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Distribution of *Cryptosporidium parvum* subtypes in calves in Germany

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Abstract

Cryptosporidium DNA was extracted from 134 faecal specimens from pre-weaned calves from different German Federal States (age range, 3–15 days old), which tested positive for oocysts by microscopic analysis. The 18S rDNA gene and the oocyst wall protein gene (COWP) were used as targets for PCR and RFLP techniques. *Cryptosporidium* species were identified by using SspI, MboII and RsaI endonucleases for the digestion of 18S rDNA and COWP amplified fragments, respectively. In all samples, restriction patterns corresponding to *Cryptosporidium parvum* were identified, which is in agreement with abundant literature data indicating *C. parvum* as the most common species in pre-weaned calves. In order to estimate the genetic heterogeneity among *C. parvum* calf isolates, 53 samples chosen to represent different German Federal States were successfully subtyped by sequence analysis of the highly polymorphic 60-kDa glycoprotein gene. All isolates belonged to the allele IIa (with seven subtypes), with the exception of one isolate that belonged to the allele IId. Moreover, three novel subtypes of the allele family IIa have been found. This study confirms the utility of genotyping and subtyping tools in characterizing the transmission of *Cryptosporidium* spp. This is the first molecular epidemiological report about subtyping of *Cryptosporidium* bovine isolates in Germany.

Keywords: *Cryptosporidium*; Calf; Germany; Molecular typing; Subtypes

1. Introduction

Cryptosporidiosis is a widespread intestinal infection of both humans and livestock (Fayer et al., 1997; Cacciò and Pozio, 2006). In Germany, human cryptosporidiosis is notifiable since 2001, and around 1000 cases are reported every year. However, little is known about the transmission routes and the role played by animals in the epidemiology of human infection.

The main animal reservoir of *Cryptosporidium* (*C.*) is cattle, in particular calves, in which cryptosporidiosis is responsible for significant neonatal morbidity (De la Fuente et al., 1999; Naciri et al., 1999; McDonald, 2000). The prevalence of cryptosporidiosis in cattle in Germany has been investigated in the last decades using mainly direct coproscopical methods (Aurich et al., 1990; Siebert and Gründer, 1991; Joachim et al., 2003; Epe et al., 2004), and the surveillance programmes in Germany indicate a high prevalence above all in young animals. Nevertheless, classical coproscopical methods have a low sensitivity for detection of *Cryptosporidium* oocysts, and do not allow to differentiate among the *Cryptosporidium* species and genotypes, which are known to show different epidemiological patterns (i.e. host specificity and pathogenicity), with important consequences for public health (Fayer et al., 1997; Sunnotel et al., 2006; Xiao et al., 2002, 2004).

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Over the last two decades the increasing numbers of cryptosporidiosis outbreaks (in particular water related) recorded in developed countries (Craun et al., 2005), raised the interest of many researchers towards this pathogen, with the development of molecular techniques for species and genotype characterization of *Cryptosporidium* (Xiao and Ryan, 2004). Using a combination of genetic, biologic and morphologic data, the taxonomy of the genus has been revised, and currently 16 *Cryptosporidium* species are recognized as valid, among which seven have a zoonotic potential (Sunnotel et al., 2006).

In particular, cattle are infected with at least four *Cryptosporidium* species, including *C. parvum*, *C. bovis*, *C. andersoni*, and the *Cryptosporidium* deer-like genotype (Feng et al., 2007; Fayer et al., 2006), although *C. parvum* is the only zoonotic species.

Further molecular analysis aimed at a better investigation of the transmission routes and source tracking of *C. parvum* include subtyping tools such as that based on sequence analysis of the 60-kDa glycoprotein gene (GP60). The GP60 gene contains a very polymorphic microsatellite sequence with variable numbers of TCA and TCG repeats, which allows the discrimination of up to eight allele families. This marker allows to differentiate *C. parvum* to several subtypes within each family (Strong et al., 2000; Peng et al., 2001, 2003; Alves et al., 2003, 2006; Wu et al., 2003; Sulaiman et al., 2005; Chalmers et al., 2005; Abe et al., 2006; Trotz-Williams et al., 2006). Of the two major *C. parvum* subtype families, the IIa subtype family is zoonotic in nature and is found in both humans and calves, whereas the IIc subtype family is anthroponotic and is only found in humans (Alves et al., 2003; Xiao and Ryan, 2004).

This is the first molecular epidemiological study in Germany to report the subtyping of *C. parvum* in calf isolates.

2. Materials and methods

2.1. Sources of positive isolates and DNA extraction

One hundred thirty four faecal samples from naturally infected neonatal calves (age range 3–15 days) from different German Federal States were provided by regional laboratories and veterinary faculties. The diagnosis of cryptosporidiosis was performed by the different laboratories by Ziehl–Neelsen staining method (36%), Heine staining method (10%) copro ELISA test (30%), and phase contrast microscopy (24%). Isolates were tested by molecular techniques at the Federal Institute for Risk Assessment in Berlin. To confirm *Cryptosporidium* positivity, all specimens were re-checked using an immunofluorescence test (IFT) with a commercial kit (Merifluor, Meridian), and stored at 4 °C in potassium dichromate 2.5% until DNA extraction. Oocyst disruption and DNA extraction were performed using the Qiamp Stool kit (Qiagen). Eluted DNA samples were stored at −20 °C.

2.2. PCR-RFLP

PCR amplification of the oocyst wall protein (COWP) gene fragment was performed using the nested primer set developed by Pedraza-Díaz et al. (2001), using BCOWPF (5′-ACCGCTTCTCAACAACCATCTTGTCCTC-3′) and BCOWPR primers (5′-CGCACCTGTTCCCACTCAATGTAAACCC-3′) for primary amplification, and Cry9 (5′-GTAGATAATGGAAGAGATTGTG-3′) and Cry15 primers (5′-GGACTGAAATACAGGCATTATCTTG-3′) for secondary amplification (Spano et al., 1997). The restriction analysis was performed with 10 μl of the COWP amplified products digested with 10 units of endonucleases RsaI and 2 μl of 10× buffer in a total volume of 20 μl, at 37 °C for 3 h.

18S rDNA nested PCR amplification followed the protocol described by Xiao et al. (2000). Restriction patterns of the 18S rDNA amplicons were obtained by digestion of 10 μl of amplified products respectively with 10 units of endonuclease SspI and 5 units of MboII endonucleases and their respective buffers. The MboII restriction allows differentiating *C. parvum* from other *Cryptosporidium* bovine-related species, in particular *C. bovis* and *Cryptosporidium* deer-like genotype (Feng et al., 2007). Restriction products were separated on ethidium bromide-stained 3% agarose gel.

2.3. Sequencing of GP60 gene fragment

A GP60 gene fragment was amplified using the nested PCR protocol of Peng et al. (2001) from 67 samples chosen to represent eight German Federal States.

Sequences were aligned by Clustal W method with reference sequences retrieved from the GenBank and phylogenetic analysis was performed using neighbour-joining method as implemented in the software MEGA 3.1 (Kumar et al., 2004). Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers: EU106857,