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Short Communication

Infections with multiple *Cryptosporidium* species and new genetic variants in young dairy calves on a farm located within a drinking water catchment area in New Zealand

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ABSTRACT

Several *Cryptosporidium* species are known to infect cattle. However, the occurrence of mixed infections with more than one species and the impact of this phenomenon on animal and human health are poorly understood. Therefore, to detect the presence of mixed *Cryptosporidium* infections, 15 immunoflourescence-positive specimens obtained from 6-week-old calves' faeces ($n = 60$) on one dairy farm were subjected to PCR-sequencing at multiple loci. DNA sequences of three *Cryptosporidium* species: *C. parvum* (15/15), *C. bovis* (3/15) and *C. andersoni* (1/15), and two new genetic variants were identified. There was evidence of mixed infections in five specimens. *C. parvum*, *C. bovis* and *C. andersoni* sequences were detected together in one specimen, *C. parvum* and *C. bovis* in two specimens, and *C. parvum* and *C. parvum*-like variants in the remaining two specimens. Sequencing of gp60 amplicons identified the IIaA19G4R1 (8/15) and IIaA18G3R1 (4/15) *C. parvum* subgenotypes. This study provides evidence of endemic mixed infections with the three main *Cryptosporidium* species of cattle and new genetic variants, in calves at the transition age of six weeks. The results add to the body of evidence describing *Cryptosporidium* isolates as genetically heterogeneous populations, and highlight the need for iterative genotyping to explore their genetic makeup.

1. Introduction

Gastro-intestinal infections with *Cryptosporidium* parasites, in particular the species *C. parvum*, are well recognised in cattle. In addition, cattle are also an important infection source for humans (Fayer and Xiao, 2008).

Many aspects of the epidemiology of cryptosporidiosis are not well understood due to the inability to differentiate between *Cryptosporidium* taxa by their morphology or phenotype. Therefore, molecular genetics tools targeting taxonomically informative loci are widely used retrospectively, to distinguish between taxa (Caccio et al., 2005; Sulaiman et al., 2000). To date, about 30 *Cryptosporidium* species have been recognised, many of which are associated with disease in humans, domesticated livestock, companion animals and wildlife (Santín,

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2012; Šlapeta, 2013). The species so far identified in cattle are the gastric species *Cryptosporidium andersoni*, and the intestinal species *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium rynae*, *Cryptosporidium ubiquitum*, *Cryptosporidium suis*, *Cryptosporidium scrofarum* and *Cryptosporidium hominis* (Fayer, 2010; Ng et al., 2011; Ryan and Power, 2012; Smith et al., 2005; Tanriverdi et al., 2003). Whereas *C. parvum* is a frank pathogen and zoonotic (Xiao and Feng, 2008), the clinical and zoonotic potential of the other species is not understood. In cattle, infections with the different *Cryptosporidium* species tend to follow specific temporal patterns, with *C. parvum* cycling mostly during the first month of life, and the other species more commonly found in post-weaned calves (*C. bovis*; *C. ryanae*), or yearlings and adult cattle (*C. andersoni)* (Fayer et al., 2007a, 2007b; Santin et al., 2004). The reasons for this age-specific pattern of infection are not well understood, and the level of cross-immunity between the species is not known.

Mixed infections with multiple *Cryptosporidium* species could originate from co-infections or, in the case of chronic infections, super-infections with multiple taxa. Globally, mixed *Cryptosporidium* infections have been described in humans, in particular in HIV-AIDS patients (Cama et al., 2006; Kurniawan et al., 2013), and also in animals (Rzeżutka and Kaupke, 2013; Silverlås et al., 2013; Tanriverdi et al., 2003). However, such infections are usually considered an exception, rather than the rule, and exhaustive investigations of the nature of mixed infections in animal populations are not commonly pursued in molecular epidemiological studies. Thus, the prevalence and impact of this phenomenon on animal or public health are unknown.

