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Short communication

Genotypes and subtypes of *Cryptosporidium* spp. in diarrheic lambs and goat kids in northern Greece

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ABSTRACT

Inconsistent data exist on the distribution of zoonotic *Cryptosporidium* species and subtypes in sheep and goats in European countries, and few such data are available from Greece. In this study, 280 fecal specimens were collected from 132 diarrheic lambs and 148 diarrheic goat kids aged 4 to 15 days on 15 farms in northern Greece, and examined for *Cryptosporidium* spp. using microscopy of Ziehl-Neelsen-stained fecal smears. *Cryptosporidium* spp. in 80 microscopy-positive fecal specimens (39 from lambs and 41 from goat kids) were genotyped by PCR-RFLP analysis of the small subunit rRNA gene and subtyped by sequence analysis the 60 kDa glycoprotein gene. Among the 33 specimens successfully genotyped, *C. parvum* was found in 32 and *C. xiaoi* in one. Seven subtypes belonging to two subtype families (IIa and IId) were identified among the 29 *C. parvum* specimens successfully subtyped, including IIaA14G2R1 (1/29), IIaA15G2R1 (6/29), IIaA20G1R1 (7/29), IIdA14G2 (1/29), IIdA15G1 (9/29), IIdA16G1 (3/29), and IIdA23G1 (2/29). Lambs were more commonly infected with *C. parvum* IIa subtypes, whereas goat kids were more with IId subtypes. The results illustrate that *C. parvum* is prevalent in diarrheic lambs and goat kids in northern Greece and these animals could potentially play a role in epidemiology of human cryptosporidiosis.

1. Introduction

Cryptosporidium spp. are common protozoan parasites, causing moderate to severe diarrhea in humans, domestic animals and wild vertebrates [1]. Human infection can occur via several transmission routes, such as direct or indirect contact with infected persons or animals and ingestion of contaminated food and water [2]. Infected small ruminants and cattle are important reservoirs of *Cryptosporidium* spp., as they shed a large number of oocysts in the environment [3].

Thus far, there have been over 30 established species and more than 50 named genotypes in the genus *Cryptosporidium* [4,5]. Molecular techniques have identified three major *Cryptosporidium* species in sheep including *C. parvum, C. ubiquitum* and *C. xiaoi*, although *C. hominis, C. suis, C. scrofarum, C. andersoni,* and *C. fayeri* are occasionally detected [4,6–8]. Among them, *C. hominis, C. parvum,* and *C. ubiquitum* are major human pathogens [3,4]. Studies on molecular characterization of *Cryptosporidium* species in goats are fewer, but a similar distribution of *Cryptosporidium* species has been obtained [9–17]. The dominant *Cryptosporidium* species in sheep or goats, however, is different among different studies, even those conducted in Europe [15,18–21].

While the transmission of *Cryptosporidium* spp. has been characterized in many European countries using molecular epidemiologic tools, genotypic characterization of *Cryptosporidium* spp. in Greece has been reported only in a recent study of small ruminants in the island of Crete [13]. The aim of the present study was to identify the distribution of *Cryptosporidium* species and *C. parvum* subtypes in pre-weaned lambs and goat kids with diarrhea in northern Greece.

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2. Materials and methods

2.1. Specimen collection and examinations

The study was conducted with specimens collected from 10 sheep and 5 goat farms in northern Greece during October 2013 to March 2014. These farms had diarrhea problems in newborn animals at the time of the study. A total of 280 fecal specimens were collected from the rectum of 132 diarrheic lambs and 148 diarrheic goat kids aged 4 to 15 days, using rectal swabs (live animals) or single–use latex gloves (dead animals). Before laboratory tests the specimens were kept at 4 °C for less than 24 h. They were examined for *Cryptosporidium* oocysts using microscopy of fecal smears strained by the modified Ziehl-Neelsen technique [22]. Microscopy-positive specimens from 39 lambs and 41 goat kids were sent to the Centers for Disease Controls and Prevention (CDC), Atlanta, USA for molecular characterization of *Cryptosporidium* spp.

2.2. Genotyping of Cryptosporidium spp.

Upon arrival at CDC, fecal specimens were washed twice with distilled water by centrifugation. DNA was extracted from up to 0.5 g of fecal concentrate using the Fast DNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA), and analyzed for *Cryptosporidium* spp. by using a small subunit (SSU) rRNA-based nested PCR. *Cryptosporidium* species presented were determined by restriction fragment length polymorphism (RFLP) analysis of the secondary PCR products, using restriction enzymes *Ssp*I and *Mbo*II [23]. The relative number of *Cryptosporidium* oocysts among groups of PCR-positive specimens was compared using Ct values in the newly established 18S-LC2 qPCR [24], with the use of EvaGreen instead of FRET probes for detection of the amplification product.

