Prevalence and zoonotic potential of Cryptosporidium spp and Giardia duodenalis in different host species in Greece

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Dissertation submitted in fulfillment of the requirements for the degree of Doctor in Philosophy (PhD) in Veterinary Sciences.

2018
This research was funded by a grant of the State Scholarships Foundation (IKY)
“Reach what you cannot”

N. Kazantzakis
To my parents
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<th>Description</th>
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<tbody>
<tr>
<td>bg</td>
<td>beta giardin</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CD4⁺</td>
<td>cluster of differentiation 4</td>
</tr>
<tr>
<td>COWP</td>
<td>cryptosporidium oocyst wall protein</td>
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<tr>
<td>CPG</td>
<td>cysts per gram of faeces</td>
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<tr>
<td>DALYs</td>
<td>disability-adjusted life years</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ef1a</td>
<td>elongation factor 1 alpha</td>
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<tr>
<td>EIAs</td>
<td>enzyme immunoassays</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ESCCAP</td>
<td>european scientific counsel companion animal parasites</td>
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<tr>
<td>gdh</td>
<td>glutamate dehydrogenase</td>
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<tr>
<td>GLORF-C4</td>
<td><em>giardia lamblia</em> open reading frame –C4</td>
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<tr>
<td>gp60</td>
<td>60-kDa glycoprotein</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HSP70</td>
<td>70kDa heat shock protein</td>
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<tr>
<td>IFA</td>
<td>immunofluorescence assay</td>
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<tr>
<td>IFN-γ</td>
<td>γ-interferon</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>ITS</td>
<td>internal transcribed spacers of ribosomal DNA</td>
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<tr>
<td>miRNA</td>
<td>micro ribonucleic acid</td>
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<tr>
<td>MLB</td>
<td>mannose-binding lectin</td>
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<tr>
<td>MLG</td>
<td>multilocus genotyping</td>
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<tr>
<td>NGS</td>
<td>next generation sequencing</td>
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<tr>
<td>OPG</td>
<td>oocysts per gram of faeces</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SIgA</td>
<td>anti-<em>Giardia</em> secretory immunoglobulin A</td>
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<tr>
<td>SSU rRNA</td>
<td>small subunit ribosomal RNA</td>
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<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<tr>
<td>TF-test</td>
<td>Three Faecal test</td>
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<tr>
<td>tpi</td>
<td>triose phosphate isomerase</td>
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<tr>
<td>vsp</td>
<td>surface protein</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Chapter I

Literature review
Worldwide the parasitic protozoa *Giardia* spp and *Cryptosporidium* spp are a major cause of diarrhoea in humans and several animal species. The last few decades a public health concern referring to these protozoa has arisen, not only for the significance of the infection they cause to their hosts, but mainly for their routes of transmission and their potential zoonotic risk. In this context, in the text to follow, we aimed to present an up-to-date literature review providing critical information that deals with those parasites and the disease they cause in both animals and humans; focusing especially on their epidemiology and zoonotic potential which were analysed in greater depth.

**CHAPTER I.1. *Giardia* spp**

1.1 Introduction

The genus *Giardia* is a single-celled protistan parasite which comprises many species that inhabit the intestinal tract of a series of vertebrate hosts including humans, domestic animals, rodents and wildlife. However, one species, *Giardia duodenalis* (synonymous with *G. intestinalis* and *G. lamblia*), is known to infect and cause giardiosis in humans and mammals, suggesting a zoonotic transmission (Di Genova and Tonelli, 2016).

Although *Giardia* was first observed in 1681 by Antonie van Leeuwenhoek, the first detailed description of this protist was not published until 1859 and its zoonotic significance was controversial until the World Health Organization (WHO) recognized it as a zoonotic agent in 1979 (Abeywardena et al. 2015).

