High occurrence of zoonotic subtypes of *Cryptosporidium parvum* in Cypriot dairy farms

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Abstract

Cryptosporidium parvum is one of the major causes of neonatal calf diarrhoea resulting in reduced farm productivity and compromised animal welfare worldwide. Livestock act as a major reservoir of this parasite, which can be transmitted to humans directly and/or indirectly, posing a public health risk. Research reports on *Cryptosporidium* prevalence from the east Mediterranean region are scarce with even less originating from Cyprus. This study is the first to explore occurrence of *Cryptosporidium* spp. in young dairy calves on the island. A total of 242 faecal samples were collected from 10 dairy cattle farms in Cyprus, all of which were screened for *Cryptosporidium* spp. using nested-PCR amplification targeting the small subunit of the ribosomal RNA (*18S* rRNA) gene. The 60 kDa glycoprotein (*gp60*) gene was also sequenced for the samples identified as *C. parvum* positive to determine the subtypes present. Occurrence of *Cryptosporidium* was 43.8% (106/242) with at least one positive isolate in each farm sampled. Prevalence per farm ranged from 20–64%, while *C. bovis, C. ryanae* and *C. parvum* were the only species identified. Amongst those, the latter was the predominant species, representing 51.8% of all positive samples. Four zoonotic subtypes were identified – IIaA14G1R1, IIaA15G1R1, IIaA15G2R1 and IIaA18G2R1. IIaA14G1R1 was the most abundant; representing 48.2% of all *C. parvum* positive samples and was also the most widespread. This is the first report of zoonotic subtypes of *C. parvum* circulating in Cyprus. These results highlight the need for further research into the parasite focusing on its diversity, prevalence, host-range and transmission dynamics on the island.

Introduction

Cryptosporidium is an apicomplexan parasite and the causative agent of cryptosporidiosis [1]. Clinical signs of this disease include watery diarrhoea and dehydration, which can prove fatal, particularly in immunocompromised individuals and infants [2,3]. The transmission form of the parasite is the oocyst, which can be transmitted directly via the faecal-oral route or indirectly, through contaminated food and water [1]. Oocysts are very robust, highly resistant to environmental changes and can remain dormant for up to six months [1,4]. Millions of oocysts can be shed through bowel movements of infected hosts, both humans and other animals. Coupled with the lack of effective therapeutic agents and vaccines [5,6], it has proven difficult to manage outbreaks and the spread of *Cryptosporidium*. The outbreaks are commonly of zoonotic nature with farms constituting a major reservoir of the parasite [7].

Of the 49 described *Cryptosporidium* species, around 20 of them are zoonotic [8,9]. Molecular surveillance of *Cryptosporidium* outbreaks indicates that *C. parvum* is responsible for the majority of human cryptosporidiosis cases [10,11]. Amongst farm animals, goats and cattle are the main hosts of the organism. In cattle farms, *C. andersoni, C. bovis, C. ryanae* and *C. parvum* are the infectious species. Age-related variance of infecting *Cryptosporidium* species has been shown, with *C. parvum* typically infecting neonatal calves, while the others are typically found in cows, heifers and post-weaned calves [12]. *Cryptosporidium parvum* is the most clinically significant as the most widespread species in cattle [13–15], a major cause of enteritis, causing neonatal diarrhoea and subsequent long-term adverse effects on weight gain [6,13,16]. The disease leads to significant economic losses for cattle farmers due to reduced production efficiency of meat and milk [17–19]. Aside from the financial impact of *Cryptosporidium* infection, cattle have been well established as a key source of zoonotic cryptosporidiosis giving an epidemiological dimension to the issue [5,20,21].

Prevalence investigations have frequently been carried out in almost all continents [22,23]. Studies of *Cryptosporidium* occurrence in Europe point towards a high prevalence [24–26]. These investigations have primarily focused on mainland Europe, while island nations have been overlooked with the exception of the UK [27]. Island settings are ideal for studying epidemiological and ecological aspects of parasitic organisms due to restricted boundaries and subsequent limited movement of hosts. Regrettably, there is lack of information on the prevalence and subtypes of *Cryptosporidium* spp. circulating in small European island nations.

