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Molecular characterization of *Giardia intestinalis* and *Cryptosporidium parvum* from calves with diarrhoea in Austria and evaluation of point-of-care tests

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ABSTRACT

To obtain information about the occurrence and genotype distribution of *G. intestinalis* and *C. parvum* in Austrian cattle, faecal samples from diarrhoeic calves younger than 180 days of age originating from 70 farms were examined. Of the 177 faecal samples, 27.1% were positive for *Giardia* cysts (immunofluorescence microscopy) and 55.4% for *Cryptosporidium* oocysts (phase-contrast microscopy). Positive samples were characterized by nested PCR for *Giardia*, 83.3% (*triosephosphate isomerase; tpi*) and 89.6% (*β-giardin; bg*) were positive, while the *Cryptosporidium* nested PCR returned 92.5% (60-kDa glycoprotein) positive results. Sequence analysis revealed one assemblage A-positive sample and 30 (*bg*) respectively 29 (*tpi*) assemblage E-positive samples for *G. intestinalis*. For *C. parvum* four subtypes within the IIa family (IIaA15G2R1, n = 29; IIaA19G2R2, n = 3; IIaA21G2R1, n = 2; IIaA14G1R1, n = 1) could be differentiated. Validation of two immunochromatographic point-of-care tests resulted in a sensitivity of 29.2% and 77.6%; a specificity of 98.4% and 91.1% for the detection of *Giardia intestinalis* and *Cryptosporidium parvum*, respectively. Results confirm the widespread occurrence of both protozoa in diarrhoeic calves in Austria.

1. Introduction

Cryptosporidium parvum and *Giardia intestinalis* (syn. *G. duodenalis, G. lamblia*) are protozoal intestinal parasites which cause mild to severe diarrhoea in humans and infect a wide range of animals including, livestock, pets and wild animals [1,2]. The resilient environmental stages of the parasites (oocysts and cysts, respectively) are transmitted by faecal-oral route via contaminated food or water [3,4]. Mammalian infections with *G. intestinalis* are caused by different genotype assemblages (A to H) [5]. Humans can become infected by a wide range of *Cryptosporidium* and *Giardia* species and genotypes, and cattle are considered to be a major contributor to zoonotic transmission [6,7].

G. intestinalis genotype assemblages A (*G. intestinalis* sensu stricto) and B (*G. enterica*) have a low host specificity, infecting a wide range of mammals including primates, dogs, cats, livestock, rodents and other wild mammals, while genetic characterization of genotype assemblages C–H revealed a host-specific occurrence [8]. Cattle are primarily infected with *G. intestinalis* assemblage E [9–11] but assemblages A and B can also be detected [9,12,13]. The latter cause the majority of human infections worldwide [5,14,15] but assemblage E can also occasionally be found in humans [16–18]. *G. intestinalis* can be found both in diarrhoeic and non-diarrhoeic calves so its role as a pathogen in cattle is unclear [13,19].

Molecular investigations based on multiple genetic loci for sequencing showed variations within the assemblages described as sub-assemblages [8]. In assemblage A several host-specific sub-assemblages occur. Sub-assemblage AIII is presumably host-specific and occurs predominantly in wild animals [20,21].

Currently, 31 *Cryptosporidium* species are recognized, and *C. hominis* and *C. parvum* are responsible for the majority of human cryptosporidiosis cases [6,22]. Cattle have been described as a main host for *C. bovis, C. andersoni*, *C. parvum*, *C. ryanae*, and the *Cryptosporidium* deer-like genotype [23] and show age-related susceptibility to the different species. *C. bovis* and *C. andersoni* occur primarily in post-weaned calves, *Cryptosporidium* deer-like genotype in older calves and adults [22,24]. *C. parvum* infects pre-weaned calves and can cause severe disease [25,26]. *C. parvum* subtype family IIa is the predominant subtype family in calves worldwide in countries with intensive cattle farming, such as Canada [27], USA [28], England [29], Spain [25], New Zealand [30] and Italy [31], and subtype IIaA15G2R1 is the most frequently

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reported subtype worldwide [32]. Subtype family IId was described from Sweden [33], Egypt [34], China [35] and Malaysia [36].

In Austria only limited information about *Giardia* and *Cryptosporidium* infections of cattle is available from conventional flotation examination without genotyping data [19,37].

