Presence and distribution of *Cryptosporidium* infection amongst European dairy farms

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DECLARATION

I declare that this thesis was composed by myself, that the work contained herein is my own except where explicitly stated otherwise in the text. No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other institution of learning.

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ABSTRACT

Cryptosporidium parvum is a major contributor to gastroenteritis in cattle and has a significant impact on livestock health and farm productivity worldwide. As a zoonotic parasite, it poses a public health risk and can be transmitted both directly and indirectly to humans from livestock.

A total of 1369 faecal samples were collected from 68 dairy farms across Belgium, Cyprus, the Czech Republic, France and the Netherlands. Following DNA extraction, these were screened for *Cryptosporidium* spp. using nested-PCR amplification targeting the 18S rRNA gene. Infecting *Cryptosporidium* species were accurately identified through phylogenetic analysis of sequences. *C. parvum* subtypes were identified through sequencing of the 60kDa glycoprotein (*gp60*) gene.

Our findings show prevalence of *Cryptosporidium* ranged from 23.8% to 43.8%, across the five countries surveyed. The parasite was found in most of the farms sampled, with 86.8% of farms testing positive. *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* were identified, with *C. parvum* being the most predominant, representing 66.6% of all infections. Age-related pattern of infection was observed where infections predominantly occurred in calves. We also found *C. parvum* was associated with pre-weaned calves, while *C. bovis*, *C. ryanae* and *C. andersoni* were associated with older animals. *gp60* subtyping revealed 11 subtypes, 10 of which have been previously reported to cause clinical disease in humans.

This study confirms the role of dairy cattle as a major carrier of zoonotic disease and highlights the need for further research into the parasite focusing on its prevalence and transmission dynamics to better understand how to control disease spread.

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CHAPTER 1: LITERATURE REVIEW

1.1 *Cryptosporidium* – what is it?

Cryptosporidiosis is a major diarrheal disease, the etiological agent being the apicomplexan enteric parasite *Cryptosporidium* [1]. Global disease burden is difficult to estimate due to underreporting and lack of laboratory diagnoses in most countries. Despite this, *Cryptosporidium* is considered a leading contributor to global disease burden among the zoonotic parasites [2]. The parasite interferes with intestinal barrier function and the subsequent malabsorption leads to the main clinical sign of cryptosporidiosis – profuse, watery diarrhoea [3,4]. In immunocompetent individuals, cryptosporidiosis usually presents with self-limiting diarrhoea. Other symptoms include abdominal cramps, nausea, weight loss and fever. Some may not experience any disease symptoms, with only asymptomatic shedding of oocysts. The disease generally resolves itself without intervention, but when compounded with other factors, such as age, pre-existing illnesses and immune status, outcomes can be far more severe. Immunocompromised individuals are particularly at risk for serious outcomes as persistent diarrhoea can lead to severe dehydration and malnutrition [1]. Indeed, *Cryptosporidium* is considered a major opportunistic pathogen, particularly in developing countries with high numbers of HIV/AIDS sufferers [5]. Infected children can have long-term health consequences with studies showing retarded growth in children who contracted the disease in infancy [6–9]. This is particularly troubling for developing countries, where children face other significant health challenges, such as malnutrition, lack of access to clean water and inadequate healthcare.

The infectious form of the parasite is the oocyst, which is primarily transmitted via the faecal-oral route [1] The parasite has a monoxenous life cycle, whereby it takes place entirely in a single host [10–12], with both sexual and asexual reproduction taking place (**Figure 1**). Following ingestion of oocysts by a host, oocysts travel through the digestive system until reaching the GI tract. Here, excystation occurs and four sporozoites are released from the oocyst. Sporozoites invade the mucosal cells of the GI tracts, developing into trophozoites. Through disruption of host cell's actin machinery [13] and adherens/tight junctions [14], the host's apical cell membranes are modified [11,12], eventually forming extracytoplasmic structures known as parasitophorous vacuoles. Within this structure, trophozoites undergo schizogeny to become a type I meront containing eight merozoites. Once released, merozoites can either propagate the asexual reproduction cycle by becoming type I meronts, or form type II meronts in sexual reproduction. In sexual reproduction, these type II meronts undergo gametogony, differentiating into either male microgamonts or female macrogamonts. Microgametes rupture from the macrogamont and go on to fertilise the macrogamonts. After fertilisation, a zygote develops, eventually forming oocysts through sporogony. Two types of oocysts may arise – thick-walled and thin-walled. Thick-walled oocysts form a two-layered, environmentally robust wall and are expelled from the host through excretion, to be ingested by another host. Thin-walled oocysts are responsible for autoinfection. These have only a single-membrane and remain within the host where they rapidly excyst to start the cycle again. These are believed responsible for persistent reoccurring infections in hosts despite a lack of ongoing oocyst challenge [12].

Cryptosporidium oocysts are particularly robust and able to withstand many environmental stresses without impacting viability [1,15]. Decontamination is challenging as they are highly resistant to chlorination and can remain dormant for up to six months [1,15]. While some oocyst inactivation methods have been shown effective, such as ultraviolet radiation [16], these can be difficult to employ wide scale in a cost-effective manner. Excreted oocysts are immediately infectious, millions may be shed in host bowel movements and continue to be passed up to two months after initial symptoms subside. Additionally, infectious dose is low, with minimal levels of oocysts still being sufficient to establish infection in healthy adults [17,18]. Furthermore, there is a lack of effective therapeutic agents and vaccines [19,20]. As such, it is very difficult to manage outbreaks and contain spread of infection.

Cryptosporidium has been discovered in many host species worldwide. While several zoonotic species have been described, *Cryptosporidium parvum* and *Cryptosporidium hominis* are the most important as they are the predominant species responsible for human cryptosporidiosis cases. Unlike most *Cryptosporidium* species which tend to be host-specific, *C. parvum* has a vast host-range, including humans, ruminants, fish, birds, insects and reptiles. This wide host-range is significant as they act as a disease reservoir and can all contribute to disseminating the parasite further. This is particularly troubling when we consider the ever-increasing overlap between human and animal territories.

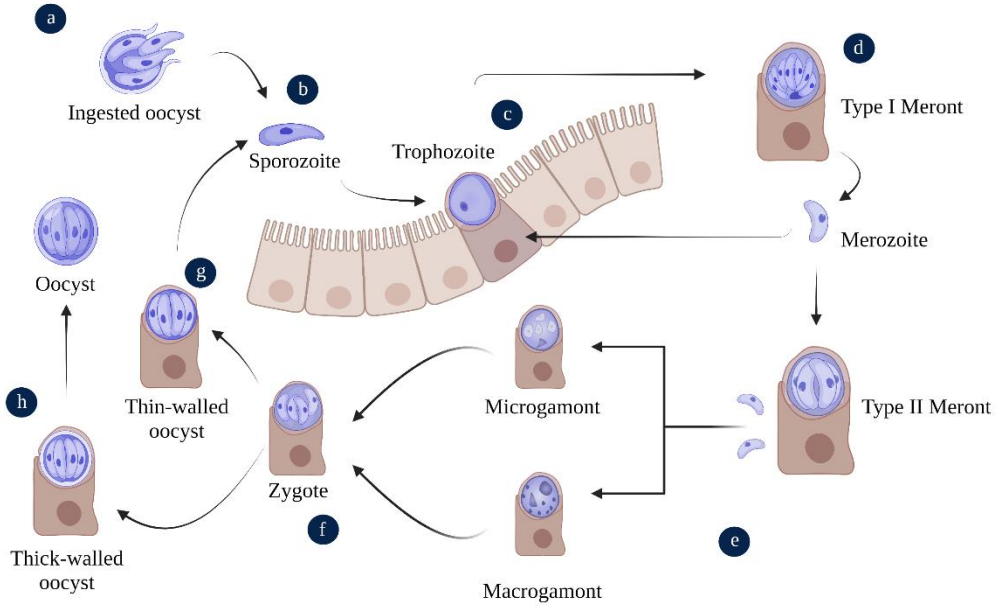

Figure 1. Life cycle of *Cryptosporidium.*
(a) Following ingestion by a suitable host, excystation of oocysts occurs. (b) Sporozoites emerge from the oocyst and (c) invade host mucosal cells. Within these cells, the parasites undergo (d) asexual reproduction and then (e) sexual reproduction. (f) Upon fertilization, zygotes develop and sporulate in the infected host. Zygotes give rise to two different types of oocysts. (g) thin-walled oocysts are responsible for autoinfection, while (h) thick-walled oocysts are excreted from the host into the environment.
Created with BioRender.com

1.2 *Cryptosporidium* in cattle

Of the 49 described *Cryptosporidium* species, around 20 are zoonotic [21–24] and outbreaks are commonly of zoonotic nature [25]. Most zoonotic cryptosporidiosis cases are associated with livestock. Cattle, in particular, have long been established as a key reservoir of infection with numerous global reports of human cryptosporidiosis linked to close contact with cattle [26,27]. In cattle, *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* are the main infectious species, with the *C. parvum* being the most widespread [28–30]. Molecular surveillance of *Cryptosporidium* outbreaks indicates that *C. parvum* is responsible for the majority of human cryptosporidiosis cases [27,31]. Furthermore, oocysts isolated from cattle were able to infect a wide variety of other species [32], so cattle may also contribute to *Cryptosporidium* occurrence in other animals.

Modes of transmission between cattle and humans are not entirely known. While direct contact between individuals and cattle have been implicated, infections on farms may contribute to waterborne transmission too. Water runoff from farms may contaminate drinking water sources, recreational water sources used for swimming or sources of irrigation for crops, leading to foodborne illness. It is possible all these modes of transmission play a role in cryptosporidiosis spread though it remains to be seen which is most important. Reduction of transmission will require better clarity on routes of transmission and sources of infection.

Aside from the epidemiological significance of *Cryptosporidium* in cattle, bovine cryptosporidiosis also has a major financial impact on this sector [19,26,33]. *C. parvum* is a major cause of acute enteritis in cattle, causing neonatal diarrhoea. In most cases, the disease is self-limiting, though persistent diarrhoea can lead to

emaciation, fatigue, severe dehydration, and eventually prove fatal. While mortality is generally low, cryptosporidiosis in young animals can lead to long-term adverse health consequences [20,28]. A recent study found a 34kg difference in weight gain between calves who had severe cryptosporidiosis as neonates and calves that had no infections [34]. Thus, this disease leads to significant economic losses for cattle farmers through dead livestock, treatment of sick animals and reduced production efficiency of meat and milk due to retarded growth [35–37]. Additionally, cryptosporidiosis may exacerbate concurrent infections with other pathogens [38,39] as health compromised animals will be more susceptible to developing severe cryptosporidiosis. Furthermore, it is difficult for farmers to know how to manage spread of infection, as there is insufficient data on risk factors for *Cryptosporidium* infection in cattle. A recent systematic review found no consistent risk or protective factors for preventing *C. parvum* infection [40].

1.3 Methods of detection of *Cryptosporidium* – with emphasis on molecular techniques

As cryptosporidiosis symptoms are non-pathognomonic, cases must be confirmed through laboratory testing. Several methods have been developed to identify *Cryptosporidium* infections, the most common being direct detection of the parasite in faecal samples though indirect detection of infection through serological studies has also been used. Methods of diagnosis can vary greatly between countries and not all have standard protocols with regards to testing patients' specimens [41]. In the UK, clinicians are recommended to screen faecal specimens in all patients suffering with diarrhoea. However, even within countries, different laboratories may

have different screening protocols [42]. The conventional method of diagnosis has been through stool microscopy, though in recent times there has been a shift to utilising molecular methods for the reasons outlined below [41].

Historically, *Cryptosporidium* detection has relied on microscopic examination of faecal smears for the presence of oocysts. These methods typically require some form of differential staining to distinguish oocysts from any background faecal debris [43]. While these methods are generally low-cost, they unfortunately suffer from low sensitivity and specificity [43], meaning *Cryptosporidium* infection may not be detected in samples with low oocyst levels. As even low levels of oocysts can still establish infections in the host [17,18], it is important to use more sensitive methods of detection. Furthermore, these microscopic methods are often time-consuming and require skilled, experienced microscopists to interpret results [43]. Besides these, the major drawback of microscopic studies is the lack of species and subtype identification as *Cryptosporidium* oocysts are not morphologically distinct enough to make accurate species classifications [44].

Different *Cryptosporidium* spp. cause varying disease severities – of the 4 species found in cattle. *C. parvum* is the only one to cause clinical disease, with the other species typically asymptomatic [20]. Species and subtype identifications are vital for outbreak surveillance, as well as tackling infection spread. For example, *C. parvum* and *C. hominis* were historically classified as one species. Molecular testing allowed for the distinction between *C. parvum* human genotype and bovine genotype, later named *C. hominis* and *C. parvum* [45,46]. This distinction allowed researchers to find dichotomies in distribution and transmission dynamics between these two species. *C. hominis* is predominantly associated with anthroponotic

transmission in urban areas, while *C. parvum* was associated with animal contact in rural areas [47,48].

Nucleic-acid based diagnostic techniques, such as polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP), nested-PCR and qPCRs, have allowed for sensitive detection of *Cryptosporidium*. Aside from increased sensitivity, the key advantage of these molecular investigations is the ability to identity infecting *Cryptosporidium* species and subtypes.

Nested-PCR is a highly sensitive method, able to detect as little as a single oocyst in a faecal sample [49–52]. Compared to conventional PCR, nested-PCRs are far more specific as non-specific binding from the first set of primers is decreased in the second amplification step. Concurrently, however, two amplification steps mean there is a greater potential for contamination than in conventional PCR. Some comparative studies have shown sensitivity of nested-PCR varies according to the gene target used, with assays targeting the COWP gene being the most sensitive [52]. Even when targeting the same loci, different nested-PCR protocols have been shown to preferentially amplify one species over another [53]. Quantitative PCR (qPCR) is also an effective detection method and has also shown similar or higher sensitivity than nested-PCR [54–56]. A key advantage qPCR has over nested-PCR is the real-time monitoring of DNA amplification which can be used to assess oocyst load in a sample.

These PCR-based assays have been used extensively to genotype *Cryptosporidium* in epidemiological studies. The genotypic data gained is useful for comparison of studies of clinical, veterinary and environmental sources that allows for outbreak sourcing and identification of potential transmission pathways. However, as of yet there is no established standardised protocol for stool DNA

extraction, primer design and ideal cycling conditions. Furthermore, source and condition of stool samples can impact the efficacy of these methods, with a study showing sensitivity and specificity of diagnostic tests differed between cattle, horse and sheep stool samples [53]. Development of a standardised protocol would allow for better comparisons between studies.

Various genetic loci have been targeted in molecular studies, though the most common is the 18S small subunit (SSU) rRNA gene. The SSU gene is a useful marker for species identification due to its high interspecies variation and low intraspecies variation [41,57]. Multiple copies of the SSU rRNA gene are present in each oocyst [58], enabling sensitive detection of the parasite even in samples with low levels of oocysts. The widespread use of sequencing in the past decade has expanded the database of available sequences for comparison on GenBank. When typing at the SSU gene, there is little genetic variation within species. The *gp60* gene has been well established as a genetic marker for *C. parvum* [59] and is used to further subtype this species, through assessment of the repeat serine residues in this gene. Globally, IIaA15G2R1 is the most prevalent subtype by far, and considered "hypertransmissible" [23]. Its dominance worldwide has led it to be considered a fitness marker [60], though it is unclear what the mechanism of action is behind its success. Subtype analysis can shed further light on potential zoonotic transmission by identifying which *C. parvum* subtypes occur in both humans and cattle. With increased molecular typing studies, distinctions in epidemiology between different subtypes may be discovered also. This information can enable us to ascertain how best to tackle infection spread in certain areas.

1.4 Bovine cryptosporidiosis in Europe

Prevalence investigations have frequently been carried out in almost all continents [61,62]. Studies of *Cryptosporidium* occurrences in Europe point towards a high prevalence [63–65]. Since the aim of this thesis is to investigate the presence of *Cryptosporidium* in dairy cattle from Belgium, Cyprus, Czech Republic, France and the Netherlands, in the following subsections I will introduce the status quo of *Cryptosporidium* in dairy farms from the aforementioned countries.

Belgium

In Belgium, several studies have been conducted to assess *Cryptosporidium spp.* infection, though only a limited number have been in cattle. In 2007, a prevalence study on 832 calves from 150 beef and dairy farms found 37% of dairy calves were positive for *Cryptosporidium* [66]. Prevalence peaked in calves 2-4 weeks old. This may be an underestimation of *Cryptosporidium* as occurrence was measured through examination of faecal smears for presence of oocysts, which is a less sensitive detection technique and may have discounted samples with low oocyst loads. Nonetheless, 18S SSU PCR and sequencing and was conducted on positive samples and found the majority of infections were caused by *C. parvum*, with most *C. bovis* infections occurring in calves older than one month. However, as only one isolate was selected from each farm for sequencing, this may not be an accurate reflection of the numbers of each *Cryptosporidium* species present. In dairy farms, only *C. bovis and C. parvum* were identified but, interestingly, one case of *C. suis* was identified among the beef farms. This species is predominantly associated with infections in pigs, though has been previously reported in cattle [67,68]. Its presence

here may simply be sporadic or could suggest a difference in *Cryptosporidium* species between beef and dairy calves. Five *C. parvum gp60* subtypes were identified, all of which have previously been reported in humans, with IIaA15G2R1 the most prevalent.

Molecular genotyping in human cryptosporidiosis cases indicated *C. parvum* was responsible for just under half of all cryptosporidiosis in East Flanders [69]. *gp60* subtyping identified several genotypes that have previously been reported in cattle [70].

Aside from cattle, other livestock species in Belgium have also been found to carry *C. parvum*. In a small study on goat and sheep farms, *C. parvum* was responsible for all infections in goats, as well as a sporadic case in one lamb [71]. Subtype analysis identified these as IIaA15G2R1 and IIdA22G1, both of which have been reported in human patients [72]. This indicates goats may also act as a major reservoir of zoonotic infection, in addition to cattle.

Cryptosporidium oocysts have also been found in environmental samples. A study across four water catchments found presence of *C. hominis, C. parvum, C. andersoni, C. suis* and *Cryptosporidium* horse genotype [73]. The presence of these particular species supports the idea that livestock may be a key source of contamination as these predominantly occur in cattle, pigs and horses. *C. andersoni* was isolated from recreational lakes [74], providing further evidence of potential contamination of the environment via livestock.

Cyprus

Though numerous prevalence investigations have been conducted worldwide, with the exception of the UK, studies in island nations are rare [75]. The limited movement of hosts in island settings can provide insights into parasite epidemiology. However, investigations into *Cryptosporidium* presence in Cyprus have been largely absent. Regardless of host species, there have been no molecular investigations to identify species and genotypes of *Cryptosporidium* circulating in Cyprus. To date, no studies have been carried out in cattle, though studies in goats and sheep have indicated a high presence of *Cryptosporidium* [76]. In 2015, 76.4 % of diarrheic goat kids were found to be infected with *Cryptosporidium* [77]. However, as these studies were only carried out in diarrheic animals, they do not indicate overall prevalence. Furthermore, they relied on microscopic examination of faecal samples and commercial ELISA tests, without identification of the particular *Cryptosporidium* species and subtypes present. As such, very little is known about the zoonotic potential of *Cryptosporidium* species in Cyprus. Oocyst examination cannot discern well between different *Cryptosporidium* species and subtypes so molecular tools are vital for ascertaining public health risk.

Previous molecular studies in the surrounding Mediterranean region do indicate presence of *Cryptosporidium* infection amongst cattle. 18S rRNA PCR investigation in Turkey [78–82], Egypt [83–87] and Jordan [88] have identified *Cryptosporidium* on cattle farms. Discrepancies in the observed prevalence from these studies are likely due to a variety of factors, including but not limited to age of animal sampled, farm location, farm management and method of detection. Additionally, some studies only targeted diarrheic animals [80,89], ignoring the presence of *Cryptosporidium* in asymptomatic individuals. Presence of the parasite in non-

diarrheic samples is still of clinical significance, as asymptomatic animals can still shed oocysts to their environment and contribute to spread of infection [90].

The complete absence of reported human cryptosporidiosis cases [91] is likely due to the lack of research in this region. However, studies in other countries do indicate presence of *Cryptosporidium* on this island - *C. hominis* was identified in UK patients with recent travel history to Cyprus [91,92].

The Czech Republic

In the Czech Republic, *Cryptosporidium* prevalence studies on cattle have only been conducted using microscopic analysis of faecal smears. In pre-weaned dairy calves, *Cryptosporidium* oocysts were found in 21.2% [93]. Molecular characterisation of positive samples indicated *C. parvum* accounted for the vast majority of positive samples at 87.2%. Curiously, *C. andersoni* was next most prevalent at 12.2%, although this species is typically only found in adult cows [94]. Furthermore, PCR analysis on a subset of microscopically negative samples found they were positive for *Cryptosporidium*, demonstrating the higher sensitivity of molecular techniques. It is possible a higher incidence would have been reported if all samples had been screened using PCR. *gp60* subtyping revealed presence of zoonotic subtypes, previously shown to cause disease in humans.

Another study in calves reported a similar prevalence of 25.8% positive [95]. Through morphology of oocysts, 30.9% of positive samples were assessed to be *C. parvum* and 69.1% were identified as *C. andersoni*. However, identities made without molecular analysis cannot be considered accurate. While there is an observed size difference between *C. parvum* and *C. andersoni*, other common cattle

species – *C. bovis* and *C. ryanae* – cannot be distinguished this way. In a study on calves over six months old [96], both prevalence and species composition were very different. Only 4.9% of samples were positive, with majority of infections identified as *C. andersoni*. This marked difference is likely due to the age of calves sampled. Analysis of blood sera has suggested that exposure to *Cryptosporidium* had been increasing over time [97], however, no human cryptosporidiosis cases have yet been linked to cattle contact.

France

In France, investigations into *Cryptosporidium* infection have been far more extensive, in both cattle and humans. However, molecular testing has generally been limited to the more recent studies. All *Cryptosporidium* investigations in French cattle have been conducted specifically in calves and appear to indicate high burden of *Cryptosporidium*. These investigations indicate cryptosporidiosis is a major threat to health of neonatal calves in this country and that there is significant risk of zoonotic disease transmission.

As early as 1999, *Cryptosporidium* was considered a major cause of neonatal diarrhoea [98], with up to 51.8% of dairy calves across six farms excreting *Cryptosporidium* oocysts. A later study further established its importance in neonatal bovine cryptosporidiosis, finding *Cryptosporidium* oocysts in 17.9-43.4% of diarrheic calves [99]. While these were claimed to be *C. parvum*, oocyst morphology cannot be used to distinguish between species so its possible other species were present as well. A 2015 molecular prevalence study found 41.5% of dairy calves sampled across Normandy farms were positive [100]. At the farm level, incidence

was extremely high with only 7% of farms testing negative. Additionally, *C. parvum* was associated with a high risk of mortality. Numerous longitudinal studies have been carried out where calves were sampled multiple times over several months. Over the course of these investigations, almost all (70.4-100%) would excrete oocysts at least once during the sampling periods [101–104]. While most studies have measured *Cryptosporidium* occurrence through conventional microscopy methods, several investigations did at least genotype microscopy-positive samples. *C. parvum* has been the predominant species found, particularly amongst diarrheic animals [101,103–107].