1.2 Taxonomy

According to Plutzer et al., 2010, *Giardia’s* taxonomy is as follows:

- Phylum: Metamonada
- Subphylum: Trichozoa
- Superclass: Eopharyngia
- Class: Trepomonadea
- Subclass: Diplozoa
- Order: Giardiida
- Family: Giardiidae
<table>
<thead>
<tr>
<th><strong>Giardia</strong></th>
<th><strong>Species</strong></th>
<th><strong>Major hosts</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species</strong></td>
<td><strong>G. duodenalis</strong> (= Assemblage A)</td>
<td>Humans and other primates, dogs, cats, livestock, rodents and other wild mammals</td>
</tr>
<tr>
<td></td>
<td><strong>G. enterica</strong> (= Assemblage B)</td>
<td>Humans, ruminants and other hoofed livestock, dogs, rabbits, marsupials, marine mammals, rodents, ferrets, rock hyrax, non-human primates, chicken, ostrich, gull</td>
</tr>
<tr>
<td></td>
<td><strong>G. canis</strong> (= Assemblage C, D)</td>
<td>Dogs</td>
</tr>
<tr>
<td></td>
<td><strong>G. bovis</strong> (= Assemblage E)</td>
<td>Cattle and other hoofed livestock</td>
</tr>
<tr>
<td></td>
<td><strong>G. cati</strong> (= Assemblage F)</td>
<td>Cats</td>
</tr>
<tr>
<td></td>
<td><strong>G. simondi</strong> (= Assemblage G)</td>
<td>Rats, mice</td>
</tr>
<tr>
<td></td>
<td>Assemblage H</td>
<td>Marine mammals</td>
</tr>
<tr>
<td></td>
<td><strong>G. agilis</strong></td>
<td>Amphibians</td>
</tr>
<tr>
<td></td>
<td><strong>G. muris</strong></td>
<td>Rodents</td>
</tr>
<tr>
<td></td>
<td><strong>G. psittaci</strong></td>
<td>Birds</td>
</tr>
<tr>
<td></td>
<td><strong>G. ardeae</strong></td>
<td>Birds</td>
</tr>
<tr>
<td></td>
<td><strong>G. microti</strong></td>
<td>Voles and muskrats</td>
</tr>
<tr>
<td></td>
<td><strong>G. varani</strong></td>
<td>Lizards</td>
</tr>
</tbody>
</table>

Table 1. Proposed *Giardia* species classification (see Thompson and Monis, 2004; Thompson et al., 2008; Heyworth, 2016).

*G. duodenalis* is now considered a multispecies complex. Multigenic sequence analyses confirmed *Giardia*’s subdivision into eight distinct genetic assemblages as follows: Assemblages A and B were found in humans and other mammals, assemblages C and D in dogs, assemblage E in hoofed animals, assemblage F in cats, assemblage G in rodents and assemblage H in marine mammals (Heyworth, 2016).

Within assemblages A and B further genetic variation exists and sub-assemblages AI, AII, AIII and AIV (Monis et al., 2003) as well as BI, BII, BIII and BIV have been described respectively. Sub-
assemblage AII has been isolated mainly from humans whereas AI, AIII and AIV have been found mainly in animals. Similarly, sub-assemblages BIII and BIV have been described in human isolates whereas BI and BII belonged to animals (Ryan and Caccio, 2013).

1.3 Morphology

Morphologically, *Giardia* consists of two stages: the motile one, the trophozoite and the non-motile environmentally resistant stage, the cyst. Trophozoites are pear-shaped and are approximately 12 to 15 μm long and 5 to 9 μm wide. Their cytoskeleton includes a median body, four pairs of flagella (anterior, posterior, caudal and ventral), and a ventral adhesive disk with which they attach to the intestinal wall, where they obtain the necessary nutrients and avoid transport beyond the jejunum. They have two nuclei without nucleoli that are located at the anterior part and are symmetric with respect to the long axis. Cysts are egg shaped, approximately 5 by 7 to 10 μm in diameter and are covered by a wall that is 0.3 to 0.5 μm thick and composed of an outer filamentous layer and an inner membranous layer with two membranes. The internal portion includes two trophozoites with four nuclei, which are released at the excystation phase. Cysts are excreted with the faeces to the environment where they can survive for a long time period (Adam, 2001).
1.4 Life cycle