Whereas mixed infections can be suspected by PCR-restriction fragment length polymorphism (RFLP) and other methods, they may not be easily confirmed by Sanger sequencing (SaSq), as this method tends to detect only the predominant genetic variant present in the sequenced sample (Grinberg et al., 2013; Reed et al., 2002). Thus, the detection of mixed infections is likely to be improved by the use of iterative approaches and multiple molecular diagnostic tools (Xiao, 2010). In this study, we characterised *Cryptosporidium* parasites from calves using an iterative PCR-sequencing approach on multiple genes, to detect mixed *Cryptosporidium* species infections in calves.

2. Materials and methods

2.1. Study design and faecal samples

This study was part of a larger study aimed at assessing the presence of waterborne enteropathogens in two water catchment areas within the Manawatu region of New Zealand. Fresh faecal specimens were sampled from the ground of a paddock on a dairy farm located in one of the catchment areas. The faecal sampling was performed once a month, from 31 August to 18 November 2011, for a total of four sampling events. This period of the year coincided with the spring calving season in New Zealand, usually starting in July and ending in October. The calves grazing on the paddock were between five and six weeks of age on the first sampling occasion. In New Zealand, calves of this age are usually weaned and held on pasture. The calves were kept in barns and fed with reconstituted milk replacer from day 2 to the first month of their life, then moved to the paddock. About 35 calves were present on the paddock and adult cattle were not present on this paddock during the sampling period. Fifteen faecal specimens were collected in plastic containers on each sampling occasion, for a total of 60 specimens. In order to prevent cross-contamination of specimens, faeces located at least 2 m apart were sampled, and disposable gloves were changed between specimens. Specimens were transported on ice to Massey University and stored at 4 °C for a maximum of four days until analysed.

2.2. Laboratory analysis

Laboratory analysis included the identification of *Cryptosporidium* oocysts by immunofluoresent microscopy (IFA) using a commercial kit (Aqua-Glo G/C Direct, Fl, Comprehensive Kit; Waterborne, Inc., New Orleans, USA). The fluorescent conjugate was diluted five-fold with molecular grade water and the stained smears were observed in epiflourescence microscope using a 460–490 nm excitation wavelength. The specimens showing more than one IFA-positive oocysts on slides were considered positive. In order to identify only cycling-parasites and prevent amplification of naked DNA originating from a contaminated farm environment, only IFA-positive specimens were subjected to genotyping. Genomic DNA was extracted from the specimens using faecal DNA extraction kits (Bioline, Sydney, Australia), following the manufacturer's instructions.

Cryptosporidium taxa were identified using multilocus PCR-sequencing, followed by subgenotyping. For the taxon identification, a nested PCR targeting a ~825 bp fragment of the small-subunit ribosomal DNA (18S SSU rDNA) was used. In addition, a single step PCR was applied to amplify a ~400 bp fragment of the *Cryptosporidium* 70 kDa heat shock protein gene (HSP70). For subgenotyping, a ~850 bp fragment of the 60 kDa glycoprotein gene (gp60) was amplified using a nested PCR. The PCR conditions were optimised in-house using the previously described primer sequences (Alves et al., 2003; Grinberg et al., 2008; Learmonth et al., 2004). Agarose gel electrophoresis was used to verify PCR products. Positive amplicons were purified using an ethanol precipitation protocol and submitted to a commercial sequencing provider for bidirectional Sanger sequencing, using the same PCR primers. Consensus sequences were created by manual editing of forward and reverse sequences using Geneious 6.5 software (Biomatters, Auckland, New Zealand; http://www.geneious.com). The consensus sequences were aligned on line with sequences deposited in GenBank (National Institutes of Health, Bethesda, MD, USA) using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi, National Center for Biotechnology Information, Bethesda, MD, USA).

Initial results indicated that some specimens contained *C. bovis* 18S SSU rDNA and *C. parvum* HSP70 and gp60 sequences. In order to rule out laboratory