Age-related species associations have been observed - infections in younger calves are typically identified as *C. parvum*, while *C. bovis* and *C. ryanae* are usually found in older calves [100,101,104]. While IIaA15G1R1 is usually the predominant *gp60* identified, subtyping has revealed a very high diversity in *C. parvum* subtypes present amongst French cattle farms [101,103–107], including zoonotic subtypes in both the IIa and IId family. Interestingly, with regards to zoonosis, *C. hominis* has also been identified in cattle farms [107]. This species was previously thought to be infect humans only, though sporadic cases of *C. hominis* in other hosts have been observed worldwide [108,109]. Its presence here indicates significant risk of zoonotic transmission from French cattle farms.

C. parvum also plays a significant role in human cryptosporidiosis cases in this country, responsible for the majority of cases from 2015 to 2019 [110,111]. Four outbreaks of gastroenteritis were linked to *Cryptosporidium* spp., including *C. parvum* [112]. IIaA15G2R1 was responsible for a diarrheal disease in an infant [113]. Furthermore, this patient was discovered to have had close contact with diarrheic calves before the onset of illness, suggesting zoonotic transmission.

IIaA13G1R2 was found in a patient with persistent diarrhoea [114]. Both of these subtypes have previously been reported in cattle [115].

Cryptosporidium has also been found to infect other livestock species in France. *C. parvum* has been identified in goat kids, lambs and ewes [116–118]. *gp60* typing revealed zoonotic subtypes amongst these infections. Presence of *C. parvum* in other livestock may pose a risk of cross-species transmission in farms housing multiple livestock species. Furthermore, another potential indirect path of transmission has been proposed, where manure runoff from farms leads to contamination of bodies of water. Two studies in French lakes and seas found *C. parvum* infection in fish, and identified subtypes typically reported in livestock, including cattle [119,120].

The Netherlands

While little data on the burden of *Cryptosporidium* in cattle in the Netherlands exists, there is some evidence to suggest potential risk of zoonotic transmission. Nested PCR of the 18S rRNA gene on 20 farms across the Netherlands found occurrence of 17.3% [63]. As is seen in other countries, *C. parvum* was the predominant species present, comprising 85.5% of total infections. Age-related variance of infection was also observed with 32.5% of calves infected, compared to only 2% of heifers and dams. This is in line with farm studies in other countries. Presence of zoonotic *gp60* subtypes was also found. The research comprised in Chapter 3.2 of this thesis is a follow up on the farms sampled in this study. Prior to this, there have been no prevalence studies utilising molecular tools to screen farms. Molecular genotyping was carried out on human and cattle isolates previously determined *Cryptosporidium*-positive [121]. *C. parvum* was the only species found,

with IIaA15G2R1 identified in 69% of cases. A small-scale prevalence study on a single farm found *Cryptosporidium* oocyst shedding was most prevalent in animals 1-3 weeks old [122]. Further evidence of *Cryptosporidium* in Dutch cattle was found in heifers imported to a Polish farm from the Netherlands. Analysis of faecal smears found 30.7% of the cows imported as positive for *Cryptosporidium*. This study highlights the importance in screening to reduce spread of infection to other countries, especially considering no *Cryptosporidium* oocysts were detected in a previous investigation on this farm [123,124]. Import/export of cattle carries potential risk of introducing infection to previously uncontaminated areas, particularly troubling when considering the oocyst's robustness in the environment. While *C. parvum* has been implicated in numerous human cryptosporidiosis cases [121,125], none of these cases have been specifically linked to direct or indirect contact with cattle.

1.5 Thesis aims

Cryptosporidium is a major cause of diarrheal disease globally. Cattle are well established reservoirs of infection, and as such, should be monitored for assessment of zoonotic threat. This study was conducted to assess prevalence of *Cryptosporidium* in dairy farms across Belgium, Cyprus, the Czech Republic, France and the Netherlands. Herein, amplification of the 18S SSU rRNA and *gp60* genes and subsequent phylogenetic analysis has been used accurately characterise *Cryptosporidium* at the species and subtype level. The use of these molecular techniques will shed light on transmission dynamics, *C. parvum* species diversity

and zoonotic potential. This will complement previous molecular work and contribute to future studies aiming to discern transmission networks.

CHAPTER 2: METHODOLOGY

2.1 Sample collection

Belgium, France and the Netherlands

923 faecal samples were collected from September 2020 to July 2021. In total, forty-nine farms were surveyed - 17 in Belgium, 13 in France and 19 in the Netherlands. Veterinarians collected faecal samples from up to 10 calves and their respective mothers. Fewer samples were collected in cases where farms had less than 10 calves. Both diarrheic and asymptomatic animals were included. Faeces were taken directly from the rectum, using a single pair of disposable gloves.

This study was conducted as a cross-border collaboration under the Health for Dairy Cows (H4DC) project, funded by the Interreg-2-seas programme. This is a European Territorial Cooperation program covering the Flanders region of Belgium, the Hautes-de-France region in France and the western part of the Netherlands. These regions experience similar maritime temperate climates. The main objective of the project is to reduce the disease burden and economic impact of *Cryptosporidium* spp. on dairy farms. Inclusion criteria for selection of pilot farms were:

a) Farms should be located in 2-seas area
b) Farms should have a history of clinical diarrhoea observed in its animals
c) Farmers agree to provide samples each year, for the total three years of the study

Cyprus

242 faecal samples were collected in November 2019. Animals were randomly selected with age of calves ranging from pre-weaned calves up to three

months. Stool was collected directly from the rectum of animals and stored at -20°C until further processed. Samples were collected from ten farms located in two districts of Cyprus, namely Larnaca and Nicosia **(Figure 2)**. 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 farms in Nicosia are at a relatively low altitude (200–400 m) experiencing hot, dry and humid summers and cold winters with minimal precipitation. Two farms were chosen in the Larnaca district, specifically 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). As this was just a preliminary study into presence of *Cryptosporidium* in Cyprus, no selection criteria were used for inclusion into study. Cows from all selected farms were of the dairy producing Holstein Friesian breed.

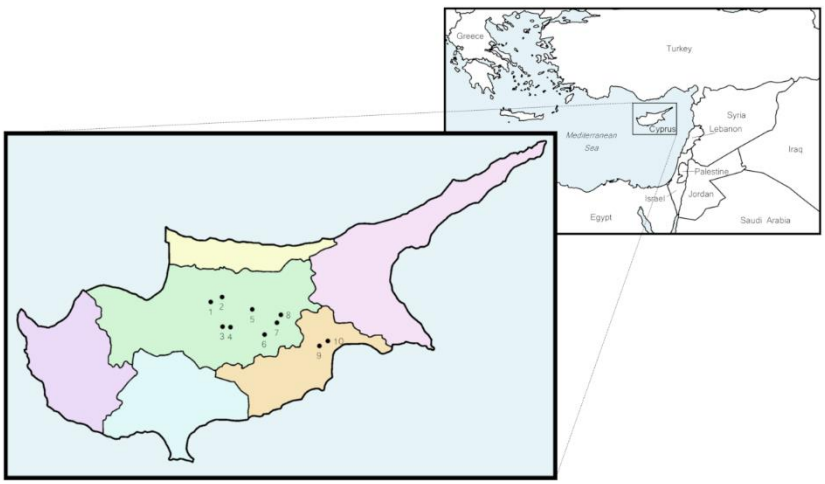

Figure 2. Geographic location of Cyprus in the East Mediterranean region and distribution of the cattle farms sampled.
Farms 1–8 were located across the Nicosia district (green), while farms 9–10 were located in the Larnaca district (orange).

The Czech Republic

204 faecal samples were collected in February to March 2020. Nine farms were surveyed across the South Bohemia region, in the České Budějovice, Český Krumlov, Prachatice and Tábor districts. Stool was collected directly from the rectum of animals and stored at -20°C until further processed. Age of calf was noted for each sample, ranging from neonatal to ~2 months old. Farms were selected at random with no prior knowledge of *Cryptosporidium* presence. Only farms exclusively housing cattle were included in the study.

2.2 DNA extraction

Faeces were collected directly from the rectum, placed in sterile tubes and stored in ice on site. 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 (ThermoFisher) 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 for 10 min to optimise removal of downstream contaminants. After addition of S6 Elution Buffer, samples were incubated at room temperature for 3 min before centrifugation to improve DNA yield. Genomic DNA was stored at -20°C until 18S rRNA and *gp60* PCR reactions were carried out.

2.3 *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 [126]. The external primers used were 5´- GATTAAGCCATGCATGTCTAA-3´ (forward) and 5´-TTCCATGCTGGAGTATTCAAG3´ (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 each of forward and reverse primers, 12.5 µl of 2× PCRBIO Taq Mix Red (PCR Biosystems) and 9.5 µl nuclease-free water. Cycling conditions for the external PCR were 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 (sterile 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 (ThermoFisher Scientific). Samples were bidirectionally sequenced through an external company (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.

2.4 *gp60* subtyping of *Cryptosporidium parvum*

To determine the subtype of *Cryptosporidium* 18S rRNA PCR-positive samples, nested PCR of the *gp60* gene was carried out [127]. 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 (ThermoFisher Scientific). 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 [128]. 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.

2.5 Phylogenetic analyses

The sequences generated in this study were aligned with each other and with reference sequences from GenBank by MAFFT v.7 (https://mafft.cbrc.jp/alignment/server). Sequence alignment was manually inspected with BioEdit v7.0.5.3 (https://bioedit.software.informer.com). Phylogenetic analyses were conducted and best DNA/Protein phylogeny models were selected using the MEGA6 software [129]. The Tamura 3-parameter model [130], was selected for 18S SSU and *gp60* alignments. Phylogenetic trees were inferred using maximum likelihood (ML), with the substitution model that best fit the alignment selected using the Bayesian information criterion. Bootstrap support for branching was based on 1,000 replications. An 18S sequence from *Monocystis agilis* (Accession number: AF457127) was used to root ML tree.

CHAPTER 3: EXPERIMENTS

3.1 *Cryptosporidium* in Belgium, France and the Netherlands

3.1.1 Incidence of Cryptosporidium in Belgian, French and Dutch dairy farms

In Belgium, 324 stool samples across 17 farms were screened. Amplification of the 18S rRNA and *gp60* genes showed an occurrence of 25% with 81/324 specimens positive for *Cryptosporidium* spp. **(Table 1)**. Occurrence varied across farms from 0% to 50% (5/10). The majority of the positive samples were identified as *C. parvum* (65.4%, 53/81) with 43 of them showing 100% nucleotide identity to the reference sequence AH006572.2, and 4 samples showing 99% nucleotide identity to the same sequence. We were unable to obtain good quality sequences for six 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 14/17 farms, with occurrence ranging from 5% to 45%. The next most common species found was *C. bovis* (21%, 17/81). Additionally, 3 samples had co-infections of *C. bovis* and *C. parvum*. 19 samples showed 100% nucleotide identity to reference sequence AB777173.1 while one sample showed 100% nucleotide identity to reference sequence MZ021459.1. The next most prevalent species was *C. ryanae* (6.17%, 5/81). Additionally, one sample showed mixed infection of *C. ryanae* and *C. parvum*. Four samples showed 100% nucleotide identity to reference sequence FJ463193.1, one sample had 100% nucleotide identity to reference sequence KT922233.1 and another showed 99% identity to the same sequence. The least common species was *C. andersoni* (2.47%, 2/81). One sample showed 100% nucleotide identity to reference sequence AB513856.1 while the other showed 100% nucleotide identity to FJ463171.1.

In France, 223 stool samples across 13 farms were screened. Amplification of the 18S rRNA and *gp60* genes showed an occurrence of 23.8% with 53/223 specimens positive for *Cryptosporidium* spp. (Table 4). Occurrence varied across farms from 0% to 45.5% (5/11). The majority of the positive samples were identified as *C. parvum* (66%, 35/53) with 33 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 one 18S rRNA PCR-positive sample but *C. parvum* identity was confirmed through positive *gp60* PCR and subsequent sequencing. At the farm level, *C. parvum* was present in 9/13 farms, with occurrence ranging from 5% to 45.5%. The next most common species was *C. bovis* (13.2%, 7/53). Additionally, two samples had co-infections of *C. bovis* and *C. parvum*. Eight samples showed 100% nucleotide identity to reference sequence AB777173.1 while one sample showed 100% nucleotide identity to reference sequence MZ021459.1. The next most prevalent species was *C. ryanae* (9.43%, 5/53). Two variants were found with three samples showing 100% identity to KT922233.1 and two samples showing 100% nucleotide identity to reference sequence FJ463193.1. The least common species was *C. andersoni* (5.66%, 3/53), with all three showing 100% nucleotide identity to reference sequence FJ463171.1.

In the Netherlands, 376 stool samples across 19 farms were screened. Amplification of the 18S rRNA and *gp60* genes showed an occurrence of 24.2% with 91/376 specimens positive for *Cryptosporidium* spp. (Table 4). Occurrence varied across farms from 0% to 42.1% (8/19). The majority of the positive samples were identified as *C. parvum* (81.3%, 74/91) with 73 isolates showing 100% nucleotide identity to the reference sequence AH006572.2. We were unable to obtain good quality sequences for one 18S rRNA PCR-positive sample but *C. parvum*

identity was confirmed through positive *gp60* PCR and subsequent sequencing. At the farm level, *C. parvum* was present in 18/19 farms, with occurrence ranging from 10% to 42.1%. The next most prevalent species was *C. bovis* at (16.5%, 15/91), with all 15 isolates showing 100% nucleotide identity to reference sequence MZ021459.1. One sample each was found positive for *C. ryanae* and *C. andersoni*, with 99% sequence identity to FJ463193.1 and 100% nucleotide identity to AB513856.1, respectively. No mixed infections were found. **Figure 3** shows phylogenetic relationship of *C. bovis*, *C. ryanae* and *C. andersoni* isolates to reference sequences from GenBank.

Table 1. Incidence of *Cryptosporidium* spp. and *C. parvum gp60* subtypes in dairy cattle from farms across Belgium, France and the Netherlands.

Country	Farm	Prevalence % (no. positives/ total screened)	*Cryptosporidium* spp. present (no. isolates identified)						*C. parvum* subtypes
			C. parvum	*C. bovis*	*C. ryanae*	*C. andersoni*	*C. parvum/ C. bovis (co-infection)*	*C. parvum/ C. ryanae (co-infection)*	
Belgium	BE1	25% (5/20)	5	-	-	-	-	-	IIaA15G2R1 (5)
	BE2	50% (5/10)	3	1	1	-	-	-	IIaA15G2R1 (3)
	BE3	45% (9/20)	9	-	-	-	-	-	IIaA15G2R1 (5)
	BE4	0% (0/19)	-	-	-	-	-	-	-
	BE5	38.9% (7/18)	3	3	1	-	-	-	IIaA15G2R1 (1)
	BE6	20% (4/20)	3	-	-	-	-	1	IIaA15G2R1 (3) IIaA17G1R1 (1)
	BE7	20% (4/20)	4	-	-	-	-	-	IIaA15G2R1 (4)
	BE8	45% (9/20)	8	-	1	-	-	-	IIaA15G2R1 (8)
	BE9	20% (4/20)	2	1	1	-	-	-	-
	BE10	25% (5/20)	4	-	-	1	-	-	IIaA15G2R1 (1)
	BE11	20% (4/20)	3	-	-	1	-	-	IIaA15G2R1 (2)
	BE12	33.3% (7/21)	3	3	1	-	-	-	IIaA14G1R1 (1) IIaA13G2R1 (1)
	BE13	29.4% (5/17)	1	2	-	-	2	-	IIaA14G1R1 (2) IIaA13G2R1 (1)
	BE14	5% (1/20)	-	1	-	-	-	-	-
	BE15	20% (4/20)	1	3	-	-	-	-	IIaA15G2R1 (1/1)
	BE16	5.3% (1/19)	-	1	-	-	-	-	-
	BE17	35% (7/20)	4	2	-	-	1	-	IIaA15G2R1 (3) IIaA15G1R1 (1)

France	FR1	10% (2/20)	1	-	-	1	-	-	IIa15G2R1 (1)
	FR2	10% (2/20)	-	1	1	-	-	-	-
	FR3	45.5% (5/11)	5	-	-	-	-	-	IIaA15G2R1 (3)
	FR4	29.4% (5/17)	5	-	-	-	-	-	IIaA16G2R1 (3)
	FR5	35% (7/20)	6	-	-	1	-	-	IIaA16G2R1 (5)
	FR6	27.3% (3/11)	3	-	-	-	-	-	IIaA15G2R1 (2) IIaA16G1R1 (1)
	FR7	25% (5/20)	2	1	1	-	1	-	IIaA16G2R1 (2)
	FR8	38.9% (7/18)	3	-	2	2	-	-	IIaA15G2R1 (3)
	FR9	38.9% (7/18)	6	-	1	-	-	-	IIaA15G2R1 (4) IIaA16G2R1 (1)
	FR10	0% (0/14)	-	-	-	-	-	-	-
	FR11	0% (0/14)	-	-	-	-	-	-	-
	FR12	10% (2/20)	-	2	-	-	-	-	-
	FR13	40% (8/20)	4	3	-	-	1	-	IIaA16G2R1 (4)
The Netherlands	NL1	35% (7/20)	7	-	-	-	-	-	IIaA15G2R1 (6)
	NL2	16.7% (3/18)	3	-	-	-	-	-	IIaA15G2R1 (2)
	NL3	15% (3/20)	-	1	1	1	-	-	-
	NL4	10% (2/20)	-	2	-	-	-	-	-
	NL5	25% (5/20)	5	-	-	-	-	-	IIaA14G1R1 (5)
	NL6	20% (4/20)	4	-	-	-	-	-	IIaA15G2R1 (4)
	NL7	20% (4/20)	2	2	-	-	-	-	IIaA15G2R1 (2)
	NL8	25% (5/20)	-	5	-	-	-	-	-
	NL9	15% (3/20)	3	-	-	-	-	-	IIaA13G2R1 (3)
	NL10	25% (5/20)	1	4	-	-	-	-	-
	NL11	31.6% (6/19)	6	-	-	-	-	-	IIaA15G2R1 (4)
	NL12	40% (8/20)	7	1	-	-	-	-	IIaA15G2R1 (5)
	NL13	0% (0/20)	-	-	-	-	-	-	-
	NL14	40% (8/20)	8	-	-	-	-	-	IIaA15G2R1 (6)
	NL15	20% (4/20)	4	-	-	-	-	-	IIaA15G2R1 (3)
	NL16	20% (4/20)	4	-	-	-	-	-	IIaA17G2R1 (4)
	NL17	25% (5/20)	5	-	-	-	-	-	IIaA15G2R1 (3)
	NL18	42.1% (8/19)	8	-	-	-	-	-	IIaA15G2R1 (2)
	NL19	35% (7/20)	7	-	-	-	-	-	IIaA17G1R1 (6)

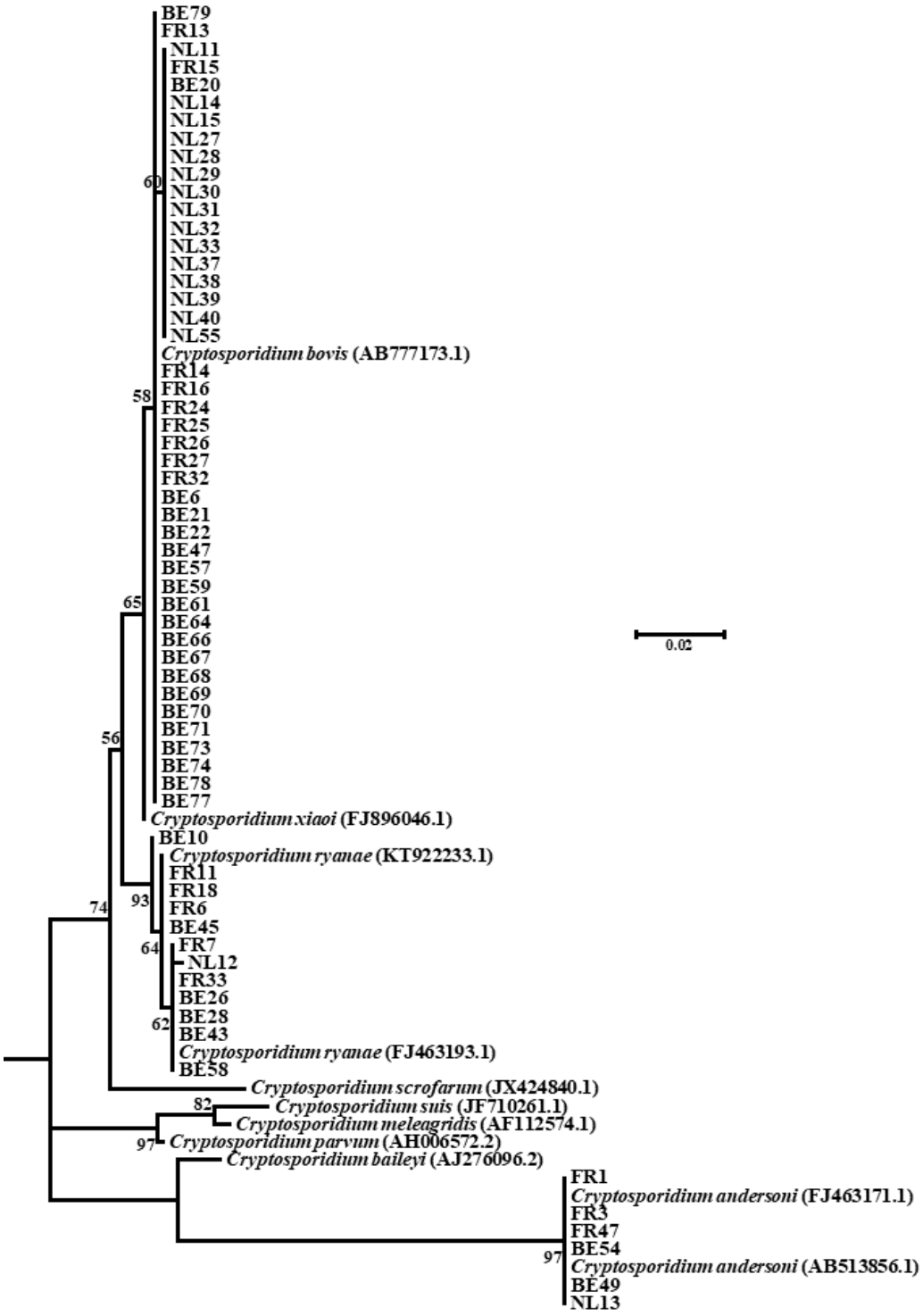

Figure 3. A maximum likelihood (ML) tree based on the 18S rRNA gene sequences of *C. bovis*, *C. ryanae* and *C. andersoni* obtained in Belgium, France and the Netherlands.
Bootstrap values for the nodes with more than 50% support are shown.
The ML tree was rooted with an 18S rRNA sequence from *Monocystis agilis* (AF457127).

