward and reverse primer (Generi Biotech, Czech Republic) in a 50 µl reaction volume. For the secondary PCR step, the

PCR mixture was identical except that BSA was excluded and 100 µl reactions were done. For both PCR steps a total of 35 cycles, each consisting of 94°C for 45 s, 55°C (SSU) or 50°C (GP60) for 45 s, and 72°C for 60 s were performed. An initial incubation at 94°C for 3 min., final extension at 72°C for 7 min., and final soak 4°C until samples removal were included.

As a positive controls DNA isolated from oocysts of *C. muris* TS03 or *C. hominis* were used. The PCR products were visualized in a 1% agarose gel containing 0.2 µg/ml ethidium bromide.

2.3. Genotyping using RFLP analysis

The secondary PCR products of SSU rRNA were analyzed by restriction fragment length polymorphism (RFLP) according to Xiao et al. (2001) and Feng et al. (2007). The RFLP mixture consisted of 20 µl of secondary PCR product, 10U of the *Ssp*I, *Vsp*I, or *Mbo*II enzyme (Fermentas, Ontario, Canada), 3 µl of 10× corresponding buffer (Fermentas, Ontario, Canada), and PCR water to final volume 30 µl. After subsequent incubation at 37°C overnight the fragments were visualized in a 1.5% agarose gel containing 0.2 µg/ml ethidium bromide. The species or genotypes of *Cryptosporidium* were determined by band patterns.

2.4. DNA sequence analysis

The secondary PCR products were sequenced using ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI3130 Genetic Analyzer (Applied Biosystems). GP60 PCR products of *C. parvum* were further sequenced with an intermediary sequencing primer (5´-GAGATATATCTTGTTGCG-3´) (Feng et al., 2007). All sequences from each target were assembled using ChromasPro (Technelysium, Queenland, Australia). The consensus sequences were aligned with reference sequences from the GenBank database using ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/), and the multiple-sequence alignments were edited using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.5. Statistical analyses

For statistical evaluation of results, Statistica, Release 5.1 Software (Statsoft, Tulsa, OK, USA, 1997) was used. The Chi-square statistic for evaluation of significant differences was utilized.

3. Results

3.1. Prevalence of *Cryptosporidium* spp. by microscopy, PCR and gene sequencing

A total of 995 fecal samples were examined and 49 were microscopically positive for *Cryptosporidium* spp. The average prevalence at the 79 farms was 4.9%. No PCR positivity from 200 randomly microscopically negative samples was detected. Out of 49 microscopically positive fecal specimens, 44 were PCR positive and namely *Cryptosporidium parvum*, *C. andersoni* and *C. bovis* were found in 2.3%, 93.2% and 4.5%, respectively. The sequence analysis results corresponded 100% to RFLP and 100% homology with GenBank-listed species was recorded as follows: 1 *C. parvum* (GenBank accession number: AF093490), 41 *C. andersoni* (GenBank accession number: AB089285) and 2 *C. bovis* (GenBank accession number: AY120911). Moreover *C. parvum* was identified as the IIaA16G1R1 subtype. No mixed infection either on the basis of microscopy or PCR-RFLP was detected.

3.2. Prevalence of *Cryptosporidium* spp. as related to age

Although the highest *Cryptosporidium* prevalence (18.2%) was observed among 12-18 months old cattle followed by the 6-12 months old cattle (4.6%) and the lowest prevalence was recorded among 18 months and older cattle, no statistical differences were observed among these age categories ($p > 0.05$) (Table 1). While the highest prevalence of *C. andersoni* (3.9%, 38 positive samples) was found in group of cattle older than 18 months, in younger animals (12-18 and 6-12 months) only 2 and 1, respectively, *C. andersoni*-positive samples were found. Only two cows positive for *C. bovis* oocysts were identified in the group of cattle older then 18 months. Surprisingly, one heifer at the age of 14 months was found with *C. parvum* infection. Although both intestinal species were found on the farms where *C.*

andersoni was detected, no mixed infections of gastric and intestinal species were detected on the basis of oocyst morphology.

