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PROTOZOOLOGY - SHORT COMMUNICATION

Molecular identification of *Cryptosporidium* species from domestic ruminants and wild reptiles in Cyprus

Chad Schou[1] · Kyriacos Hasapis[1] · Panagiotis Karanis[1]

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Abstract
The presence of *Cryptosporidium* species in faecal samples of 32 sheep (*Aries bovis*), 10 goats (*Capra circus*), 1 blunt nose viper (*Macrovipera lebetina lebetina*), 3 Kotschy's geckos (*Mediodactylus kotschyi*) and 6 wild stellagamas (*Stellagama stellio cypriaca*) in Cyprus were investigated by polymerase chain reaction (PCR) and sequencing. *Cryptosporidium* species were found in 9/32 sheep, 5/10 goats, 2/3 Kotschy's geckos and 2/6 stellagamas faecal samples based on the sequencing of the *18S* rRNA gene. Subtyping was achieved based on the sequencing of the *gp60* gene. Four different species have been identified: *Cryptosporidium parvum* in goats (subtype IIaA15G1R1), *C. xiaoi* (subtypes XXIIId and XXIIIl) and *C. ubiquitum* (subtype XIIa) in sheep and *C. varanii* and *C. parvum* in lizards; the viper snake sample was negative. This is the first report on the molecular identification of a variety of *Cryptosporidium* species from domestic ruminants and wild reptiles in the Republic of Cyprus.

Keywords *Cryptosporidium* · Epidemiology · Ruminants · Reptiles · Cyprus

Introduction

Cryptosporidium is a ubiquitous protozoan parasite that can cause diarrheal illnesses and death in vulnerable groups of people and livestock (Karanis et al. 2007; Kotloff et al. 2013; Caccio and Chalmers 2016; Carter et al. 2020). *Cryptosporidium* is frequently found in many wildlife habitats and farm areas (Ryan et al. 2021). It has environmentally stable oocysts that are resistant to chlorine disinfection and can easily be spread in the environment or in the food and water supply by infected faecal material (Karanis et al. 2007; Ryan et al. 2021). *Cryptosporidium* is known to infect ruminants (cattle, camels, deer, elk, goats and sheep) and lead to economic losses from decreased milk production and animal death (Plutzer and Karanis 2009; Giadinis et al. 2012; Arsoy 2020; Ursini et al. 2020).

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Chad Schou and Kyriacos Hasapis contributed equally.

✉ Panagiotis Karanis
karanis.p@unic.ac.cy

[1] Department of Basic and Clinical Sciences, University of Nicosia Medical School, 21 Ilia Papakyriakou, 2414 Engomi, P.O. Box 24005, 1700 Nicosia, Cyprus

Cryptosporidium infection can cause a severe diarrheal disease called cryptosporidiosis that is particularly life-threatening to neonate animals, children and immunocompromised individuals (Giadinis et al. 2012; Kotloff et al. 2013; Caccio and Chalmers 2016; Alsmark et al. 2018; Carter et al. 2020).

Humans and animals are at risk of infection from some of the more pathogenic species (Carter et al. 2020; Ryan et al. 2021). Heavy rainfall can flush *Cryptosporidium* oocysts from farms into lakes, rivers and reservoirs, which can have a negative impact on public and animal health (Xiao et al. 2004; Karanis et al. 2007; Plutzer and Karanis 2009; Robertson 2009; Baldursson and Karanis 2011).

Few studies from Cyprus have indicated the infection frequency of several pathogens including *Cryptosporidium* in young ruminants might be similar to other geographical areas (Giadinis et al. 2012; Arsoy 2020; Schou et al. 2020). Using a commercial ELISA test and the acid-fast Ziehl–Neelsen (ZN) technique, *Cryptosporidium* infections were detected in 4–15-day-old lambs and goat kids in the Larnaca area of Cyprus and > 20% of the investigated goat and sheep flocks suffered from neonatal diarrhoea (Giadinis et al. 2012). Surveyed dairy goat farms in the northern part of Cyprus reveal that 75% of goat kids experienced episodes of diarrhoea and attributed the main cause to common internal parasites

(Arsoy 2020). However, the molecular identification of the *Cryptosporidium* species was not determined in Cyprus.

