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International Journal for Parasitology 43 (2013) 155–161

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Is there a need for improved *Cryptosporidium* diagnostics in Swedish calves?

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ARTICLE INFO

Article history:
Received 27 June 2012
Received in revised form 21 August 2012
Accepted 5 October 2012
Available online 8 November 2012

Keywords:
Cryptosporidium parvum
Cryptosporidium bovis
Calf diarrhoea
ssrRNA
GP60

ABSTRACT

Cryptosporidium parvum is a common pathogen in preweaned calves but in Sweden *Cryptosporidium bovis*, which is considered apathogenic, is the most common species in this age group and it has been identified in diarrhoeal samples, indicating that it could be a cause of diarrhoea. In routine diagnostic procedures, infection is determined by microscopy, which is not sufficient to differentiate these species. We investigated whether routine *Cryptosporidium* diagnostic procedures need improvement to include species determination. The relation of *Cryptosporidium* spp. and subtype with the clinical picture and other pathogens was also investigated. A total of 782 diarrhoeal calf samples were analysed and *Cryptosporidium* infection was diagnosed in 198 samples. *Cryptosporidium parvum* was identified in 178, *C. bovis* in six and mixed *C. bovis*/*C. parvum* in seven samples. Twenty-seven *C. parvum* subtypes were identified, of which 16 were newly described. Except for three herds, only one subtype per herd was identified. *Cryptosporidium parvum*-positive calves were younger than *C. bovis*-positive calves and most *C. parvum* infections were seen at 1–3 weeks of age. Oocyst counts were higher in *C. parvum* samples. Yellow faecal colour was associated with *C. parvum* infection. Watery faeces had no greater association with *C. parvum* infection, but *C. parvum* subtype family IIa was more common than subtype family IId in watery faecal samples. No other pathogens were detected in the six *C. bovis*-infected calves, indicating a pathogenic potential. Our results show that species determination does not need to be included in routine *Cryptosporidium* diagnostic procedures in order to estimate the clinical relevance of infection in diarrhoeal calves. The maximum age when analysis for clinical cryptosporidiosis is performed can be lowered to 6 weeks of age. However, the indicated pathogenic potential of *C. bovis* warrants further attention.

1. Introduction

Cryptosporidium spp. are ubiquitous protozoan parasites of vertebrates. Globally, the zoonotic species, *Cryptosporidium parvum,* is a common species in, and a cause of diarrhoea in, preweaned calves. Infection is most prevalent between 1and 3 weeks of age (Nydam et al., 2001; Santín and Trout, 2008) and related symptoms are watery diarrhoea, inappetence, depression and sometimes death (Santín and Trout, 2008). Calf diarrhoea is a multifactorial disease and co-infection with other pathogens or the presence of non-infectious diarrhoeal causes can lead to more severe disease and higher mortality rates. Traditionally, *C. parvum* has been diagnosed by microscopy of faecal smears or concentrated samples, with or without staining. However, two other species, *Cryptosporidium bovis* and *Cryptosporidium ryanae*, with an oocystmorphology which is similar to *C. parvum*, can be identified in cattle using DNA analysis. Differentiation between these three species cannot be made by microscopy. *Cryptosporidium bovis* and *C. ryanae* are considered to be a pathogenic and are most prevalent after 1–2 months of age (Santín et al., 2004; Fayer et al., 2006, 2007; Langkjaer et al., 2006), but possible pathogenic effects of *C. bovis* on *Cryptosporidium* naïve calves have not been investigated. *Cryptosporidium parvum* has been identified as the main species in young calves, responsible for >80% of *Cryptosporidium* infections (Santín et al., 2004; Trotz-Williams et al., 2006; Fayer et al., 2007; Plutzer and Karanis, 2007; Broglia et al., 2008; Brook et al., 2009). However, in Sweden and some other countries, *C. bovis* is the most prevalent species in preweaned calves (Silverlås, 2010; Wang et al., 2011; Budu-Amoako et al., 2012; Silverlås and Blanco-Penedo, 2012). In Sweden, *C. bovis* constituted 50% of the infections during the second week of life and was the most common species from 2 weeks of age. The species was also detected in a number of diarrhoeal calf samples (Silverlås et al., 2010a,b). *Cryptosporidium* analysis has been offered as a part of routine diagnostic services of diarrhoeal calf faecal samples at the Swedish National Veterinary Institute (SVA) for many years,

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0020-7519/$36.00
http://dx.doi.org/10.1016/j.ijpara.2012.10.009

but diagnostic procedures do not include species determination. If the *Cryptosporidium* spp. distribution in diarrhoeal calves is the same as in Swedish dairy calves in general, i.e. there is a high *C. bovis* prevalence, species determination should be included in routine diagnostic procedures in order to make it possible to estimate the clinical relevance of *Cryptosporidium* infection.

