Veterinary Parasitology 196 (2013) 301–306

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

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

Occurrence and molecular identification of *Cryptosporidium* species isolated from cattle in Poland

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ARTICLE INFO

Article history:
Received 6 July 2012
Received in revised form 6 March 2013
Accepted 12 March 2013

Keywords:
Cryptosporidium
Molecular detection
Cattle
Prevalence
Species distribution

ABSTRACT

Cryptosporidiosis is a disease reported in both humans and animals and is caused by a protozoan parasite *Cryptosporidium*. In most cases calves are infected by different *Cryptosporidium* species adapted to this animal host. Most infections are subclinical; however in some cases the watery diarrhea appears. The rapid development of new molecular diagnostic tools has provided an opportunity for better recognition of *Cryptosporidium* species and genotypes isolated from infected hosts. The aim of this study was an assessment of the prevalence of *Cryptosporidium* species in cattle herds in Poland. In total, 700 cattle fecal samples were tested. The examined cattle were at the age of 1 day to 6 years old. Overall 194 farms were monitored for parasite presence. *Cryptosporidium* detection in animal feces was performed using only molecular methods. Species identity of oocyst-positive samples were defined on the basis of PCR-RFLP and nucleotide sequence analysis of the amplified 18 SSU rRNA and COWP gene fragments. *Cryptosporidium* oocysts were detected in 119 (17%) of cattle feces. Most of the positive feces 55 (19.7%) were derived from young animals at the age of 1–4 weeks. The tested samples were positive for *Cryptosporidium bovis*, *Cryptosporidium parvum*, *Cryptosporidium andersoni*, and *Cryptosporidium ryanae*. *C. parvum* was not the most frequently detected parasite species, but calves below the age of 1 month were the major host for this parasite. The overall prevalence of *C. parvum* in Polish cattle herds was estimated at 5.1%. *C. andersoni* was the only species occurring in adult calves. The infected animals were housed in 67 (34.5%) of the monitored homesteads. Although *C. bovis* and *C. ryanae* were previously detected in cattle populations in many countries, this is the first report describing their presence in Polish cattle. Moreover, this is the first report on the prevalence of *Cryptosporidium* species in Polish cattle herds.

1. Introduction

Cryptosporidiosis is a frequent disease of neonatal livestock including cattle, lambs and piglets. The first case of bovine cryptosporidiosis was reported more than thirty years ago (Panciera et al., 1971) and since then the significance of this protozoan parasite as a causative agent of diarrheal disease in farm animals has been widely studied (de Graaf et al., 1999; Sréter and Varga, 2000). In most cases calves are infected by different *Cryptosporidium* species adapted to this animal host such as *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium andersoni*, *Cryptosporidium ryanae* (previously known as *Cryptosporidium* Deer-like genotype), but occasionally other species or genotypes such as *Cryptosporidium suis*, *Cryptosporidium suis*-like genotype, *Cryptosporidium* Pig genotype II, *Cryptosporidium meleagridis*, *Cryptosporidium hominis* and *Cryptosporidium felis* (Bornay-Llinares et al., 1999; Fayer et al., 2008; Santín et al., 2008; Imre and Dărăbus, 2011) have been isolated from the bovine host. *Cryptosporidium* infections have been reported in cattle worldwide (de Graaf et al., 1999) with the prevalence ranging from 3.5%

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http://dx.doi.org/10.1016/j.vetpar.2013.03.009

Table 1
The distribution of farms across each province and the number of collected feces.

Province	Number of sampled farms	Number of tested feces
Lower Silesia	15	50
Kujawy-Pomerania	7	35
Lublin	17	129
Lubuskie	7	34
Łódź	8	40
Małopolska	12	38
Mazovia	8	36
Opole	11	30
Podkarpacie	15	35
Podlasie	15	39
Pomerania	9	39
Silesia	28	40
Świętokrzyskie	13	30
Warmia-Masuria	8	40
Wielkopolska	9	44
West Pomerania	12	41
Total	194	700