3.1.2 Cryptosporidium parvum subtyping through gp60 analysis

PCR amplification and sequencing of the *gp60* gene was utilised to identify the specific *C. parvum* subtypes present in these three countries. All 18S rRNA PCR-positive samples were further screened using nested-PCR of the *gp60* gene. Of these, the *gp60* gene was successfully amplified and sequenced in 127 samples. 41 *C. parvum* positive sequences could not be successfully subtyped. Sequence analysis revealed the presence of 8 subtypes belonging to the IIa subtype family **(Figure 4)**. Thirty-nine of the farms sampled contained at least one *C. parvum* subtype.

The most abundant subtype found overall was IIaA15G2R1. 85 isolates were found - 83 with 100% nucleotide identity to reference sequence DQ630518.1 and two with 99% nucleotide identity to the same sequence. This subtype was also the most widely distributed, occurring in 26/39 *C. parvum*-positive farms. This subtype represented 63.2%, 35.1% and 50% of all *C. parvum* positive samples, in Belgium, France and the Netherlands, respectively. It was the most prevalent subtype in Belgium and the Netherlands. In all three countries, this subtype was only found in calves

The second most common subtype in this study was IIaA16G2R1. 15 isolates were found with 100% nucleotide identity to reference sequence DQ192505.1, with all isolates found in France. This subtype was found in four farms and represented 40.5% of total *C. parvum* infections in the French farms. This subtype predominantly occurred in calves, with only one isolate occurring in a dam. Eight isolates of IIaA14G1R1 were identified - six with 100% nucleotide identity to reference sequence AM937017.1 and two with 99% nucleotide identity to the same sequence. This subtype occurred in three farms, representing 5.26% and 6.76% of *C. parvum* infections in Belgium and the Netherlands, respectively. This subtype predominantly occurred in calves, with only one isolate occurring in a dam.

Seven isolates of IIaA17G1R1 were identified - six with 100% nucleotide identity to reference sequence GQ983359.1 and one with 99% nucleotide identity to the same sequence. This was the sole subtype present in two farms, representing 1.75% and 8.11% of *C. parvum* infections in Belgium and the Netherlands, respectively.

Five isolates of IIaA13G2R1 were identified – four with 100% nucleotide identity to reference sequence DQ192502.1 and one isolate with 99% homology to the same sequence. This was the sole subtype present in three farms, representing 3.52% and 4.05% of *C. parvum* infections in Belgium and the Netherlands, respectively.

IIaA17G2R1 was found exclusively in the Netherlands. Four isolates were identified with 100% nucleotide identity to DQ630516.1. This subtype occurred on one farm and represented 5.41% of *C. parvum* infections. The remaining subtypes were IIaA15G1R1 (99% nucleotide identity to AB777872) and IIaA16G1R1 (100% nucleotide identity to DQ192504.1), each occurring once in Belgium and France, respectively. IIaA17G1R1, IIaA13G2R1, IIaA17G2R1, IIaA15G1R1 and IIaA16G1R1 were all found exclusively in calves. Geographical distribution of subtypes is visualised in **Figure 5**.

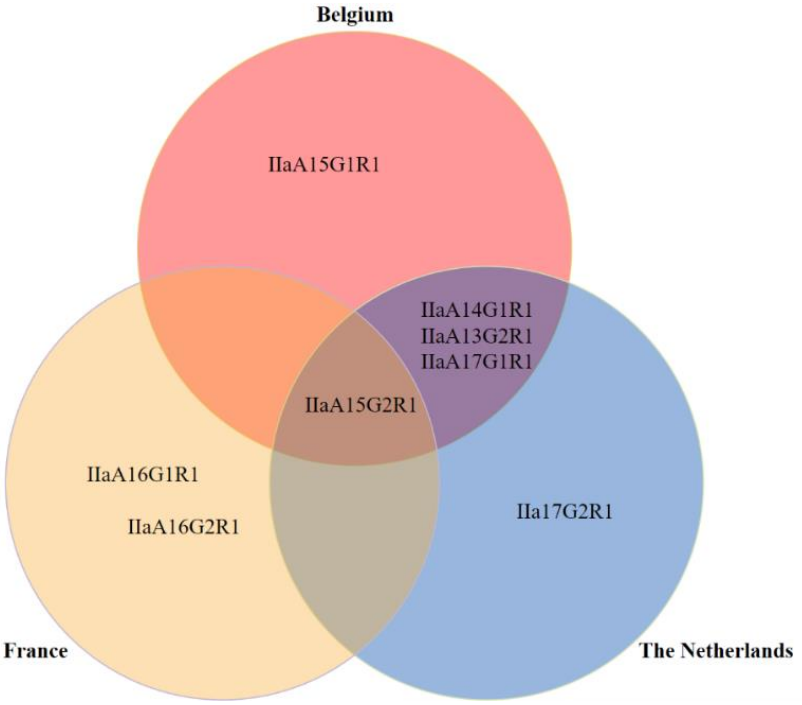

Figure 4. Venn diagram with all observed *C. parvum gp60* subtypes across Belgium, France, and the Netherlands

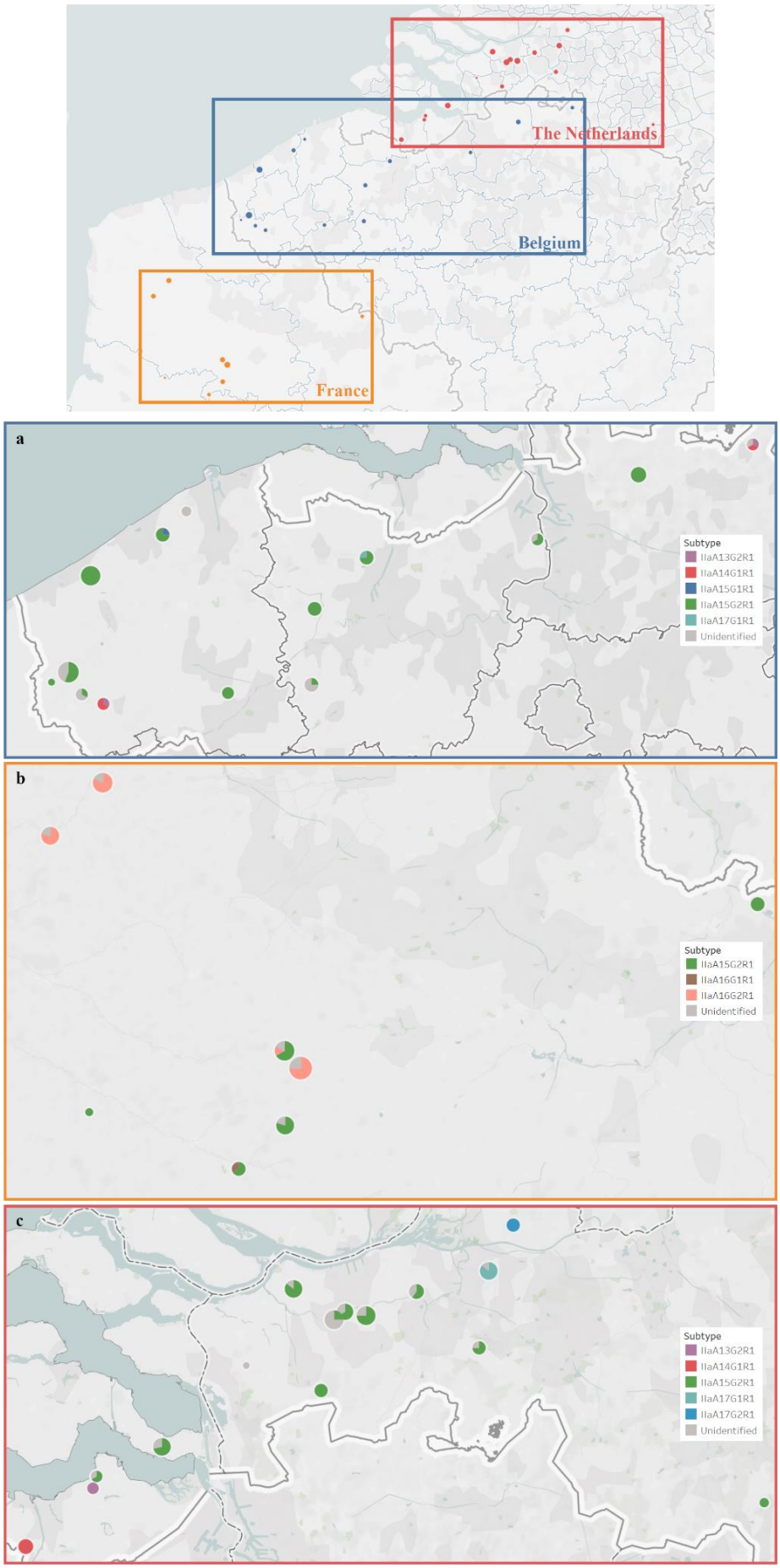
The Netherlands
Belgium
France
a
Subtype
IIaA13G2R1
IIaA14G1R1
IIaA15G1R1
IIaA15G2R1
IIaA17G1R1
Unidentified
b
Subtype
IIaA15G2R1
IIaA16G1R1
IIaA16G2R1
Unidentified
c
Subtype
IIaA13G2R1
IIaA14G1R1
IIaA15G2R1
IIaA17G1R1
IIaA17G2R1
Unidentified

Figure 5. Geographical distribution of *gp60* subtypes in dairy farms across a) Belgium, b) France and c) the Netherlands.
IIaA13G12R1 (purple), IIaA14G1R1 (red), IIaA15G1R1 (dark blue), IIaA15G2R1 (green), IIaA16G1R1 (brown), IIaA16G2R1 (peach), IIaA17G1R1 (turquoise), IIaA17G2R1 (blue). *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.

3.1.3 Age-associated variation in Cryptosporidium infection

In all three countries, infection occurred at considerably greater levels in calves than in adult cows **(Table 2)**. In Belgium, 40.61% of all calves were infected with *Cryptosporidium*, whereas only 8.81% of adult cows were infected. In France, 41.1% of calves were infected, whereas only 7.5% of adult cows were infected. In the Netherlands, 43.2% of all calves were infected, whereas only 4.8% of adult cows were infected. Overall, calves represented 86.2% of all positive samples, while adult cows represented 13.1% of all positive samples.

In calves, the infecting *Cryptosporidium* species correlated with age, with the majority of *C. parvum* infections occurring in the first 1-3 weeks of life **(Figure 6).** *C. bovis* and *C. ryanae* typically occurred at relatively low levels over the first few months of life. This trend held true for all three countries.

Table 2. *Cryptosporidium* spp. incidence in calves and dams in Belgium, France and the Netherlands.

Country	Age	*Cryptosporidium* spp.					
		C. parvum	***C. bovis***	***C. ryanae***	***C. andersoni***	***C. parvum/ C. ryanae***	***C. parvum/ C. bovis***
Belgium	**Calves**	49	12	2	-	1	3
	Dams	4	5	3	2	-	-
France	**Calves**	33	5	5	1	-	2
	Dams	2	2	-	3	-	-
The Netherlands	**Calves**	71	11	-	-	-	-
	Dams	3	4	1	1	-	-

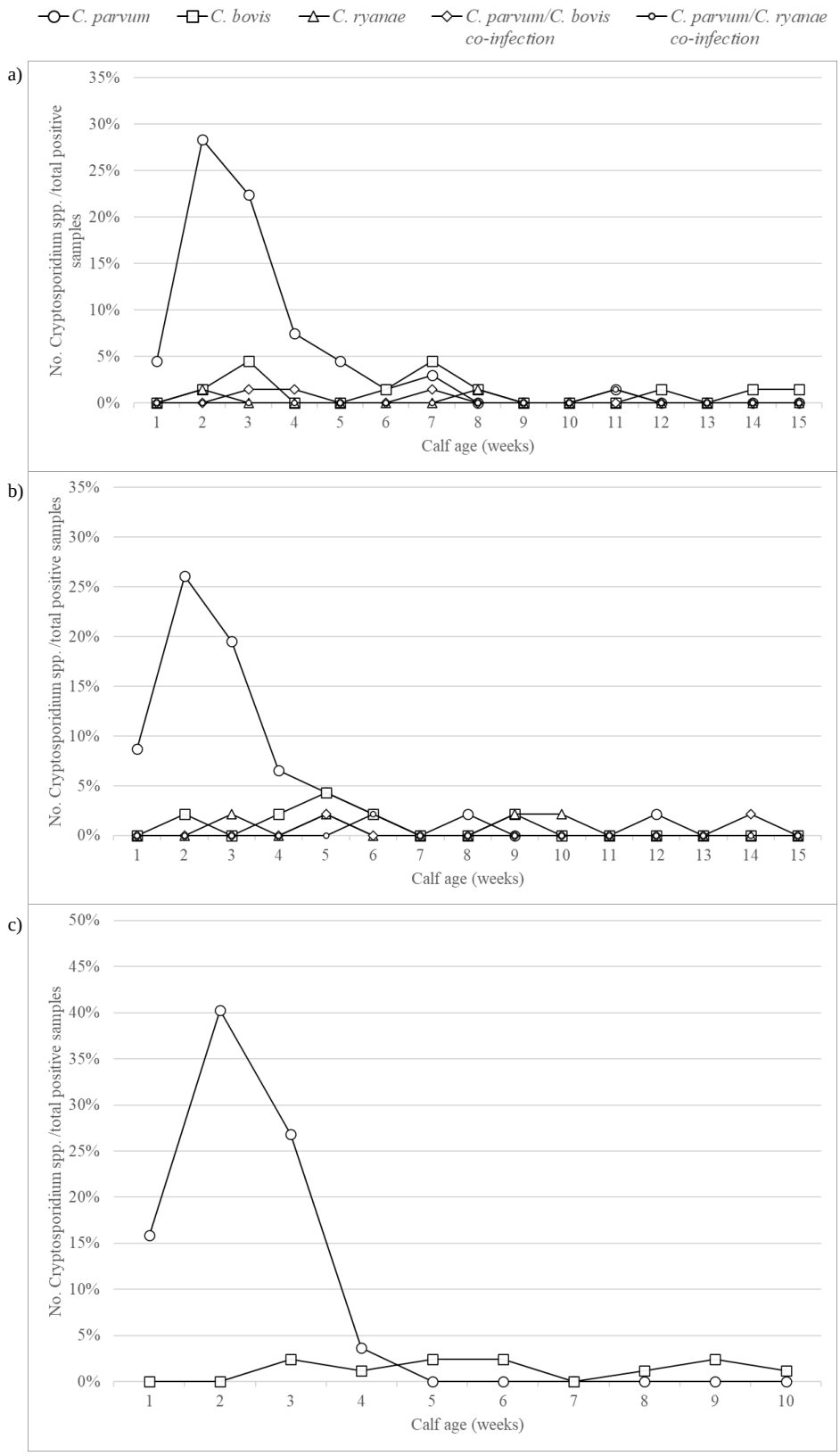

Figure 6. Prevalence of *Cryptosporidium* spp., *C. parvum*, *C. bovis*, *C. ryanae*, *C. andersoni* and coinfections in calves from 1-15 weeks.
a) Belgium [n=165], b) France [n=113], c) The Netherlands [n=190].

3.1.4 Comparison to previous sampling study

This study is a follow-up to a prevalence study carried out in 2019 [63]. Methodology was slightly altered between the two studies. In the preceding study, *gp60* PCR and subtyping was only carried out on samples confirmed as *C. parvum* positive through 18S SSU PCR and sequencing. As such, co-infections with *C. parvum* were not detected and *gp60* sequencing was not used to confirm *C. parvum* identity when quality 18S sequences were not able to be obtained. For comparability between the cohorts, prevalence figures in the 2nd cohort have been adjusted accordingly as though the same method had been used. Likewise, results from FR9 have been omitted as this farm did not participate in the initial study.

Cryptosporidium incidence was higher in all three countries in the follow-up study **(Table 3)**, particularly in the Netherlands, where incidence increased over 6% points. Age variation of infections is similar between the two, with *Cryptosporidium* predominantly occurring in calves.

In both cohorts, *C. parvum* is consistently the predominant species present in all countries. However, composition of *Cryptosporidium* spp. does differ between the years. The first cohort in France reported similar numbers of *C. parvum* and *C. bovis* - 50% and 41.6% of total infections, respectively. However, the second cohort reports a markedly higher incidence of *C. parvum* at 70.2%.

C. parvum, C. bovis, C. ryanae and *C. andersoni* were all found in both years, though the follow-up study does report higher incidence of *C. andersoni. C. andersoni* comprised 1.1% of infections across all three countries in cohort 1, which rose to 2.9% in cohort 2. No isolates of *C. xiaoi* were detected in the follow-up study.

The difference in *Cryptosporidium* prevalence between the two studies was not seen uniformly over all farms (**Figure 7**). In Belgium, six farms had a two-fold

or higher increase in prevalence, while the incidence in three farms had reduced by at least half. While BE11 initially was negative for *Cryptosporidium*, 20% of cows sampled tested positive in the following year. The opposite effect happened in BE4 and BE7, where no infection was found despite presence of the parasite in the cohort 1 study. In France, prevalence doubled in FR3 and increased six-fold in FR13. No infection was found in FR10 and FR11 in the follow-up study, despite presence of the parasite in the first study. In the Netherlands, prevalence in four farms had a two-fold or higher increase. While NL1, NL2 and NL9 initially were negative for *Cryptosporidium*, 15-16.7% of cows sampled tested positive in the following year. The opposite effect happened in NL13, where no infection was found despite presence of the parasite in cohort 1.

Table 3. Comparison of *Cryptosporidium* spp. prevalence and *gp60* subtypes present in Belgian, French and Dutch dairy farms over 2 years. Data from the Year 1 cohort has been previously published [63].

Country	Cohort	Prevalence (%)			*Cryptosporidium* spp. present					*C. parvum*
		Overall	Calves	Dams	*C. parvum*	*C. bovis*	*C. ryanae*	*C. andersoni*	*C. xiaoi*	*gp60 subtypes*
Belgium	Year 1	18.8	31.2	6.06	45	14	4	-	-	IIa15G2R1 IIa13G2R1 IIa16G3R1 IIa17G2R1
	Year 2	21.9	30.9	8.81	47	17	5	2	-	IIa15G2R1 IIa14G1R1 IIa13G2R1 IIa15G1R1 IIa16G2R1
France	Year 1	22.1	39.4	4.6	24	20	2	1	1	IIa15G2R1 IIa16G1R1 IIa16G2R1
	Year 2	22.4	36.5	5.94	33	7	4	3	-	IIa15G2R1 IIa16G1R1 IIa16G2R1
Netherlands	Year 1	17.4	32.6	2.1	56	6	4	-	-	IIa15G2R1 IIa14G1R1 IIa13G2R1
	Year 2	23.9	42.6	4.8	73	15	1	1	-	IIa15G2R1 IIa14G1R1 IIa13G2R1 IIa17G1R1 IIa17G2R1

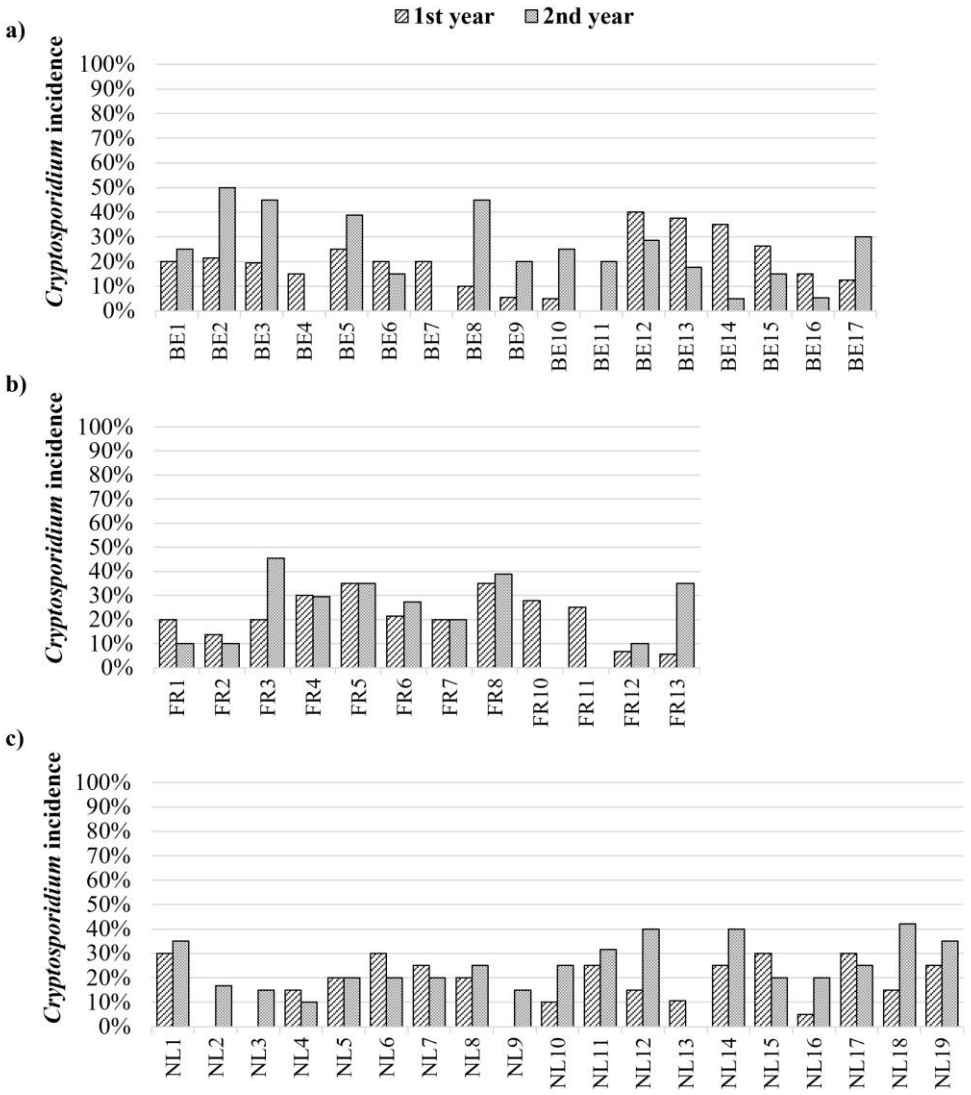

Figure 7. Comparison of *Cryptosporidium* incidence in dairy farms across two sampling studies in Belgium, France and the Netherlands.
a) Belgium [1st year: n=335, 2nd year: n=324], b) France [1st year: n=217, 2nd year: n=205], c) The Netherlands [1st year: n=379, 2nd year: n=376].
Data from 1st year has been previously published [63].