The aim of this study was to obtain detailed information about the occurrence of *Giardia* and *Cryptosporidium* in diarrhoeic calves from Austria and to further characterize the pathogens at the molecular level. Additionally, commercially available point-of-care tests for the detection of *Giardia* and *Cryptosporidium* in cattle were validated.

It was hypothesized that diarrhoeic calves from Austria harbour *Giardia* and *Cryptosporidium* genotypes/subtypes which have the potential to cause human infection, and that immunochromatographic point-of-care tests are valid methods for the detection of these parasites in calf faeces.

2. Material and methods

2.1. Samples and sample processing

Convenience diarrhoeic faecal samples were collected per rectum from dairy and beef calves less than 180 days of age from November 2017 to July 2018. Farmers and veterinarians from all over Austria were invited to participate in the study. All participating farms were visited once. On the farm two commercial immunochromatographic point-of-care tests for the detection of *G. intestinalis* (FASTest® Giardia Strip, Megacor, Austria) and *C. parvum* (FASTest® Crypto Strip, Megacor, Austria) were carried out according to the manufacturer's instructions by the local veterinarian or one of the authors (KL). Before testing, all samples were homogenized with a wooden spatula in a 20 ml faecal collection cup. Faecal samples were transferred to the University Clinic for Ruminants Vienna and processed immediately. The SAF (sodium acetate-acetic acid-formalin solution) method was used for purifying faecal samples [38]. Briefly, 1 *g* of homogenized sample material was diluted with 10 ml SAF solution, filtered, centrifuged (2 min, 500 x *g*) and the supernatant was discarded. After mixing of 8 ml of 0.9% sodium chloride solution and 3 ml diethyl ether, the sample was centrifuged again. The supernatant was then discarded and the pellet was re-suspended in 1 ml PBS (phosphate-buffered saline; pH 7.2), vortexed and used for further microscopical examination.

2.2. Microscopical detection of Giardia and Cryptosporidium

For the detection of *Giardia* the immunofluorescence assay (IFA) Merifluor® (Merifluor® *Cryptosporidium/Giardia* Meridian Bioscience Inc., USA) test was performed according to the manufacturer's instructions. Briefly, 60 µl of each purified sample was transferred to the slide cavity and air-dried. After incubation with the staining and counterstaining reagents, the slide was washed, covered and screened for *Giardia* cysts (appearing bright green) under a fluorescence microscope (Olympus AX 70, Olympus Optical Co., LTD., Japan) with 200 x magnification. Cysts were counted using a hand counter. The amount of cysts per gram of faeces (cpg) was calculated (cpg = counted cysts divided by 0.06 as the counted volume was 60 µl). Excretion rates were categorized as low ($< 10^3$ cpg), moderate (10^3-10^4 cpg) or high ($> 10^4$ cpg).

For the detection of *Cryptosporidium* the purified sample was transferred to the chamber of a disposable haemocytometer (C-Chip, NanoEnTek Inc., USA). After 5 min the slide was screened under a phase-contrast microscope (PCM) (Nikon Labophot-2, Nikon Instruments Inc., Japan) with 200 × magnification for oocysts. Based on the mean value, the amount of oocysts per gram of faeces (opg) was calculated (opg = counted oocysts × 10^4 as the counted volume was 0.1 µl) and categorized as low ($\leq 10^4$ opg) or high ($> 10^4$ opg) excretion rates.

2.3. DNA extraction

For molecular analysis of *Giardia* (all IFA positive samples, n = 48) approximately 200 mg of purified flotate from flotation with standard saturated sugar solution (specific gravity 1.3), washed three times with tap water in a 50 ml tube were subjected to DNA extraction. For DNA extraction of selected PCM-positive *C. parvum* samples (n = 40), whole faeces was used. Extraction was performed using the NucleoSpin® Soil kit (Macherey-Nagel GmbH & Co. KG, USA) according to the manufacturer's specifications. In brief, lysis buffer (SL1) was used for sample preparation without lysis condition adjustment, and DNA elution was carried out using 100 µl of elution buffer. The remaining steps were performed according to the manufacturer's instructions.

