Cryptosporidium parvum, Cryptosporidium ryanae, and Cryptosporidium bovis in samples from calves in Austria

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Abstract
Fecal samples of 177 calves of up to 180 days of age with diarrhea from 70 farms in Austria were examined to obtain information on the occurrence of Cryptosporidium species. Initially, all samples were examined by phase-contrast microscopy. Cryptosporidium-positive samples (55.4%; n = 98) were screened by gp60 PCR, resulting in 68.4% (n = 67) C. parvum-positive samples. The remaining 31 gp60-PCR-negative and the phase-contrast microscopy negative samples (n = 79) were screened by PCR targeting a 700 bp fragment of the 18S rRNA gene. Sequencing of the PCR products revealed the presence of C. parvum (n = 69), C. ryanae (n = 11), and C. bovis (n = 7). The latter two species have never been described in Austria. C. parvum-positive samples were genotyped at the gp60 gene locus, featuring four subtypes (IIaA15G2R1, IIaA21G2R1, IIaA19G2R1, IIaA14G1R1). The most frequently detected subtype IIaA15G2R1 (n = 52) was present in calves from 30 different farms. IIaA14G1R1 (n = 5) occurred on a single farm, subtype IIaA21G2R1 (n = 4) on two farms, and subtype IIaA19G2R1 (n = 4) on three farms. The results confirm the widespread occurrence of zoonotic C. parvum in diarrheic calves.

Keywords  Protozoal infection • gp60 • 18S • Fecal consistency

Introduction
To date, 38 Cryptosporidium species are known, of which four—C. parvum, C. bovis, C. ryanae, and C. andersoni—can be found in cattle. Cryptosporidium parvum and C. bovis are responsible for over 90% of bovine infections (Feng et al. 2018; Widmer et al. 2020). Cryptosporidium parvum is associated with diarrhea in neonatal calves and intra-herd prevalence extends up to 100% (Avendano et al. 2018; Holzhausen et al. 2019; Thompson et al. 2017). Cryptosporidium bovis and C. ryanae are primarily found in the feces of pre-weaned calves and C. andersoni in the abomasum of adult cattle (Ryan et al. 2014). Nevertheless, C. ryanae and C. bovis were isolated from pre-weaned diarrheic and healthy calves in certain areas of Sweden, China, and Sudan (Silverlås et al. 2010; Taha et al. 2017; Wang et al. 2011). Human cryptosporidiosis is primarily caused by C. parvum and C. hominis, and diarrheic and healthy calves are considered major reservoirs for human infections (Razakandrainibe et al. 2018; Ryan et al. 2014). Cryptosporidium oocysts are microscopically indistinguishable due to their similar size and shape. Therefore, molecular methods are indispensable for species differentiation (Ryan et al. 2014). Previous investigations focusing on the typing of C. parvum by sequencing a section of the 60-kD glycoprotein (gp60) gene in calves primarily report the occurrence of the subtype families IIA and IId with some geographical differences (Feng et al. 2018; Ryan et al. 2014). Subtype family IIA dominates in industrialized nations such as Italy (Díaz et al. 2018), the USA (Xiao et al. 2007), New Zealand (Abeywardena et al. 2012), and Austria (Lichtmannsperger et al. 2019). Subtype family IId was commonly reported from less industrialized countries (Ryan et al. 2014) such as Sudan (Taha et al. 2017), Malaysia (Muhid...
et al. 2011), Egypt (Amer et al. 2013), and China (Wang et al. 2011). Subtype IaA15G2R1 has been described as the predominant subtype in symptomatic and asymptomatic calves worldwide (Feng et al. 2018; Holzhausen et al. 2019).

Differentiation of Cryptosporidium on species and subtype level is apparently lacking in Austria. The aim of this study was to determine the occurrence of different Cryptosporidium species and genotypes in calves with diarrhea less than 180 days of age. It was hypothesized that besides C. parvum, other Cryptosporidia species occur in feces of diarrheic calves in Austria.