It has been found that sheep and goats worldwide are commonly infected with three *Cryptosporidium* species, *C. parvum*, *C. ubiquitum* and *C. xiaoi* (Guo et al. 2021). The dominant species found in small ruminants from European countries was *C. parvum* which is a species that has a wide range of hosts, including humans (Cacciò and Chalmers 2016). This has led to zoonotic infections and petting farm outbreaks due to contact with lambs and goat kids (Conrad et al. 2017). Outbreaks of cryptosporidiosis associated with cattle spring pasture events have been also reported (Alsmark et al. 2018). Historically, *Cryptosporidium* infection has been thought to be host-specific, and species have been named according to the host in which they were identified. The development of molecular detection and typing tools has resulted in the identification of 45 recognised *Cryptosporidium* species and > 120 genotypes, 19 species and four genotypes have been reported in humans with *C. hominis*, *C. parvum*, *C. meleagridis*, *C. canis* and *C. felis* being the most prevalent (Ježková et al. 2021; Ryan et al. 2021; Zahedi et al. 2021).

Cyprus is an island in the eastern Mediterranean Sea. Tourism and agriculture are the main industries on the island. Goat and sheep livestock represent another important industry on the island. The milk is mainly used by the diary and haloumi cheese industries. Although there are indications of good welfare level in terms of animal care in the country, protozoan infections, such as *Cryptosporidium*, can negatively affect milk production on the island and subsequently lead to economic loss from diarrheal associated mortality in young ruminants (Giadinis et al. 2012; Arsoy 2020; Ursini et al. 2020). The present preliminary study aims to investigate the molecular identity of *Cryptosporidium* species of the Republic of Cyprus. The inclusion of snake and lizard samples in this study was intended to encompass a One Health approach on *Cryptosporidium* infection in the immediate environment. The One Health concept shows how the health of people, animals and the environment are linked to one another.

Materials and methods

Study area and sample collection

Between January to September 2021, a total of 52 faecal samples from domestic sheep (*Aries bovis*), domestic goats (*Capra circus*) and wild reptiles (Stellagama lizards/*Stellagama stellio cypriaca*; Gecko lizards/*Mediodactylus kotschyi*; Blunt nose viper snake/*Macrovipera lebetina lebetina*) were randomly collected from a single farm and its surroundings in the Larnaca district of the Republic of Cyprus (see Table 1). The farm owner was asked to participate in the study after a discussion with the researchers in December 2020 about annual diarrhoeal incidents in the new borne sheep and goats. The owner mentioned that sick animals were isolated from healthy ones, and it was common that some of the lambs and goat kids died from diarrhoeal infection, which was the reason that this particular farm was selected for this preliminary study dealing with the molecular identification of *Cryptosporidium* species in Cyprus.

Sheep were separated from the goats and the young animals were separated from the adults in different areas of the shelters until they matured or were sold for meat. Fresh samples were collected from the animal shelter floors early in the morning before the ruminants had access to pastures for several hours. There were 3 diarrhoeal samples collected in January and 3 diarrhoeal samples collected May 2021 from young lambs less than 5 months old. The rest of the ruminant samples were collected from shelter floors for the 2–3-year-old goats and sheep. Due to the COVID-19 lockdown situation in Cyprus, sampling opportunities were limited in this preliminary study. In addition, many young animals were sold for meat before sample collection in May 2021. The wild lizard samples were collected near the animal shelters and landscape since they were difficult to locate inside the shelters. The snake sample was taken from the road that led approximately 1500 m to the farm where it was found dead and presupposed to be crushed by a recently passing car.