This study fills the gap of scant molecular epidemiological data on *Cryptosporidium* spp. in dairy cattle in Cyprus. The island is the third largest in the Mediterranean region and is in the cross paths of three continents. Using molecular techniques and analyses, we were able to

identify *Cryptosporidium* species and subtypes circulating within the examined cattle farms. This work will spearhead further studies on investigating the role and effect of the parasite in this unique region of the world.

Methods

Study area

Samples were collected from ten randomly selected farms located in two districts of Cyprus, namely Larnaca and Nicosia (**Figure 1**). These two districts were chosen due to the high density of cattle farms. Eight farms were located in Nicosia and two in Larnaca. The Nicosia district lies in the centre of the island and samples were taken from the Dali, Tseri, Ayia Varvara, Akaki and Arediou regions. Most of the selected farms in Nicosia are at a relatively low altitude (200–400 m) experiencing hot, dry and humid summers and cold winters with minimal precipitation. The two farms in the Larnaca district were located in the Aradippou region, which has a great confluence of farms. The area experiences a similar climate to that of Nicosia with slightly elevated humidity due to a closer proximity to the sea and a lower altitude (80 m). Farmers were invited to participate to the study by the employed veterinarian, who also explained the research project and its overall aims and objectives. The study was under the umbrella of the national Veterinary Services. Cows from all selected farms were of the dairy producing Holstein Friesian breed. All participating farms raised their cows semi-intensively.

Sample collection and DNA extraction

242 faecal samples were collected in November 2019. Samples ranged from pre-weaned calves up to three months of age and animals were randomly selected. Faeces were collected immediately after defecation, placed in sterile tubes and stored on ice. Tubes were stored at -20 °C upon arrival to the laboratory. DNA extraction was carried out using 200 mg of faeces per sample and the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, California, USA) according to manufacturer's instructions, with slight modifications. Specifically, 650 µl of S1 Lysis Buffer was used for each sample. After addition of S2 Lysis enhancer, samples were incubated for 13 min at 65 °C, and homogenised for a further 13 min. Following addition of S3 Clean-up Buffer, samples were incubated at 4 °C to optimise removal of proteins. After addition of 100 µl S6 Elution Buffer, samples were incubated at room temperature for 3 min before centrifugation to improve DNA yield. Genomic

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DNA was stored at -20 °C until amplification reactions of the small subunit ribosomal RNA (*18S* rRNA) and 60 kDa glycoprotein (*gp60*) genes were carried out.

Cryptosporidium *spp. screening and molecular genotyping*

Samples were screened for *Cryptosporidium* spp. using nested-PCR amplification of a 631 bp region of the *18S* rRNA gene [28]. The external primers used were 5´-GATTAAGCCATGCATGTCTAA-3´ (forward) and 5´-TTCCATGCTGGAGTATTCAAG-3´ (reverse). The internal primers were 5´-CAGTTATAGTTTACTTGATAATC-3´ (forward) and 5´-CCTGCTTTAAGCACTCTAATTTTC-3´ (reverse). Each PCR mixture contained 1 µl of DNA, 0.4 µM of each forward and reverse primers, 12.5 µl of 2× PCRBIO Taq Mix Red (PCR Biosystems, London, UK) and 9.5 µl nuclease-free water. Cycling conditions for the external PCR were as follows: a denaturation step for 2 min at 94 °C, followed by 24 cycles at 94 °C for 50 s, annealing at 53 °C for 50 s and extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min.

Cycling conditions for the internal PCR were a denaturation step for 2 min at 94 °C, followed by 30 cycles at 94 °C for 50 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Positive (genomic DNA from a pure culture of *C. parvum*) and negative (water was used as template instead of DNA) controls were included in both reactions. PCR products were separated on a 2% gel and extracted using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, California, USA). Samples were bidirectionally sequenced (Eurofins Genomics) using the internal PCR primers. Chromatograms were manually assessed for quality and ambiguous bases were trimmed on both ends of the reads. For species level identification, sequences were used as queries to perform BLAST searches against the nucleotide database in GenBank followed by alignment with reference sequences.