3.2 *Cryptosporidium* in Cyprus

The results outlined in this chapter have been previously published [131].

3.2.1 Cryptosporidium spp. occurrence across Cypriot farms

Amplification of the 18S rRNA gene found 43.8% (106/242) samples tested positive for *Cryptosporidium* spp. **(Table 4)**. Occurrence ranged across farms, with the lowest at 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. Good quality sequences were unable to be obtained 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 identified (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. **Figure 8**

shows phylogenetic relationship of *C. bovis* and *C. ryanae* isolates to reference sequences from GenBank.

Table 4. *Cryptosporidium* spp. prevalence in Cypriot dairy farms. Data is from a previously published study [131]

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**

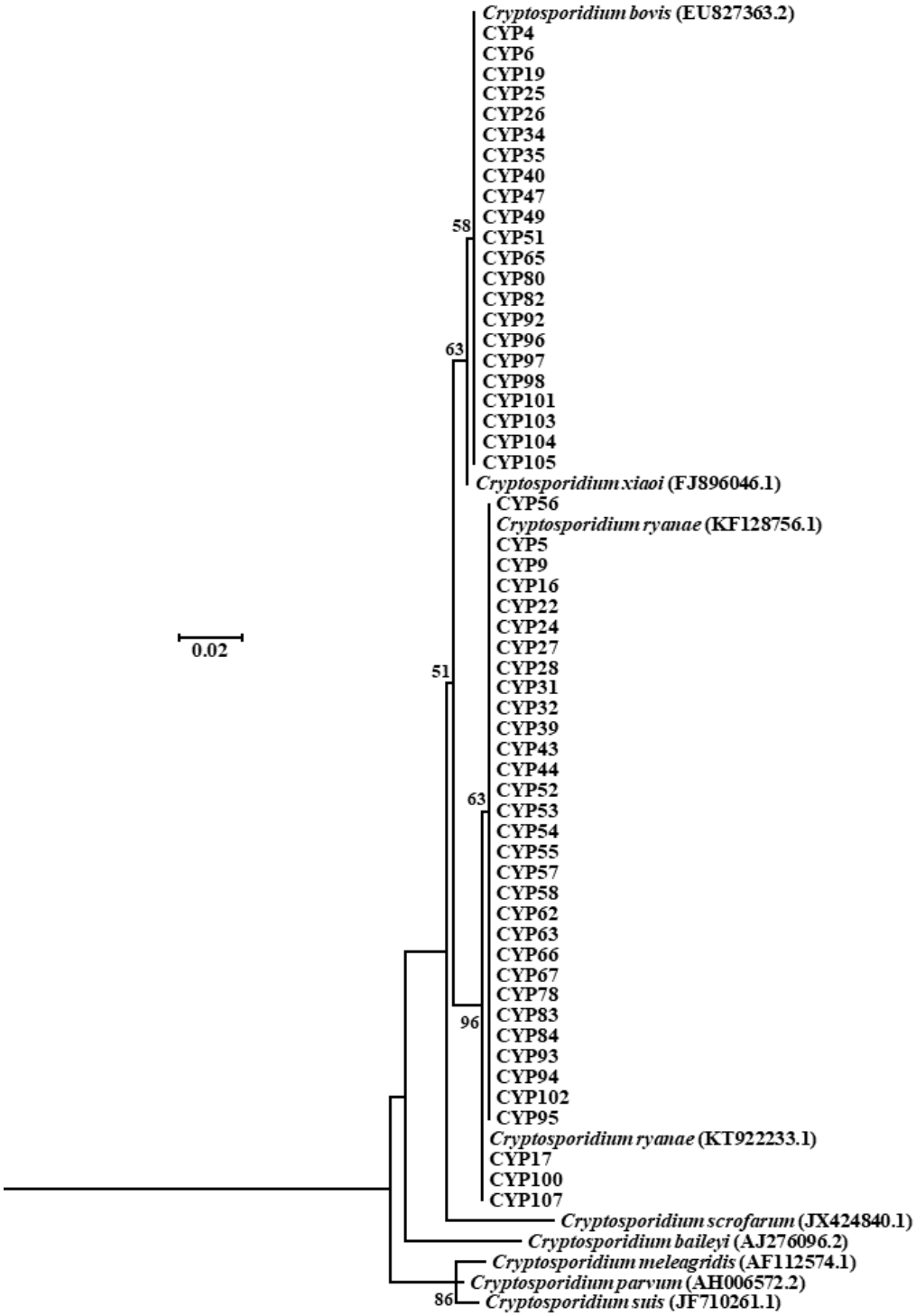

Figure 8. A maximum likelihood (ML) tree based on the 18S rRNA gene sequences of *C. bovis* and *C. ryanae* obtained in Cyprus.
Bootstrap values for the nodes with more than 50% support are shown. The ML tree was rooted with an 18S rRNA sequence from *Monocystis agilis* (AF457127).
Data is from a previously published study [131].

3.2.2 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 5)**. 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. Representative nucleotide sequences of *gp60* have been deposited in GenBank under accession numbers OL462897- OL462943. Polymorphisms are detailed in **Table 6**.

Table 5. Number of *Cryptosporidium parvum gp60* subtypes identified out of total *C. parvum* positive samples per farm.
Data is from a previously published study [131]

Farm	Subtypes (no. of subtype/total *C. parvum*-positive 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 (1/16)
10	IIaA14G1R1 (6/8), Unidentified (2/8)

Table 6. Polymorphisms in *Cryptosporidium* 18S SSU rRNA and *gp60* gene sequences showing intra-species and intra-subtype genetic variability.
Data is from a previously published study [131]

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[c] and 581[c] A→T, position 639 A→G, position 687[c]	DQ630518.1
gp60	*C. parvum* (IIaA15G2R1)	OL462917	T→C, positions 454[c] and 469[c]	
gp60	*C. parvum* (IIaA15G1R1)	OL462922	C→T, position 753	AB777872.1
gp60	*C. parvum* (IIaA18G2R1)	OL462903	T→G, position 375[c]	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.
[c] Polymorphisms result in an amino acid change.

3.2.3 Geographical distribution of subtypes

For easy visualization, subtype names are also indicated by their colour as depicted in **Figure 9**. 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 contained only IIaA14G1R1 (red) subtype, while the other had both IIaA12G1R1 (orange) and IIaA15G2R1 (green).

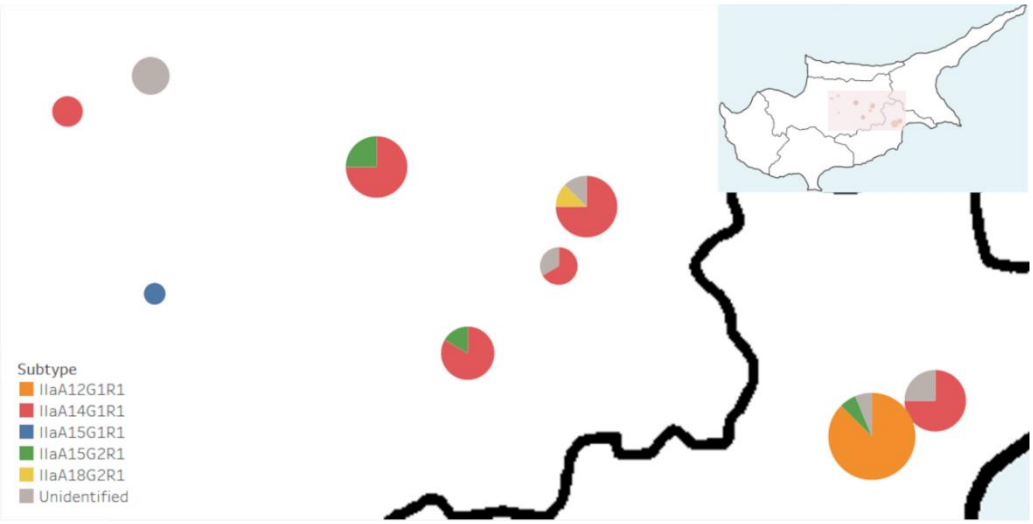

Figure 9. Geographical distribution of *gp60* subtypes across Cyprus cattle farms. IIaA12G1R1 (orange), IIaA14G1R1 (red), IIaA15G1R1 (blue), IIaA15G2R1 (green), IIaA18G2R1 (yellow). *C. parvum* positive samples with unsuccessful *gp60* sequencing are indicated in grey. Pie charts are proportional to number of *C. parvum* positive samples identified per farm.
Data is from a previously published study [131]

3.3 *Cryptosporidium* in the Czech Republic

3.3.1 Incidence of Cryptosporidium in Czech dairy farms

204 stool samples across nine farms were processed. Amplification of the 18S rRNA and *gp60* genes showed an occurrence of 43.1% with 88/204 specimens positive for *Cryptosporidium* spp. **(Table 7)**. *Cryptosporidium* was found in all farms, with occurrence varying from 19.2% (5/26) to 67.7% (23/34). The majority of positive samples were identified as *C. parvum* (76.1%, 67/88) with 60 isolates showing 100% nucleotide identity to the reference sequence AH006572.2. We were unable to obtain good quality sequences for seven 18S rRNA PCR-positive samples. Nonetheless, *C. parvum* identity was confirmed through *gp60* PCR and subsequent sequencing.

The next most common species found was *C. bovis* (15.9%, 14/88). Additionally, two samples had co-infections of *C. bovis* and *C. parvum*. All 14 isolates had 100% nucleotide identity to reference sequence AB777173. The least prevalent species found was *C. ryanae* (5.68%, 5/88), with all five samples showing 100% nucleotide identity to reference sequence FJ463193.1. **Figure 10** shows phylogenetic relationship of *C. bovis* and *C. ryanae* isolates to the aforementioned reference sequences from GenBank**.**

Table 7. Incidence of *Cryptosporidium* spp. and *C. parvum gp60* subtypes in dairy cattle from farms in the South Bohemia region of the Czech Republic.

Farm	Prevalence % (no. positives/ total screened)	*Cryptosporidium* spp. present				*C. parvum gp60* subtypes
		C. parvum	*C. bovis*	*C. ryanae*	*C. bovis/ C. parvum* co-infection	
CZ1	23.5% (4/17)	4	-	-	-	IIdA20G1 (2)
CZ2	54.5% (6/11)	6	-	-	-	IIa15G2R1 (6)
CZ3	22.7% (5/22)	5	-	-	-	IIa15G2R1 (5)
CZ4	67.6% (23/34)	19	3	-	1	IIa16G1R1 (19)
CZ5	54.5% (12/22)	12	-	-	-	IIa15G2R1 (9)
CZ6	19.2% (5/26)	5	-	-	-	IIa15G2R1 (5)
CZ7	30% (3/10)	-	3	-	-	-
CZ8	51.5% (17/33)	11	4	2	-	IIa15G2R1 (11)
CZ9	44.8% (13/29)	5	4	3	1	IIa15G2R1 (1) IIa16G1R1 (5)

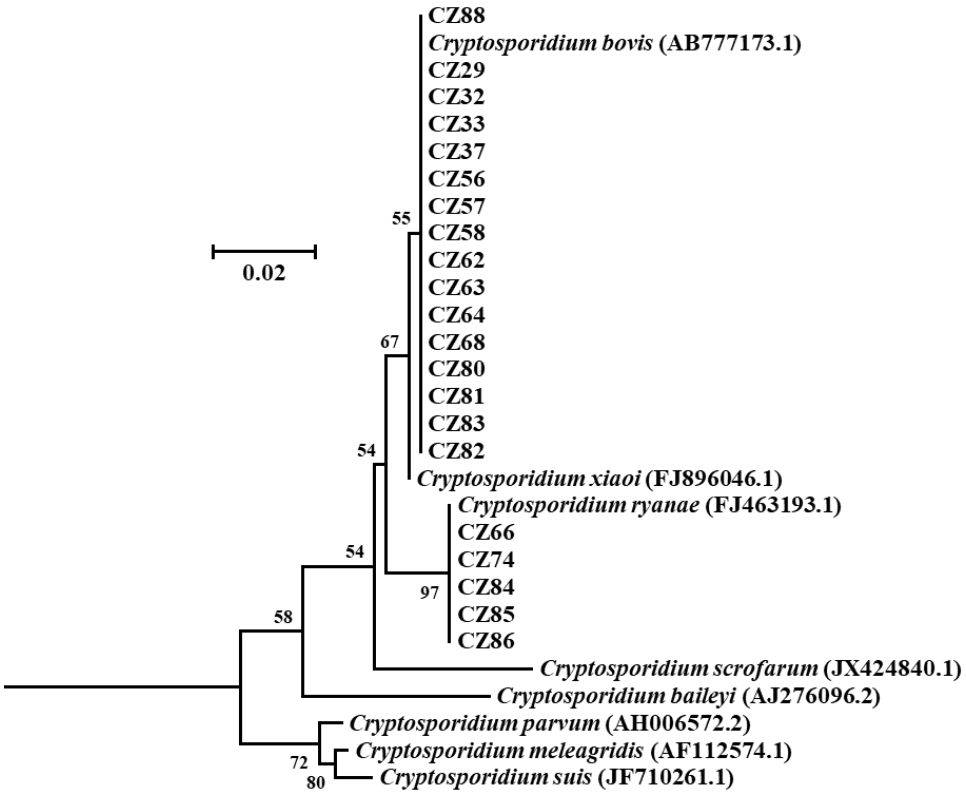

Figure 10. A maximum likelihood (ML) tree based on the 18S rRNA gene sequences of *C. bovis* and *C. ryanae* obtained in the Czech Republic. Bootstrap values for the nodes with more than 50% support are shown. The ML tree was rooted with an 18S rRNA sequence from *Monocystis agilis* (AF457127).

3.3.2 Cryptosporidium parvum subtyping through gp60 analysis

PCR amplification and sequencing of the *gp60* gene was utilised to identify the specific *C. parvum* subtypes present. All 18S rRNA PCR-positive samples were further screened using nested-PCR of the *gp60* gene. Of these, the *gp60* gene was successfully amplified and sequenced in 63 samples. Six *C. parvum* positive sequences could not be successfully subtyped. Sequence analysis revealed the presence of three subtypes, two of which belong to the IIa subtype family and one belonging to the IId subtype family. Eight of the nine farms sampled contained at least one *C. parvum* subtype.

The most abundant subtype found overall was IIaA15G2R1. 37 isolates were found - 36 with 100% nucleotide identity to reference sequence DQ630518.1 and one with 99% nucleotide identity to the same sequence. This subtype was also the most widely distributed, occurring in 6/8 *C. parvum*-positive farms. This subtype represented 53.6% of all *C. parvum* positive samples.

The second most common subtype in this study was IIaA16G2R1. 24 isolates were found, representing 34.8% of all *C. parvum* positive samples. This subtype occurred in 2/8 *C. parvum*-positive farms. Each farm had a different variant present - 19 isolates were found on CZ4 with 99% nucleotide identity to DQ192504.1, while five isolates were found on CZ9 with 99% nucleotide identity to the same sequence.

The least common subtype identified in this study was IIdA20G1. It occurred on only one farm, representing 2.9% of *C. parvum* positive samples. Two isolates were found with 100% nucleotide identity to reference sequence KU852714.1. Geographical distribution of subtypes is visualised in **Figure 11**. All farms had a single subtype present with the exception of CZ9.

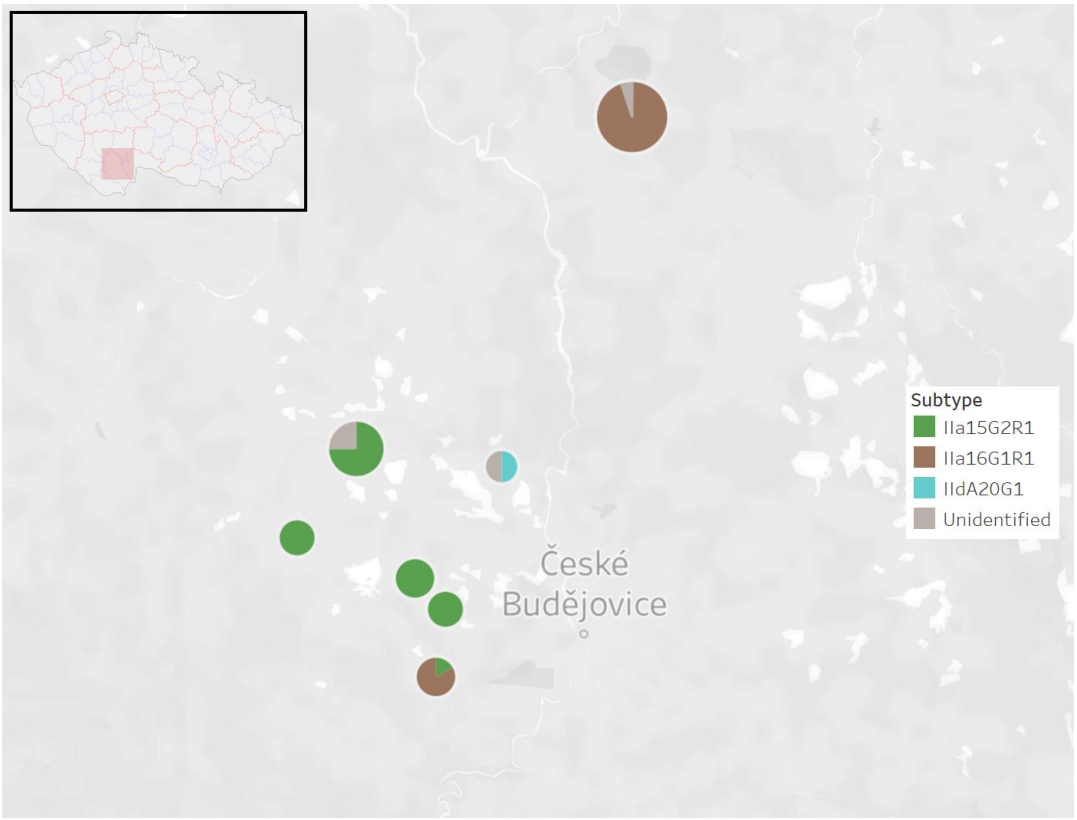

Figure 11. Geographical distribution of *gp60* subtypes in Czech dairy farms. IIaA15G2R1 (green), IIaA16G1R1 (brown), IIdA20G1 (blue). *C. parvum* positive samples with unsuccessful *gp60* sequencing are indicated in grey. Pie charts are proportional to number of *C. parvum* positive samples identified per farm.

3.3.3 Age-associated differences in Cryptosporidium infection

Infecting *Cryptosporidium* species were associated with age. *C. parvum* was most prevalent in neonatal calves with the majority of *C. parvum* infections occurring in the first 1-3 weeks of life **(Figure 12)**. *C. bovis* and *C. ryanae* typically occurred at much lower levels over the first few months of life.

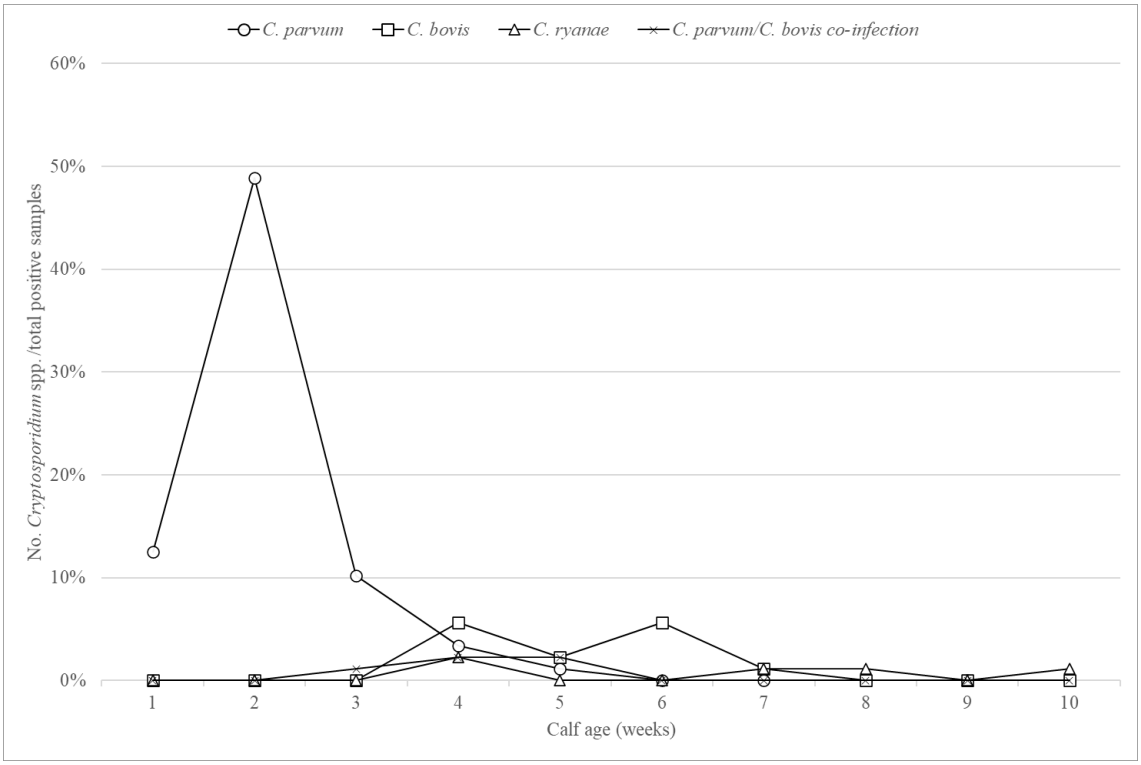

Figure 12. Prevalence of *Cryptosporidium* spp., *C. parvum*, *C. bovis*, *C. ryanae*, and coinfections in calves from 1-10 weeks in Czech dairy farms.

CHAPTER 4: DISCUSSION AND CONCLUDING REMARKS

4.1 *Cryptosporidium* species in cattle

Amplification and sequencing of 18S rRNA gene revealed high presence of *Cryptosporidium* at the farm level, with 84.6-100% of farms containing at least one positive sample in the five countries surveyed. Cyprus and the Czech Republic were highest with *Cryptosporidium* present in every farm. While no bovine cryptosporidiosis research has been carried out in Cyprus, flock-level studies also reported high distribution of *Cryptosporidium* across sheep and goats herds [76]. A Czech study in dairy calves reported farm level incidence at 70%. However, this lower figure may be due to the use of microscopy to screen samples. Additionally, the calves sampled did not include any young neonatal calves (i.e., 0-3 weeks). Our results are in accordance with a previous cattle studies in France, reporting 88.4-100% at the farm level [104,106]. Conversely, a study in Belgium reported just 32% of farms sampled as containing *Cryptosporidium*. This could be attributed to the less sensitive method of detection used (IFA of faecal smears). However, other molecular studies utilising 18S PCR amplification in Europe have also reported lower *Cryptosporidium* at the farm level, ranging from 44.5-66% [132,133]. On the other hand, as the cows in this study were only sampled at one time point, the number of infections may be underestimated. Oocyst shedding can be intermittent [134], and numerous longitudinal studies in farms have observed that almost all calves will shed oocysts over the course of the sampling period [102,103,117].