The first aim of this study was to determine whether *Cryptosporidium* routine diagnostic procedures need to be supplemented with *Cryptosporidium* spp. analysis. A second aim was to investigate the spectrum of *C. parvum* subtypes present in herds with diarrhoeal problems, and whether any subtype seems to be associated with more severe symptoms. Finally, the presence of other pathogens and characterisation of the clinical picture at sample and herd levels were investigated and related to *Cryptosporidium* infection.

2. Materials and methods

2.1. Study design, sample collection and primary analyses

"Kalvpaketet" is a service package offered to farmers by The Swedish Animal Health Services (Svenska Djurhälsovården AB, Sweden). The package includes sampling, laboratory analysis and advice for herds with diarrhoeal or respiratory disease problems in young calves. Samples are collected by veterinarians in the field and analysed at SVA, Uppsala, Sweden. Submissions can comprise up to five diarrhoeal samples or nasal swabs from diseased calves, and blood samples from up to five healthy 1- to 7-day-old calves. All faecal and blood samples sent via "Kalvpaketet" from herds asking for *Cryptosporidium* analysis from 30 March 2010 to 29 March 2012were included in this study.

As soon as samples arrived at SVA, a questionnaire asking about calf management and the clinical picture of the calf diarrhoeal problems was sent to the farmer (Supplementary data S1). If a herd submitted samples more than once, a new questionnaire was sent if at least 1 month had passed since the last submission or if the farmer had not answered the previous questionnaire.

"Kalvpaketet" referrals for diarrhoeal samples can include analysis of *Cryptosporidium* spp., *Eimeria* spp., *Escherichia coli* F5+, *Salmonella* spp., rotavirus and coronavirus (BCV). *Giardia* cannot be requested but is analysed together with *Cryptosporidium* spp., as the immunofluorescent stain used in routine diagnostics (AquaGlo™ G/C Direct, Waterborne, Inc., USA) identifies both pathogens. Blood samples from clinically healthy 1- to 7-day-old calves were analysed for serum total protein (TP) as a measure of passive transfer. The age of sampled calves should be noted on the referral.

2.2. Cryptosporidium

Infection is usualy diagnosed by direct microscopy after immunofluorescent staining. The presence of *Eimeria* spp. is analysed in calves $\geqslant$3 weeks of age by the MacMaster flotation method. The presence of *E. coli* F5+ is analysed in calves $\leqslant$2 weeks of age by cultivation on blood agar plates followed by agglutination of one to two representative colonies to determine whether they are F5+. For rotavirus, BCV and *Salmonella* analyses, up to five diarrhoeal samples per herd are pooled at the laboratory and analyses are thus performed at herd level. Rotavirus is detected by antigen-ELISA, coronavirus by PCR and *Salmonella* by cultivation on specific agars. TP is analysed by refractometry.

2.3. Extended sample analyses

Faecal consistency and colour was registered on arrival or samples at SVA. Samples diagnosed as *Cryptosporidium*-positive by routine diagnostic methods were further analysed to verify the presence of *Cryptosporidium* and determine the oocyst count, *Cryptosporidium* sp. and subtype.

One gram of each *Cryptosporidium*-positive faecal sample was cleaned and concentrated by sodium chloride flotation (Silverlås et al., 2009) within 1 week of arrival at the laboratory. Out of the final 1.5 mL volume; a subsample of 30 μL was used to determine the oocyst count. Oocysts were enumerated at 200× magnification by epifluorescence microscopy after staining with FITC-labelled monoclonal anti-*Cryptosporidium* antibodies (CryptoCel IF test kit, CelLabs, Australia). The entire 30 μL sub-sample was examined if there were $\leqslant$6 oocysts per field of vision ($\leqslant$1000 per 30 μL). If there were >6 oocysts per field of vision, oocysts in 10 fields were counted and the average number was used to estimate the total oocyst count in the sample. Samples were stored at 4 °C until further analysis.