2.4. PCR for genotyping

A 530 bp fragment of the triosephosphate isomerase (*tpi)* gene for *Giardia* was amplified by nested PCR [39]. The cycling protocol for both reactions included an initial cycle of 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 60 s and a final extension of 72 °C for 10 min. The reaction volume was 50 µl containing 1 µl of genomic DNA template, standard PCR buffer (5xGreen GoTaq® Reaction Buffer, Promega USA), 10 mM of each dNTP, 50 mM $MgCl_2$, 1.25 U of Taq polymerase (GoTaq® G2 DNA Polymerase, Promega, USA) and 25 pmol of each oligonucleotide primer (Table 1).

A region within the *β*-giardin (*bg*) gene of *Giardia* was amplified using an established nested PCR protocol (by Lalle et al., 2005) [40]. Briefly, the cycling protocol for the first reaction included one cycle of

Table 1
Primers utilized in nested PCR reactions amplifying *tpi*, *bg* and SSU rRNA of *Giardia* and *gp60* of *Cryptosporidium parvum* from faecal samples.

	Primer	Primer sequence (5'-3')	Amplicon size (bp)	Annealing (°C)	Reference
tpi	AL3543	for: AAATTATGCCTGCTCGTCG	605	50	Sulaiman et al., 2003 [39]
	AL3546	rev: CAAACCTTTTCCGCAAACC			
	AL3544	for: CCCTTCATCGGIGGTAACTT	530	50	
	AL3545	rev: GTGGCCACCACICC CGTGCC			
bg	G7	for: AAGCCCGACGACCTCACCCGCAGTGC	753	65	Lalle et al., 2005 [40]
	G759	rev: GAGGCCGCCCTGGATCTTCGAGACGAC			
	GiarF	for: GAACGAGATCGAGGTCCG	511	55	
	GiarR	rev: CTCGACGAGCTTCGTTGTT			
SSU rRNA	RH 11	for: CATCCGGTCGATCCTGCC	292	59	Hopkins et al., 1997 [41]
	RH 4	rev: AGTCGAACCCTGATTCTCCGCCCAGG			
	GiarFor	for: GACGCTCTCCCCAAGGAC	130	59	Read et al., 2002 [42]
	GiarRev	rev: CTGCGTCACGCTGCTCG			
gp60	AL3531	for: ATAGTCTCCGCTGTATTC	850	56	Peng et al., 2001 [43]
	AL3534	rev: GCAGAGGAACCAGCATC			
	AL3532	for: TCCGCTGTATTCTCAGCC	450	60	
	AL3533	rev: GAGATATATCTTGGTGCG			

95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 65 °C for 30 s, 72 °C for 60 s and a final extension of 72 °C for 7 min. The annealing temperature of the secondary reaction was reduced to 55 °C for 30 s. Five microliters of genomic DNA was used for both reactions in a final volume of 25 µl containing standard PCR buffer (5xGreen GoTaq® Reaction Buffer, Promega USA), 1.25 U of Taq polymerase (GoTaq® G2 DNA Polymerase, Promega, USA), 25 mM dNTPs and 10 pmol of each primer (Table 1).

In cases when PCR results were negative or inconclusive, PCR was repeated with native faecal material (*tpi*: n = 16; *bg*: n = 17).

Tpi negative *Giardia* samples were further analysed amplifying a fragment of the small subunit rRNA (SSU rRNA) using nested PCR [41,42]. In a 20 µl reaction volume 5 µl of genomic DNA, standard PCR buffer (5xGreen GoTaq® Reaction Buffer, Promega USA), 25 mM dNTPs, 1.25 U Taq polymerase (GoTaq® G2 DNA Polymerase, Promega, USA) and 25 pmol of each primer was used (Table 1). The cycling protocol for both reactions included one cycle of 95 °C for 2 min, followed by 35 cycles of 96 °C for 20 s, 59 °C for 20 s, 72 °C for 30 s and a final extension of 72 °C for 7 min.

For genotype analysis of *C. parvum,* a 60-kD glycoprotein (*gp60*) gene fragment was amplified [43]. One microliter of genomic DNA was used in a 20 µl reaction volume with standard PCR buffer, 25 mM dNTPs, 25 mM $MgCl_2$, 1.25 U Taq (GoTaq® G2 DNA Polymerase, Promega, USA) and 20 pmol of each primer. The cycling protocol included one cycle of 94 °C for 2 min, followed by 30 cycles of 95 °C for 50 s, 56 °C for 50 s, 65 °C for 60 s, and a final extension of 65 °C for 5 min. For nested PCR annealing temperature was increased to 60 °C and 0.5 µl DNA template from the previous PCR round was used.