Cryptosporidium infections were predominantly identified as *C. parvum*, comprising 51.8-81.3% of total infections across the five countries. These results are

reflected in previous studies in Belgium [66], France [104], the Netherlands [121] and the Czech Republic [93]. While there is no previous molecular data on *Cryptosporidium* spp. circulating Cyprus, our results on the predominance of *C. parvum* are in agreement with PCR-based studies from surrounding regions in both healthy and diarrheic animals [78,82,83,135–137]. Beyond these countries, the ubiquity of *C. parvum* in cattle has been reported extensively worldwide. 18S PCR amplification has been previously shown to selectively amplify the dominant species in a sample [138]. As *C. parvum* infections have been suggested to result in higher oocyst shedding density, mixed infections with *C. bovis*, *C. ryanae* and *C. andersoni* are effectively concealed from detection.

Our findings indicate this parasite is widespread throughout the regions studied. The wide variation in occurrence per farm could indicate differences in farm management practices. While this study focussed on molecular characterisation of *Cryptosporidium* in these countries, information on potential risk factors should also be reported – e.g., calf husbandry practices to gain insight to role in parasite spread. Studies have shown seasonal variation in *Cryptosporidium* prevalence on farms [139], indicating climate variables may also play a role. As warm weather is positively correlated with *Cryptosporidium* prevalence [140,141], this partly explains why *Cryptosporidium* incidence in Cyprus was significantly higher than seen in Belgium, France and the Netherlands.

4.2 High occurrence of zoonotic *C. parvum gp60* subtypes circulating in European dairy farms

In order to assess zoonotic risk of *Cryptosporidium* infection in cattle, *C. parvum* subtype identification was carried through amplification and sequencing of the *gp60* gene. Eleven subtypes, belonging to the IIa and IId family, were identified across the five countries sampled.

The most prevalent and widespread subtype in Belgium, the Czech Republic, France and the Netherlands was IIaA15G2R1. In this study, it was identified in 68.1% of *C. parvum*-positive farms. The IIaA15G2R1 subtype is the most predominant worldwide [142] and considered "hypertransmissable" [29]. However, it occurred at relatively low levels in the Cypriot farms studied (8.5%). The zoonotic risk of IIaA15G2R1 has been well established, being responsible for the majority of human cryptosporidiosis cases [142,143]. Further studies will be required to better assess its role in human disease in Cyprus.

The IIaA16G2R1 subtype was the second most common, and only found in Czech and French farms. Incidence in both countries was high, representing 34.8% and 40.5% of *C. parvum* infection in the Czech Republic and France, respectively. This subtype has previously been reported in French calves [104], albeit at a much lower incidence, as well as in fish collected from a French lake [119]. Interestingly, there have been no prior reports of this subtype in the Czech Republic despite the majority of previous molecular investigations in the Czech Republic having also been carried out in farms from the South Bohemia region. In cattle, IIaA16G2R1 has been found worldwide in the Netherlands [121], Belgium [66], Spain [57], Estonia [132], Colombia [144] , Algeria [145,146], China [108] and Iraq [147]. It has been

implicated in numerous human cryptosporidiosis cases globally[148–153], pointing to potential risk of zoonotic transmission.

IIaA14G1R1 was next most common subtype found. Though only found sporadically in Belgian and Dutch farms, it was the most abundant and widespread subtype in Cyprus, comprising 57.4% of the *C. parvum* infections in the country. There is no previous molecular data from Cyprus for comparison, however reported subtypes in the surrounding Mediterranean region do not align with this result. IIaA13G2R1 is the endemic subtype in Turkey, while IIdA20G1 appears endemic to Egypt [84–87,135,154]. This subtype has been reported previously in farms across Europe [78,89,125,132,155–159]. Additionally, outbreaks of human cryptosporidiosis in Norway, New Zealand and the UK [160–162] have been attributed to IIaA14G1R1.

The IIaA12G1R1 subtype appeared only in Cyprus, comprising 25.4% of *C. parvum* positive samples and was only found in a single farm. The only previous report of this subtype was in Israel [136], also in cattle, making this report the second one worldwide. Having only been reported in calves, zoonotic potential of this subtype remains to be seen.

IIaA17G1R1 was found in Belgium and the Netherlands, at a prevalence of 1.75% and 8.11%, respectively. This is in line with a previous study in the Netherlands, which found a slightly higher prevalence at 11% [121]. This higher incidence is also seen in numerous other cattle studies reporting this subtype in Argentina [163], Israel [136], Poland [159], Slovakia [164] and the UK [75,165]. Zoonotic risk appears high, with this subtype appearing in human cryptosporidiosis cases in Ethiopia [166], Iran [167], the Netherlands [121], Slovakia [168,169],

Slovenia [170], Sweden [115] and the UK[171,172]. In a UK study, infections with IIaA17G1R1 were strongly associated with exposure to cattle prior to illness onset.

Subtype IIaA13G2R1 was also found solely in Belgium and the Netherlands, at a prevalence of 3.52% and 4.05%, respectively. This subtype has been reported previously in both Belgium and the Netherlands at similarly low incidence [66,121]. Most reports in cattle are similarly low [75,136,173], with the exception of Turkey where this subtype appears endemic [78,89,174]. Zoonotic potential appears low as only sporadic cases in humans have been reported [151,175–177].

Subtype IIaA17G2R1 was only found on a single farm in the Netherlands, comprising 5.41% of *C. parvum* infections, in line with a previous study in this country [121]. This subtype has been found in cattle in several European countries including in Germany [158], Italy [178], Northern Ireland [179], Poland [180] and Slovakia [181]. Outside Europe, it has been found in cattle in Australia [152,182,183], Canada [184], Israel [136], Uruguay [185] and the USA [68]. IIaA17G2R1 incidence in humans appears mostly sporadic [186–189], with only one outbreak reported in Jordan [149].

IIaA15G1R1 was only identified in one isolate each from Belgium and Cyprus. In cattle, IIaA15G1R1 has been found in the Czech Republic [190], Egypt [83,86] and Sweden [191]. It has been linked to numerous instances of human cryptosporidiosis globally. A Scottish study found IIaA15G1R1 was responsible for 47% of human cases there [192]. The subtype has also been identified in humans presenting with diarrhoea from Australia [187], Egypt [135], England [193], Lebanon [194] and Slovenia [195].

A single isolate of each IIaA16G1R1 and IIaA18G2R1 was detected in France and Cyprus, respectively. IIaA16G1R1 has been widely reported in cattle,

including France [104], Germany [158], Sweden [196], Hungary [197], Serbia [198], Romania [199], Estonia [132], Slovenia [170], Argentina [200] and Jordan [88]. Despite previous reports in Netherlands [121] and the Czech Republic [93,96], it was not detected there in this study. IIaA18G2R1 has been previously identified in cattle in the France [63], USA [68], Northern Ireland [179], Germany [158] and Italy [201], though was typically one of the less common subtypes found. Zoonotic potential of both these subtypes appears to be low having only been identified sporadically in humans from England [193], USA [151,202], Mexico [203] and Australia [152,204].

IIdA20G1 was the only subtype found from the IId *C. parvum* family. Two isolates were identified in a single farm in the Czech Republic. This subtype has commonly been found in cattle in China [205–209] and Egypt [84,86,135,210], appearing somewhat endemic to these regions. To our knowledge, it has not previously been reported in the Czech Republic. Human outbreaks linked to this subtype have occurred in Sweden [211,212], Egypt [135,213] and Qatar [214,215].

Amongst the 286 *C. parvum* positive samples identified with 18S rRNA, only 231 were successfully subtyped. The rest of the samples could not be subtyped due to unclear sequence chromatograms. It is possible this is due to the cattle carrying multiple subtypes of *C. parvum*. As mentioned previously, a significant disadvantage nested-PCR and Sanger sequencing is the preferential amplification of only the more abundant species, while mixed or less abundant species are essentially hidden. It is still clinically important to identify presence of mixed species and subtypes within a host, as due to the sexual stage of the parasite's lifecycle this may generate variation via recombination. This, in turn, could potentially result in altered parasite fitness, which could impact cryptosporidiosis disease pathology. Future studies could utilise

next-generation sequencing instead, which has been used to identify mixed infections as well as less abundant species and subtypes [216]. As a cheaper alternative, albeit more labour-intensive, cloning can also be utilised to distinguish species and subtypes in mixed infections.

Several polymorphisms were identified in the sequenced *gp60* genes that resulted in a change to the amino acid sequence. At present, it is unknown whether these have any biochemical significance, though future genome-wide association studies could be carried out to shed some light on this.

4.3 Age-associated differences in *Cryptosporidium* spp. infection

Age-related variance of infection was observed with 41.7% of calves infected, compared to just 6.6% of dams across Belgium, France and the Netherlands. This infection rate in calves was similar to the overall prevalence in both Cyprus and the Czech Republic, where sampled animals were predominantly pre-weaned calves. The inverse correlation of parasite burden with age has been observed numerous times in cattle [95,121,217,218], and suggests calves are particularly at risk for bovine cryptosporidiosis. However, while the methods used in this study are far more sensitive than conventional microscopic detection, they may not be sufficient when processing stool samples from adult cows. Efficient DNA extraction is vital for accurate detection of infection. Adult cows' faecal pats are much larger and contain more fibrous material [219]. As such, oocysts can be "diluted" in these samples and DNA extracts may contain higher levels of PCR inhibitors [220]. In this study, DNA extraction was carried out on just 0.2g of each faecal sample so reported infection in adults may be underestimated. Indeed, when

utilising a method to concentrate oocysts prior to PCR screening, 91% of sampled adult cows were positive for *Cryptosporidium* [221].

We observed the proportion of infecting *Cryptosporidium* spp. differed across age groups. *C. parvum* was most commonly detected in young neonatal calves up to 3 weeks old, while *C. bovis* and *C. ryanae* became more prevalent as age increased. The age-related variation in infecting *Cryptosporidium spp.* has been observed extensively including within these same countries and elsewhere throughout Europe [66,104,106,132,133,170]. As *C. parvum* is the main 'cattle' species that causes clinical disease, this shows neonatal calves are particularly at risk for bovine cryptosporidiosis.

Co-infections were only observed in calves, however, this is likely attributable to the method in which mixed infections were identified. Co-infections were detected through a positive non-*C. parvum* 18S rRNA identity and positive *gp60* identity. As such, only mixed infections containing *C. parvum* were able to be detected which would tend to discount older animals as *C. parvum* is associated with neonates. It is possible other mixed infections were present in the dams, but combinations of *C. bovis, C. ryanae* and *C. andersoni* could not be recognised. As suggested in Chapter 4.2, future molecular work can utilise techniques to better uncover mixed infections.

4.4 Follow-up study reveals increase in *Cryptosporidium* infection and diversity in Belgium, France and the Netherlands

Comparison of *Cryptosporidium* infection Belgian, French and Dutch dairy farms over two years reveals apparent variation in effective infection control. In France, *Cryptosporidium* infection remained stable across the two years, while an

increase was observed in Belgium and the Netherlands. This indicates infection control measures may be more effective in the French farms. Furthermore, circulating *gp60* subtypes in French farms did not change year to year, further suggesting effective prevention of parasite entry into these farms. While disease burden has not reduced, oocysts are highly robust so it is to be expected that some infection would remain, particularly in the brief period between samplings. Contrastingly, in Belgium and the Netherlands, more subtype diversity was observed in the following year. This could suggest some source of parasite inflow into these farms. However, it is possible these subtypes were present in the initial study but simply not detected as the method we used could not identify mixed subtypes. Curiously, there were more mutual subtypes between Belgium and the Netherlands than was observed in the previous year, possibly suggesting a flow of transmission between these countries. Analysis of *Cryptosporidium* in the surrounding environment could reveal potential sources of infection as well as routes of transmission.

While overall prevalence did not significantly differ in France, there were some differences in species present. A single isolate of *C. xiaoi* was detected in the first study, while none were found in the follow-up. This points to it likely being an isolated case, especially considering this species is typically observed in sheep [222]. The source of infection does not appear to have persisted to the subsequent study.

A major shortcoming of this study is a lack of statistical analyses for the year-to-year comparisons. Given the variation in age of calves sampled, the reported prevalence are likely not an accurate indication of the efficacy of infection control practices. This work comprises the 2nd year of a 3-year study, by the end of which,

statistical analyses will be used to determine how prevalence changed between years, considering the age of calves sampled.

4.5 Future avenues

Further monitoring of this parasite will be paramount in reducing disease burden in cattle. Development of a standardised molecular protocol for species and subtype identification would allow for better comparability between studies for tracking spread of different subtypes. While *gp60* does reveal large subtype diversity, additional typing at other loci may better reveal intra-subtype variability. This is particularly important regarding the "hypertransmissable" IIaA15G2R1 subtype. Despite being the most prevalent and widespread, isolates showed little genetic variation, even across countries, so transmission networks are difficult to discern.

While age has been identified as a key risk factor for infection, other risk factors regarding animal husbandry should be explored. This includes factors such as age of separation of calves from their mothers, administration of colostrum, feed type, housing and bedding. Additionally, as there were large variations in prevalence between farms, investigations into how farm management practices differ may reveal effective infection control strategies. This would help ease the significant economic impact on farmers. Furthermore, cattle breeds should be noted in future investigations as studies have found significant differences in prevalence from between different breeds [108]. Further studies could help indicate which breeds are more resistant to cryptosporidiosis disease.

A One Health approach will be needed to properly tackle disease burden of this parasite. Import and export of cattle across borders should be monitored to prevent

infection spread to uncontaminated areas [123,124]. As outbreaks are commonly associated with waterborne transmission, sampling of the surrounding water bodies may reveal other reservoirs of disease as well as possible indirect routes of transmission through contaminated water runoff from farms.

4.6 Concluding remarks

The high levels of *Cryptosporidium* detected in this study reinforces the role of dairy farms as a key reservoir of this parasite. Our findings point to a high risk of zoonotic transmission with zoonotic *C. parvum* as the predominant species found across all five countries while the majority of *gp60* subtypes identified here have previously been detected in human cryptosporidiosis cases and outbreaks. Though this report contributes to the molecular knowledge of circulating *Cryptosporidium* species and genotypes in Europe, significant knowledge gaps on transmission dynamics, sources of infection and effective interventions remain, calling into attention the need for a One Health approach in the immediate future.

REFERENCES

1. Shirley, D.-A.T.; Moonah, S.N.; Kotloff, K.L. Burden of Disease from Cryptosporidiosis. *Curr. Opin. Infect. Dis.* **2012**, *25*, 555–563, doi:10.1097/QCO.0b013e328357e569.
2. Pisarski, K. The Global Burden of Disease of Zoonotic Parasitic Diseases: Top 5 Contenders for Priority Consideration. *Trop. Med. Infect. Dis.* **2019**, *4*, 44, doi:10.3390/tropicalmed4010044.
3. Carter, B.L.; Chalmers, R.M.; Davies, A.P. Health Sequelae of Human Cryptosporidiosis in Industrialised Countries: A Systematic Review. *Parasit. Vectors* **2020**, *13*, 1–14, doi:10.1186/S13071-020-04308-7.
4. Vanathy, K.; Parija, C.; Mandal, J.; Hamide, A.; Krishnamurthy, S. Cryptosporidiosis: A Mini Review. *Trop. Parasitol.* **2017**, *7*, 72–72, doi:10.4103/tp.TP_25_17.
5. O'Connor, R.M.; Shaffie, R.; Kang, G.; Ward, H.D. Cryptosporidiosis in Patients with HIV/AIDS. *AIDS* **2011**, *25*, 549–560, doi:10.1097/QAD.0b013e3283437e88.
6. Dillingham, R.A.; Lima, A.A.; Guerrant, R.L. Cryptosporidiosis: Epidemiology and Impact. *Microbes Infect.* **2002**, *4*, 1059–1066, doi:10.1016/S1286-4579(02)01630-1.
7. Khalil, I.A.; Troeger, C.; Rao, P.C.; Blacker, B.F.; Brown, A.; Brewer, T.G.; Colombara, D.V.; De Hostos, E.L.; Engmann, C.; Guerrant, R.L.; et al. Morbidity, Mortality, and Long-Term Consequences Associated with Diarrhoea from Cryptosporidium Infection in Children Younger than 5 Years: A Meta-Analyses Study. *Lancet Glob. Health* **2018**, *6*, e758–e768, doi:10.1016/S2214-109X(18)30283-3.
8. Checkley, W.; Epstein, L.D.; Gilman, R.H.; Black, R.E.; Cabrera, L.; Sterling, C.R. Effects of Cryptosporidium Parvum Infection in Peruvian Children: Growth Faltering and Subsequent Catch-up Growth. *Am. J. Epidemiol.* **1998**, *148*, 497–506, doi:10.1093/oxfordjournals.aje.a009675.
9. Guerrant, R.L.; Oriá, R.B.; Moore, S.R.; Oriá, M.O.; Lima, A.A. Malnutrition as an Enteric Infectious Disease with Long-Term Effects on Child Development. *Nutr. Rev.* **2008**, *66*, 487–505, doi:10.1111/j.1753-4887.2008.00082.x.
10. Gerace, E.; Presti, V.D.M.L.; Biondo, C. Cryptosporidium Infection: Epidemiology, Pathogenesis, and Differential Diagnosis. *Eur. J. Microbiol. Immunol.* **2019**, *9*, 119–123, doi:10.1556/1886.2019.00019.
11. Bouzid, M.; Hunter, P.R.; Chalmers, R.M.; Tyler, K.M. Cryptosporidium Pathogenicity and Virulence. *Clin. Microbiol. Rev.* **2013**, *26*, 115–134, doi:10.1128/CMR.00076-12.
12. Current, W.L.; Garcia, L.S. Cryptosporidiosis. *Clin. Microbiol. Rev.* **1991**, *4*, 325–358.
13. Guérin, A.; Striepen, B. The Biology of the Intestinal Intracellular Parasite Cryptosporidium. *Cell Host Microbe* **2020**, *28*, 509–515, doi:10.1016/j.chom.2020.09.007.

14. Kumar, A.; Chatterjee, I.; Anbazhagan, A.N.; Jayawardena, D.; Priyamvada, S.; Alrefai, W.A.; Sun, J.; Borthakur, A.; Dudeja, P.K. Cryptosporidium Parvum Disrupts Intestinal Epithelial Barrier Function via Altering Expression of Key Tight Junction and Adherens Junction Proteins. *Cell. Microbiol.* **2018**, *20*, e12830, doi:10.1111/cmi.12830.

15. King, B.J.; Monis, P.T. Critical Processes Affecting Cryptosporidium Oocyst Survival in the Environment. *Parasitology* **2007**, *134*, 309–323, doi:10.1017/S0031182006001491.

16. Matsubayashi, M.; Teramoto, I.; Urakami, I.; Naohara, J.; Sasai, K.; Kido, Y.; Kaneko, A. Evaluation of Cryptosporidium Parvum Oocyst Inactivation Following Exposure to Ultraviolet Light-Emitting Diodes by in Vitro Excystation and Dye Staining Assays. *Parasitol. Int.* **2022**, *88*, 102557, doi:10.1016/j.parint.2022.102557.

17. DuPont, H.L.; Chappell, C.L.; Sterling, C.R.; Okhuysen, P.C.; Rose, J.B.; Jakubowski, W. The Infectivity of Cryptosporidium Parvum in Healthy Volunteers. *N. Engl. J. Med.* **1995**, *332*, 855–859, doi:10.1056/NEJM199503303321304.

18. Chappell, C.L.; Okhuysen, P.C.; Langer-Curry, R.; Widmer, G.; Akiyoshi, D.E.; Tanriverdi, S.; Tzipori, S. Cryptosporidium Hominis: Experimental Challenge of Healthy Adults. *Am. J. Trop. Med. Hyg.* **2006**, *75*, 851–857.

19. Mosier, D.A.; Oberst, R.D. Cryptosporidiosis: A Global Challenge. *Ann. N. Y. Acad. Sci.* **2000**, *916*, 102–111, doi:10.1111/J.1749-6632.2000.TB05279.X.

20. Thomson, S.; Hamilton, C.A.; Hope, J.C.; Katzer, F.; Mabbott, N.A.; Morrison, L.J.; Innes, E.A. Bovine Cryptosporidiosis: Impact, Host-Parasite Interaction and Control Strategies. *Vet. Res.* **2017**, *48*, doi:10.1186/S13567-017-0447-0.

21. Ježková, J.; Limpouchová, Z.; Prediger, J.; Holubová, N.; Sak, B.; Konečný, R.; Květoňová, D.; Hlásková, L.; Rost, M.; McEvoy, J.; et al. Cryptosporidium Myocastoris n. Sp. (Apicomplexa: Cryptosporidiidae), the Species Adapted to the Nutria (Myocastor Coypus). *Microorganisms* **2021**, *9*, 813–813, doi:10.3390/MICROORGANISMS9040813.

22. Prediger, J.; Ježková, J.; Holubová, N.; Sak, B.; Konečný, R.; Rost, M.; McEvoy, J.; Rajský, D.; Kváč, M. Cryptosporidium Sciurinum n. Sp. (Apicomplexa: Cryptosporidiidae) in Eurasian Red Squirrels (Sciurus Vulgaris). *Microorganisms* **2021**, *9*, 2050–2050, doi:10.3390/MICROORGANISMS9102050.

23. Ryan, U.; Zahedi, A.; Feng, Y.; Xiao, L. An Update on Zoonotic Cryptosporidium Species and Genotypes in Humans. *Anim. Open Access J. MDPI* **2021**, *11*, 3307, doi:10.3390/ani11113307.

24. Ryan, U.; Fayer, R.; Xiao, L. Cryptosporidium Species in Humans and Animals: Current Understanding and Research Needs. *Parasitology* **2014**, *141*, 1667–1685, doi:10.1017/S0031182014001085.

25. Hatam-Nahavandi, K.; Ahmadpour, E.; Carmena, D.; Spotin, A.; Bangoura, B.; Xiao, L. Cryptosporidium Infections in Terrestrial Ungulates with Focus

on Livestock: A Systematic Review and Meta-Analysis. *Parasit. Vectors* **2019**, *12*, 1–23, doi:10.1186/S13071-019-3704-4.

26. Chalmers, R.M.; Giles, M. Zoonotic Cryptosporidiosis in the UK – Challenges for Control. *J. Appl. Microbiol.* **2010**, *109*, 1487–1497, doi:10.1111/J.1365-2672.2010.04764.X.

27. Xiao, L.; Feng, Y. Zoonotic Cryptosporidiosis. *FEMS Immunol. Med. Microbiol.* **2008**, *52*, 309–323, doi:10.1111/J.1574-695X.2008.00377.X.