Table 1. Age distribution of *Cryptosporidium* spp. infection in cattle

Age group (months)	No. of screened samples	No. of positive samples	Prevalence (%)
6-12	22	1	4.6
12-18	33	6	18.2
> 18	940	37	3.9

3.3. Seasonal prevalence of *Cryptosporidium* spp. infection in cattle

The samples were collected throughout the whole year (Table 2). While *Cryptosporidium* prevalence was up during the winter (5.6%) followed by summer and autumn (4.7 and 4.3%, respectively), the lowest number of infected animals was observed during the spring (2.3%). However, no significant differences ($p > 0.05$) of prevalence were detected among the seasons.

Table 2. Seasonal prevalence of *Cryptosporidium* spp. infection in cattle

Season	No. of screened samples	No. of positive samples	Prevalence (%)
Winter	306	17	5.6
Spring	174	4	2.3
Summer	193	9	4.7
Autumn	322	14	4.3

3.4. Infection intensity

Generally low *Cryptosporidium* spp. infection intensity was recorded. The infection intensity of *C. andersoni* in different age groups of animals is shown in Table 3. Sporadic infections of *C. bovis* were detected in both positive animals. In the case of the heifer with *C. parvum* infection a weak level of infection intensity was determined. Moreover, the feces were diarrheal as inferred from its liquid consistency.

Table 3. Age-related level of *Cryptosporidium andersoni* infection intensity

Level of infection intensity		Age group (months)		
		6-12	**12-18**	**> 18**
Positive	Sporadic infection	1	3	17
	Weak infection	0	2	8
	Medium infection	0	0	9
	Strong infection	0	0	1
	Total	1	5	35
	Negative	21	27	903

4. Discussion

Nine hundred and ninety five fecal samples which originated from slaughtered cattle 6 months and older were examined for the presence of *Cryptosporidium* oocysts. Out of 249 PCR tested samples including both microscopically positive and randomly selected samples, only 44 samples were identified as the *Cryptosporidium* spp. positive. Moreover, all these positive samples were also microscopically positive beforehand. Generally, the PCR-based detection of parasites is considered to be more sensitive than microscopical examination (Santín et al., 2004, Sak et al., 2008), however, surprisingly, we obtained different results. The unsuccessful amplification of expected DNA fragment in the case of the rest of microscopically positive samples may be explained by the low concentration, or poor quality, or both of the template DNA.

Cryptosporidial infection of cattle was reported worldwide. In our study the overall prevalence of 4.9% was recorded. This is in agreement with other studies; *Cryptosporidium* oocyst prevalence of 4.7% was recorded in a study carried out at a slaughterhouse in Japan (Kaneta and Nakai, 1998). Similar prevalence (4-6%) was reached in the United States (Maryland and North Dakota) and Portugal (Santín et al., 2004, 2008; Fayer et al., 2006, 2007; Mendonca et al., 2007; Feltus et al., 2008). Although the higher prevalence in adult cattle (10-14%) was detected in other studies performed in Japan, India and Denmark (Maddox-Hyttel et al., 2006; Amer et al., 2009; Paul et al., 2009), generally the prevalence values in older animals are several-times lower as compared to calves (Xiao and Herd, 1994; Sischo et al., 2000; Wade et al., 2000; Huetink et al., 2001; Sturdee et al., 2003; Olson et al., 2004; Santín et al., 2004; Fayer et al., 2006, 2007; Kváč et al., 2006; Maddox-Hyttel et al., 2006; Langkjær et al., 2007; Mendoca et al., 2007).