Cryptosporidium *screening and gp60 subtyping*

To determine the subtype of *Cryptosporidium 18S* rRNA PCR-positive samples, nested PCR of the *gp60* gene was carried out [29]. The external primers used were 5´-ATAGTCTCCGCTGTATTC- 3´ (forward) and 5´-GGAAGGAACGATGTATCT-3´ (reverse). The internal primers were 5´-TCCGCTGTATTCTCAGCC- 3´ (forward) and 5´-GCAGAGGAACCAGCATC-3´ (reverse). Each PCR mixture contained 2 µl of DNA, 0.2 µM each of forward and reverse primers, 15 µl of 2× PCRBIO Taq Mix Red (PCR Biosystems) and 11.8 µl nuclease-free water. Cycling conditions for both internal and external PCR

reactions were: a denaturation step for 3 min at 94 °C, followed by 35 cycles at 94 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Positive and negative controls were as described above and included in both reactions. PCR products were separated on a 2% gel and extracted using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, California, USA). Samples were bidirectionally sequenced (Eurofins Genomics) using the internal PCR primers. Chromatograms were manually assessed for quality and ambiguous bases were trimmed on both ends of the reads. Subtypes were determined using established standard nomenclature [30]. Newly-generated sequences were used as queries to perform BLAST searches against the nucleotide database in GenBank followed by alignment with reference sequences. Polymorphisms were identified using these alignments.

Results

Cryptosporidium *spp. occurrence across Cypriot farms*

Amplification of the *18S* rRNA gene showed an occurrence of 43.8% with 106/242 specimens positive for *Cryptosporidium* spp. (**Table 1**). Occurrence varied across farms from 20% (3/15) to 64% (16/25).

The majority of the positive samples were identified as *C. parvum* (47.2%, 50/106) with 41 of them showing 100% nucleotide identity to the reference sequence AH006572.2, and one sample showing 99% nucleotide identity to the same sequence. We were unable to obtain good quality sequences for eight *18S* rRNA PCR-positive samples. Nonetheless, *C. parvum* identity was confirmed through positive *gp60* PCR and subsequent sequencing. At the farm level, *C. parvum* was present in 9/10 farms, with occurrence ranging from 9.1% to 34.8%.

The next most common species present was *C. ryanae* (25.5%, 27/106). Additionally, six samples from three farms had co-infections of *C. ryanae* and *C. parvum*, with their presence determined through *18S* rRNA and *gp60* amplification, respectively. Twenty-nine samples had 100% nucleotide identity to the reference sequence KF128756.1, while one was 99% identical to the same sequence. Another variant of *C. ryanae* was identified with three samples having 100% nucleotide identity to the reference sequence KT922233.1. *Cryptosporidium bovis* was the least prevalent species present (21.7%, 23/106) with all 23 samples showing 100% nucleotide identity to the reference sequence EU827363.2. Representative nucleotide sequences of *18S* rRNA have been deposited in GenBank under accession numbers OL348064-OL348160.

Cryptosporidium parvum subtyping through gp60 analysis

All 50 of *18S* rRNA PCR-positive samples were screened using nested-PCR of the *gp60* gene. Of these, the *gp60* gene was successfully amplified and sequenced in 42 samples and then subtyped for *C. parvum*. A further five *gp60* positive samples were identified as *C. ryanae.* Sequence analysis revealed the presence of five subtypes, all belonging to the IIa *C. parvum* family (**Table 2**). Nine of the farms sampled contained at least one *C. parvum* subtype.

The IIaA14G1R1 subtype was the most numerically prevalent occurring in 60% of the farms, making it also the most widely distributed. It was the sole subtype present on three farms and represented 57.4% (27/47) of all *C. parvum* positive samples with successful *gp60* sequencing. All 27 showed 100% nucleotide identity to the reference sequence MN815774.1. The IIaA12G1R1 subtype was the next most prevalent and was only found in one farm, accounting for 29.7% (14/47) of all *C. parvum* infections. All 14 samples were identical and showed 99% nucleotide identity to the reference sequence MW411017.1. Four samples were identified as IIaA15G2R1, with two of them showing 100% nucleotide identity to the reference sequence DQ630518.1, while the other two were 99% identical with the same sequence. One sample was identified as IIaA15G1R1, with 99% nucleotide identity to the reference sequence AB777872.1 and another was identified as IIaA18G2R1, with 99% nucleotide identity to the reference sequence DQ630515.1 (Table 3). Representative nucleotide sequences of *gp60* have been deposited in GenBank under accession numbers OL462897- OL462943.