28. Cho, Y.-I.; Yoon, K.-J. An Overview of Calf Diarrhea - Infectious Etiology, Diagnosis, and Intervention. *J. Vet. Sci.* **2014**, *15*, 1–17, doi:10.4142/JVS.2014.15.1.1.

29. Feng, Y.; Ryan, U.M.; Xiao, L. Genetic Diversity and Population Structure of Cryptosporidium. *Trends Parasitol.* **2018**, *34*, 997–1011, doi:10.1016/J.PT.2018.07.009.

30. De Graaf, D.C.; Vanopdenbosch, E.; Ortega-Mora, L.M.; Abbassi, H.; Peeters, J.E. A Review of the Importance of Cryptosporidiosis in Farm Animals. *Int. J. Parasitol.* **1999**, *29*, 1269–1287, doi:10.1016/S0020-7519(99)00076-4.

31. Leitch, G.J.; He, Q. Cryptosporidiosis-an Overview. *J. Biomed. Res.* **2011**, *25*, 1–1, doi:10.1016/S1674-8301(11)60001-8.

32. Fayer, R.; Ungar, B.L. Cryptosporidium Spp. and Cryptosporidiosis. *Microbiol. Rev.* **1986**, *50*, 458–483.

33. Widmer, G.; Carmena, D.; Kváč, M.; Chalmers, R.M.; Kissinger, J.C.; Xiao, L.; Sateriale, A.; Striepen, B.; Laurent, F.; Lacroix-Lamandé, S.; et al. Update on Cryptosporidium Spp.: Highlights from the Seventh International Giardia and Cryptosporidium Conference.; EDP Sciences, 2020; Vol. 27, pp. 14–14.

34. Shaw, H.J.; Innes, E.A.; Morrison, L.J.; Katzer, F.; Wells, B. Long-Term Production Effects of Clinical Cryptosporidiosis in Neonatal Calves. *Int. J. Parasitol.* **2020**, *50*, 371–376, doi:10.1016/J.IJPARA.2020.03.002.

35. Stott, A.; Gunn, G. A Comparison of Economic Losses Due to Calf Enteritis and Calf Pneumonia in Northern Scotland. *Epidémiologie Santé Anim.* **1997**.

36. Hawkins, A.; Burdine, K.; Amaral-Phillips, D.; Costa, J.H.C. An Economic Analysis of the Costs Associated with Pre-Weaning Management Strategies for Dairy Heifers. *Anim. Basel* **2019**, *9*, 471–471, doi:10.3390/ANI9070471.

37. Bennett, R. The "direct Costs" of Livestock Disease: The Development of a System of Models for the Analysis of 30 Endemic Livestock Diseases in Great Britain. *J. Agric. Econ.* **2003**, *54*, 55–71, doi:10.1111/J.1477-9552.2003.TB00048.X.

38. Delling, C.; Daugschies, A. Literature Review: Coinfection in Young Ruminant Livestock—Cryptosporidium Spp. and Its Companions. *Pathogens* **2022**, *11*, 103, doi:10.3390/pathogens11010103.

39. García, A.; Ruiz-Santa-Quiteria, J.A.; Orden, J.A.; Cid, D.; Sanz, R.; Gómez-Bautista, M.; de la Fuente, R. Rotavirus and Concurrent Infections with Other Enteropathogens in Neonatal Diarrheic Dairy Calves in Spain. *Comp. Immunol. Microbiol. Infect. Dis.* **2000**, *23*, 175–183, doi:10.1016/S0147-9571(99)00071-5.

40. Brainard, J.; Hooper, L.; McFarlane, S.; Hammer, C.C.; Hunter, P.R.; Tyler, K. Systematic Review of Modifiable Risk Factors Shows Little Evidential Support for Most Current Practices in Cryptosporidium Management in Bovine Calves. *Parasitol. Res.* **2020**, *119*, 3571–3584, doi:10.1007/s00436-020-06890-2.

41. O'Leary, J.K.; Sleator, R.D.; Lucey, B. Cryptosporidium Spp. Diagnosis and Research in the 21st Century. *Food Waterborne Parasitol.* **2021**, *24*, e00131, doi:10.1016/j.fawpar.2021.e00131.

42. Chalmers, R.M.; Davies, A.P. Minireview: Clinical Cryptosporidiosis. *Exp. Parasitol.* **2010**, *124*, 138–146, doi:10.1016/j.exppara.2009.02.003.

43. Adeyemo, F.E.; Singh, G.; Reddy, P.; Stenström, T.A. Methods for the Detection of Cryptosporidium and Giardia: From Microscopy to Nucleic Acid Based Tools in Clinical and Environmental Regimes. *Acta Trop.* **2018**, *184*, 15–28, doi:10.1016/j.actatropica.2018.01.011.

44. Fall, A.; Thompson, R.C.A.; Hobbs, R.P.; Morgan-Ryan, U. Morphology Is Not a Reliable Tool for Delineating Species Within Cryptosporidium. *J. Parasitol.* **2003**, *89*, 399–402, doi:10.1645/0022-3395(2003)089[0399:MINART]2.0.CO;2.

45. Morgan-Ryan, U.M.; Fall, A.; Ward, L.A.; Hijjawi, N.; Sulaiman, I.; Payer, R.; Thompson, R.C.A.; Olson, M.; Lal, A.; Xiao, L. Cryptosporidium Hominis n. Sp. (Apicomplexa: Cryptosporidiidae) from Homo Sapiens. *J. Eukaryot. Microbiol.* **2002**, *49*, 433–440, doi:10.1111/j.1550-7408.2002.tb00224.x.

46. Morgan, U.M.; Xiao, L.; Fayer, R.; Lal, A.A.; Thompson, R.C.A. Variation in Cryptosporidium: Towards a Taxonomic Revision of the Genus. *Int. J. Parasitol.* **1999**, *29*, 1733–1751, doi:10.1016/S0020-7519(99)00109-5.

47. Lake, I.R.; Harrison, F.C.D.; Chalmers, R.M.; Bentham, G.; Nichols, G.; Hunter, P.R.; Kovats, R.S.; Grundy, C. Case-Control Study of Environmental and Social Factors Influencing Cryptosporidiosis. *Eur. J. Epidemiol.* **2007**, *22*, 805, doi:10.1007/s10654-007-9179-1.

48. Loeck, B.K. Genotyping and Subtyping Cryptosporidium To Identify Risk Factors and Transmission Patterns — Nebraska, 2015–2017. *MMWR Morb. Mortal. Wkly. Rep.* **2020**, *69*, doi:10.15585/mmwr.mm6912a4.

49. Sulaiman, I.M.; Xiao, L.; Lal, A.A. Evaluation of Cryptosporidium ParvumGenotyping Techniques. *Appl. Environ. Microbiol.* **1999**, *65*, 4431–4435, doi:10.1128/AEM.65.10.4431-4435.1999.

50. Bhat, S.A.; Dixit, M.; Juyal, P.D.; Singh, N.K. Comparison of Nested PCR and Microscopy for the Detection of Cryptosporidiosis in Bovine Calves. *J. Parasit. Dis. Off. Organ Indian Soc. Parasitol.* **2014**, *38*, 101–105, doi:10.1007/s12639-012-0201-5.

51. Uppal, B.; Singh, O.; Chadha, S.; Jha, A.K. A Comparison of Nested PCR Assay with Conventional Techniques for Diagnosis of Intestinal Cryptosporidiosis in AIDS Cases from Northern India. *J. Parasitol. Res.* **2014**, *2014*, e706105, doi:10.1155/2014/706105.

52. Yu, J.-R.; Lee, S.-U.; Park, W.-Y. Comparative Sensitivity of PCR Primer Sets for Detection of Cryptosporidium Parvum. *Korean J. Parasitol.* **2009**, *47*, 293–297, doi:10.3347/kjp.2009.47.3.293.

53. Ezzaty Mirhashemi, M.; Zintl, A.; Grant, T.; Lucy, F.E.; Mulcahy, G.; De Waal, T. Comparison of Diagnostic Techniques for the Detection of Cryptosporidium Oocysts in Animal Samples. *Exp. Parasitol.* **2015**, *151–152*, 14–20, doi:10.1016/j.exppara.2015.01.018.

54. Stroup, S.E.; Roy, S.; Mchele, J.; Maro, V.; Ntabaguzi, S.; Siddique, A.; Kang, G.; Guerrant, R.L.; Kirkpatrick, B.D.; Fayer, R.; et al. Real-Time PCR Detection and Speciation of Cryptosporidium Infection Using Scorpion Probes. *J. Med. Microbiol. 55*, 1217–1222, doi:10.1099/jmm.0.46678-0.

55. Hadfield, S.J.; Robinson, G.; Elwin, K.; Chalmers, R.M. Detection and Differentiation of Cryptosporidium Spp. in Human Clinical Samples by Use of Real-Time PCR. *J. Clin. Microbiol.* **2011**, *49*, 918–924, doi:10.1128/JCM.01733-10.

56. De Waele, V.; Berzano, M.; Berkvens, D.; Speybroeck, N.; Lowery, C.; Mulcahy, G.M.; Murphy, T.M. Age-Stratified Bayesian Analysis To Estimate Sensitivity and Specificity of Four Diagnostic Tests for Detection of Cryptosporidium Oocysts in Neonatal Calves. *J. Clin. Microbiol.* **2011**, *49*, 76–84, doi:10.1128/JCM.01424-10.

57. Quilez, J.; Torres, E.; Chalmers, R.M.; Robinson, G.; Cacho, E.D.; Sanchez-Acedo, C. Cryptosporidium Species and Subtype Analysis from Dairy Calves in Spain. *Parasitology* **2008**, *135*, 1613–1620, doi:10.1017/S0031182008005088.

58. Xiao, L.; Alderisio, K.; Singh, A. *Development and Standardization of a Cryptosporidium Genotyping Tool for Water Samples*; American Water Works Association, 2006; ISBN 978-1-58321-441-1.

59. Luka, G.; Samiei, E.; Tasnim, N.; Dalili, A.; Najjaran, H.; Hoorfar, M. Comprehensive Review of Conventional and State-of-the-Art Detection Methods of Cryptosporidium. *J. Hazard. Mater.* **2022**, *421*, 126714, doi:10.1016/j.jhazmat.2021.126714.

60. Feng, Y.; Torres, E.; Li, N.; Wang, L.; Bowman, D.; Xiao, L. Population Genetic Characterisation of Dominant Cryptosporidium Parvum Subtype IIaA15G2R1. *Int. J. Parasitol.* **2013**, *43*, 1141–1147, doi:10.1016/j.ijpara.2013.09.002.

61. Dong, S.; Yang, Y.; Wang, Y.; Yang, D.; Yang, Y.; Shi, Y.; Li, C.; Li, L.; Chen, Y.; Jiang, · Qingwu; et al. Prevalence of Cryptosporidium Infection in the Global Population: A Systematic Review and Meta-Analysis. *Acta Parasitol.* **1234**, *65*, doi:10.2478/s11686-020-00230-1.

62. Abeywardena, H.; Jex, A.R.; Gasser, R.B. A Perspective on Cryptosporidium and Giardia, with an Emphasis on Bovines and Recent Epidemiological Findings. *Adv. Parasitol.* **2015**, *88*, 243–301, doi:10.1016/BS.APAR.2015.02.001.

63. Pinto, P.; Ribeiro, C.A.; Hoque, S.; Hammouma, O.; Leruste, H.; Détriché, S.; Canniere, E.; Daandels, Y.; Dellevoet, M.; Roemen, J.; et al. Cross-Border

Investigations on the Prevalence and Transmission Dynamics of Cryptosporidium Species in Dairy Cattle Farms in Western Mainland Europe. *Microorganisms* **2021**, *9*, 2394, doi:10.3390/microorganisms9112394.

64. Plutzer, J.; Lassen, B.; Jokelainen, P.; Djurković-Djaković, O.; Kucsera, I.; Dorbek-Kolin, E.; Šoba, B.; Sréter, T.; Imre, K.; Omeragić, J.; et al. Review of Cryptosporidium and Giardia in the Eastern Part of Europe, 2016. *Eurosurveillance* **2018**, *23*, 16–00825, doi:10.2807/1560-7917.ES.2018.23.4.16-00825.

65. Cacciò, S.M.; Chalmers, R.M. Human Cryptosporidiosis in Europe. *Clin. Microbiol. Infect.* **2016**, *22*, 471–480, doi:10.1016/J.CMI.2016.04.021.

66. Geurden, T.; Berkvens, D.; Martens, C.; Casaert, S.; Vercruysse, J.; Claerebout, E. Molecular Epidemiology with Subtype Analysis of Cryptosporidium in Calves in Belgium. *Parasitology* **2007**, *134*, 1981–1987, doi:10.1017/S0031182007003460.

67. Geurden, T.; Goma, F.Y.; Siwila, J.; Phiri, I.G.K.; Mwanza, A.M.; Gabriel, S.; Claerebout, E.; Vercruysse, J. Prevalence and Genotyping of Cryptosporidium in Three Cattle Husbandry Systems in Zambia. *Vet. Parasitol.* **2006**, *138*, 217–222, doi:10.1016/j.vetpar.2006.02.009.

68. Xiao, L.; Zhou, L.; Santin, M.; Yang, W.; Fayer, R. Distribution of Cryptosporidium Parvum Subtypes in Calves in Eastern United States. *Parasitol. Res.* **2006**, *100*, 701–706, doi:10.1007/S00436-006-0337-2.

69. Geurden, T.; Levecke, B.; Cacció, S.M.; Visser, A.; Groote, G.D.; Casaert, S.; Vercruysse, J.; Claerebout, E. Multilocus Genotyping of Cryptosporidium and Giardia in Non-Outbreak Related Cases of Diarrhoea in Human Patients in Belgium. *Parasitology* **2009**, *136*, 1161–1168, doi:10.1017/S0031182009990436.

70. Rahmouni, I.; Essid, R.; Aoun, K.; Bouratbine, A. Glycoprotein 60 Diversity in Cryptosporidium Parvum Causing Human and Cattle Cryptosporidiosis in the Rural Region of Northern Tunisia. *Am. J. Trop. Med. Hyg.* **2014**, *90*, 346–350, doi:10.4269/ajtmh.13-0522.

71. Geurden, T.; Thomas, P.; Casaert, S.; Vercruysse, J.; Claerebout, E. Prevalence and Molecular Characterisation of Cryptosporidium and Giardia in Lambs and Goat Kids in Belgium. *Vet. Parasitol.* **2008**, *155*, 142–145, doi:10.1016/J.VETPAR.2008.05.002.

72. Tarekegn, Z.S.; Tigabu, Y.; Dejene, H. Cryptosporidium Infection in Cattle and Humans in Ethiopia: A Systematic Review and Meta-Analysis. *Parasite Epidemiol. Control* **2021**, *14*, e00219, doi:10.1016/j.parepi.2021.e00219.

73. Ehsan, A.; Geurden, T.; Casaert, S.; Paulussen, J.; De Coster, L.; Schoemaker, T.; Chalmers, R.; Grit, G.; Vercruysse, J.; Claerebout, E. Occurrence and Potential Health Risk of Cryptosporidium and Giardia in Different Water Catchments in Belgium. *Environ. Monit. Assess.* **2015**, *187*, 1–12, doi:10.1007/S10661-014-4157-Z/TABLES/3.

74. Ehsan, A.; Casaert, S.; Levecke, B.; Van Rooy, L.; Pelicaen, J.; Smis, A.; De Backer, J.; Vervaeke, B.; De Smedt, S.; Schoonbaert, F.; et al.

Cryptosporidium and Giardia in Recreational Water in Belgium. *J. Water Health* **2015**, *13*, 870–878, doi:10.2166/wh.2015.268.

75. Smith, R.P.; Clifton-Hadley, F.A.; Cheney, T.; Giles, M. Prevalence and Molecular Typing of Cryptosporidium in Dairy Cattle in England and Wales and Examination of Potential On-Farm Transmission Routes. *Vet. Parasitol.* **2014**, *204*, 111–119, doi:10.1016/J.VETPAR.2014.05.022.

76. Giadinis, N.D.; Symeoudakis, S.; Papadopoulos, E.; Lafi, S.Q.; Karatzias, H. Comparison of Two Techniques for Diagnosis of Cryptosporidiosis in Diarrhoeic Goat Kids and Lambs in Cyprus. *Trop. Anim. Health Prod. 2012 447* **2012**, *44*, 1561–1565, doi:10.1007/S11250-012-0106-4.

77. Giadinis, N.D.; Papadopoulos, E.; Lafi, S.Q.; Papanikolopoulou, V.; Karanikola, S.; Diakou, A.; Vergidis, V.; Xiao, L.; Ioannidou, E.; Karatzias, H. Epidemiological Observations on Cryptosporidiosis in Diarrheic Goat Kids in Greece. *Vet. Med. Int.* **2015**, *2015*, doi:10.1155/2015/764193.

78. Yildirim, A.; Adanir, R.; Inci, A.; Yukari, B.A.; Duzlu, O.; Onder, Z.; Ciloglu, A.; Simsek, E. Prevalence and Genotyping of Bovine Cryptosporidium Species in the Mediterranean and Central Anatolia Region of Turkey. *Comp. Immunol. Microbiol. Infect. Dis.* **2020**, *69*, 101425, doi:10.1016/J.CIMID.2020.101425.

79. Guven, E.; Avcioglu, H.; Balkaya, I.; Hayirli, A.; Kar, S.; Karaer, Z. Prevalence of Cryptosporidiosis and Molecular Characterization of Cryptosporidium Spp. in Calves in Erzurum. *Kafkas Üniversitesi Vet. Fakültesi Derg.* **2013**, *19*, 969–974.

80. Yildirim, A.; Sevinc, F.; Onder, Z.; Duzlu, O.; Ekici, O.D.; Isik, N.; Ciloglu, A.; Simsek, E.; Yetismis, G.; Inci, A. Comparison of Three Diagnostic Methods in the Diagnosis of Cryptosporidiosis and Gp60 Subtyping of Cryptosporidium Parvum in Diarrheic Calves in Central Anatolia Region of Turkey. *Eurobiotech J.* **2021**, *5*, 63–69, doi:10.2478/EBTJ-2021-0010.

81. Ayan, A.; Orunc Kilinc, O.; Yuksek, N.; Basbugan, Y. Detection of Cryptosporidium Spp. in Calves through Nested PCR and Kinyoun's Acid-Fast Methods in Van, Turkey. *Int. J. Ecosyst. Ecol. Sci. IJEES* **2020**, *10*, 271–276, doi:10.31407/ijees.

82. Kabir, M.H. Bin; Ceylan, O.; Ceylan, C.; Shehata, A.A.; Bando, H.; Essa, M.I.; Xuan, X.; Sevinc, F.; Kato, K. Molecular Detection of Genotypes and Subtypes of Cryptosporidium Infection in Diarrheic Calves, Lambs, and Goat Kids from Turkey. *Parasitol. Int.* **2020**, *79*, 102163, doi:10.1016/J.PARINT.2020.102163.

83. Naguib, D.; El-Gohary, A.H.; Mohamed, A.A.; Roellig, D.M.; Arafat, N.; Xiao, L. Age Patterns of Cryptosporidium Species and Giardia Duodenalis in Dairy Calves in Egypt. *Parasitol. Int.* **2018**, *67*, 736–741, doi:10.1016/J.PARINT.2018.07.012.

84. Amer, S.; Honma, H.; Ikarashi, M.; Tada, C.; Fukuda, Y.; Suyama, Y.; Nakai, Y. Cryptosporidium Genotypes and Subtypes in Dairy Calves in Egypt. *Vet. Parasitol.* **2010**, *169*, 382–386, doi:10.1016/J.VETPAR.2010.01.017.

85. Ibrahim, M.A.; Abdel-Ghany, A.E.; Abdel-Latef, G.K.; Abdel-Aziz, S.A.; Aboelhadid, S.M. Epidemiology and Public Health Significance of Cryptosporidium Isolated from Cattle, Buffaloes, and Humans in Egypt. *Parasitol. Res.* **2016**, *115*, 2439–2448, doi:10.1007/S00436-016-4996-3.

86. Amer, S.; Zidan, S.; Adamu, H.; Ye, J.; Roellig, D.; Xiao, L.; Feng, Y. Prevalence and Characterization of Cryptosporidium Spp. in Dairy Cattle in Nile River Delta Provinces, Egypt. *Exp. Parasitol.* **2013**, *135*, 518–523, doi:10.1016/J.EXPPARA.2013.09.002.

87. Mahfouz, M.E.; Mira, N.; Amer, S. Prevalence and Genotyping of Cryptosporidium Spp. in Farm in Egypt. *J. Vet. Med. Sci.* **2014**, *76*, 1569, doi:10.1292/JVMS.14-0272.

88. Hijjawi, N.; Mukbel, R.; Yang, R.; Ryan, U. Genetic Characterization of Cryptosporidium in Animal and Human Isolates from Jordan. *Vet. Parasitol.* **2016**, *228*, 116–120, doi:10.1016/J.VETPAR.2016.08.015.

89. Kabir, M.H.B.; Ceylan, O.; Ceylan, C.; Shehata, A.A.; Bando, H.; Essa, M.I.; Xuan, X.; Sevinc, F.; Kato, K. Molecular Detection of Genotypes and Subtypes of Cryptosporidium Infection in Diarrheic Calves, Lambs, and Goat Kids from Turkey. *Parasitol. Int.* **2020**, *79*, 102163–102163, doi:10.1016/J.PARINT.2020.102163.

90. Thomson, S.; Hamilton, C.A.; Hope, J.C.; Katzer, F.; Mabbott, N.A.; Morrison, L.J.; Innes, E.A. Bovine Cryptosporidiosis: Impact, Host-Parasite Interaction and Control Strategies. *Vet. Res.* **2017**, *48*, doi:10.1186/S13567-017-0447-0.

91. European Centre for Disease Prevention and Control Cryptosporidiosis - Annual Epidemiological Report for 2018. *Annu. Epidemiol. Rep. Commun. Dis. Eur.* **2021**.

92. Chalmers, R.M.; Hadfield, S.J.; Jackson, C.J.; Elwin, K.; Xiao, L.; Hunter, P. Geographic Linkage and Variation in Cryptosporidium Hominis. *Emerg. Infect. Dis.* **2008**, *14*, 496, doi:10.3201/EID1403.071320.

93. Kváč, M.; Hromadová, N.; Květoňová, D.; Rost, M.; Sak, B. Molecular Characterization of Cryptosporidium Spp. in Pre-Weaned Dairy Calves in the Czech Republic: Absence of C. Ryanae and Management-Associated Distribution of C. Andersoni, C. Bovis and C. Parvum Subtypes. *Vet. Parasitol.* **2011**, *177*, 378–382, doi:10.1016/J.VETPAR.2010.11.048.

94. Wang, R.; Ma, G.; Zhao, J.; Lu, Q.; Wang, H.; Zhang, L.; Jian, F.; Ning, C.; Xiao, L. Cryptosporidium Andersoni Is the Predominant Species in Post-Weaned and Adult Dairy Cattle in China. *Parasitol. Int.* **2011**, *60*, 1–4, doi:10.1016/j.parint.2010.09.002.

