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Short communication

Dynamics of excretion and molecular characterization of *Cryptosporidium* isolates in pre-weaned French beef calves

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ABSTRACT

Studies on excretion and molecular characterization of *Cryptosporidium* have been mostly conducted in dairy calves, both diarrhoeic and non-diarrhoeic. Little is known about *Cryptosporidium* in beef calves, especially in non-diarrhoeic ones. This study was conducted in a herd of Parthenais beef cattle (France) with no history of clinical cryptosporidiosis. Twenty-five calves were sampled once a week from birth to one month of age (age range: 5–34 days). At each sampling date, presence of clinical signs of cryptosporidiosis (diarrhoea) was recorded. Oocyst excretion was assessed using the Heine staining method and a direct immunofluorescence method (Merifluor® C/G) which allowed quantification (oocysts per gram of faeces, opg). All samples were subjected to a two-step nested PCR protocol to amplify the 18S rRNA gene and amplification products were sequenced. None of the calves presented diarrhoea. Twenty-three of them excreted oocysts at least one sampling date. Prevalence of excretion was maximal when calves were 27–34 days old, with a percentage of excretion of 85% in this age category [95% CI: 70; 100]. Mean excretion was maximal when calves were 20 to 26 days old, with a mean excretion of 7.6×10^5 opg (range: 0–8×10^6 opg). 32 isolates were successfully identified: 27 as *Cryptosporidium bovis*, 4 as *Cryptosporidium ryanae* and 1 as *Cryptosporidium parvum*. *C. bovis* was isolated from samples of calves between 11 and 33 days old. *C. ryanae* was isolated from samples of calves between 17 and 34 days old. *C. parvum* was isolated from one calf aged 13 days. This survey demonstrated the high infection rate of non-diarrhoeic beef calves by *Cryptosporidium* species other than *C. parvum*.

Cryptosporidium is one of the main agents of neonatal diarrhoea in calves (de Graaf et al., 1999). Cattle have been reported to be infected with four species of *Cryptosporidium*: *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae* and *Cryptosporidium andersoni*. Each of these four species presents a different prevalence pattern relative to the age of the animal (Wyatt et al., 2010). This has been well-described in dairy calves: nearly 100% of the calves become infected before 1 month of age, with *C. parvum* being the predominant species. In young stock, *C. bovis* followed by *C. ryanae* are the most prevalent species. In adult cows, most of the infections are due to *C. andersoni* (Santín et al., 2008).

Cryptosporidium infection is usually considered as less prevalent in beef calves than in dairy calves (Kváč et al., 2006; Geurden et al., 2007). Because of this, fewer studies on this parasite have been conducted in beef cattle than in dairy cattle. In some of these studies, microscopic techniques used for the detection of *Cryptosporidium* did not distinguish between species (Naciri et al., 1999; Atwill et al., 2003; Kváč et al., 2006). The remaining studies (Feltus et al., 2008; Fayer et al., 2010) involved weaned calves or cows.

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Only 3 studies used pre-weaned beef calves and molecular methods to characterize the isolates (Geurden et al., 2007; Budu-Amoako et al., 2012; Murakoshi et al., 2012). These authors reported contrasting results: while *C. parvum* was the most prevalent species in calves less than 1 month old in the Belgian study (Geurden et al., 2007) as in dairy calves, Budu-Amoako et al. (2012) reported the same prevalence for both *C. parvum* and *C. bovis* in Canada, without any significant link between species and age of the calves. In contrast, Murakoshi et al. (2012) reported a predominance of *C. bovis* and the absence of *C. parvum* in grazing beef calves in Japan with a significantly higher prevalence of *C. bovis* in calves under 3 months of age.

Little information has been published on the molecular epidemiology of *Cryptosporidium* infection in calves in France. One longitudinal study conducted in dairy calves reported the same pattern as previously described elsewhere (Follet et al., 2011).

The present longitudinal study aims to describe the dynamics of excretion of *Cryptosporidium* sp in pre-weaned beef calves during their first month of life in one French farm.