(Epe et al., 2004) to 60.2% (Mišic and Abe, 2007). Under natural conditions calves acquire infections shortly after birth but the highest rates are reported in animals at the age of 1 to 3 weeks (Santín and Trout, 2008). The disease usually lasts up to 2 weeks and its clinical course depends on the species and pathogenicity of individual parasite strains (Santín and Trout, 2008) as well as the animal breed (Olson et al., 2003). Infection by *C. parvum* can result in the development of clinical signs with watery diarrhea predominating, whereas those initiated by *C. andersoni*, *C. bovis* and *C. ryanae* are usually asymptomatic (O'Handley and Olson, 2006; Kváč et al., 2008). On the other hand some infections are characterized by low intensity of invasion in which low numbers of oocysts were shed in animal feces. In these cases infection can go unrecognized if solely traditional microscopic methods are applied.

The aim of the present study was identification and assessment of the prevalence of *Cryptosporidium* species isolated from cattle in Poland using molecular methods.

2. Materials and methods

2.1. Sample collection

Over the period of 4 months from September to December 2009, 700 fecal samples were collected from cattle at the age of 1 day to 6 years. The animals were housed in 194 farms located in all 16 administrative provinces in Poland (Table 1). From each province 7–28 commercial farms were randomly chosen for sampling. They represented different administrative locations across each province. Average herd size differs in the sampled farms, although a minimum number of heads per farm was 20. Animals at the age over 4 months were housed in the small farms with traditional, grazing based husbandry system. Feces were collected mostly from dairy cattle as 96.2% of housed cattle under the age of 1 year in Poland represent dairy breeds (GUS, 2011). The number of animals sampled per farm ranged from 1 to 5. Upon collection, approximately 10–15 g of feces were placed individually into plastic containers, labeled and sent to the laboratory. Most of the sampled cattle were in good health, as only 25 fecal samples were derived from diarrheic animals. These samples were sent by vets to the Parasitology Department of the National Veterinary Research Institute for general parasitological screening. The number of sampled animals divided into age categories are shown in Table 2.

Table 2
Age and health status of sampled cattle.

Age	Number of cattle		Total
	Not diarrheic	Diarrheic	
<1 week	50	–	50
>1 week–1 month	278	1	279
>1–4 months	331	20	351
>4–12 months	6	4	10
>1 year	10	–	10
Total	675	25	700

Cryptosporidium oocysts, Iowa strain (Waterborne™, Inc., New Orleans, LA, USA) were used as positive control during nucleic acid isolation and molecular detection.

2.2. Extraction and purification of parasite DNA

Cryptosporidium DNA was extracted from 0.1 g (100 μl) of animal feces using Millar et al. (2001) method with slight modifications. The temperature of thawing during repeated freeze–thawing cycles was changed replacing the temperature of 100 °C into 65 °C and the number of cycles was extended to 15 according to the recommendation of Nichols and Smith (2004). Nucleic acid extracts that gave positive results at 18 SSU rRNA PCR were further purified using GeneMATRIX PCR/DNA Clean-Up Purification Kit (EURx Ltd) according to manufacturer's instruction. They were used during selective amplification of COWP and Lib 13 gene fragments. The extracts containing parasite DNA were stored at −20 °C until use.

2.3. Amplification of Cryptosporidium 18 SSU rRNA, COWP and LIB 13 gene fragments

The presence of the parasite DNA in feces was confirmed by a nested-PCR method allowing amplification of the 18 SSU rRNA *Cryptosporidium* gene fragment using primers and conditions previously described by Xiao et al. (1999). To increase amplification efficiency and to reduce amplification inhibition each reaction mixture contained a higher concentration of *Taq* polymerase (Nichols et al., 2006) and was supplemented with bovine serum albumin (BSA) (Kreader, 1996).

All DNA extracts positive at 18 SSU rRNA gene locus were further investigated using methods enabling selective amplification of the COWP gene fragment (Homan et al., 1999) and unknown *C. parvum* genomic sequence using species-specific Lib 13 PCR assay (Tanriverdi et al., 2003) with modified temperature profile of the reaction (Nichols R.A., pers. communication). The COWP PCR was a two-step modification of the single-tube nested PCR. All reactions were performed in a Biometra thermocycler (TProfessional BASIC) using 0.2 ml thin walled tubes. The 50 μl reaction