DNA analyses were performed every sixth month during the study as described previously (Silverlås et al., 2010b). Briefly, DNA was extracted from concentrated samples by a combined freeze–thawing and QIAamp DNA stool mini kit (QIAGEN, Germany) protocol (Quilez et al., 2008). Samples were subjected to nested PCR protocols to amplify ~800 bp each of the ssrRNA (Santín et al., 2004) and 60 kDa Glycoprotein (GP60) (Chalmers et al., 2005) gene loci. Species and subtype were determined by sequencing in both directions on an ABI 3100 Genetic Analyzer (Applied Biosystems, USA) using the internal primers and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Vector NTI software (Invitrogen, USA) was used to assemble consensus sequences and manually correct mismatches. Derived sequences were compared with sequences deposited in GenBank using BLAST (Basic Local Alignment Search Tool, NCBI http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

At the end of the study period, all samples that contained *C. bovis* were run through ssrRNA-restriction fragment length polymorphism (RFLP) to identify whether co-infection with *C. parvum* was present. The secondary PCR products were digested with *Mbo*II (New England BioLabs, UK) and separated by gel electrophoresis on a 3% Metaphor® Agarose gel (Lonza, ME USA) according to Feng et al. (2007).

2.4. Statistical analyses

Data were entered into Microsoft Office Excel 2007 spreadsheets (©2006Microsoft Corporation). Specific datasets were created for (i) extended microscopy and DNA analysis, and (ii) questionnaires, as data were entered on sample versus herd levels. In addition, the Excel spread sheets that SVA creates for "Kalvpaketet" samples, with information about all referrals, were used because these added information about other pathogens, TP and disease history data for the included herds. The spreadsheets were transferred to Stata 11 (©1985–2009 Statacorp LP, College Station, Texas, USA) and merged for statistical analysis. χ^2 Test, Fisher's exact test (F) and Mann–Whitney test (MW) were used as appropriate.

3. Results

3.1. Included referrals, samples and Cryptosporidium results from routine diagnostic procedures

From 30 March 2010 to 30 March 2012, SVA received 268 referrals that requested analysis of diarrhoeal calf samples for the presence of *Cryptosporidium*. The referrals included 801 samples, of which 782 were sent for analysis of *Cryptosporidium* (and sometimes other pathogens), whereas 29 faecal samples were sent for analysis of other pathogens but not *Cryptosporidium*. The referrals also included 125 blood samples for control of TP. Referrals were

from 192 herds and 51 herds sent samples on two (n = 33), three (n = 15), five (n = 2) or six (n = 1) occasions.

Cryptosporidium oocysts were detected by routine diagnostic methods in 242 (31.0%) samples from 91 (48.7%) herds. The sample prevalence was 32.7% (141 of 431) in year 1 and 28.8% (101 of 351) in year 2 (χ^2 = 1.4, degrees of freedom (df) = 1, P > 0.05). For herds with multiple referrals, *Cryptosporidium* oocysts were identified zero to three times.

3.2. Extended microscopy and molecular analysis results

Of 242 *Cryptosporidium*-positive samples, 218 samples from 82 herds contained enough faeces to allow further analysis, and 198 samples were positive by microscopy after concentration. Estimated oocysts per gram (OPG) in these samples ranged from 100 to 11 $\times$ 10^7. Samples determined as *Cryptosporidium*-negative after concentration were considered as false positives.

Species was successfully determined by ssrRNA analysis in 187 of 198 samples (94.4%). Four of the 11 samples with undetermined species contained *C. parvum* subtypes in GP60 analyses, giving a total of 191 samples (96.5%) with successful species determination. *Cryptosporidium parvum* and *C. bovis* were identified in 178 (93.2%) and 13 (6.8%) samples, respectively. *Cryptosporidium ryanae* was not found. Of the 13 *C. bovis* samples, seven were determined as mixed *C. bovis/C. parvum* by ssrRNA-RFLP. Sixty-eight herds were positive for *C. parvum* only, five for *C. bovis* only and both species were detected in six herds. In three herds with one referral and one *Cryptosporidium*-positive sample each, neither species nor subtype could be determined.