*Giardia* spp has a direct life cycle. Infection initiates when a host ingests viable cysts (through the faecal-oral route) through direct contact or via contaminated water or food. After passing through the stomach, cysts begin the excystation process and release two trophozoites in the upper part of the small intestine. The released trophozoites attach with their adhesive ventral disk to the epithelial cells of the small intestine where they multiply by binary fission. Under suitable environmental conditions (i.e., increased bile salt concentration and cholesterol deprivation), trophozoites transform into cysts (encystation process) which then are excreted and passed with the faeces to the environment (Di Genova and Tonelli, 2016). The pre-patent period of *Giardia* spp is usually around 10 days, however, in dogs and cats, a prepatent period of 5 days has been reported (Jokipii and Jokipii, 1977; Kirkpatrick and Farrell, 1984; Rosa et al., 2007; Araujo et al., 2008).
1.5 Pathogenesis

As the parasite enters the small intestine lumen the trophozoites adhere into the intestinal epithelium. During this process, the metabolic enzymatic activity of the enterocytes is deranged. The apoptosis of the epithelial cells initiates and the intercellular junctions break, leading to the loss of the mucosal barrier and the dysfunction of the gut microflora. Through these alterations the colonization of Giardia is facilitated and prolonged. Besides, the intestinal microvilli are shortened which leads to the malabsorption of electrolytes and disaccharides. Intestinal permeability is caused and the production of diarrhoea is induced (Einarsson et al., 2016; Certad et al., 2017).
1.6 Immunity

Despite a number of studies which provide valuable data about immune responses against *Giardia* spp mainly based on studies in mice, still, host defense mechanisms against this parasite have not been totally understood. Similarly to other infections, the composition of intestinal microbiota can play a significant role in the establishment of giardiosis. Thus, enriched commensal microbiota can either eliminate *Giardia*’s colonization by for instance, providing a toxic environment to trophozoites or may have a protective role through the maintenance of the gut integrity during infection (Lopez-Romero et al., 2015; Fink and Singer, 2017). Apart from the mucus and the peristaltic movements of the intestine which constitute mechanical barriers to trophozoites’ attachment, several cytokines or other proteins and compounds are produced by the intestinal epithelial and immune cells during infection (Lopez-Romero et al., 2015). For example, the release of antimicrobial peptides can reduce the number of *G. muris* trophozoites or the increased production of nitric oxide inhibits the excystation process and also prevents the *G. duodenalis* trophozoites’ multiplication (Lopez-Romero et al., 2015).

Not only innate defense mechanisms but also acquired humoral and cell-mediated immune responses protect against *Giardia* infections (Faubert 2000), including CD4⁺ T cells and their cytokines as well as B cells and their antibodies i.e. specific IgA or IgG (Abdul-Wahid and Faubert, 2008; Singer and Nash, 2000; Lopez-Romero et al., 2015). In fact, IgA is considered a major contributor to protective immunity against giardiosis (Taherkhani et al., 2009; Grit et al., 2012) with the anti-*Giardia* secretory IgA being detected in human saliva and breast milk (El-Gebaly et al., 2012). Elimination of the parasite has been achieved in animal models at approximately 3-5 weeks post-infection via humoral immunity. Also, it has been reported that repeated infections in both humans and animals lead to the development of immunity or to the display of less severe clinical manifestations (Lopez-Romero et al., 2015). Besides, the IL-17 production by CD4⁺ T cells seems to play significant a role in the development of protective immunity against *G. duodenalis* (Grit et al., 2014; Paerewijck et al., 2017). Specifically, *Giardia* infection upregulates IL-17A cytokine which activates the IL-17RA gene and as a result induces the production of a wide array of antimicrobial proteins and complement factors, which in combination with an intestinal IgA response seems to be important to confer protection against *Giardia* (Paerewijck et al., 2017).
1.7 Giardiosis in dogs and cats

*Giardia* is considered one of the most common intestinal parasites of dogs and cats in developed countries (Palmer et al., 2008; Thompson et al., 2008; Claerebout et al., 2009; Scaramozzino et al., 2009; Ballweber et al., 2010; Epe et al., 2010; Barutzki et al., 2011; Polak et al., 2014; Zanzani et al., 2014; Osman et al., 2015; Pallant et al., 2015).