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

2.5. Sequencing of PCR products

Twenty microliter of the PCR products and 5 pmol of the appropriate primer were prepared on a mirror plate for subsequent sequencing in both directions (LGC Genomics GmbH, Berlin, Germany). The quality of each sequence was assessed and edited using ChromasLite® 2.1.1 (Technelysium, Brisbane, Australia) and trimmed as required. Sequences were compared with reference sequences in GenBank® using the Basis Local Alignment Searching Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast). The obtained sequences are deposited in GenBank® under MK202953-MK202973 (Tables 2 and 3).

Table 2
Results of *G. intestinalis* genotype analysis and corresponding Genbank® accession numbers. Six different sequences were obtained at a *β*-giardin (*bg*) and triosephosphate isomerase (*tpi*).

Assemblages	N Samples	Generated accession numbers	Most similar sequence in BLAST; Identity in percent
A (*tpi*)	1	MK202973	KU531717; 99.7%
A (*bg*)	1	MK202958	KR051225; 100%
E (*tpi*)	1	MK202968	MH158498; 100%
	1	MK202972	MH158491; 100%
	4	MK202964	MH158495; 100%
	1	MK202969	MH158505; 99.5%
	1	MK202971	MH158497; 100%
	21	MK202965-MK202967; MK202970	MH158505; 100%
E (*bg*)	2	MK202953	KY633466; 100%
	21	MK202954	AY655703; 100%
	1	MK202955	MH158454; 100%
	4	MK202956	DQ116624; 99.8%
	1	MK202957	MH158455; 99.8%
	1	MK202959	MH158454; 99%

Table 3
C. parvum (*gp60)* typing, generated GenBank® accession numbers and homologous references sequence accession numbers in GenBank®.

Subtype	N samples	Generated accession numbers	Most similar sequence in BLAST; Identity in percent
IIaA15G2R1	29	MK202963	MK095339; 100%
IIaA19G2R1	3[a]	MK202962	HQ149039; 100%
IIaA21G2R1	2	MK202961	DQ648535; 100%
IIaA14G2R1	1	MK202960	JQ026103; 100%

[a] incl. two animals from the same farm.

2.6. Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics Version 24 (IBM®, New York, USA) and Microsoft Excel 2010. Descriptive statistics were performed on all completed records and results were expressed as mean, median, standard deviation (SD) and range (minimum to maximum). IFA results were used as a reference for the calculation of performance criteria of the FASTest® Giardia and PCM results for the validation of FASTest® Crypto. Cohen's Kappa was used to evaluate agreement between the reference and the point-of-care tests. Kappa values range from 1 to 0 and were interpreted as follows: > 0.81 very good agreement, 0.61–0.80 good agreement, 0.41–0.60 moderate agreement, 0.21–0.40 fair agreement and ≤0.2 poor agreement [44].

To describe the differences in parasite occurrence between age groups a Chi-square test was applied. Kolmogorov-Smirnov tests were performed to test for normal distribution of cyst and oocyst shedding between age groups. For normally distributed values a *t*-test and for not normally distributed variables a Mann-Whitney U test was used. All tests were calculated with a significance level of $p < 0.05$.

3. Results

3.1. Sample distribution

In total 177 faecal samples from calves with diarrhoea were collected from 70 farms located in Burgenland (n = 6), Upper Austria (3), Lower Austria (24), Salzburg (26), Styria (7) and Tyrol (4). The majority of the calves belonged to Simmental breed (n = 140), the other were Holstein (8), Brown Swiss (1) or cross breeds (27). Samples originated from 57 dairy farms (135), three cow-calf operations (8), three calf-rearing operations (6), three beef farms (14) and four mixed farms (beef and dairy operation) (14). On average 2.5 calves were sampled per farm (range = 1–10, median = 2). The youngest calf was one day old, the oldest 164 days (mean = 27, median = 12). The majority (66.7%) of sampled calves was ≤ 21 days old. Five calves were older than 17 weeks. Two calves were 18 weeks, two 19 weeks and one calf was 23 weeks old (Fig. 1).