By GP60 analyses, subtypes were successfully determined in 171 of 198 (86.4%) samples. Of these, 166 were *C. parvum* and five were mixed *C. bovis/C. parvum* (Table 1). The subtype family (SF) but not subtype could be identified in another five samples, because the sequence started within the repetitive region. A total of 27 *C. parvum* subtypes were identified (Table 1). Sixteen new subtypes were identified and submitted to GenBank (Accession Numbers JX183796–JX183811) (Table 1). Six subtypes had a unique number of TCA/TCG repeats, whereas 10 had the same number of repeats as previously reported subtypes but shifts in the TCG position within the repetitive region and/or one or two single nucleotide polymorphisms in the post-repetitive region compared with reported sequences. IIa subtypes were most common, with a dominance of subtype IIaA16G1R1 (Table 1). For IId subtypes there was a slight dominance of IIdA20G1e (Table 1), a subtype variant first reported by us (Silverlås et al., 2010b). Three other subtypes, IIaA21G1R1, IIdA22G1c and IIdA23G1, which were first reported in Sweden (Silverlås et al., 2010b) were also identified, as well as a variant of one of those (IIaA21G1b). With the exception of three herds, only one subtype per herd was identified when multiple samples (n = 2–9) were analysed; also in herds with repeated samplings. In one of those three herds, only *C. parvum* was detected and samples with different subtypes were sent on the same occasion. In the other two herds, subtypes were identified in both *C. parvum* and mixed *C. bovis/C. parvum* samples. In one herd, samples were taken 8 months apart and subtypes differed in the *C. parvum* and mixed *C. bovis/C. parvum* samples. In the other herd, all samples were sent on the same occasion and two different subtypes were identified in the *C. bovis/C. parvum* sample, one of those being identical to the *C. parvum* subtype of that herd. One was a dairy herd, one a fattening herd and for the third herd there was no information about herd type.

3.3. Characteristics of Cryptosporidium-positive calves and Cryptosporidium-positive faecal samples

For statistical analysis of individual samples, calves with mixed *C. bovis/C. parvum* infection were kept in the *C. bovis* group at species level and in their *C. parvum* SF group at SF level. Due to the many subtypes present, with few isolates per subtype, data for subtype association with severity of disease was analysed at the SF level. There were 136sample observations for SF IIa and 40 observations for SFIId.

Age had been registered for 173of the 198 *Cryptosporidium*-positive calves. The oldest positive calf was 90 days old. *Cryptosporidium parvum*-positive calves were significantly younger than *C. bovis*-positive calves (Table 2). Of all *C. parvum* infections, 138 of 156 (88.5%) were seen at 1–3 weeks of age and 93.9% of all *Cryptosporidium*-positive samples from this age group contained *C. parvum*. *Cryptosporidium bovis* infections and *Cryptosporidium*-negative samples were more evenly distributed (Fig. 1). Oocyst counts differed significantly by species and there was a tendency towards higher oocyst counts for SF IIa compared with SF IId (Table 2).

Colour and consistency was registered in the laboratory for 239 *Cryptosporidium*-positive samples (Table 2).Yellow colour was significantly associated with *C. parvum*, whereas there was no difference in faecal consistency between *C. parvum* and *C. bovis* samples (Table 2). SF IIa was more common in samples with watery faeces, whereas there was no SF difference related to faecal colour (Table 2).

3.4. Other pathogens and passive transfer

Of the 13 *C. bovis*-infected calves, sixappeared to be mono infections, of which one calf was from a BCV-positive herd. The other seven samples were co-infections with *C. parvum*. In addition, one of the *C. bovis/C. parvum* infected calves was co-infected with *Giardia* and five were co-infected with *Eimeria bovis/zuerni*or *Eimeria* spp. One of the latter five calves was from a rotavirus-positive herd.

Because *Cryptosporidium* infection was not detected by routine diagnostic methods after 90 days of age, data for older calves were not included when the presence of multiple calf diarrhoeal pathogens in herds were examined. The presence of one to four other pathogens was detected in 77 of the 82 herds with *Cryptosporidium*-positive samples (Table 3). *Eimeria* spp. was detected in 72, rotavirus in 34, *Giardia* in 21 and *E. coli* F5+ in four herds. Coronavirus was only requested to be examined for three herds and detected in two of those, both *Cryptosporidium*-positive (Table 3). *Salmonella* was requested to be examined for 20 herds but not detected at all.