Prevalence rates for *Giardia* infection in dogs and cats vary substantially from one study to another. The study population, the study area, the diagnostic method used and the health status of the animal are factors which contribute to this variation. The prevalence rates most often range between 5.0% and 15.0% in healthy or clinically ill dogs or cats (Tangtrongsup and Scorza, 2010) however, recent studies in Europe reported infection rates between 20.0% and 25.0% in both species (Baneth et al., 2016).

*Giardia* infection is common in companion animals presented mainly as a sub-clinical, asymptomatic disease. According to textbooks *Giardia* infections can cause chronic diarrhoea and clinical manifestations similar to other species like abdominal pain, malodorous and soft to watery diarrhoea (which can be self-limiting in immunocompetent animals), and weight loss. The severity of such signs depends on several factors such as the *Giardia* assemblage, the immune condition and the age of the animal, its’ lifestyle, physical stress, malnutrition and the co-presentation of other pathogens (Thompson et al., 2008; Tangtrongsup and Scorza, 2010; Tysnes et al. 2014; Adell-Aledón et al. 2018). However, there are limited data proving that *Giardia* infection was the main cause of diarrhoea in the infected animals (Westermarck, 2016).

1.8 Giardiosis in ruminants

Giardiosis is also common in ruminants. In both cattle and small ruminants (sheep and goats), significant variability in the prevalence of *Giardia* is observed, depending on the age of the animals, the housing, feeding and management practices as well as the diagnostic methods used, reaching up to 100.0% infection in some cases (Robertson, 2009; Geurden et al., 2010a; Feng and Xiao, 2011).
Young animals seem to be more susceptible to the disease (Geurden et al., 2010b; Abeywardena et al., 2015). Infections are mostly asymptomatic but can be associated with mild diarrhoea and ill thrift leading to production losses (Feng and Xiao, 2011; Abeywardena et al., 2015).

1.9 Giardiosis in horses

The prevalence of *Giardia* has been reported in horses from various locations with considerable variation (0.5-35.0%) (Pavlasek et al., 1995; Olson et al., 1997; Atwill et al., 2000; De Souza et al., 2009; Veronesi et al., 2010; Traversa et al., 2012; Santin et al., 2013).

Although the presence of this protozoan parasite in horses with diarrhoea has been reported (Kirkpatrick and Skand, 1985), horses infected with *Giardia* rarely show any associated clinical signs of diarrhoea, colic, lethargy and anorexia (Manahan, 1970; Santin et al., 2013).

As with other animals, foals seem to be more prone to infection (Xiao and Herd, 1994; Johnson et al., 1997; Veronesi et al., 2010). However, there are studies which showed that the prevalence of *Giardia* was not significantly associated with age (Olson et al., 1997; Santin et al., 2013; Qi et al., 2015).

1.10 Giardiosis in humans

*G. duodenalis* is one of the most common human intestinal parasites. About 280 million people are being infected every year (Lane and Lloyd, 2002). According to World Health Organisation (WHO) estimates, in 2010, giardiosis had a burden of 171,100 disability-adjusted life years (DALYs) (Kirk et al., 2015). Infection of humans by *Giardia* are reported globally, showing lower rates in developed countries (ranging from 0.4% to 7.5%), compared to the ones in the developing world where the infection rates can reach up to 30.0% (Feng and Xiao, 2011).