3.2. Microscopical analysis (IFA and PCM)

Upon IFA and PCM examination of the 177 faecal samples, 48 (27.1%) were positive for *Giardia* cysts and 98 (55.4%) for *Cryptosporidium* oocysts. *Giardia* and *Cryptosporidium* co-infections were observed in 21 calves originating from 21 different farms.

Cryptosporidium was significantly more frequent in the first two weeks of age (70.6% of 102 samples compared to 38.7% of 75 samples from calves older than two weeks; Fig. 1) ($p < 0.001$) while *Giardia* occurred significantly more often in animals of three weeks and older (58.7% compared to 6.9% in younger animals; Fig. 1) ($p < 0.001$).

Giardia excretion was low in 43.8%, moderate in 33.3% and high in 22.9% of the positive samples, with 17–76,333 cpg (mean = 10,108; median = 1308; SD = 19,244). There was no significant difference in cyst shedding between calves younger or older than three weeks of age ($p = 0.68$).

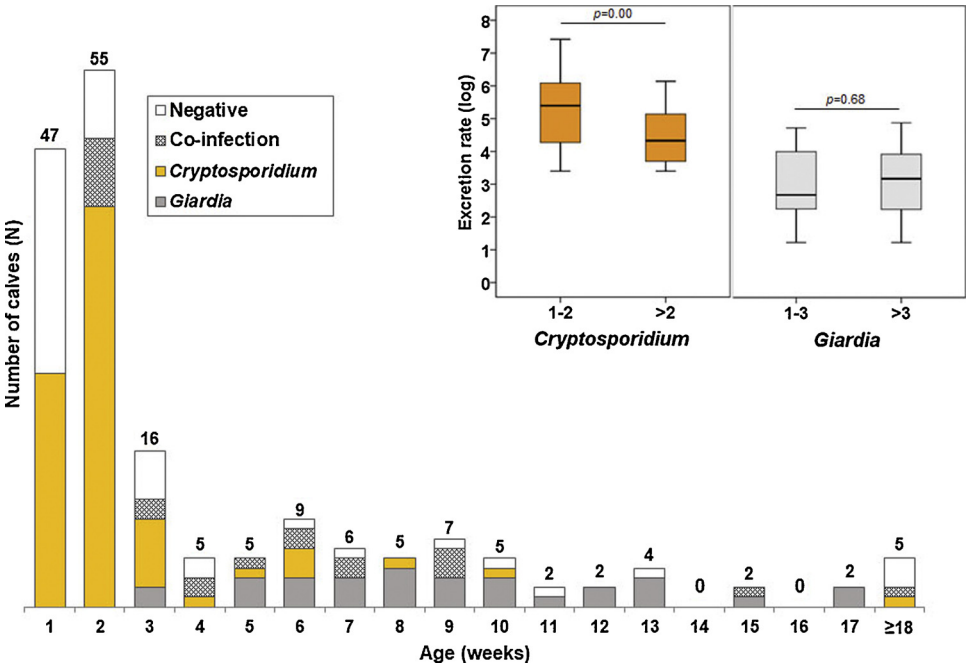

Fig. 1. Age distribution categorized in weeks of life of the sampled calves (n = 177). The bars show the absolute number of animals in relation to their infection status (immunofluorescence microscopy for *Giardia* and phase-contrast microscopy for *Cryptosporidium*). Boxplots show oocyst and cyst excretion rates for *Cryptosporidium* and *Giardia,* respectively, in logarithmic representation. Calves younger than two weeks shed significantly more oocysts than older calves ($p = 0.00$); differences were not significant for *Giardia* cyst excretion ($p = 0.68$).

Cryptosporidium oocyst shedding was high in 70.4% of the positive samples, and opg values ranged between 3×10^3 and 3×10^7 opg (mean = 1×10^5; median = 1×10^6; SD = 3×10^6). Calves younger than two weeks of age shed significantly more oocysts than older calves ($p = 0.00$) (see Fig. 1).

3.3. Point-of-care test validation

In total 14/48 (29.2%) and 83/98 (84.7%) of the analysed samples yielded positive results in the FASTest® Giardia Strip and FASTest® Crypto Strip compared to IFA and PCM. The FASTest® Giardia Strip test validation yielded a sensitivity of 29.2% and a specificity of 98.4%. Fig. 2a shows the correlation between three different detection methods for *Giardia*. For interrater reliability of the IFA and the FASTest® Giardia Strip, Cohen's Kappa coefficient was calculated and yielded 0.35. Positive and negative predictive values were 87.5% and 78.9%, respectively. FASTest® Crypto Strip validation showed a sensitivity of 77.6%, a specificity of 91.1%, a positive predictive value of 91.6% and a negative predictive value of 76.6%. The agreement between PCM and FASTest® Crypto Strip was good (Cohen's Kappa value = 0.67; Fig. 2b).