Geographical distribution of subtypes

For easy visualization, subtype names are also indicated by their colour as depicted in **Figure 2**. The most broadly distributed subtype is IIaA14G1R1 (red), present in 6/9 (67%) of *C. parvum* positive farms, followed by IIaA15G2R1 (green), present in 3/9 (33%) farms. In all cases, the IIaA15G2R1 (green) subtype co-occurred with another and was the least dominant of the two. In two cases, co-occurrence was with IIaA14G1R1 (red) and in one with IIaA12G1R1 (orange). The latter subtype, along with IIaA18G2R1 (yellow) and IIaA15G1R1 (blue) occurred only in one farm each. In four farms only a single subtype was detected, IIaA14G1R1 (red) in three and IIaA15G1R1 (blue) in one. Notably, in Larnaca, the two sampled farms were in very close proximity, but the subtypes present did not overlap. One farm had only IIaA14G1R1 (red) subtype, while the other had IIaA12G1R1 (orange) and IIaA15G2R1 (green).

Discussion

This is the first study investigating *Cryptosporidium* spp. occurrence on dairy farms of Cyprus. The study focused exclusively on young calves up to three months of age. The overall occurrence of the parasite was 43.8%. Previous molecular studies in the surrounding Mediterranean region have generally reported lower incidences of *Cryptosporidium* infection amongst cattle. Specifically, studies using PCR followed by *18S* rRNA sequencing in Turkey found prevalence ranging from 3.9-53.6% [31–35]. In Egypt, subtype based prevalence was between 7-30.2% [36–40]. A small-scale PCR-based study in Jordan found 18.7% of asymptomatic cattle carried the organism [41]. The discrepancy in prevalence observed among these studies may be due to various factors, including age of cows, farm/herd location, seasonality, farm management, and method of detection. Due to time and financial constraints, microscopic analysis of faeces is a popular screening method, where only microscopically positive samples undergo molecular genotyping. While these studies do provide information on *Cryptosporidium* species and *C. parvum* subtypes present, they likely underestimate prevalence due to the reduced sensitivity of microscopy. Additionally, some studies only targeted diarrheic animals [33, 35], possibly inflating incidence of *Cryptosporidium* reported. Nonetheless, this is not an accurate representation of occurrence of the parasite in the overall cattle population, as it does not include healthy animals. Herein, we used a nested PCR approach to identify and subtype (see below) *Cryptosporidium*, which is a more sensitive and specific method. At the farm level, *Cryptosporidium* was broadly distributed across all. This finding matches previous flock-level studies in goats and sheep in Cyprus [42]. Even though this is a preliminary finding, it shows that the parasite is widespread in this region hinting at its potential of zoonotic transmission. To that end, follow up studies should expand to include farms across the whole island. The variation in *Cryptosporidium* occurrence per farm could indicate differences in farm management practices. Climate variables, such as temperature and precipitation, may also play a part in *Cryptosporidium* spread [43,44], with some studies showing seasonal variation in *Cryptosporidium* prevalence in cattle farms [45]. Nonetheless, in this study, there was no precipitation and minimal variation in temperature in the region during the sampling period (see Supplementary Material, S1). As such, future investigations should report these along with potential risk factors – e.g., calf husbandry practices, box

dynamics, bedding, colostrum feeding schedule, climate and geographical variables – to understand their impact in parasite spreading.

Cryptosporidium parvum was the most numerically predominant species present, representing 51.8% of total infections, followed by *C. ryanae* at 31.1% and *C. bovis* at 21.7%. Our results on predominance of *C. parvum* are in agreement with those from surrounding regions in both healthy and diarrheic animals [31,35,36,46–48]. The predominance of *C. parvum* specifically in pre-weaned cattle has also been reported in the USA [12]. At the farm level, *C. parvum* and *C. ryanae* were found in 90% of the farms, while *C. bovis* was present in 60%. *Cryptosporidium ryanae* and *C. bovis* infections are typically asymptomatic and occur in older animals. Since this study focused on pre-weaned and young calves, it is possible that these two species might be underrepresented. Hence, future studies should focus on a wider age range of cattle.