The parasite has been responsible for 37.0% of the waterborne disease outbreaks reported during the last 6 years worldwide (Efstratiou et al., 2017). It is estimated that only in the United States, 1.2 million cases of the disease and 3,581 hospitalizations occur annually (Scallan et al., 2011). Besides, according to the Centres for Disease Control and Prevention, 242 outbreaks had been reported from 1971 to 2011 in this country (Adam et al., 2016).
The disease is also present in Europe, with reported outbreaks in Nordic countries, the Netherlands, Belgium, UK, and Greece (Hadjichristodoulou et al., 1998; Hardie et al., 1999; Smith et al., 2006a; Braeye et al., 2015; Guzman-Herrador et al., 2015). In Denmark, Finland, Norway and Sweden the prevalence in asymptomatic and symptomatic human populations was estimated to be 3.0% (2.6-3.3) and 6.0% (5.3-6.3), respectively. It was also estimated that for each registered *Giardia* clinical case, an estimated 867 unregistered symptomatic cases occur annually, per 100,000 inhabitants in Finland, 634 in Norway, and 254 in Sweden (Escobedo et al., 2014). In Germany, on average, 3,806 notified giardiosis cases (range 3,101-4,626) were reported between 2001 and 2007, which corresponded to an average incidence of 4.6 cases/100,000 population. Much higher incidence rates were reported for some other countries. In the Netherlands, there were 11,600 cases in 2004, corresponding to 69.9 cases/100,000 population (Baneth et al., 2016) whereas in Belgium the reported cases were 10.1/100,000 population (source EPISTAT, 2018).

*Giardia* is also considered to be the most common cause of acute diarrhoea in travellers mainly those returning from developing countries (Lebbad et al., 2011; Gautret et al., 2012; Muhsen and Levine, 2012; Broglia et al., 2013; Bartelt and Sartor, 2015).

For all the above reasons, WHO regards giardiosis a Neglected disease since 2004 (WHO, 2004; Savioli et al., 2006).

*Giardia* infection is typically characterized by gastrointestinal symptoms including diarrhoea (watery, fatty or mucous), bloating, abdominal cramps, nausea, vomiting, weight loss and malabsorption (vitamins A and B12, d-xylene, iron and zinc as well as lactase deficiency in 20.0-40.0% of symptomatic cases) but asymptomatic infections can also occur (Nash et al., 1987; Huang and White, 2006; Robertson et al., 2010; Bartelt and Sartor, 2015). The first clinical signs appear after 1-2 weeks of infection and vary from mild and self-limiting to severe with no response to commonly applied treatment (Einarsson et al., 2016). In some cases, the host fails to eradicate the parasite, which leads to a chronic infection (Feng and Xiao, 2011). Children and immunodeficient patients are more vulnerable to clinical manifestations (Haliez and Buret, 2013; Soares and Tasca, 2016). However, children in developing countries seem to be protected against symptomatic disease and specifically against acute diarrhoea, either because they acquire immunity in their first weeks of age through breastfeeding, or due to malnutrition and potentially to an already developed environmental enteropathy which makes their small
intestine characterized by hypercellularity (high numbers of lamina propria lymphocytes) and bacterial overgrowth. It has been suggested that *Giardia*’s invasion may modulate the innate immune system and mucosal environment to such extent that, partially, protection is also offered against diarrhoea caused by other enteropathogens (Muhsen and Levine, 2012).

In various studies, the prevalence of *Giardia* infection has been found statistically significantly higher among HIV seropositive patients compared to HIV seronegative patients, although there are studies which support that HIV infection does not affect the frequency or severity of clinical disease (Escobedo et al., 2014). It has been also reported that previous exposure to the parasite seems to have a protective role and results in less serious manifestations (Halliez and Buret, 2013).

Furthermore, apart from the common symptoms, giardiosis may lead to post-infectious complications such as irritable bowel syndrome, chronic fatigue, malnutrition and cognitive impairment. Also extra-intestinal manifestations may be observed mostly as a result of immunologic reaction, such as food allergy, urticaria, reactive arthritis, and inflammatory ocular manifestations. These symptoms can manifest even without the presence of the parasite in the intestine (Robertson et al., 2010; Wensaas et al., 2012; Naess et al., 2012; Halliez and Buret, 2013; Mørch et al., 2013; Bartelt and Sartor, 2015). However, the mechanisms responsible for post-infectious and extra-intestinal manifestations in giardiosis remain obscure (Halliez and Buret, 2013).