TP values ranged from 39 to 87 g/L (median 56 g/L) in individual samples and from 46 to 70.5 g/L (median 56.5 g/L) at herd level.

3.5. Characteristics of Cryptosporidium-positive herds

Data on all variables were not available for all herds and thus the number of observations included in different analyses varied. Questionnaires were answered for 52 (63.4%) of the 82 *Cryptosporidium*-positive herds. Because many farmers checked several questionnaire categories for diarrhoeal prevalence, colour and type of diarrhoea, categories became overlapping and data could not be interpreted.

Farmers from52 *Cryptosporidium*-positive herds had noted in the questionnaire that 1–100% of diarrhoeal calves were given some kind of rehydration (to drink, by oesophageal tube or i.v.). In 26 herds all diarrhoeal calves needed some sort of rehydration therapy. No differences in rehydration needs were observed between *C. parvum* (median 82%, range 10–100%, n = 44), *C. bovis* (median 100%, range 25–100%, n = 4) or *C. parvum/C. bovis* (median 20%, range 20–100%, n = 3) positive herds with data about rehydration; MW = 0.2–0.4, P > 0.05. Total or oral rehydration rates did not differ by SF, but there was a tendency for forced rehydration to be more common for herds with SF IIa than for herds with SF IId (median 5%, range 0–100% (n = 36) versus median 0%, range

Table 1
Cryptosporidium spp. and subtypes identified in 198 diarrhoeal calf samples from 82 herds[a].

Species in sample	Subtype family	Subtype	GenBank ID[c]	No of herds	No of samples
Cryptosporidium parvum[d]	IIa			**57**	**131**
		IIaA13G1R1		1	3
		IIaA13G1R2[b]	JX183796	1	1
		IIaA14R1[b]	JX183797	1	2
		IIaA14G1R1b[b]	JX183798	1	1
		IIaA15G2R1		4	4
		IIaA16G1R1		26	58
		IIaA16G1R1b[b]	JX183799	1	2
		IIaA17G1R1		2	6
		IIaA17G1R1c[b]	JX183801	3	15
		IIaA18G1R1		1	3
		IIaA18G1R1c[b]	JX183802	2	7
		IIaA18G1R1d[b]	JX183803	1	2
		IIaA20G1R1[b]	JX183804	2	5
		IIaA21G1R1		4	11
		IIaA22G1R1[b]	JX183806	3	7
		IIaA23G1R1[b]	JX183807	1	1
		IIa Unknown		3	3
	IId			**20**	**40**
		IIdA16G1b[b]	JX183808	2	4
		IIdA17G1d[b]	JX183809	1	2
		IIdA19G1		1	2
		IIdA20G1e		5	14
		IIdA22G1		2	4
		IIdA22G1c		2	3
		IIdA23G1		2	3
		IIdA24G1c[b]	JX183810	2	4
		IIdA26G1b[b]	JX183811	1	2
		IId unknown		2	2
	GP60 negative			**5**	**7**
Mixed *C. parvum*/*Cryptosporidium bovis*	IIa			**4**	**5**
		IIaA17G1R1c		1	2
		IIaA17R1[b]	JX183800	1	1
		IIaA21G1R1		1	1
		IIaA21G1R1b[b], IIaA16G1R1	JX183805	1	1
	GP60 negative			**2**	**2**
C. bovis	GP60 negative			**6**	**6**
18S rRNA and GP60 negative				**3**	**7**

GP60, 60 kDa Glycoprotein.
[a] A herd could be represented in more than one cell if analysis of multiple samples from that herd gave different results, e.g. two subtypes were present, or a subtype was identified in some samples but only subtype family in others.
[b] Novel subtypes.
[c] GenBank ID for novel subtypes.
[d] Samples with undetermined species but *Cryptosporidium parvum* subtypes are reported as *C. parvum.*

Table 2
Characteristics of sampled calves and faecal samples.