Amongst the 55 *C. parvum* positive samples identified with *18S* rRNA including co-infections, only 47 were subtyped. The rest of the samples could not be subtyped due to unclear sequence chromatograms. This could suggest that the calves from which the samples were obtained carried multiple subtypes of *C. parvum*. A combination of metagenomics and upcoming methodologies will assist in tackling this common issue in the future. The predominant *C. parvum* subtypes found in this study do not correspond to those of surrounding countries. For instance, in Turkey the endemic subtype is IIaA13G2R1. In Egypt, the IId family is the most commonly found in cows, with IIdA20G1 subtype being endemic [37–40,46,49]. Herein, the most numerically abundant *C. parvum* subtype was IIaA14G1R1 (red colour, **Figure 2**) and it was also the most widespread, having been found in 6/10 farms. This subtype has been reported previously in cows from Turkey [31], Austria [50,51], Estonia [52], Poland [53], Germany [54] and the Netherlands [24], though it was typically less abundant in all cases. Outbreaks of human cryptosporidiosis in Norway and New Zealand [55,56] have been attributed to IIaA14G1R1, and the subtype has also been found in human samples from the USA [57], Ethiopia [58], Slovenia [59] and Slovakia [60]. In the UK, IIaA14G1R1 was found in animal housing premises that had been identified as potential sources of transmission [61]. This is the first report of IIaA14G1R1 as the predominant subtype circulating in a region highlighting its zoonotic potential. However, as this study had a relatively small sample size, the subtypes identified here may not be indicative of the entire cattle population in this country.

The IIaA12G1R1 (orange, **Figure 2**) comprised 25.4% of *C. parvum* positive samples and it was identified only in a single farm. Previously, IIaA12G1R1 was detected in cows in Israel, making this report the second one in the region [47]. Zoonotic potential of this subtype

remains to be determined, having so far only been reported in animals. The IIaA15G2R1 (green, **Figure 2**) subtype is the most predominant worldwide [62], including the neighbouring countries Turkey [63] and Israel [47]. However, it occurred at relatively low levels in the Cypriot farms studied (8.5%). IIaA15G2R1 is responsible for the majority of acute clinical disease in humans, hence, its zoonotic potential has been well established [14,62]. Nonetheless, our results indicate it may not play a key role in zoonotic transmission in Cyprus, though this needs further investigation with larger sample sizes.

A single isolate of each IIaA15G1R1 (blue, **Figure 2**) and IIaA18G2R1 (yellow, **Figure 2**) were also detected. In cattle, IIaA15G1R1 has been found in Egypt [36,39], Sweden [64] and Czech Republic [65]. Its zoonotic potential appears to be high, having been linked to numerous instances of human cryptosporidiosis globally. For instance, in Scotland, IIaA15G1R1 was responsible for 47% of human cryptosporidiosis cases [66]. The subtype has also been identified in humans with diarrhoea from England [67], Australia [68], Egypt [46], Slovenia [59] and Lebanon [69]. IIaA18G2R1 has been previously identified in cattle in the USA [70], Northern Ireland [71], Germany [72], Italy [73] and France [24], though typically was one of the less common subtypes found. This is a potentially zoonotic subtype having been identified sporadically in humans from England [67], USA [57,74] and Australia [75].

Conclusions

To date, there have been no studies on human cryptosporidiosis in Cyprus [76]. Despite this, our study revealed a high occurrence of *C. parvum* (90%) in dairy calves and presence of four zoonotic subtypes in cattle farms. This brings into question the circulation of the various subtypes not only in calves but also in human and other animal hosts as well as the environment. It's worth noting that Cyprus imports cattle from Austria, Czech Republic, Denmark, Germany, Italy and the Netherlands for both breeding and production purposes. Hence, screening these animals for *Cryptosporidium* spp. would help track circulation and introduction of various subtypes across the different countries. While this report represents a first step in determining *Cryptosporidium* burden in cattle in Cyprus, significant gaps on prevalence, transmission dynamics, sources of infection and effective interventions remain, calling into attention the need for a One Health approach in the immediate future.