**Material and methods**

**Sample collection and microscopic examination**

Farmers and veterinarians from all over Austria were contacted and asked to participate in the study. In total, 177 calves with diarrhea originating from 70 farms were included. The fecal samples used in this investigation were collected during the study on the occurrence of C. parvum and Giardia intestinalis in diarrheic calves in Austria (Lichtmannsperger et al. 2019). Samples were collected per rectum during a farm visit. All calves younger than 180 days of age with diarrhea (soft, liquid, or watery feces) were included and sampled once by the first author or the local veterinarian. The samples were transferred to the Institute of Parasitology at the University of Veterinary Medicine Vienna for immediate diagnostics. All samples were screened for Cryptosporidium spp. by phase-contrast microscopy (PCM) as described previously (Lichtmannsperger et al. 2019). In brief, sample purification was performed using the sodium-acetate-acetic formalin (SAF) method, and the pellet was resuspended in phosphate-buffered saline. The suspension was filled into the chamber of a disposable hemocytometer, and oocysts were counted using PCM with 200-fold magnification. The number of oocysts was given in oocysts per gram feces (opg).

**DNA extraction and PCR for genotyping**

DNA was extracted from all diarrheic fecal as described previously (Lichtmannsperger et al. 2019). For the detection of all Cryptosporidium spp. except C. parvum, a nested PCR protocol was implemented to amplify a 700 bp fragment of the nuclear 18S rRNA gene (18S). For genotype analysis of C. parvum, a 450 bp section of the gp60 gene was amplified. The 18S PCR was performed on all PCM-negative and gp60-negative samples. The primers were designed based on complete or almost complete 18S sequences of various apicomplexan parasites mined from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The quality of the primers was tested using AmplifX v.2.0.7. (Nicolas Julien; https://inp.univ-amu.fr/en/amplifx-managetest-and-design-your-primers-for-pcr) and Primer-BLAST implemented in NCBI GenBank. The primers (Table 1) are specific to the genus Cryptosporidium and do not amplify the 18S of other apicomplexan parasites. The reaction volume (25 μl) contained 1 μl of genomic DNA template, 14.375 μl nuclease free water, 5.0 μl of 5X Green GoTaq® Reaction Buffer (Promega, USA), 2.0 μl of 25 mM MgCl2, 0.5 μl of 10 mM dNTP mix, 0.125 μl of 5X Green GoTaq G2® Polymerase (5 U/μl, Promega), and 1.0 μl each of 10 mM oligonucleotide primers (Table 1). For the second PCR round, 0.5 μl template from the previous PCR was used. The cycling protocol for both reactions included an initial cycle of 94 °C for 2 min, followed by 20 (nest 1)/35 (nest 2) cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, and a final extension of 72 °C for 5 min.

The gp60 PCRs were carried out on all samples positive by phase-contrast microscopy as described previously (Lichtmannsperger et al. 2019). In brief, 1 μl of genomic DNA was used in a 25 μl reaction volume with 13.675 μl of nuclease free water, 5.0 μl of 5X Green GoTaq® Reaction Buffer (Promega, USA), 2.0 μl of 25 mM MgCl2, 0.5 μl of 10 mM dNTP mix, 0.125 μl of 5X Green GoTaq G2® Polymerase (5 U/μl, Promega), and 1.0 μl each of 10 mM oligonucleotide primers (Table 1). The second PCR round, 0.5 μl template from the previous PCR was used. The cycling protocol for both reactions included an initial cycle of 94 °C for 2 min, followed by 20 (nest 1)/35 (nest 2) cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, and a final extension of 72 °C for 5 min.

**Table 1** Primers utilized in nested PCR reactions amplifying sections of the 18S of Cryptosporidium spp. and the gp60 of Cryptosporidium parvum from fecal samples. The 18S primers and the protocol were designed for this investigation; the implemented protocol for the detection of gp60 was described previously by Peng et al. (2001)

| Primer | Primer sequence (5’-3’) | Amplicon size (bp) | Annealing (°C) |
|--------|------------------------|--------------------|---------------|
| 18S    |                        |                    |               |
| Crypto18S_ F1 | for: ACATATCATTCAAGTTTCTG ACCTATC | 766 | 56 |
| Crypto18S R1 | rev: TCTCATAAGGTGCTGAAGGAGT |                |               |
| Crypto18S F2 | for: CAGCTTTAGACGGTGTTAG | 740 | 56 |
| Crypto18S R2 | rev: TAAGGTGCTGAAGGAGTAAG GAAC |             |               |
| gp60   |                        |                    |               |
| AL3531 | for: ATAGTCTCCGCTGATTCC | 850 | 56 |
| AL3534 | rev: GCAGAGGAAACGACATC |                |               |
| AL3532 | for: TCCGCTGATTTCTCAGCC | 450 | 60 |
| AL3533 | rev: GAGATATATCTTTGTCGG |             |               |
Buffer (Promega), 0.2 μl of 25 mM dNTPs, 3.0 μl of 25 mM MgCl₂, 0.125 μl of GoTaq® G2 DNA Polymerase (5 U/μl), and 1 μl each of 20 pmol oligonucleotide primers (Table 1). For the second PCR round, 0.5 μl template from the previous PCR was used. The cycling protocol for both reactions included one cycle of 94 °C for 2 min, followed by 30 cycles of 95 °C for 50 s, 56 °C (nest1)/60 °C (nest 2) for 50 s, 65 °C for 60 s, and a final extension of 65 °C for 5 min.