2.3. Subtyping of C. parvum

All SSU rRNA PCR-positive specimens were further examined by PCR analysis of a ~850-bp fragment of the 60 kDa glycoprotein (gp60) gene [25]. The *C. parvum* subtypes presented were identified by DNA sequencing of the gp60 PCR products using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The nucleotide sequences obtained were aligned with reference sequences using Clustal X 2.1 (http://www.clustal.org/). The established subtype nomenclature was used in naming *C. parvum* subtypes [3].

3. Results

3.1. Cryptosporidium occurrence in lambs and goat kids

Cryptosporidium spp. were detected by microscopy in 39 (29.5%) of the 132 lambs and 41 (27.7%) of the 148 goat kids examined on the 15 farms. Among the 10 sheep farms examined in this study, eight were positive for *Cryptosporidium* spp. Similarly, four of the five goat farms were positive for *Cryptosporidium* spp.

3.2. Distribution of Cryptosporidium species

Among the 80 microscopy-positive specimens examined by PCR analysis of the SSU rRNA gene, 16 specimens from lambs and 17 from goat kids generated the expected PCR products. RFLP analysis of the PCR products identified *C. parvum* in 16 lambs and 16 goat kids and *C. xiaoi* in one goat kid (Table 1).

3.3. Distribution of C. parvum subtypes

In gp60 PCR, 28 of the 32*C. parvum*-positive specimens and the *C. xiaoi*-positive specimen generated the expected products. Sequence analysis of the gp60 PCR products identified the presence of seven *C. parvum* subtypes, including IIdA15G1 (in 8 goat kids and 1 lamb), IIaA20G1R1 (in 5 lambs and 2 goat kids), IIaA15G2R1 (in 4 lambs and 2 goat kids), IIdA16G1 (in 3 lambs), IIdA23G1 (in 2 goat kids), IIaA14G2R1 (in 1 goat kid), and IIdA14G2 (in 1 goat kid). They had nucleotide sequences identical to GQ121027, KJ158750, AF403166, LT556064, KP997136, KF128738, and GQ121027 (with an A to G substitution at the trinucleotide repeat region), respectively. Four *C. parvum*-positive specimens (from 3 lambs and 1 goat kid) did not produce the expected PCR product at the gp60 locus (Table 2).

Table 1
Distribution of *Cryptosporidium* species in lambs and goat kids in northern Greece.

Animal species	No. of specimens examined	No. of specimens microscopy-positive	Number of specimens successfully genotyped	*C. parvum*	*C. xiaoi*
Lambs	132	39	16	16	–
Goat kids	148	41	17	16	1
Total	280	80	33	32	1

3.4. Differences in parasite loads between C. parvum IIa and IId subtype families

In 18S-LC2 qPCR, the mean ± SD Ct value of *Cryptosporidium*-positive specimens was 28.05 ± 2.27. Among them, gp60-negative specimens (29.72 ± 1.18) had higher mean Ct value than gp60-positive specimens (27.82 ± 2.30). Specimens positive for IId subtypes (28.29 ± 2.24) had slightly higher mean Ct value than specimens positive for IIa subtypes (27.31 ± 2.34).

4. Discussion

The present study provides the first molecular identification and characterization of *Cryptosporidium* spp. in diarrheic lambs and goat kids in northern Greece. Data generated indicate that *C. parvum* is the major *Cryptosporidium* species in pre-weaned lambs and goat kids with diarrhea; it was identified in all 32 specimens successfully characterized. One of the goat kids had concurrent infection of *C. parvum* and *C. xiaoi*.

This distribution of *Cryptosporidium* spp. in lambs is comparable to the ones in studies conducted in the United Kingdom and Spain, which have shown a dominant occurrence of *C. parvum* in lambs [9,19,26,27]. Elsewhere in Europe, studies conducted in Belgium and Norway have shown a dominance of *C. ubiquitum* in lambs [18,20], and one study in Poland has shown a dominance of *C. xiaoi* [21]. In contrast, studies from Australia, China and Unites States have mostly shown a common

Table 2
Distribution of *Cryptosporidium parvum* subtypes in lambs and goat kids in northern Greece.

Subtype	Lambs		Goat kids		Subtotal
	No.	Age (days)	No.	Age (days)	
IIa subtype family					
IIaA14G2R1	0	–	1	15	1
IIaA15G2R1	4	8–10	2	8–10	6
IIaA20G1R1	5	7–10	2	10	7
IId subtype family					
IIdA14G2	0	–	1	15	1
IIdA15G1	1	10	8	10–15	9
IIdA23G1	0	–	2	15	2
IIdA16G1	3	8–10	0	–	3