3.4. Molecular characterization of Giardia positive samples

Molecular investigations on positive *Giardia* samples (n = 48) yielded positive results for 40 samples (83.3%) at the *tpi* locus and 43 (89.6%) at the *bg* locus (Fig. 3); 37 were positive for both. Eight samples negative at *tpi* locus were characterized targeting the SSU rRNA locus and revealed a further six positive samples. Two of these six samples were confirmed as *G. intestinalis* after sequencing. One sample positive by IFA yielded negative results at any locus targeted using both faeces and flotate as medium for DNA extraction. In total 23 specimen were sequenced at both loci (*bg* and *tpi*) resulting in 24 *G. intestinalis* genotype assemblage E and one *G. intestinalis* genotype assemblage A (see Table 2 for details).

3.5. Molecular characterization of Cryptosporidium positive samples

Forty *C. parvum* point-of-care test-positive samples with the highest concentration of oocysts from 39 different farms were characterized further by PCR and sequencing (Fig. 3). At the *gp60* locus 37/40 samples were confirmed as *C. parvum*. Thirty-five sequenced samples could be allocated to four subtypes within subtype family IIa, while sequencing was unsuccessful in the remaining two samples. Subtype IIaA15G2R1 was the most frequent and detected in 29 (82.9%) of the sequenced samples. Subtype IIaA19G2R1 was detected in three (8.6%) samples. Subtype IIaA21G2R1 was found in two (5.7%), subtype IIaA14G1R1 in one (2.9%) of the samples (Table 3). All samples positive for *Giardia* and *Cryptosporidium* showed *C. parvum* subtype IIaA15G2R1.

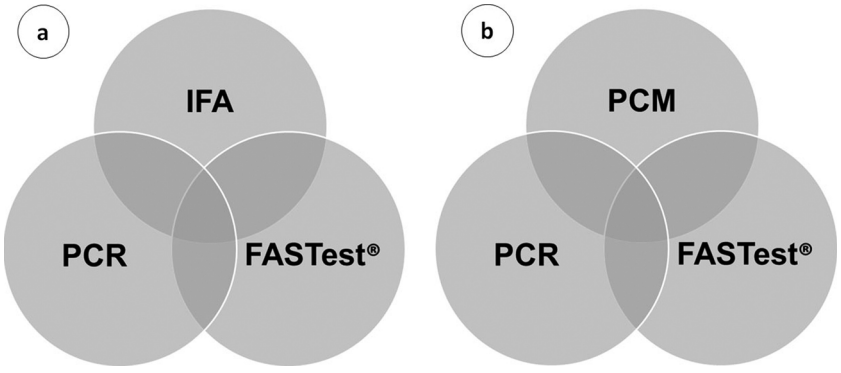

Fig. 2. a Venn diagrams illustrating results of three different detection methods. (a): Forty-eight faecal samples positive for *Giardia* in the IFA (Merifluor®) test. Samples were considered positive by PCR when amplified on any of the three investigated gene loci (*tpi, bg,* or *SSU rRNA*; for details see text). (b): Forty samples positive for *Cryptosporidium* by PCM and FASTest® (highest excretion rates) were selected for subsequent PCR analysis amplifying *gp*60.