After sampling, the material was transported 1 h to our laboratory and immediately stored at − 20 °C until thawed

Table 1 Summary of results on the prevalence of PCR-positive *Cryptosporidium* samples from various animal species in the Larnaca district

Animal host (species)	Positive	n = number of samples examined	% positive
Sheep (*Ovis aries*)	9	32	28.1%
Goats (*Capra circus*)	5	10	50%
Stellagama lizards (*Stellagama stellio cypriaca*)	2	6	33.3%
Gecko lizards (*Mediodactylus kotschyi*)	2	3	66.7%
Blunt nose viper snake (*Macrovipera lebetina lebetina*)	0	1	0%
Total	**18**	**52**	**34.6%**

for DNA extraction. Sample DNA was extracted within 2 weeks of collection and stored at − 20 °C before PCR analysis. All samples were subject to PCR technique for the detection of cryptosporidial DNA followed by sequencing to specify the species of *Cryptosporidium* as described below (Table 1).

DNA extraction and PCR

Total genomic DNA was extracted from faecal samples by QIA amp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *Cryptosporidium* species were determined by nested PCR amplification of a *18S* rRNA gene fragment (600 bp), using primers previously described (Silva et al. 2013). The primers used for the first amplification were SHP1 (forward) 5′-ACC TAT CAG CTT TAG ACG GTA GGG TAT-3′ and SHP2 (reverse) 5′-TTC TCA TAA GGT GCT GAA GGA GTA AGG-3′. The primers used for the second amplification were SHP3 (forward) 5′-ACA GGG AGG TAG TGA CAA GAA ATA ACA-3′ and SSU-R3 (reverse) 5′-AAG GAG TAA GGA ACA ACC TCC A-3′. The conditions used in both amplifications were 94 °C for 3 min, 35 cycles of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 60 s, followed by a final extension of 72 °C for 7 min.

The subtyping of *Cryptosporidium* species was achieved by nested PCR amplification of a *gp60* gene fragment. More specifically, for the subtyping of *C. parvum*, primers AL3531 (5′-ATAGTCTCCGCTGTATTC-3′) and AL3535 (5′-GGAAGGAACGATGTATCT-3′) were used for the first PCR and primers AL3532 (5′-TCCGCTGTATTCTCAGCC -3′) and AL3534 (5′-GCAGAGGAACCAGCATC-3′) were used for the nested PCR. The size of the final product was about 870 bp (Alves et al. 2003). Cycling conditions for both internal and external PCR were a denaturation step for 3 min at 94 °C, followed by 35 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min, and a final extension step at 72 °C for 7 min (Alves et al. 2003).

For the subtyping of *C. xiaoi*, primers F1 (5′-CCTCTC GGCACTTATTGCCCT-3′) and R1 (5′-ATACCTGAGATC AAATGCTGATGAA-3′) were used for the first PCR and primers F2 (5′-CCTCTTAGGGGTTCATTGTCTA-3′) and R2 (5′-TACCTTCAAAGATGACATCAC-3′) were used for the nested PCR. The size of the final product was about 900 bp (Fan et al. 2021). The PCR amplification consisted of an initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 90 s and a final extension of 72 °C for 10 min (Fan et al. 2021).

For the subtyping of *C. ubiquitum*, primers UbiF1 (5′-TTTACCCACACATCTGTAGCGTCG-3′) and UbiR1 (5′-ACGGACGGAATGATGTATCTGA-3′) were used for the first PCR and primers UbiF2 (5′-ATAGGTGATAATTAG TCAGTCTTTAAT-3′) and UbiR2 (5′-TCCAAAAGCGGC TGAGTCAGCATC-3′) were used for the nested PCR. The size of the final product was about 900 bp (Li et al. 2014). PCR cycling conditions consisted of an initial denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 45 s, 45 s at 58 °C (primary PCR) or at 55 °C (secondary PCR), 1 min at 72 °C and a final extension step for 7 min at 72 °C (Li et al. 2014).

Positive control (*Cryptosporidium parvum* DNA—Leipzig strain) was kindly provided by the Leipzig Parasitology Group and negative control (PCR-grade water instead of DNA template in the master mix) was included in each PCR analysis in parallel.