95. Kváč, M.; Kouba, M.; Vítovec, J. Age-Related and Housing-Dependence of Cryptosporidium Infection of Calves from Dairy and Beef Herds in South Bohemia, Czech Republic. *Vet. Parasitol.* **2006**, *137*, 202–209, doi:10.1016/J.VETPAR.2006.01.027.

96. Ondráčková, Z.; Kváč, M.; Sak, B.; Květoňová, D.; Rost, M. Prevalence and Molecular Characterization of Cryptosporidium Spp. in Dairy Cattle in South

Bohemia, the Czech Republic. *Vet. Parasitol.* **2009**, *165*, 141–144, doi:10.1016/J.VETPAR.2009.06.035.

97. Pumann, P.; Kožíšek, F.; Craun, G.F.; Kunde, T.R.; Malý, M.; Frost, F.J.; Čeřovská, L. High Serological Response to Cryptosporidium-Specific Antigens in the Czech Republic and Its Association with Water Supply. *J. Water Health* **2019**, *17*, 691–700, doi:10.2166/WH.2019.061.

98. Naciri, M.; Paul Lefay, M.; Mancassola, R.; Poirier, P.; Chermette, R. Role of Cryptosporidium Parvum as a Pathogen in Neonatal Diarrhoea Complex in Suckling and Dairy Calves in France. *Vet. Parasitol.* **1999**, *85*, 245–257, doi:10.1016/S0304-4017(99)00111-9.

99. Lefay, D.; Naciri, M.; Poirier, P.; Chermette, R. Prevalence of Cryptosporidium Infection in Calves in France. *Vet. Parasitol.* **2000**, *89*, 1–9, doi:10.1016/S0304-4017(99)00230-7.

100. Delafosse, A.; Chartier, C.; Dupuy, M.C.; Dumoulin, M.; Pors, I.; Paraud, C. Cryptosporidium Parvum Infection and Associated Risk Factors in Dairy Calves in Western France. *Prev. Vet. Med.* **2015**, *118*, 406–412, doi:10.1016/J.PREVETMED.2015.01.005.

101. Rieux, A.; Paraud, C.; Pors, I.; Chartier, C. Molecular Characterization of Cryptosporidium Isolates from Pre-Weaned Calves in Western France in Relation to Age. *Vet. Parasitol.* **2013**, *197*, 7–12, doi:10.1016/J.VETPAR.2013.05.001.

102. Rieux, A.; Chartier, C.; Pors, I.; Paraud, C. Dynamics of Excretion and Molecular Characterization of Cryptosporidium Isolates in Pre-Weaned French Beef Calves. *Vet. Parasitol.* **2013**, *195*, 169–172, doi:10.1016/J.VETPAR.2012.12.043.

103. Rieux, A.; Paraud, C.; Pors, I.; Chartier, C. Molecular Characterization of Cryptosporidium Isolates from Beef Calves under One Month of Age over Three Successive Years in One Herd in Western France. *Vet. Parasitol.* **2014**, *202*, 171–179, doi:10.1016/J.VETPAR.2014.03.004.

104. Follet, J.; Guyot, K.; Leruste, H.; Follet-Dumoulin, A.; Hammouma-Ghelboun, O.; Certad, G.; Dei-Cas, E.; Halama, P. Cryptosporidium Infection in a Veal Calf Cohort in France: Molecular Characterization of Species in a Longitudinal Study. *Vet. Res.* **2011**, *42*, 1–8, doi:10.1186/1297-9716-42-116/TABLES/3.

105. Rieux, A.; Chartier, C.; Pors, I.; Delafosse, A.; Paraud, C. Molecular Characterization of Cryptosporidium Isolates from High-Excreting Young Dairy Calves in Dairy Cattle Herds in Western France. *Parasitol. Res.* **2013**, *112*, 3423–3431, doi:10.1007/S00436-013-3520-2/TABLES/4.

106. Mammeri, M.; Chevillot, A.; Chenafi, I.; Julien, C.; Vallée, I.; Polack, B.; Follet, J.; Adjou, K.T. Molecular Characterization of Cryptosporidium Isolates from Diarrheal Dairy Calves in France. *Vet. Parasitol. Reg. Stud. Rep.* **2019**, *18*, 100323–100323, doi:10.1016/J.VPRSR.2019.100323.

107. Razakandrainibe, R.; Diawara, E.H.I.; Costa, D.; Le Goff, L.; Lemeteil, D.; Ballet, J.J.; Gargala, G.; Favennec, L. Common Occurrence of Cryptosporidium Hominis in Asymptomatic and Symptomatic Calves in

France. *PLoS Negl. Trop. Dis.* **2018**, *12*, e0006355–e0006355, doi:10.1371/JOURNAL.PNTD.0006355.

108. Gong, C.; Cao, X.-F.; Deng, L.; Li, W.; Huang, X.-M.; Lan, J.-C.; Xiao, Q.-C.; Zhong, Z.-J.; Feng, F.; Zhang, Y.; et al. Epidemiology of Cryptosporidium Infection in Cattle in China: A Review. *Parasite* **2017**, *24*, 1, doi:10.1051/parasite/2017001.

109. Nydam, D.V.; Lindergard, G.; Santucci, F.; Schaaf, S.L.; Wade, S.E.; Mohammed, H.O. Risk of Infection with Cryptosporidium Parvum and Cryptosporidium Hominis in Dairy Cattle in the New York City Watershed. *Am. J. Vet. Res.* **2005**, *66*, 413–417, doi:10.2460/ajvr.2005.66.413.

110. Costa, D.; Razakandrainibe, R.; Sautour, M.; Valot, S.; Basmaciyan, L.; Gargala, G.; Lemeteil, D.; Debourgogne, A.; Angoulvant, A.; Agnamey, P.; et al. Human Cryptosporidiosis in Immunodeficient Patients in France (2015–2017). *Exp. Parasitol.* **2018**, *192*, 108–112, doi:10.1016/J.EXPPARA.2018.08.001.

111. Costa, D.; Razakandrainibe, R.; Valot, S.; Vannier, M.; Sautour, M.; Basmaciyan, L.; Gargala, G.; Viller, V.; Lemeteil, D.; Ballet, J.J.; et al. Epidemiology of Cryptosporidiosis in France from 2017 to 2019. *Microorg. 2020 Vol 8 Page 1358* **2020**, *8*, 1358–1358, doi:10.3390/MICROORGANISMS8091358.

112. Beaudeau, P.; De Valk, H.; Vaillant, V.; Mannschott, C.; Tillier, C.; Mouly, D.; Ledrans, M. Lessons Learned from Ten Investigations of Waterborne Gastroenteritis Outbreaks, France, 1998–2006. *J. Water Health* **2008**, *6*, 491–503, doi:10.2166/WH.2008.051.

113. Crestia, J.; Razakandrainibe, R.; Costa, D.; Damiani, C.; Totet, A.; Le Govic, Y. 'Seven Shades of Cryptosporidium.' *Clin. Microbiol. Infect.* **2021**, *0*, doi:10.1016/J.CMI.2021.04.023.

114. Brunet, J.; Lemoine, J.P.; Pesson, B.; Valot, S.; Sautour, M.; Dalle, F.; Muller, C.; Borni-Duval, C.; Caillard, S.; Moulin, B.; et al. Ruling out Nosocomial Transmission of Cryptosporidium in a Renal Transplantation Unit: Case Report. *BMC Infect. Dis.* **2016**, *16*, 1–6, doi:10.1186/S12879-016-1661-5/PEER-REVIEW.

115. Insulander, M.; Silverlås, C.; Lebbad, M.; Karlsson, L.; Mattsson, J.G.; Svenungsson, B. Molecular Epidemiology and Clinical Manifestations of Human Cryptosporidiosis in Sweden. *Epidemiol. Infect.* **2013**, *141*, 1009–1020, doi:10.1017/S0950268812001665.

116. Bordes, L.; Houert, P.; Costa, D.; Favennec, L.; Vial-Novella, C.; Fidelle, F.; Grisez, C.; Prévot, F.; Jacquiet, P.; Razakandrainibe, R. Asymptomatic Cryptosporidium Infections in Ewes and Lambs Are a Source of Environmental Contamination with Zoonotic Genotypes of Cryptosporidium Parvum. *Parasite* **2020**, *27*, doi:10.1051/PARASITE/2020054.

117. Rieux, A.; Paraud, C.; Pors, I.; Chartier, C. Molecular Characterization of Cryptosporidium Spp. in Pre-Weaned Kids in a Dairy Goat Farm in Western France. *Vet. Parasitol.* **2013**, *192*, 268–272, doi:10.1016/J.VETPAR.2012.11.008.

118. Mammeri, M.; Cartou, L.; Chevillot, A.; Thomas, M.; Julien, C.; Vallée, I.; Polack, B.; Follet, J.; Adjou, K.T. First Identification of Cryptosporidium Parvum Zoonotic Subtype IIaA15G2R1 in Diarrheal Lambs in France. *Vet. Parasitol. Reg. Stud. Rep.* **2019**, *18*, 100355–100355, doi:10.1016/J.VPRSR.2019.100355.

119. Certad, G.; Dupouy-Camet, J.; Gantois, N.; Hammouma-Ghelboun, O.; Pottier, M.; Guyot, K.; Benamrouz, S.; Osman, M.; Delaire, B.; Creusy, C.; et al. Identification of Cryptosporidium Species in Fish from Lake Geneva (Lac Léman) in France. *PLOS ONE* **2015**, *10*, e0133047–e0133047, doi:10.1371/JOURNAL.PONE.0133047.

120. Certad, G.; Follet, J.; Gantois, N.; Hammouma-Ghelboun, O.; Guyot, K.; Benamrouz-Vanneste, S.; Fréalle, E.; Seesao, Y.; Delaire, B.; Creusy, C.; et al. Prevalence, Molecular Identification, and Risk Factors for Cryptosporidium Infection in Edible Marine Fish: A Survey across Sea Areas Surrounding France. *Front. Microbiol.* **2019**, *10*, 1037–1037, doi:10.3389/FMICB.2019.01037/BIBTEX.

121. Wielinga, P.R.; de Vries, A.; van der Goot, T.H.; Mank, T.; Mars, M.H.; Kortbeek, L.M.; van der Giessen, J.W.B. Molecular Epidemiology of Cryptosporidium in Humans and Cattle in The Netherlands. *Int. J. Parasitol.* **2008**, *38*, 809–817, doi:10.1016/j.ijpara.2007.10.014.

122. Huetink, R.E.C.; Van der Giessen, J.W.B.; Noordhuizen, J.P.T.M.; Ploeger, H.W. Epidemiology of Cryptosporidium Spp. and Giardia Duodenalis on a Dairy Farm. *Vet. Parasitol.* **2001**, *102*, 53–67, doi:10.1016/S0304-4017(01)00514-3.

123. Pilarczyk, B.; Kolodziejczyk, L.; Zajaczkowska, K.; Kuzna-Grygiel, W.; Balicka-Ramisz, A.; Tomza-Marciniak, A.; Pilarczyk, R. Prevalence of Eimeria and Cryptosporidium Sp. Protozoa in Polish Cows and in Cows Imported from the Netherlands as in-Calf Heifers. *Bull. Vet. Inst. Pulawy* **2009**, *53*, 637–640.

124. Pilarczyk, B.; Ramisz, A.; Jastrzfbski, G. [Internal parasites of cattle in select Western Pomerania farms]. *Wiad. Parazytol.* **2002**, *48*, 383–390.

125. Fournet, N.; Deege, M.P.; Urbanus, A.T.; Nichols, G.; Rosner, B.M.; Chalmers, R.M.; Gorton, R.; Pollock, K.G.; van der Giessen, J.W.B.; Wever, P.C.; et al. Simultaneous Increase of Cryptosporidium Infections in the Netherlands, the United Kingdom and Germany in Late Summer Season, 2012. *Eurosurveillance* **2013**, *18*, 20348–20348, doi:10.2807/ESE.18.02.20348-EN/CITE/PLAINTEXT.

126. Ziegler, P.E.; Santucci, F.; Lindergard, G.; Nydam, D.V.; Wade, S.E.; Schaaf, S.L.; Chang, Y.-F.; Mohammed, H.O. Evaluation of Polymerase Chain Reaction Diagnosis of Cryptosporidium Spp in Dairy Cattle and Wildlife. *Vet. Ther.* **2007**, *8*, 148–159.

127. Alves, M.; Xiao, L.; Sulaiman, I.; Lal, A.A.; Matos, O.; Antunes, F. Subgenotype Analysis of Cryptosporidium Isolates from Humans, Cattle, and Zoo Ruminants in Portugal. *J. Clin. Microbiol.* **2003**, *41*, 2744–2747, doi:10.1128/JCM.41.6.2744-2747.2003.

128. Sulaiman, I.M.; Hira, P.R.; Zhou, L.; Al-Ali, F.M.; Al-Shelahi, F.A.; Shweiki, H.M.; Iqbal, J.; Khalid, N.; Xiao, L. Unique Endemicity of Cryptosporidiosis in Children in Kuwait. *J. Clin. Microbiol.* **2005**, *43*, 2805–2805, doi:10.1128/JCM.43.6.2805-2809.2005.

129. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729, doi:10.1093/molbev/mst197.

130. Tamura, K. Estimation of the Number of Nucleotide Substitutions When There Are Strong Transition-Transversion and G+C-Content Biases. *Mol. Biol. Evol.* **1992**, *9*, 678–687, doi:10.1093/oxfordjournals.molbev.a040752.

131. Hoque, S.; Mavrides, D.E.; Pinto, P.; Costas, S.; Begum, N.; Azevedo-Ribeiro, C.; Liapi, M.; Kváč, M.; Malas, S.; Gentekaki, E.; et al. High Occurrence of Zoonotic Subtypes of Cryptosporidiumparvum in Cypriot Dairy Farms. *Microorganisms* **2022**, *10*, 531, doi:10.3390/microorganisms10030531.

132. Santoro, A.; Dorbek-Kolin, E.; Jeremejeva, J.; Tummeleht, L.; Orro, T.; Jokelainen, P.; Lassen, B. Molecular Epidemiology of Cryptosporidium Spp. in Calves in Estonia: High Prevalence of Cryptosporidium Parvum Shedding and 10 Subtypes Identified. *Parasitology* **2019**, *146*, 261–267, doi:10.1017/S0031182018001348.

133. Díaz, P.; Navarro, E.; Remesar, S.; García-Dios, D.; Martínez-Calabuig, N.; Prieto, A.; López-Lorenzo, G.; López, C.M.; Panadero, R.; Fernández, G.; et al. The Age-Related Cryptosporidium Species Distribution in Asymptomatic Cattle from North-Western Spain. *Animals* **2021**, *11*, 256, doi:10.3390/ani11020256.

134. Shaw, H.J.; Armstrong, C.; Uttley, K.; Morrison, L.J.; Innes, E.A.; Katzer, F. Genetic Diversity and Shedding Profiles for Cryptosporidium Parvum in Adult Cattle and Their Calves. *Curr. Res. Parasitol. Vector-Borne Dis.* **2021**, *1*, 100027, doi:10.1016/j.crpvbd.2021.100027.

135. Helmy, Y.A.; Krücken, J.; Nöckler, K.; von Samson-Himmelstjerna, G.; Zessin, K.H. Molecular Epidemiology of Cryptosporidium in Livestock Animals and Humans in the Ismailia Province of Egypt. *Vet. Parasitol.* **2013**, *193*, 15–24, doi:10.1016/J.VETPAR.2012.12.015.

136. Yasur-Landau, D.; Zilberberg, M.; Perry Markovich, M.; Behar, A.; Fleiderovitz, L.; Leszkowicz Mazuz, M. Cryptosporidium Parvum Subtypes from Diarrheic Dairy Calves in Israel. *Vet. Parasitol. Reg. Stud. Rep.* **2021**, *25*, 100608, doi:10.1016/J.VPRSR.2021.100608.

137. Kassouha, M.; Soukkarieh, C.; Alkhaled, A. First Genotyping of Cryptosporidium Spp. in Pre-Weaned Calves, Broiler Chickens and Children in Syria by PCR-RFLP Analysis. *Vet. Parasitol.* **2016**, *225*, 86–90, doi:10.1016/J.VETPAR.2016.06.009.

138. Cama, V.; Gilman, R.H.; Vivar, A.; Ticona, E.; Ortega, Y.; Bern, C.; Xiao, L. Mixed Cryptosporidium Infections and HIV. *Emerg. Infect. Dis.* **2006**, *12*, 1025–1028, doi:10.3201/eid1206.060015.

139. Szonyi, B.; Bordonaro, R.; Wade, S.E.; Mohammed, H.O. Seasonal Variation in the Prevalence and Molecular Epidemiology of Cryptosporidium Infection in Dairy Cattle in the New York City Watershed. *Parasitol. Res. 2010 1072* **2010**, *107*, 317–325, doi:10.1007/S00436-010-1864-4.

140. Jagai, J.S.; Castronovo, D.A.; Monchak, J.; Naumova, E.N. Seasonality of Cryptosporidiosis: A Meta-Analysis Approach. *Environ. Res.* **2009**, *109*, 465, doi:10.1016/J.ENVRES.2009.02.008.

141. Lal, A.; Baker, M.G.; Hales, S.; French, N.P. Potential Effects of Global Environmental Changes on Cryptosporidiosis and Giardiasis Transmission. *Trends Parasitol.* **2013**, *29*, 83–90, doi:10.1016/J.PT.2012.10.005.

142. Xiao, L. Molecular Epidemiology of Cryptosporidiosis: An Update. *Exp. Parasitol.* **2010**, *124*, 80–89, doi:10.1016/J.EXPPARA.2009.03.018.

143. Feng, Y.; Ryan, U.M.; Xiao, L. Genetic Diversity and Population Structure of Cryptosporidium. *Trends Parasitol.* **2018**, *34*, 997–1011, doi:10.1016/J.PT.2018.07.009.

144. Avendaño, C.; Ramo, A.; Vergara-Castiblanco, C.; Sánchez-Acedo, C.; Quílez, J. Genetic Uniqueness of Cryptosporidium Parvum from Dairy Calves in Colombia. *Parasitol. Res.* **2018**, *117*, 1317–1323, doi:10.1007/s00436-018-5818-6.

145. Baroudi, D.; Khelef, D.; Hakem, A.; Abdelaziz, A.; Chen, X.; Lysen, C.; Roellig, D.; Xiao, L. Molecular Characterization of Zoonotic Pathogens Cryptosporidium Spp., Giardia Duodenalis and Enterocytozoon Bieneusi in Calves in Algeria. *Vet. Parasitol. Reg. Stud. Rep.* **2017**, *8*, 66–69, doi:10.1016/j.vprsr.2017.02.005.

146. Ouakli, N.; Belkhiri, A.; de Lucio, A.; Köster, P.C.; Djoudi, M.; Dadda, A.; Khelef, D.; Kaidi, R.; Carmena, D. Cryptosporidium-Associated Diarrhoea in Neonatal Calves in Algeria. *Vet. Parasitol. Reg. Stud. Rep.* **2018**, *12*, 78–84, doi:10.1016/j.vprsr.2018.02.005.

147. Merdaw, M.; Thabit Salih Al-Zubaidi, M.; Al-Zubaidi, T.; Basil, D.; Khalaf, I.; Sadoon, H. Genotyping of Cryptosporidium Spp. Isolated from Human and Cattle in Baghdad Province, Iraq. **2018**.

148. Chalmers, R.M. Waterborne Outbreaks of Cryptosporidiosis. *Ann. Ist. Super. Sanita* **2012**, *48*, 429–446, doi:10.4415/ANN_12_04_10.

149. Hijjawi, N.; Zahedi, A.; Kazaleh, M.; Ryan, U. Prevalence of Cryptosporidium Species and Subtypes in Paediatric Oncology and Non-Oncology Patients with Diarrhoea in Jordan. *Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis.* **2017**, *55*, 127–130, doi:10.1016/j.meegid.2017.08.033.

150. Sharbatkhori, M.; Nazemalhosseini Mojarad, E.; Taghipour, N.; Pagheh, A.S.; Mesgarian, F. Prevalence and Genetic Characterization of Cryptosporidium Spp. In Diarrheic Children from Gonbad Kavoos City, Iran. *Iran. J. Parasitol.* **2015**, *10*, 441–447.

151. Herges, G.R.; Widmer, G.; Clark, M.E.; Khan, E.; Giddings, C.W.; Brewer, M.; McEvoy, J.M. Evidence That Cryptosporidium Parvum Populations Are

Panmictic and Unstructured in the Upper Midwest of the United States. *Appl. Environ. Microbiol.* **2012**, *78*, 8096, doi:10.1128/AEM.02105-12.

152. Nolan, M.J.; Jex, A.R.; Mansell, P.D.; Browning, G.F.; Gasser, R.B. Genetic Characterization of Cryptosporidium Parvum from Calves by Mutation Scanning and Targeted Sequencing – Zoonotic Implications. *Electrophoresis* **2009**, *30*, 2640–2647, doi:10.1002/ELPS.200900071.

153. Azcona-Gutiérrez, J.M.; de Lucio, A.; Hernández-de-Mingo, M.; García-García, C.; Soria-Blanco, L.M.; Morales, L.; Aguilera, M.; Fuentes, I.; Carmena, D. Molecular Diversity and Frequency of the Diarrheagenic Enteric Protozoan Giardia Duodenalis and Cryptosporidium Spp. in a Hospital Setting in Northern Spain. *PLoS ONE* **2017**, *12*, e0178575, doi:10.1371/journal.pone.0178575.

154. Amer, S.; Zidan, S.; Feng, Y.; Adamu, H.; Li, N.; Xiao, L. Identity and Public Health Potential of Cryptosporidium Spp. in Water Buffalo Calves in Egypt. *Vet. Parasitol.* **2013**, *191*, 123–127, doi:10.1016/J.VETPAR.2012.08.015.

155. Lichtmannsperger, K.; Harl, J.; Freudenthaler, K.; Hinney, B.; Wittek, T.; Joachim, A. Cryptosporidium Parvum, Cryptosporidium Ryanae, and Cryptosporidium Bovis in Samples from Calves in Austria. *Parasitol. Res.* **2020**, *119*, 4291–4295, doi:10.1007/S00436-020-06928-5.

156. Lichtmannsperger, K.; Hinney, B.; Joachim, A.; Wittek, T. Molecular Characterization of Giardia Intestinalis and Cryptosporidium Parvum from Calves with Diarrhoea in Austria and Evaluation of Point-of-Care Tests. *Comp. Immunol. Microbiol. Infect. Dis.* **2019**, *66*, 101333, doi:10.1016/J.CIMID.2019.101333.

157. Holzhausen, I.; Lendner, M.; Göhring, F.; Steinhöfel, I.; Daugschies, A. Distribution of Cryptosporidium Parvum Gp60 Subtypes in Calf Herds of Saxony, Germany. *Parasitol. Res.* **2019**, *118*, 1549–1558, doi:10.1007/S00436-019-06266-1.