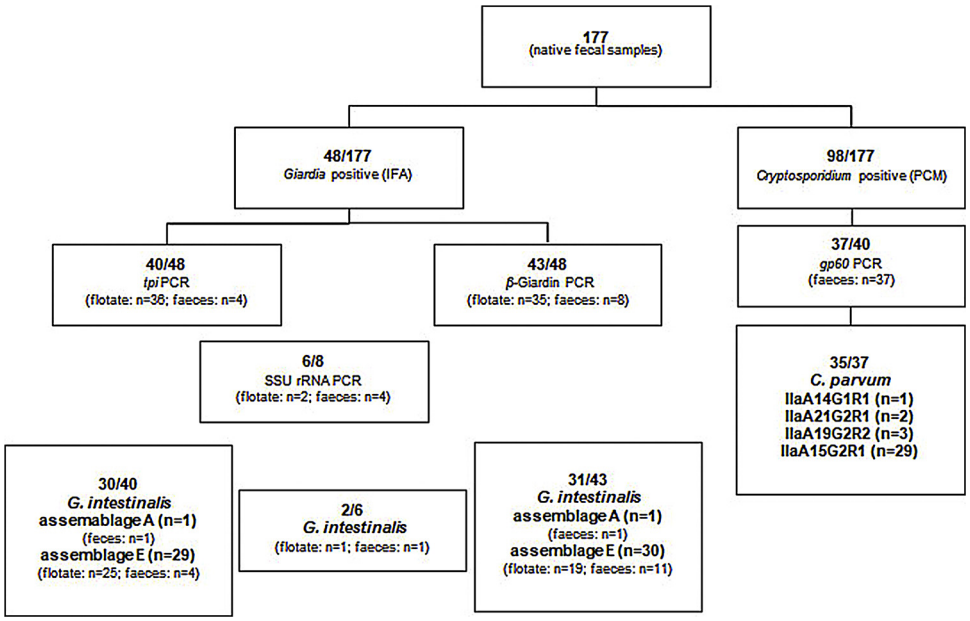

Fig. 3. Results of different methods implemented for the detection and characterization of *Giardia* and *Cryptosporidium* (PCM: phase-contrast microscopy, IFA: immunofluorescence assay, *tpi*: triosephosphate isomerase, *gp60*: 60-kD glycoprotein, SSU rRNA: small subunit ribosomal RNA). All microscopically positive *Giardia* (n = 48) and selected (rapid test-positive; n = 40) *Cryptosporidium* samples were investigated. Sequence analysis of the SSU rRNA locus only permits species but not genotype assemblage analysis so two samples could only be identified to species level by sequencing.

4. Discussion

In the present study, *Giardia* and *Cryptosporidium* were shown to be common intestinal protozoa in Austrian calves younger than six months with clinical signs of diarrhoea, and must be considered as causes of gastrointestinal disease in calves in this age group, although co-infections with other enteropathogens such as *Escherichia coli*, *Clostridium perfringens*, bovine rotavirus or bovine coronavirus cannot be excluded. Since Austrian farms are run as small-scale enterprises (with an average of 33 heads per farm in 2018; www.statistik-austria.at) the number of sampled calves per farm (2.5 on average) was limited. In addition, the protocol was restricted to single convenience samples from a wide age range of animals. Consequently, the occurrence of these protozoa on the farm level is most likely underestimated. A representative sample including diseased and healthy animals will be necessary to describe the true prevalence of *Giardia* and *Cryptosporidium* on Austrian farms and to draw conclusions on their epidemiology and their role as enteropathogens in Austrian calves.

Genetic characterization revealed the presence of the primarily host-specific *G. intestinalis* assemblage E and the zoonotic *C. parvum* subtype IIaA15G2R1 as the most frequent genotypes.

At the individual level 27.1% and 55.4% yielded positive results for *Giardia* (IFA) and *Cryptosporidium* (PCM) indicating a wide distribution of these two pathogens. This is in sharp contrast to a previous study on the possible causes of diarrhoea in calves from Austria which only detected 4.4–6.1% *Giardia*- and 11.7–25.6% *Cryptosporidium*-positive samples by sugar-flotation [19,37]. The use of more sensitive methods [45,46] in the current study is presumed to be the reason for this difference (rather than an increase in prevalence over a relatively short time), but an age-related effect cannot be excluded, since the majority of the calves sampled in the present study was three weeks or younger, while the animals formerly under study were up to six weeks old.

A strong age-dependence was observed for both pathogens. *Cryptosporidium* was detected mostly in the first two weeks of age while *Giardia* occurred more frequently in animals of three weeks and older. Age related infection rates have previously been shown with a peak at two weeks of age for *Cryptosporidium* and four to seven weeks for *Giardia* [47–49]. Consequently, double infections were observed primarily in calves of three to nine weeks of age, as previously reported [50].

