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

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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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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