158. Broglia, A.; Reckinger, S.; Cacció, S.M.; Nöckler, K. Distribution of Cryptosporidium Parvum Subtypes in Calves in Germany. *Vet. Parasitol.* **2008**, *154*, 8–13, doi:10.1016/J.VETPAR.2008.02.029.

159. Kaupke, A.; Rzeżutka, A. Emergence of Novel Subtypes of Cryptosporidium Parvum in Calves in Poland. *Parasitol. Res.* **2015**, *114*, 4709–4716, doi:10.1007/S00436-015-4719-1.

160. Robertson, L.J.; Temesgen, T.T.; Tysnes, K.R.; Eikås, J.E. An Apple a Day: An Outbreak of Cryptosporidiosis in Norway Associated with Self-Pressed Apple Juice. *Epidemiol. Infect.* **2019**, *147*, doi:10.1017/S0950268819000232.

161. Garcia-R, J.C.; Pita, A.B.; Velathanthiri, N.; French, N.P.; Hayman, D.T.S. Species and Genotypes Causing Human Cryptosporidiosis in New Zealand. *Parasitol. Res.* **2020**, *119*, 2317–2326, doi:10.1007/s00436-020-06729-w.

162. Smith, R.P.; Newton, K.; Rimdap, E.; Wight, A.; Robinson, G.; Chalmers, R.M. Review of Investigations of Premises Housing Animals That Were Linked to Human Outbreaks of Cryptosporidiosis in England and Wales between 2009 and 2019. *Vet. Rec.* **2021**, *189*, doi:10.1002/VETR.246.

163. Tomazic, M.L.; Maidana, J.; Dominguez, M.; Uriarte, E.L.; Galarza, R.; Garro, C.; Florin-Christensen, M.; Schnittger, L. Molecular Characterization of Cryptosporidium Isolates from Calves in Argentina. *Vet. Parasitol.* **2013**, *198*, 382–386, doi:10.1016/j.vetpar.2013.09.022.

164. Mravcová, K. Cryptosporidium Parvum Infection in Calves from an Animal Farm in Slovakia. **2019**.

165. Brook, E.J.; Anthony Hart, C.; French, N.P.; Christley, R.M. Molecular Epidemiology of Cryptosporidium Subtypes in Cattle in England. *Vet. J.* **2009**, *179*, 378–382, doi:10.1016/j.tvjl.2007.10.023.

166. Hailu, A.W.; Degarege, A.; Adamu, H.; Costa, D.; Villier, V.; Mouhajir, A.; Favennec, L.; Razakandrainibe, R.; Petros, B. Molecular Characterization of Cryptosporidium Spp. from Humans in Ethiopia. *PLOS ONE* **2021**, *16*, e0253186, doi:10.1371/JOURNAL.PONE.0253186.

167. Ranjbar, R.; Baghaei, K.; Nazemalhosseini Mojarad, E. Genetic Characterization of Cryptosporidium Spp. among Patients with Gastrointestinal Complaints. *Gastroenterol. Hepatol. Bed Bench* **2016**, *9*, 301–307.

168. Hatalová, E.; Valenčáková, A.; Luptáková, L.; Špalková, M.; Kalinová, J.; Halánová, M.; Bednárová, V.; Gabzdilová, J.; Dedinská, K.; Ondriska, F.; et al. The First Report of Animal Genotypes of Cryptosporidium Parvum in Immunosuppressed and Immunocompetent Humans in Slovakia. *Transbound. Emerg. Dis.* **2018**, *66*, 243–249.

169. Hatalova, E.; Guman, T.; Bednarova, V.; Simova, V.T.; Logoida, M.; Halanova, M. Occurrence of Cryptosporidium Parvum IIaA17G1R1 in Hospitalized Hemato-Oncological Patients in Slovakia. *Parasitol. Res.* **2022**, *121*, 471–476, doi:10.1007/s00436-021-07294-6.

170. Soba, B.; Logar, J. Genetic Classification of Cryptosporidium Isolates from Humans and Calves in Slovenia. *Parasitology* **2008**, *135*, 1263–1270, doi:10.1017/S0031182008004800.

171. Chalmers, R.M.; Smith, R.P.; Hadfield, S.J.; Elwin, K.; Giles, M. Zoonotic Linkage and Variation in Cryptosporidium Parvum from Patients in the United Kingdom. *Parasitol. Res.* **2011**, *108*, 1321–1325, doi:10.1007/s00436-010-2199-x.

172. Chalmers, R.M.; Robinson, G.; Elwin, K.; Elson, R. Analysis of the Cryptosporidium Spp. and Gp60 Subtypes Linked to Human Outbreaks of Cryptosporidiosis in England and Wales, 2009 to 2017. *Parasit. Vectors* **2019**, *12*, 95, doi:10.1186/s13071-019-3354-6.

173. Benhouda, D.; Hakem, A.; Sannella, A.R.; Benhouda, A.; Cacciò, S.M. First Molecular Investigation of Cryptosporidium Spp. in Young Calves in Algeria. *Parasite 24*, 15, doi:10.1051/parasite/2017014.

174. Taylan-Ozkan, A.; Yasa-Duru, S.; Usluca, S.; Lysen, C.; Ye, J.; Roellig, D.M.; Feng, Y.; Xiao, L. Cryptosporidium Species and Cryptosporidium Parvum Subtypes in Dairy Calves and Goat Kids Reared under Traditional Farming Systems in Turkey. *Exp. Parasitol.* **2016**, *170*, 16–20, doi:10.1016/j.exppara.2016.06.014.

175. Guy, R.A.; Yanta, C.A.; Muchaal, P.K.; Rankin, M.A.; Thivierge, K.; Lau, R.; Boggild, A.K. Molecular Characterization of Cryptosporidium Isolates from Humans in Ontario, Canada. *Parasit. Vectors* **2021**, *14*, 69, doi:10.1186/s13071-020-04546-9.

176. Iqbal, A.; Lim, Y.A.L.; Surin, J.; Sim, B.L.H. High Diversity of Cryptosporidium Subgenotypes Identified in Malaysian HIV/AIDS Individuals Targeting Gp60 Gene. *PLOS ONE* **2012**, *7*, e31139, doi:10.1371/journal.pone.0031139.

177. Ma, D.-W.; Lee, M.-R.; Hong, S.-H.; Cho, S.-H.; Lee, S.-E. Molecular Prevalence and Genotypes of Cryptosporidium Parvum and Giardia Duodenalis in Patients with Acute Diarrhea in Korea, 2013–2016. *Korean J. Parasitol.* **2019**, *57*, 531–536, doi:10.3347/kjp.2019.57.5.531.

178. Duranti, A.; Cacciò, S.M.; Pozio, E.; Egidio, A.D.; Curtis, M.D.; Battisti, A.; Scaramozzino, P. Risk Factors Associated with Cryptosporidium Parvum Infection in Cattle. *Zoonoses Public Health* **2009**, *56*, 176–182, doi:10.1111/J.1863-2378.2008.01173.X.

179. Thompson, H.P.; Dooley, J.S.G.; Kenny, J.; McCoy, M.; Lowery, C.J.; Moore, J.E.; Xiao, L. Genotypes and Subtypes of Cryptosporidium Spp. in Neonatal Calves in Northern Ireland. *Parasitol. Res.* **2007**, *100*, 619, doi:10.1007/S00436-006-0305-X.

180. Kaupke, A.; Rzeżutka, A. Emergence of Novel Subtypes of Cryptosporidium Parvum in Calves in Poland. *Parasitol. Res.* **2015**, *114*, 4709–4716, doi:10.1007/s00436-015-4719-1.

181. Danišová, O.; Valenčáková, A.; Petrincová, A. Detection and Identification of Six Cryptospordium Species in Livestock in Slovakia by Amplification of SSU and GP60 Genes with the Use of PCR Analysis. *Ann. Agric. Environ. Med. AAEM* **2016**, *23*, 254–258, doi:10.5604/12321966.1203886.

182. Ng, J.; Yang, R.; McCarthy, S.; Gordon, C.; Hijjawi, N.; Ryan, U. Molecular Characterization of Cryptosporidium and Giardia in Pre-Weaned Calves in Western Australia and New South Wales. *Vet. Parasitol.* **2011**, *176*, 145–150, doi:10.1016/j.vetpar.2010.10.056.

183. Zahedi, A.; Monis, P.; Gofton, A.W.; Oskam, C.L.; Ball, A.; Bath, A.; Bartkow, M.; Robertson, I.; Ryan, U. Cryptosporidium Species and Subtypes in Animals Inhabiting Drinking Water Catchments in Three States across Australia. *Water Res.* **2018**, *134*, 327–340, doi:10.1016/j.watres.2018.02.005.

184. Trotz-Williams, L.A.; Martin, D.S.; Gatei, W.; Cama, V.; Peregrine, A.S.; Martin, S.W.; Nydam, D.V.; Jamieson, F.; Xiao, L. Genotype and Subtype Analyses of Cryptosporidium Isolates from Dairy Calves and Humans in Ontario. *Parasitol. Res.* **2006**, *99*, 346–352, doi:10.1007/s00436-006-0157-4.

185. Caffarena, R.D.; Meireles, M.V.; Carrasco-Letelier, L.; Picasso-Risso, C.; Santana, B.N.; Riet-Correa, F.; Giannitti, F. Dairy Calves in Uruguay Are Reservoirs of Zoonotic Subtypes of Cryptosporidium Parvum and Pose a Potential Risk of Surface Water Contamination. *Front. Vet. Sci.* **2020**, *7*.

186. Waldron, L.S.; Ferrari, B.C.; Power, M.L. Glycoprotein 60 Diversity in C. Hominis and C. Parvum Causing Human Cryptosporidiosis in NSW,

Australia. *Exp. Parasitol.* **2009**, *122*, 124–127, doi:10.1016/j.exppara.2009.02.006.

187. Waldron, L.S.; Dimeski, B.; Beggs, P.J.; Ferrari, B.C.; Power, M.L. Molecular Epidemiology, Spatiotemporal Analysis, and Ecology of Sporadic Human Cryptosporidiosis in Australia. *Appl. Environ. Microbiol.* **2011**, *77*, 7757, doi:10.1128/AEM.00615-11.

188. Braima, K.; Zahedi, A.; Oskam, C.; Reid, S.; Pingault, N.; Xiao, L.; Ryan, U. Retrospective Analysis of Cryptosporidium Species in Western Australian Human Populations (2015–2018), and Emergence of the C. Hominis IfA12G1R5 Subtype. *Infect. Genet. Evol.* **2019**, *73*, 306–313, doi:10.1016/j.meegid.2019.05.018.

189. O' Leary, J.K.; Blake, L.; Corcoran, G.D.; Sleator, R.D.; Lucey, B. Increased Diversity and Novel Subtypes among Clinical Cryptosporidium Parvum and Cryptosporidium Hominis Isolates in Southern Ireland. *Exp. Parasitol.* **2020**, *218*, 107967, doi:10.1016/j.exppara.2020.107967.

190. Kváč, M.; Hromadová, N.; Květoňová, D.; Rost, M.; Sak, B. Molecular Characterization of Cryptosporidium Spp. in Pre-Weaned Dairy Calves in the Czech Republic: Absence of C. Ryanae and Management-Associated Distribution of C. Andersoni, C. Bovis and C. Parvum Subtypes. *Vet. Parasitol.* **2011**, *177*, 378–382, doi:10.1016/J.VETPAR.2010.11.048.

191. Silverlås, C.; Näslund, K.; Björkman, C.; Mattsson, J.G. Molecular Characterisation of Cryptosporidium Isolates from Swedish Dairy Cattle in Relation to Age, Diarrhoea and Region. *Vet. Parasitol.* **2010**, *169*, 289–295, doi:10.1016/J.VETPAR.2010.01.003.

192. Deshpande, A.P.; Jones, B.L.; Connelly, L.; Pollock, K.G.; Brownlie, S.; Alexander, C.L. Molecular Characterization of Cryptosporidium Parvum Isolates from Human Cryptosporidiosis Cases in Scotland. *Parasitology* **2015**, *142*, 318–325, doi:10.1017/S0031182014001346.

193. Chalmers, R.M.; Robinson, G.; Elwin, K.; Elson, R. Analysis of the Cryptosporidium Spp. and Gp60 Subtypes Linked to Human Outbreaks of Cryptosporidiosis in England and Wales, 2009 to 2017. *Parasit. Vectors* **2019**, *12*, doi:10.1186/S13071-019-3354-6.

194. Osman, M.; Safadi, D. El; Benamrouz, S.; Guyot, K.; Dei-Cas, E.; Aliouat, E.M.; Creusy, C.; Mallat, H.; Hamze, M.; Dabboussi, F.; et al. Initial Data on the Molecular Epidemiology of Cryptosporidiosis in Lebanon. *PLoS ONE* **2015**, *10*, doi:10.1371/JOURNAL.PONE.0125129.

195. SOBA, B.; LOGAR, J. Genetic Classification of Cryptosporidium Isolates from Humans and Calves in Slovenia. *Parasitology* **2008**, *135*, 1263–1270, doi:10.1017/S0031182008004800.

196. Silverlås, C.; Näslund, K.; Björkman, C.; Mattsson, J.G. Molecular Characterisation of Cryptosporidium Isolates from Swedish Dairy Cattle in Relation to Age, Diarrhoea and Region. *Vet. Parasitol.* **2010**, *169*, 289–295, doi:10.1016/j.vetpar.2010.01.003.

197. Plutzer, J.; Karanis, P. Genotype and Subtype Analyses of Cryptosporidium Isolates from Cattle in Hungary. *Vet. Parasitol.* **2007**, *146*, 357–362, doi:10.1016/j.vetpar.2007.02.030.

198. Misic, Z.; Abe, N. Subtype Analysis of Cryptosporidium Parvum Isolates from Calves on Farms around Belgrade, Serbia and Montenegro, Using the 60 KDa Glycoprotein Gene Sequences. *Parasitology* **2007**, *134*, 351–358, doi:10.1017/S0031182006001508.

199. Imre, K.; Lobo, L.M.; Matos, O.; Popescu, C.; Genchi, C.; Dărăbuş, G. Molecular Characterisation of Cryptosporidium Isolates from Pre-Weaned Calves in Romania: Is There an Actual Risk of Zoonotic Infections? *Vet. Parasitol.* **2011**, *181*, 321–324, doi:10.1016/j.vetpar.2011.04.042.

200. Del Coco, V.F.; Córdoba, M.A.; Bilbao, G.; de Almeida Castro, A.P.; Basualdo, J.A.; Fayer, R.; Santín, M. Cryptosporidium Parvum GP60 Subtypes in Dairy Cattle from Buenos Aires, Argentina. *Res. Vet. Sci.* **2014**, *96*, 311–314, doi:10.1016/j.rvsc.2013.12.010.

201. Duranti, A.; Cacciò, S.M.; Pozio, E.; Egidio, A. Di; Curtis, M. De; Battisti, A.; Scaramozzino, P. Risk Factors Associated with Cryptosporidium Parvum Infection in Cattle. *Zoonoses Public Health* **2009**, *56*, 176–182, doi:10.1111/J.1863-2378.2008.01173.X.

202. Feltus, D.C.; Giddings, C.W.; Schneck, B.L.; Monson, T.; Warshauer, D.; McEvoy, J.M. Evidence Supporting Zoonotic Transmission of Cryptosporidium Spp. in Wisconsin. *J. Clin. Microbiol.* **2006**, *44*, 4303, doi:10.1128/JCM.01067-06.

203. Valenzuela, O.; González-Díaz, M.; Garibay-Escobar, A.; Burgara-Estrella, A.; Cano, M.; Durazo, M.; Bernal, R.M.; Hernandez, J.; Xiao, L. Molecular Characterization of Cryptosporidium Spp. in Children from Mexico. *PLoS ONE* **2014**, *9*, e96128, doi:10.1371/journal.pone.0096128.

204. Koehler, A.V.; Whipp, M.; Hogg, G.; Haydon, S.R.; Stevens, M.A.; Jex, A.R.; Gasser, R.B. First Genetic Analysis of Cryptosporidium from Humans from Tasmania, and Identification of a New Genotype from a Traveller to Bali. *Electrophoresis* **2014**, *35*, 2600–2607, doi:10.1002/elps.201400225.

205. Zhang, Z.; Su, D.; Meng, X.; Liang, R.; Wang, W.; Li, N.; Guo, Y.; Guo, A.; Li, S.; Zhao, Z.; et al. Cryptosporidiosis Outbreak Caused by Cryptosporidium Parvum Subtype IIdA20G1 in Neonatal Calves. *Transbound. Emerg. Dis. n/a*, doi:10.1111/tbed.13976.

206. Li, N.; Zhao, W.; Song, S.; Ye, H.; Chu, W.; Guo, Y.; Feng, Y.; Xiao, L. Diarrhoea Outbreak Caused by Coinfections of Cryptosporidium Parvum Subtype IIdA20G1 and Rotavirus in Pre-Weaned Dairy Calves. *Transbound. Emerg. Dis. n/a*, doi:10.1111/tbed.14496.

207. Wu, Y.; Zhang, K.; Zhang, Y.; Jing, B.; Chen, Y.; Xu, C.; Wang, T.; Qi, M.; Zhang, L. Genetic Diversity of Cryptosporidium Parvum in Neonatal Dairy Calves in Xinjiang, China. *Pathogens* **2020**, *9*, 692, doi:10.3390/pathogens9090692.

208. Tao, W.; Li, Y.; Yang, H.; Song, M.; Lu, Y.; Li, W. Widespread Occurrence of Zoonotic Cryptosporidium Species and Subtypes in Dairy Cattle from

Northeast China: Public Health Concerns. *J. Parasitol.* **2018**, *104*, 10–17, doi:10.1645/17-140.

209. Xue, N.-Y.; Liu, F.; Tao, W.-F.; Zhao, Q.; Qiu, H.-Y.; Hu, Y.; Chen, Y.; Wei, X.-Y.; Wang, W.; Gao, D.; et al. Molecular Detection of Cryptosporidium Spp. and Enterocytozoon Bieneusi in Longjiang Wagyu Cattle in Northeastern China. *Microb. Pathog.* **2020**, *149*, 104526, doi:10.1016/j.micpath.2020.104526.

210. Ibrahim, M.A.; Abdel-Ghany, A.E.; Abdel-Latef, G.K.; Abdel-Aziz, S.A.; Aboelhadid, S.M. Epidemiology and Public Health Significance of Cryptosporidium Isolated from Cattle, Buffaloes, and Humans in Egypt. *Parasitol. Res.* **2016**, *115*, 2439–2448, doi:10.1007/S00436-016-4996-3.

211. Lebbad, M.; Winiecka-Krusnell, J.; Stensvold, C.R.; Beser, J. High Diversity of Cryptosporidium Species and Subtypes Identified in Cryptosporidiosis Acquired in Sweden and Abroad. *Pathogens* **2021**, *10*, 523, doi:10.3390/pathogens10050523.

212. Gherasim, A.; Lebbad, M.; Insulander, M.; Decraene, V.; Kling, A.; Hjertqvist, M.; Wallensten, A. Two Geographically Separated Food-Borne Outbreaks in Sweden Linked by an Unusual Cryptosporidium Parvum Subtype, October 2010. *Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull.* **2012**, *17*, 20318, doi:10.2807/ese.17.46.20318-en.

213. Naguib, D.; El-Gohary, A.H.; Roellig, D.; Mohamed, A.A.; Arafat, N.; Wang, Y.; Feng, Y.; Xiao, L. Molecular Characterization of Cryptosporidium Spp. and Giardia Duodenalis in Children in Egypt. *Parasit. Vectors* **2018**, *11*, 403, doi:10.1186/s13071-018-2981-7.

214. Boughattas, S.; Behnke, J.M.; Al-Sadeq, D.; Ismail, A.; Abu-Madi, M. Cryptosporidium Spp., Prevalence, Molecular Characterisation and Socio-Demographic Risk Factors among Immigrants in Qatar. *PLoS Negl. Trop. Dis.* **2019**, *13*, e0007750, doi:10.1371/journal.pntd.0007750.

215. Boughattas, S.; Behnke, J.M.; Al-Ansari, K.; Sharma, A.; Abu-Alainin, W.; Al-Thani, A.; Abu-Madi, M.A. Molecular Analysis of the Enteric Protozoa Associated with Acute Diarrhea in Hospitalized Children. *Front. Cell. Infect. Microbiol.* **2017**, *7*.

216. Zahedi, A.; Gofton, A.W.; Jian, F.; Paparini, A.; Oskam, C.; Ball, A.; Robertson, I.; Ryan, U. Next Generation Sequencing Uncovers Within-Host Differences in the Genetic Diversity of Cryptosporidium Gp60 Subtypes. *Int. J. Parasitol.* **2017**, *47*, 601–607, doi:10.1016/j.ijpara.2017.03.003.

217. Santín, M.; Trout, J.M.; Xiao, L.; Zhou, L.; Greiner, E.; Fayer, R. Prevalence and Age-Related Variation of Cryptosporidium Species and Genotypes in Dairy Calves. *Vet. Parasitol.* **2004**, *122*, 103–117, doi:10.1016/j.vetpar.2004.03.020.

218. Maddox-Hyttel, C.; Langkjær, R.B.; Enemark, H.L.; Vigre, H. Cryptosporidium and Giardia in Different Age Groups of Danish Cattle and Pigs—Occurrence and Management Associated Risk Factors. *Vet. Parasitol.* **2006**, *141*, 48–59, doi:10.1016/j.vetpar.2006.04.032.

219. Wells, B.; Thomson, S.; Ensor, H.; Innes, E.A.; Katzer, F. Development of a Sensitive Method to Extract and Detect Low Numbers of Cryptosporidium Oocysts from Adult Cattle Faecal Samples. *Vet. Parasitol.* **2016**, *227*, 26–29, doi:10.1016/j.vetpar.2016.07.018.

220. Schrader, C.; Schielke, A.; Ellerbroek, L.; Johne, R. PCR Inhibitors – Occurrence, Properties and Removal. *J. Appl. Microbiol.* **2012**, *113*, 1014–1026, doi:10.1111/j.1365-2672.2012.05384.x.

221. Wells, B.; Shaw, H.; Hotchkiss, E.; Gilray, J.; Ayton, R.; Green, J.; Katzer, F.; Wells, A.; Innes, E. Prevalence, Species Identification and Genotyping Cryptosporidium from Livestock and Deer in a Catchment in the Cairngorms with a History of a Contaminated Public Water Supply. *Parasit. Vectors* **2015**, *8*, 66, doi:10.1186/s13071-015-0684-x.

222. Fayer, R.; Santín, M. Cryptosporidium Xiaoi n. Sp. (Apicomplexa: Cryptosporidiidae) in Sheep (Ovis Aries). *Vet. Parasitol.* **2009**, *164*, 192–200, doi:10.1016/j.vetpar.2009.05.011.