Sequencing

The DNA bands of the positive PCR products were gel extracted and purified using the Blirt ExtractMe DNA kit (Blirt, Gdansk, Poland) and the purified PCR products were sent for sequencing (using the forward primer of the nested PCR reaction) to Macrogen Ltd. Europe, Amsterdam. For the determination of *Cryptosporidium* species and subtypes, sequences were subjected to BLAST (https://blast.ncbi.nlm.nih.gov/Blast) searches at NCBI GenBank. All sequences were deposited in NCBI GenBank under the accession numbers: OK335816—OK335826, OL378286—OL378292 and OM876972—OM876981 (Table 2 and Table 3).

Table 2 Sequencing results of the positive *Cryptosporidium* DNA extracted samples, *Cryptosporidium* species and GenBank accession numbers

Sample	Animal host	Age	*Cryptosporidium* species	GenBank accession number
G1	Goat	< 3 mo	*C. parvum*	OK335816.1
G4	Goat	< 3 mo	*C. parvum*	OK335817.1
50A	Goat	< 5 mo	*C. parvum*	OK335818.1
50B	Goat	< 5 mo	*C. parvum*	OL378292
50C	Goat	< 5 mo	*C. parvum*	OK335819.1
49A	Sheep	< 5 mo	*C. xiaoi*	OK335820.1
49B	Sheep	< 5 mo	*C. xiaoi*	OK335821.1
49C	Sheep	< 5 mo	*C. xiaoi*	OK335822.1
49D	Sheep	< 5 mo	*C. xiaoi*	OK335823.1
49E	Sheep	< 5 mo	*C. xiaoi*	OK335824.1
S01	Sheep	> 24 mo	*C. ubiquitum*	OL378286
S05	Sheep	> 24 mo	*C. parvum*	OL378287
S06	Sheep	> 24 mo	*C. ubiquitum*	OL378288
SA3	Sheep	> 12 mo	*C. ubiquitum*	OL378291
15B	Lizard	> 12 mo	*C. varanii*	OK335826.1
16A	Lizard	> 12 mo	*C. varanii*	OL378290
16C	Lizard	> 12 mo	*C. varanii*	OL378289
17A	Lizard	> 12 mo	*C. parvum*	OK335825.1

Note: *C. varanii* (synonym of *C. saurophilum*)

Table 3 Subtyping of *Cryptosporidium* species based on sequencing of a part of the *gp60* gene and GenBank accession numbers

Sample	Animal host	Age	*Cryptosporidium* species	*Cryptosporidium* subtype	GenBank accession number
G1	Goat	< 3 mo	*C. parvum*	IIaA15G1R1	OM876972
G4	Goat	< 3 mo	*C. parvum*	IIaA15G1R1	OM876973
50A	Goat	< 5 mo	*C. parvum*	IIaA15G1R1	OM876974
50B	Goat	< 5 mo	*C. parvum*	IIaA15G1R1	OM876975
50C	Goat	< 5 mo	*C. parvum*	IIaA15G1R1	OM876976
49D	Sheep	< 5 mo	*C. xiaoi*	XXIIId	OM876980
49E	Sheep	< 5 mo	*C. xiaoi*	XXIIIl	OM876981
S01	Sheep	> 24 mo	*C. ubiquitum*	XIIa	OM876977
S06	Sheep	> 24 mo	*C. ubiquitum*	XIIa	OM876978
SA3	Sheep	> 12 mo	*C. ubiquitum*	XIIa	OM876979

Ethics statement

Before collecting faecal samples, informed written consent to perform and anonymously publish the present study was obtained from the sheep and goat owner. No invasive, traumatic or clinical procedures were used to collect the faecal samples. No specific ethical approval was required.

Results

Fifty-two faecal samples from sheep, goats and reptiles (3 Kotschy's geckos, 6 stellagamas and 1 blunt nose viper snake) were collected from a single farm in the Larnaca district and evaluated for the presence of *Cryptosporidium* species (Table 1). Eighteen out of the 52 samples were PCR positive for *Cryptosporidium* and were further identified by DNA sequencing (Table 2). Sequence analysis of a 600 bp part of the *18S* rRNA gene revealed 4 different species from the farm location site. All of the positive goat samples (5/10) contained 100% *C. parvum*. The positive sheep samples (9/32) contained 55.6% *C. xiaoi*, 33.3% *C. ubiquitum* and 11.1% *C. parvum*. The Kotschy's geckos (2/3) all had *C. varanii*. However, the stellagamas (2/6) contained 50% *C. parvum* and 50% *C. varanii* (Table 2).