PCR products were subjected to electrophoresis on 2.0% agarose gels and visualized with ultraviolet light (LumiBIS 1.4, DNR Bio-Imaging Systems Ltd., Israel).

### Sequencing of PCR products

Purification and sequencing in both directions was done at LGC Genomics GmbH (Berlin, Germany). The raw forward and reverse sequences (and electropherograms) were carefully checked and aligned with Bioedit v.7.0.8.0 (Hall 1999). Sequences were subjected to BLAST (https://blast.ncbi.nlm.nih.gov/Blast) searches at NCBI GenBank to identify the respective gp60 and 18S variants. All sequences were deposited in NCBI GenBank under the accession numbers (18S: MT611069–MT611099; gp60: MT637080-MT637083).

### Statistical analysis

The data were organized using IBM® SPSS® Statistics Version 24 (IBM, New York, USA). Normal distribution was calculated using the Kolmogorov-Smirnov test. A chi² test was implemented for the comparison of categorical variables (fecal consistency). The mean C. parvum, C. ryanae, and C. bovis shedding was only calculated with samples confirmed by gp60 or 18S PCR. The age differences in calves shedding C. parvum, C. ryanae, and C. bovis were analyzed by using a one-way ANOVA and the post hoc Bonferroni correction for multiple testing. Differences were considered statistically significant if $p \leq 0.05$.

### Results and discussion

One to 10 animals were sampled per farm (median = 2; mean = 2.5). The fecal consistency of the diarrheic calves appeared soft ($n=72$), liquid ($n=82$), or watery ($n=23$). The age ranged from 1 to 164 days (median = 12; mean = 27). The average oocyst shedding ($n=98$) was $1 \times 10^5$ opg (range = $3.0 \times 10^3$–$3.0 \times 10^7$; median = $1.0 \times 10^6$; SD = $3.0 \times 10^6$) (Lichtmannsperger et al. 2019).

Previously, the widespread occurrence of Cryptosporidium spp. in diarrheic calves from Austria was described, but without differentiation at the species and subtype levels (Lichtmannsperger et al. 2019). All PCM-positive samples ($n=98$) were screened using the gp60 PCR, of which 68.4% ($67/98$) yielded positive results. The remaining gp60-negative samples ($n=31$) were screened for Cryptosporidium spp. using the 18S PCR assay (see Fig. 1 for details). All gp60-positive samples were further sequenced to determine C. parvum subtypes. Four subtypes (IlaA15G2R1, IlaA14G1R1, IlaA21G2R1, IlaA19G2R1) were detected.
The most frequently detected subtype was IIA15G2R1 (n = 52) which was found on 30 farms. IIA14G1R1 (n = 5) occurred on a single farm, subtype IIA21G2R1 (n = 4) on two farms, and subtype IIA19G2R1 (n = 4) on three farms. Subtype IIA15G2R1 is the predominant subtype in symptomatic and asymptomatic calves worldwide, which was in accordance with our findings (Feng et al. 2018). A high subtype diversity but endemicity of a single subtype within herds or regions has previously been found in areas where animal movement is limited (Brook et al. 2009; Silverlås et al. 2010). Due to the implemented study design (sample size calculation, randomization), information concerning on-farm prevalence of subtypes is limited. Authors from Sweden reported similar observations of the on-farm-specific occurrence of C. parvum subtypes and assume that this was due to the dominating closed herd management systems (Silverlås et al. 2010). Closed herd management systems are common due to the small structured agriculture in Austria, which might be the reason for the similar results.