Only 29.2% (14/48) of the IFA positive *Giardia* samples were positive in the FASTest® Giardia Strip results. Cohen's Kappa coefficient was 0.35, yielding a fair agreement between IFA and the FASTest® Giardia. However, the specificity of the FASTest® was high (98.4%), which is in accordance with a previous investigation which determined a low sensitivity (28% and 26%) but a high specificity (92% and 93%) of two point-of-care tests in comparison with the IFA for the detection of *Giardia* in presumably infected calves [51]. Geurden and coworkers [51] put down the lower sensitivity to the smaller amounts of faeces used in the point-of-care tests since the IFA method utilises a concentration of faeces. More than three-quarters (77.1%) of the calves showed low or moderate cyst excretion. It is hypothesized that the amount of cysts was beyond the detection limit of the FAStest® Giardia since it was developed for calves shedding higher numbers of cysts. Comparing PCM with the FASTest® Crypto, 84.7% of the PCM positive samples revealed positive results in the point-of-care test. Test validation showed a sensitivity of 77.6% and a specificity of 91.1% which is in accordance to previous investigations focusing on point-of-care tests for *Cryptosporidium* [52].

Genotyping of *Giardia* revealed 30 (*bg*) respectively 29 (*tpi*) *G. intestinalis* genotype assemblage E-positive and one (both for *bg* and *tpi*) assemblage A-positive sample. In Southern Germany 101/110 *Giardia intestinalis*-positive faecal samples from calves were positive for genotype assemblage E, eight for A and one had a mixed infection with A and E [13]. Similar assemblage distributions are described for the UK and the USA, where the predominant genotype was assemblage E (which is suggested to be host-specific), followed by assemblage A which is considered as zoonotic [10,53]. Recent investigations showed that *G. intestinalis* genotype assemblage E is frequently found in diarrhoeic but also in non-diarrhoeic calves [11,13,54]. Assemblage co-infections are reported worldwide [10,14], but were not detected in this study. The single sample positive for genotype A was 100% identical with sequences published in GenBank® isolated from cervids (fallow deer, moose, red deer). The assumed host-specific sub-assemblage AIII is primarily detected in wild ungulates [20,55,56], but occasional appearances of sub-assemblage AIII in cattle [57] have been reported. The 75 days-old calf harbouring this genotype was housed indoors with another calf of the same age. The farmer used bedding material from fields located in a forest, and it can be speculated that wild deer droppings containing cysts of *G. intestinalis* assemblage A were introduced to the calves' housing. Infection was confirmed by IFA visualization of parasite stages, so mere DNA detection without infection, possibly confounding results of genotyping [5], can be ruled out in this case. In samples from human patients *Giardia* genotype assemblage B

was found in 65% of the positive samples, while the rest belonged to assemblage A (25% AII, 10% AI) [15]. The diversity of *Giardia* genotypes between humans and calves in Austria indicates that zoonotic transmission is limited.

All of the sequenced *Cryptosporidium* samples were confirmed as *C. parvum* subtype family IIa, which is in accordance with results from other countries as Canada [27], USA [28], England [29], Spain [25], New Zealand [30], Italy [31] and Germany [58]. Subtype family IId, common in Italian calves [31], was not detected in the present study. Specifically, 82.9% of the sequenced samples revealed *C. parvum* subtype IIaA15G2R1, the most prevalent subtype in calves worldwide [32,58]. Calves with symptoms of diarrhoea excrete *C. parvum* genotype IIaA15G2R1 more frequently than asymptomatic animals [25]. This subtype is also considered the most common in humans, indicating its high zoonotic potential and the risk of human infections originating from infected calves [7,27]. Subtypes IIaA19G2R1, IIaA21G2R1 and IIaA14G1R1, detected in samples from five different farms, also occur occasionally in humans and cattle [28,59,60].

Due to the widespread occurrence of the two parasites (along with other causes of diarrhoea e.g. viral or bacterial enteropathogens), examination of diarrhoeic faecal samples from young calves is essential for etiological diagnosis, and the strong age relation must be taken into consideration for clinical appraisal. While flotation is a cheap and fast method for screening the animals' parasite infection status, accurate and detailed results can only be achieved by implementing more sensitive and specific methods (PCM, IFA, PCR), and conclusive details regarding genotypes and possible transmission can only be obtained by multilocus PCR and sequencing.

Author contribution

All authors jointly planned the study and analysis. KL took part in the sample collection, KL and BH analysed the samples. KL drafted the first version of the manuscript. TW, AJ and BH revised it. All authors approved of the final version.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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