Variable	*Cryptosporidium*-positive						Statistics (df)
	All	*Cryptosporidium* spp. undetermined	*Cryptosporidium bovis*	*Cryptosporidium parvum*			
				All	IIa	IId	
Age in days median; range; (*n*)	11; 2–75;(173)	13; 2–70; (7)	24.5; 14–75; (12)[b]	11; 6–42; (154)[b]	–	–	MW: −5.5[b], $P < 0.001$
Oocyst count (OPG) median; range; (*n*)	5.6×10^6; 100–11×10^7 ; (198)	50,100; 100–3.2×10^7 ; (7)[d]	350,700; 500–5.2×10^6 ; (13)[c]	6.8×10^6; 100–11×10^7 ; (178)[c,d]	8.2×10^6; 100–11×10^7 ; (136)	5.2×10^6; 500–7.6×10^7 (40)	MW 4.4[c], $P < 0.001$; MW 3.7[d], $P < 0.001$
Faecal colour, *n* (% in column)[a]							
Yellow	192 (80.3)	5 (71.4)	7 (53.8)[e]	147 (83.1)[e]	109 (80.7)	35 (87.5)	F[e], $P \leqslant 0.05$
Other	47 (19.7)	2 (28.6)	6 (46.2)[e]	30 (16.9)[e]	26 (19.3)	5 (12.5)	
Faecal consistency, *n* (% in column)[a]							
Watery	114 (47.7)	3 (42.9)	4 (30.8)	90 (50.8)	75 (55.6)[f]	15 (37.5)[f]	$\chi^2 = 4.0$ (1)[f], $P < 0.05$
Pasty or loose	125 (52.3)	4 (57.1)	9 (69.2)	87 (49.2)	60 (44.4)[f]	25 (62.5)[f]	

df, Degrees of freedom; MW, Mann–Whitney test; F, Fisher's exact test.
[b-f] Cells with identical superscripts differ significantly from each other by the test parameter given in the right-hand column.
[a] Data missing for one sample with subtype.

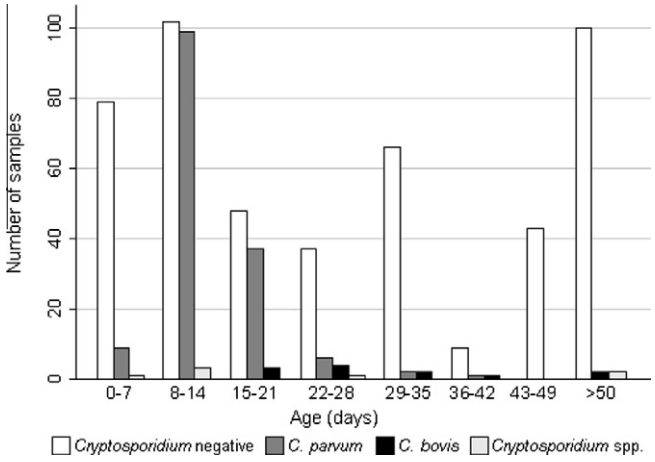

Fig. 1. Age distribution in 660 calves with diarrhoeal samples for *Cryptosporidium* analysis. The graph shows results for *Cryptosporidium parvum*, *Cryptosporidium bovis* or *Cryptosporidium* spp. Bars included 173 calves, whereas 487 calves were *Cryptosporidium*-negative. Seven samples reported as *C. bovis* samples in this figure were determined as mixed *C. bovis*/*C. parvum* infections by ssrRNA-restriction fragment length polymorphism. The *Cryptosporidium* spp. group included samples where the species was not defined.

Table 3
Presence of other diarrhoeal pathogens in 82 *Cryptosporidium*-infected herds with diarrhoeal problems.

No of herds	*Cryptosporidium* spp.	*Giardia*[a]	*Escherichia coli* F5+	Rotavirus	*Eimeria* spp.	BCV
5	X					
1	X	X				
4	X			X		
28	X				X	
10	X	X			X	
3	X		X		X	
20	X			X	X	
8	X	X		X	X	
1	X		X	X	X	
1	X	X			X	X
1	X	X		X	X	X

BCV, Bovine Coronavirus.
[a] Analysis cannot be requested but is automatically included when looking for *Cryptosporidium* oocysts.

0–20% (n = 10)); MW = 1.8, P = 0.07. There were no observations on rehydration rates for the herds with mixed IIa/IId SFs.