Institutional Review Board Statement: This study was only focusing on the collection of the faecal samples from selected dairy cows under the supervision of expert veterinarians and thus there were no ethical implications. Consequently, there was no need for perusal of any further ethical approvals.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data have been submitted to GenBank as shown in the methods section.

Conflicts of Interest: The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Geographic location of Cyprus in the East Mediterranean region and distribution of the cattle farms sampled.

Farms 1 – 8 were located across the Nicosia district (green), while farms 9 – 10 were located in the Larnaca district (orange).

Figure 2. *Cryptosporidium parvum* subtype prevalence across Cypriot cattle farms.

Geographical distribution of *gp60* subtypes across Cyprus cattle farms: IIaA12G1R1 (orange), IIaA14G1R1 (red), IIaA15G1R1 (blue), IIaA15G2R1 (green), IIaA18G2R1 (yellow). *C. parvum* positive samples with unsuccessful gp60 sequencing are indicated in grey.

Pie charts are proportional to number of *C. parvum* positive samples identified per farm.

Table 1. *Cryptosporidium* spp. prevalence in Cypriot dairy farms.

Farm	No. of specimens	*Cryptosporidium* spp. present			
		C. parvum	*C. bovis*	*C. ryanae*	*C. ryanae/ C. parvum co-infection*
1	13	3	-	2	-
2	11	2	2	-	-
3	11	1	2	4	-
4	15	-	-	3	-
5	25	7	3	5	1
6	23	5	3	2	1
7	19	3	2	1	-
8	23	8	-	3	-
9	41	13	3	2	3
10	61	8	8	6	-
Overall	242	50	23	28	5

Table 2. Number of *Cryptosporidium parvum gp60* subtypes identified out of total *C. parvum* positive samples per farm.
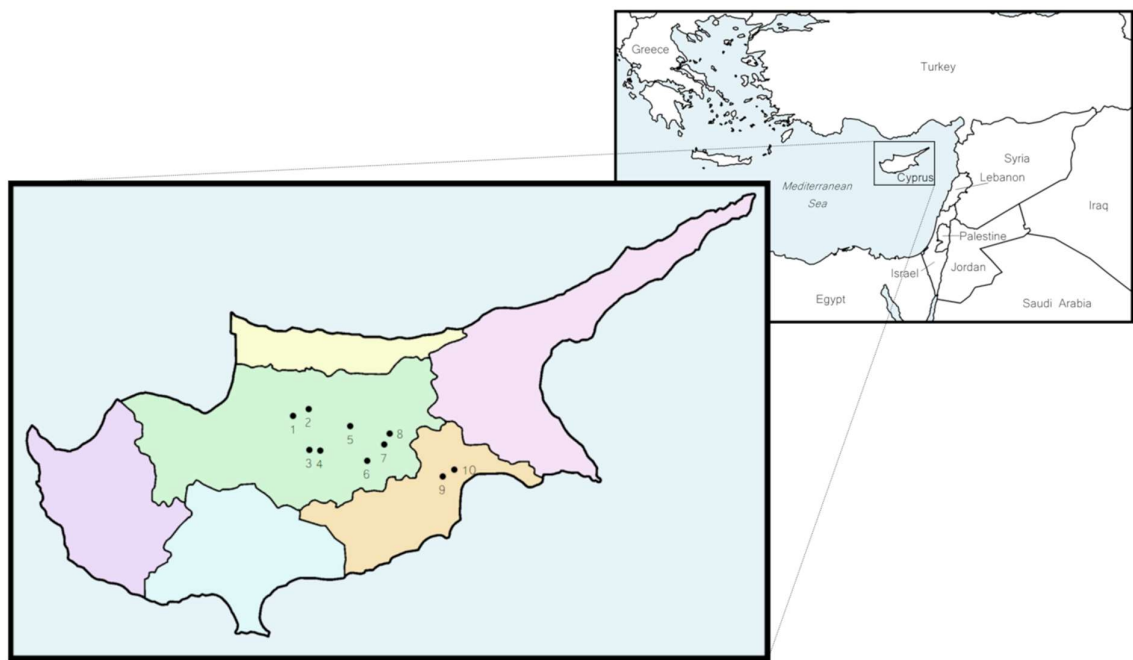
Farm	Subtypes (no. of subtype/total *C. parvum* samples per farm)
1	Unidentified (3/3)
2	IIaA14G1R1 (2/2)
3	IIaA15G1R1 (1/1)
4	-
5	IIaA14G1R1 (6/8), IIaA15G2R1 (2/8)
6	IIaA14G1R1 (5/6), IIa15G2R1 (1/6)
7	IIaA14G1R1 (6/8), IIaA18G2R1 (1/8), Unidentified (1/8)
8	IIaA14G1R1 (2/3), Unidentified (1/3)
9	IIaA12G1R1 (14/16) IIa15G2R1 (1/16), Unidentified (2/16)
10	IIaA14G1R1 (6/8), Unidentified (2/8)