1. Materials and methods

1.1. Animals

The study was set up in a herd of 50 cows of the Parthenais breed (local beef breed) located in western France. The calving season lasted from September 2009 to April 2010. During this period, all the cows and their calves were housed indoors in a single barn. They were allowed to go to pasture starting in April during the daytime when the weather was good. 25 calves born between November 2009 and April 2010 were included in the study. The first samplings took place when calves were 5–12 days old and thereafter were performed once a week for 4 weeks.

1.2. Faecal samplings

Individual faecal samples were collected directly from the rectum into a plastic cup. Cups were labelled with the calf's ear tag number and the sampling date and placed at +4 °C until processing, which was done within 3 days of collection.

1.3. Laboratory analyses

1.3.1. Heine staining method

Demonstration of oocysts was made by staining faecal smears with Ziehl fuschin and observing at ×200 magnification under a phase-contrast microscope according to Heine (1982). The intensity of excretion was evaluated semi-quantitatively according to the average number of oocysts in 10 randomly selected fields. Six scores were defined: 0: absence of oocyst, 1: <1 oocyst per field, 2: 1–10 oocysts per field, 3: 11–20 oocysts per field, 4: 21–30 oocysts per field and 5: >30 oocysts per field. One person performed and read all tests.

1.3.2. Oocyst concentration

Oocysts were concentrated from faeces as previously described (Castro-Hermida et al., 2005). Briefly, 2 g of faeces from each specimen cup were diluted in distilled water and filtered through a sieve (mesh size: 45 μm) into conical centrifuge tubes. Diethyl ether was then added at a proportion of 2:1, the tubes were shaken vigorously and centrifuged at 1000 × *g* for 5 min at 4 °C. The top three layers were decanted off. The sediment was removed and washed in distilled water by centrifuging at 1000 × *g* for 5 min at 4 °C. The volume of the oocyst suspension was adjusted to 1 ml using distilled water.

1.3.3. Immunofluorescence examination

Cryptosporidium oocysts were detected by a direct immunofluorescent antibody test (IFAT) (MeriFluor® *Cryptosporidium*/*Giardia*, Meridian Bioscience Europe, Nice, France) in all the samples. 10 μl of oocyst suspension was fixed on the slides using acetone at 4 °C for 10 min. The slides were then processed according to the manufacturer's instructions. The samples were observed by fluorescence microscopy using 400 × magnification. The number of oocysts per gram of faeces (opg) was determined by applying the following formula: [number of oocysts seen in the well × 100/2].

1.3.4. Molecular characterization

All samples were submitted to DNA extraction. 250 μl of the oocyst suspension was processed using the UltraClean Faecal DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, USA) according to the manufacturer's instructions. The eluted DNA was dissolved in 50 μl.

A two-step nested PCR protocol was used to amplify an 830 bp segment of the 18S rRNA gene using primers 5′-TTCTAGAGCTAATACATGCG-3′ and 5′-CCCATTTCCTTCGAAACAGGA-3′ for primary PCR and 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5′-AAGGAGTAAGGAACAACCTCCA-3′ for secondary PCR (Xiao et al., 1999, 2001). The amplification runs were performed in a Bio-Rad ICycler thermal cycler with the following protocol: 10 min at 94 °C, 40 cycles of 45 s at 94 °C, 55 s at 55 °C and 1 min s at 72 °C, with a final 5-min elongation step at 72 °C. Positive and negative control samples were included in each PCR reaction. When no amplification occurred, all the samples were submitted to a new PCR cycle, with dilution at 1/10e if the sample was positive by IFAT. PCR products were sequenced using an ABI 3730XL sequencer (Applied Biosystems, Warrington, UK). Sequence accuracy was ensured by two-directional sequencing, and all electrophoregrams were manually checked and edited as deemed necessary. Sequences were compared with known sequences by BLAST-analysis against the NCBI database.

1.4. Statistical analysis

Prevalences of excretion were compared for the different sampling dates using a Cochran's Q trend test ($p < 0.05$). A repeated measure analysis of variance model with one within factor (date) was used to compare oocyst excretion after transformation of individual figures as log (opg+1) between dates ($p < 0.05$). Statistical analyses were