4. Discussion

The most prominent finding was that *C. parvum* is the dominant species in diarrhoeal calf samples from herds with calf diarrhoeal problems. The dominance of *C. parvum* in *Cryptosporidium*-positive samples found here is in contrast to the results of our previous studies, where this species was only identified in 20% of samples from preweaned calves (Silverlås, 2010). However, that study material was based on a random selection of dairy herds in Sweden, giving an estimate of species distribution in the average dairy herd and calf. In the current material, only diarrhoeal samples from herds with diarrhoeal problems were included, and species distribution was similar to what has been reported from other countries (Santín et al., 2004; Langkjaer et al., 2006; Trotz-Williams et al., 2006; Plutzer and Karanis, 2007; Thompson et al., 2007). The fact that *C. parvum*-infected calves were younger than *C. bovis*-infected calves indicates that in herds harbouring *C. parvum* this is the primary species infecting newborn calves, or that if infection occurs at approximately the same time in *Cryptosporidium* naïve calves, *C. parvum* will conquer *C. bovis*. *Cryptosporidium parvum* was not identified by ssrRNA sequencing after 42 days of age, and could only be detected as a minor species in older calves (up to 75 days of age) by ssrRNA-RFLP or GP60 analysis of *C. bovis* positive-samples. Age can thus be used to estimate clinical relevance and it seems that *Cryptosporidium* analysis is not relevant in diarrhoeal calves older than 6 weeks. The cut-off currently used is 2 months of age. In epidemiological studies, species determination is still an important tool, because species cannot be determined by microscopy and species distribution obviously differs with each study population.

Because *C. parvum* is pathogenic on its own, co-infection with other pathogens were only analysed at an individual level for calves infected with the considered apathogenic *C. bovis*. The presence of *C. bovis* without co-infection with other pathogens in six diarrhoeal samples indicates that this species also has pathogenic potential.

"Kalvpaketet" analyses are primarily important to determine pathogen and TP statuses at herd level and thus multiple infections at herd level were investigated to see whether *Cryptosporidium* infection alone was a common cause of diarrhoeal problems. The identification of up to four other pathogens in most herds with *Cryptosporidium* infections confirms the complex aetiology of diarrhoeal disease, and this has to be taken into consideration when dealing with diarrhoeal problems. Calves with a TP of $\geqslant$55 g/L are considered to have sufficient passive transfer (Radostits, O.M., 2000. The principles of control of infectious diseases of calves under 30 days of age. In: Proceedings from the XXI World Buiatrics Congress in Punta del Este, Uruguay, 4–8 December). Our results thus indicate that failure of passive transfer could be a part of the herds' problems, because half of the calves and herds fell below that level.

Oocyst counts in individual samples taken at a single point in infection are affected by when in the course of infection a sample is taken, because excretion rates will follow a bell shaped curve over time (Nydam et al., 2001). However, when looking at a large sample size, these effects are hopefully distributed evenly in the material, and oocyst counts could provide a measure of pathogenicity (Santín and Trout, 2008). Indeed, *C. parvum*-positive samples had significantly higher oocyst counts than *C. bovis*-positive samples.

The yellow faecal colour associated with *C. parvum* infection could reflect the time period when calves are infected – i.e. when milk is their dominant feed source, but yellow watery faeces has previously been associated with *Cryptosporidium* infection (Sanford and Josephson, 1982). Faecal colour changes as the proportion of hay/silage and grain in the feed increases. Thus brown or other colours than yellow would be more common in diarrhoeal samples from older calves, as was the case for *C. bovis*-positive samples.

There are a high number of *C. parvum* subtypes, determined by differences in the GP60 gene, and new ones are frequently reported. In this study we identified 27 subtypes, of which 16 have not been reported previously. With three exceptions, only one subtype was present per herd. This indicates a stable within herd clonal pattern in the *C. parvum* population. Similar patterns have been identified in areas with closed herd management (Misic et al., 2006; Thompson et al., 2007; Brook et al., 2008; Soba and Logar, 2008). In areas with animal exchange between herds, fewer subtypes but two or more subtypes per herd is a common pattern (Peng et al., 2003; Trotz-Williams et al., 2006; Brook et al., 2008). Sweden generally has closed herd management, with the exception of fattening herds where calves are bought from different herds from about 2 weeks of age. It is thus not surprising that one of the herds with two subtypes was a fattening herd. The identification of two subtypes in a dairy herd is, however, unexpected. If animal exchange occurs in dairy herds, moved animals are

usually heifers or cows, where *C. parvum* infection is not likely to be present (Silverlås et al., 2010a,b). However, dairy herd size is increasing in Sweden, and thus animal exchange is likely to increase, which could affect the subtype clonality of *C. parvum* in the future.