Table 3. Polymorphisms in *Cryptosporidium* 18S SSU rRNA and gp60 gene sequences showing intra-species genetic variability.

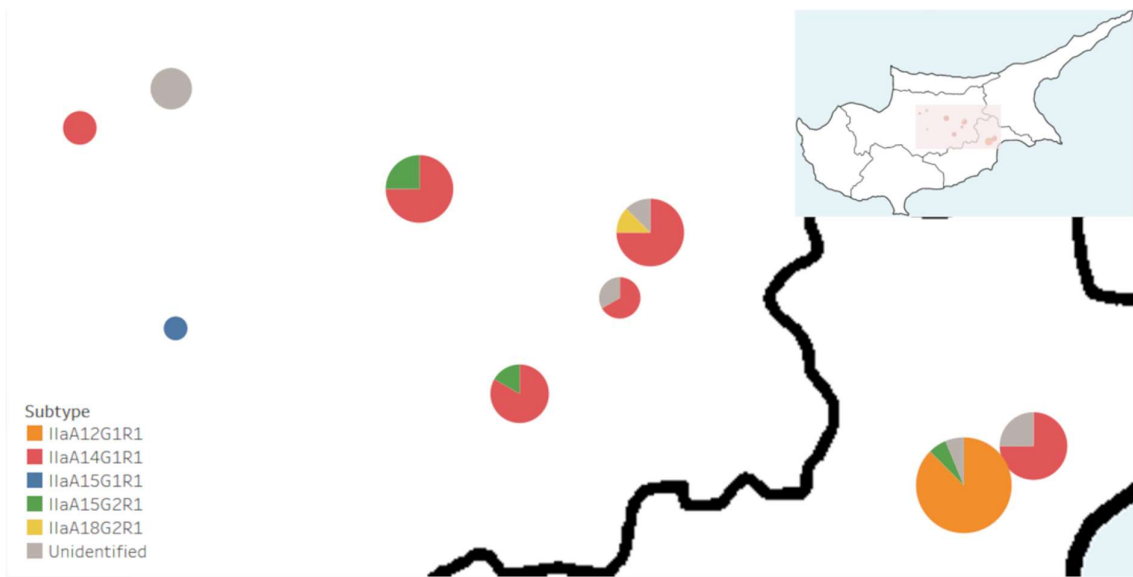| Gene	*Cryptosporidium* species/subtype	GenBank accession number	Polymorphisms[b]	Reference sequence
18S	*C. parvum*	OL348120	G→A, position 701	AH006572.2
18S	*C. ryanae*	OL348112	T insertion, position 490	KF128756.1
Gp60	*C. parvum* (IIaA12G1R1)	OL462923[a]	A→G, position 183 C→T, position 721	MW411017.1
Gp60	*C. parvum* (IIaA15G2R1)	OL462910	G→A, positions 163 and 581 A→T, position 639 A→G, position 687	DQ630518.1
Gp60	*C. parvum* (IIaA15G2R1)	OL462917	T→C, positions 454 and 469	
Gp60	*C. parvum* (IIaA15G1R1)	OL462922	C→T, position 753	AB777872.1
Gp60	*C. parvum* (IIaA18G2R1)	OL462903	T→G, position 375	DQ630515.1

[a] Though multiple identical sequences were found, only one accession number is given for simplicity

[b] Positions indicate differences from the reference sequence

Figure 1. Geographic location of Cyprus in the East Mediterranean region and distribution of the cattle farms sampled.

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