For the subtyping of the *Cryptosporidium* species, five out of seven *C. parvum* isolates were successfully sequenced for *gp60* gene and they were all identified as subtype IIaA15G1R1 (Table 3). Also, three out of four *C. ubiquitum* isolates were successfully sequenced and were all identified as subtype XIIa (Table 3). Finally, two out of five *C. xiaoi* isolates were successfully sequenced and one of them was identified as subtype XXIIIl while the second one was identified as subtype XXIIId (Table 3).

Discussion

Apart from the investigation of Giadinis et al. (2012), no other studies have been published about *Cryptosporidium* in Cyprus and limited data exists on parasitic diseases in Cyprus (Schou et al. 2020). The levels of pathogen contamination in the water supplies and within the island ecosystem are unknown. However, no water or foodborne protozoan outbreaks have been reported in the area yet (Efstratiou et al. 2017; Ahmed and Karanis 2018). In Cyprus, *Cryptosporidium* is not yet on the diagnostic focus of clinicians and information about this pathogen in relation to human infections is also scarce.

We report the first molecular identification of *Cryptosporidium* species collected from 32 sheep, 10 goats, 1 blunt nose viper, 3 Kotschy's geckos and 6 wild stellagamas from a farm in the Larnaca district of the Republic of Cyprus. One stellagama was infected with *C. parvum*, which may have been possibly carried by an insect that the lizard ingested (Graczyk et al. 2000; Szostakowska et al. 2004; Conn et al. 2007). Similar findings in captive reptiles have been reported previously (Xiao et al. 2004). In our study, the snake sample was negative; however, it is well known that snakes can carry cryptosporidial infections (Xiao et al. 2004; Plutzer and Karanis 2007). Ruminants in this study had access to pastures, where they had the ability to cross-contaminate the local environment. Herding ruminants in local pastures is a frequent practice in Cyprus. In the present study, both young and older sheep and goats showed *Cryptosporidium* infections based on the PCR and DNA sequencing analysis. In another selective sampling study (Morgan et al. 1998), a small number of goat samples from both Australia and Cyprus all exhibited the 'calf' genotype. The isolate from Australia was from an outbreak of diarrhoea in 1- to 2-week-old goats. The goat isolates from Cyprus were reported as

asymptomatic cases that were collected at random by the Cyprus Ministry of Agriculture, Department of Veterinary Services (Morgan et al. 1998).

The results of our preliminary study represent a small cross section of the molecular identify of *Cryptosporidium* species on the island. It provides a link to earlier veterinarian work and sets the stage for further investigation into the molecular epidemiology of *Cryptosporidium* species and transmission ways of this zoonotic pathogen in Cyprus.

Conclusions

First report of the molecular identification of *Cryptosporidium* species in domestic ruminants and wild reptiles in Cyprus. This research has revealed a high percentage of *Cryptosporidium* positive cases in domestic ruminants in Cyprus from a single farm selected for this preliminary study. *Cryptosporidium* can have a negative impact on agricultural activities and milk production of domestic animals. The results have indicated that human and animal pathogenic *Cryptosporidium* are present in the country. More work needs to be conducted in the area based on the One Health approach to facilitate our understanding of the zoonotic transmission of this important gastrointestinal parasite. Routine diagnostic and surveillance systems should become an important part of the public health system for the control of infectious diseases like cryptosporidiosis in Cyprus.

Author contribution All authors have read and agree to the final draft of the manuscript. Conceptualization and methodology, P. K., C. S. and K. H.; C. S. and K. H.; investigation, C. S., K. H. and P. K.; writing—original draft preparation, C. S., K. H. and P. K.; writing—review and editing, C. S., K. H. and P. K.; supervision, P. K.

Declarations

Conflict of interest The authors declare no competing interests.

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