The predominance of GP60 SF IIa could reflect a higher pathogenicity or simply better host adaptation. In line with the results of this study, SF IIa has been predominant in ruminants and humans in other studies from around the world (Alves et al., 2003, 2006; Peng et al., 2003; Wu et al., 2003; Misic et al., 2006; Trotz-Williams et al., 2006; Thompson et al., 2007; Xiao et al., 2007; Silverlås, 2010). A difference in pathogenicity between SFs has been identified for *Cryptosporidium hominis*, where SF Id, but not SFs Ia, Ib or Ie, was associated with diarrhoea (Cama et al., 2007). In the present study, *C. parvum* SF IIa had a significant association with watery faeces compared with SF IId, There was also a tendency towards a higher oocyst count for SF IIa.

For the GP60 locus, no universal *Cryptosporidium* primer (as for the ssrRNA locus) exists, and the primers used to subtype *C. parvum* and *C. hominis* fails to replicate most other species. Thus, the presence of *C. parvum* subtypes in five *C. bovis* samples indicated co-infection with *C. parvum*. This was investigated further by ssrRNA-RFLP of all *C. bovis* samples at the end of the study period. Indeed, mixed infection was confirmed in these five samples as well as in two samples negative at the GP60 locus. Interestingly, two of the mixed samples contained other subtypes (both new) than the *C. parvum* samples of those herds. This could indicate that *C. bovis* subtypes can sometimes be identified using the *C. parvum*/*C. hominis* primers, as is the case for *Cryptosporidium meleagridis* (Glaberman et al., 2001; Abe, 2010).

It could be argued that samples with mixed *C. bovis*/*C. parvum* should have been considered a separate group in our statistical analyses. They were put in the *C. bovis* group for three reasons. First, *C. bovis* was the dominant species as shown by ssrRNA sequencing. Second, it is not known whether *C. bovis* was a minor species in any of the *C. parvum* samples because RFLP was not performed and thus the *C. parvum* group could include mixed samples as well. Third, a subgroup analysis of age and oocyst counts showed that the mixed samples belonged to the *C. bovis* group (data not shown).

Our extended analysis method with concentration and cleaning of samples can detect oocysts at shedding rates of 50–100 OPG (Andersson, 2004. *Cryptosporidium* infection in cattle – Evaluation of a new method for identification of subclinical infection. In Swedish, English summary. Master of Veterinary Medicine thesis. Swedish University of Agricultural Sciences, Uppsala Sweden). The sensitivity of the method used in routine diagnostic procedures has not been established but our results show that it is less sensitive than our concentration method. In addition, the skills of the person performing the analysis will affect the result. At routine diagnostic services, several persons are involved in analysis, whereas in the extended analysis performed here, all samples were analysed by the same person, producing a more consistent result. These are the reasons why samples positive by routine diagnostic methods but negative after concentration and cleaning were considered false positives. However, the routine diagnostic method is obviously sensitive enough to evaluate clinically relevant samples.

In conclusion, *C. parvum* is the dominant *Cryptosporidium* sp.in diseased calves in diarrhoeal problem herds, in contrast to the average species distribution in Swedish preweaned calves. Thus, species determination does not need to be included in routine *Cryptosporidium* diagnostic procedures in order to estimate the clinical relevance if this parasite genus is found in samples from diarrhoeal herds.

The dominance of *C. parvum*, especially during the period when clinical cryptosporidiosis is most common, and higher oocyst counts, supports that this species is more pathogenic than *C. bovis*. However, the possible pathogenic potential of *C. bovis* warrants further attention. The age span when analysis for clinical cryptosporidiosis is performed could be shortened from 2 months to 6 weeks of age.

Acknowledgements

We would like to thank all of the farmers who returned the questionnaires and thereby improved the data available for their herds. This project was funded by the Swedish Farmers' Foundation for Agricultural Research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2012.10.009.

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