For the detection of further C. parvum species, the 18S PCR assay was performed on all PCM-negative and all gp60-negative samples (Fig. 1). In total, C. parvum (n = 5), C. ryanae (n = 11), and C. bovis (n = 7) were detected. One sample contained both C. ryanae and C. bovis. The presence of both species in this sample was evident by double peaks in the electropherograms. Since the 18S section analyzed was of same length in C. ryanae and C. bovis, the distinction of the two haplotypes was straightforward. Sequence analysis failed in four samples, which could be due to the presence of multiple Cryptosporidium strains.

The age of calves positive for C. parvum (n = 72) ranged from 3 to 127 days (mean = 14.5; median = 10.0), 9 to 126 (mean = 43.5; median = 35.0) for C. ryanae (n = 10), and 11 to 119 (mean = 64.6; median = 60.0) for C. bovis (n = 7). The age of the calves shedding C. parvum was significantly lower than from calves shedding C. bovis or C. ryanae (p = 0.00; p = 0.022). Between C. bovis and C. ryanae shedding calves, the age difference was not statistically significant (p = 0.866). The average number of oocysts shed by diarrheic calves was 1.7 × 10^6 (range = 5.0 × 10^3 to 2.6 × 10^7; median = 4.4 × 10^5) for C. parvum (n = 69), 1.1 × 10^4 (range: 2.5 × 10^3 to 2.3 × 10^4; median = 10^4) for C. ryanae (n = 4), and 1.3 × 10^4 (range = 2.5 × 10^3 to 2.8 × 10^4; median = 1.0 × 10^4) for C. bovis (n = 3). Oocysts of C. bovis and C. ryanae were shed in lower numbers in comparison to C. parvum. However, the number of excreting animals was too low for statistical comparison.

Some authors report a higher prevalence of Cryptosporidium spp. when using light microscopy, which is in accordance with our findings (Taha et al. 2017). A potential explanation might be that some of the microscopy-positive samples were wrongly classified due to the morphologically similar appearance of other particles such as yeast spores (Taha et al. 2017).

Genetic characterization of diarrheic fecal samples using the 18S PCR assay showed the occurrence of C. ryanae and C. bovis in young diarrheic calves. The youngest animals were 9 and 11 days old, respectively. The results are in accordance with other studies, where C. bovis was found in calves from 5 days of age and C. ryanae from the second week of live (Åberg et al. 2019; Wang et al. 2011). Considering Cryptosporidium PCR results (gp60 or 18S; n = 93), the occurrence of C. parvum (77.4%), C. ryanae (11.8%), and C. bovis (7.5%) is comparable to an investigation from the Sudan on young (<6 months) diarrheic calves, which featured C. parvum (73.5%), C. ryanae (13.2%), and C. bovis (1.8%) (Taha et al. 2017). Additionally, C. andersoni was detected, which was not the case in the present study. Since only gp60-negative samples were screened with the 18S PCR assay, the occurrence of C. ryanae and C. bovis was likely underestimated in the present sample.

C. parvum occurred significantly more often (p = 0.007) in animals with liquid or watery diarrhea (n = 105) versus animals showing softened feces (n = 72). Cryptosporidium ryanae and C. bovis exclusively occurred in animals with soft (n = 8) respectively liquid (n = 9) fecal consistency; none of the animals showed watery diarrhea. Cryptosporidium bovis was discussed as potentially pathogenic in a study in Sweden, where C. bovis was found in diarrheic calves as the only pathogen (Silverlås et al. 2013). Another investigation found no association between the presence of diarrhea and C. bovis or C. ryanae shedding (Åberg et al. 2019).

The results show the common occurrence of the zoonotic species C. parvum and the host-specific C. ryanae and C. bovis in diarrheic calves in Austria. C. parvum–infected calves are shedding high numbers of oocysts which leads to severe environmental contamination and further transmission. Young calves suffering from liquid or watery diarrhea must be considered C. parvum shedders and therefore have the potential to cause human infection. Due to the implemented methodology, the simultaneous occurrence of Cryptosporidium species cannot be excluded. The number of C. bovis and C. ryanae positive samples was probably underestimated in the examined samples. Molecular methods such as genotype-specific or multiplex PCR procedures should shed more light on the occurrence of coinfections with different Cryptosporidium species or genotypes.

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**Compliance with ethical standards**

This trial was evaluated and approved by the institutional Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna.

**Conflict of interest** The authors declare that they have no conflict of interest.

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