The molecular finding of *C. andersoni* as a dominant *Cryptosporidium* species in this study confirmed previous results from the Czech Republic obtained on the basis of oocyst

morphology determination (Kváč et al., 2006). The same result was reported in India with *C. andersoni* as the most frequent species in adult cattle (Paul et al., 2009). In contrast to our study, *C. andersoni* was identified as a minor species in the USA and Japan (Santín et al., 2004, Feltus et al., 2008; Amer et al., 2009). Although *C. bovis* is in the many studies one of the dominant *Cryptosporidium* species in cattle older than 6 months (Santín et al., 2004; Fayer et al., 2006, 2007; Geurden et al., 2006, 2007; Coklin et al., 2007; Feng et al., 2007; Langkjær et al., 2007; Siwila et al., 2007; Thomaz et al., 2007; Thompson et al., 2007; Brook et al., 2008; Feltus et al., 2008), only sporadic cases of *C. bovis* infection was detected in this study. Moreover, no finding of *C. ryanae*, the second most prevalent species (Santín et al., 2004; Feltus et al., 2008; Amer et al., 2009), was identified in our study. Since both of the aforementioned intestinal species were reported in dairy cattle worldwide, including European countries (Santín et al., 2004; Fayer et al., 2006, 2007; Langkjær et al., 2007; Plutzer and Karanis, 2007; Thompson et al., 2007), the low prevalence or absence of both intestinal *Cryptosporidium* spp. on the dairy farms in the Czech Republic is surprising. The result could be explained by the fact, that *C. ryanae* predominantly infects cattle at an early age (3-11 months old) (Santín et al., 2004; Fayer et al., 2006, 2007), while only low number of samples from this age category were examined in our study. Moreover, no *C. ryanae* was found in calves up to 6 months of age in the Czech Republic (unpublished data). Different breeding management could be another explanation, but no data for analysis is available.

In spite of the fact that *C. parvum* predominantly infects juvenile animals, sporadic infections have been recorded in adult animals of various species, e.g. cattle and pigs (Scott et al., 1995; Maddox-Hyttel et al., 2006; Amer et al., 2009; Kváč et al., 2009a). Alignment of the obtained sequences revealed that the isolate belonged to the *C. parvum* subtype family IIa, the major zoonotic family of *C. parvum* found worldwide in cattle (Feng et al., 2000; Strong et al., 2000; Alves et al., 2003, 2006; Peng et al., 2003; Stantic-Pavlinic et al., 2003; Wu et al.,

2003; Chalmers et al., 2005; Abe et al., 2006; Thompson et al., 2006; Trotz-Williams et al., 2006; Xiao et al., 2007). While the most common worldwide distributed subtype is IIaA15G2R1, which is very frequently found in dairy calves (Alves et al., 2003, 2006; Peng et al., 2003; Stantic-Pavlinic et al., 2003; Xiao et al., 2004; Chalmers et al., 2005; Trotz-Williams et al., 2006), in our study IIaA16G1R1 subtype was found in heifer. This finding is in agreement with the previous studies from the Czech Republic, where the IIaA16G1R1 subtype was found in sows (Kváč et al., 2009b), and Slovenia and Hungary, where this subtype was identified in calves (Plutzer and Karanis, 2007; Soba and Logar, 2008). Moreover, experimentally infected adult gerbils were susceptible to infection with mentioned subtype (Kváč et al., 2009c). These results imply that some subtypes of *C. parvum* could be infectious to both juvenile and adult animals.

The variable occurrence of *Cryptosporidium* infections in cattle according to the season is not usually observed. No difference either among the four seasons in central Europe, or between the wet and dry part of the year, were found. Regarding the indoors housing of cows during the entire year, prevalence variability was not expected. Infection intensity of both *C. bovis* and *C. parvum* was low. Although various infection intensities of *C. andersoni* were described, sporadic infections were recorded most often. The data of infection intensity is not usually mentioned in other studies, but it is an important indicator considering the evaluation of the course of animal disease. In the future more attention should be given to this matter.

Cryptosporidium bovis, *C. ryanae* and *C. andersoni* are not significant causes of human diseases. Therefore the older calves and old cows are not likely to be a public health concern. As stated previously (Atwill et al., 1999), it is likely that the contribution of cattle (both dairy and beef) to human cryptosporidiosis is limited to calves less than 2 months of age.

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