The broad spectrum of the clinical symptoms the disease shows could be attributed to various factors. One of them is the difference in virulence among *Giardia* assemblages. Genotyping of *G. duodenalis* isolates obtained from humans with *Giardia* infection has shown that assemblages A and B are predominantly associated with human infections (Lebbad et al., 2011; Muhsen and Levine, 2012; Heyworth, 2016), although there have been occasional reports of the isolation, from human subjects, of *G. duodenalis* organisms that have genetic markers characteristic of non-A, non-B, assemblages (Lebbad et al., 2011; Heyworth, 2016). However, the association between clinical disease and the assemblage type is rather vague (Cotton et al., 2015; Einarsson et al., 2016). Some studies reported that assemblage A isolates were more virulent than assemblage B isolates. Assemblage A is often associated with the presence of symptoms, while assemblage B is more often associated with asymptomatic infection (Feng and Xiao, 2011). On the other hand, according to other studies, assemblage B isolates are linked to disease
appearance (Bartelt and Sartor, 2015). Some investigations support that assemblage A is associated with intermittent diarrhoea whereas assemblage B with duodenal inflammation, nausea and persistent symptoms (Feng and Xiao, 2011) as well as flatulence in children under 5 years of age (Lebbad et al., 2011). However, others claim that symptoms’ development does not necessarily depend on Giardia’s strains (Lebbad et al., 2011), since even among family members who share the same genotypes not all individuals developed symptoms (Lebbad et al., 2011).

The host nutritional status also influences the symptoms display. For example, protein energy malnutrition, zinc deficiency and vitamin A deficiency may increase susceptibility to Giardia. Besides, the parasite infective dose (in humans, ingestion of ten Giardia cysts has been shown to cause infection), the age and the immunity status of the patient, possible co-existing infections as well as the composition and function of resident microbiota contribute to the variability of clinical manifestation of giardiosis (Lebbad et al., 2011; Bartelt and Sartor, 2015; Adam et al., 2016).

1.11 Diagnosis

Over the years, several diagnostic techniques have been proposed for the diagnosis of Giardia spp derived from either animals or humans. Traditionally, the identification of the parasite’s trophozoites or cysts has been performed by microscopic examination of faeces through direct smears or wet mounts.

Passive faecal flotation, sedimentation (acid/ether) technique, centrifugal faecal flotation (zinc sulphate or sugar solutions are the most commonly used flotation liquids) and most recently IFA (direct immunofluorescence assay) are the most common diagnostic methods used (Tangtrongsup and Scorza, 2010; Koehler et al., 2014).

Among these procedures, IFA is considered the most sensitive assay for the detection of Giardia spp (Gotfred-Rasmussen et al., 2016). It is a fluorescein-labeled monoclonal antibody system that contains monoclonal antibodies that react with Cryptosporidium spp oocysts and Giardia spp cysts (Tangtrongsup and Scorza 2010). The sensitivity and specificity of this test have been evaluated through various studies in humans and in different animal species. According to the manufacturer, sensitivity and specificity of IFA in human samples is estimated to 100.0% and 99.8% respectively (Tangtrongsup and Scorza, 2010). Immunofluorescence is considered the
reference standard assay for the detection of *Giardia* spp in dog and cat faeces (Tangtrongsup and Scorza, 2010). Geurden et al., 2008b, have confirmed using a Bayesian approach, that IFA is one of the most sensitive and specific tests for the detection of *Giardia* spp in dogs. Specifically, they demonstrated 90.0% sensitivity and 94.0% specificity as well as 91.0% sensitivity and 94.0% specificity in epidemiological and clinical studies respectively. Similarly in calves, IFA proved to be a highly sensitive technique (Se=88.0%) and an ideal diagnostic key for clinical giardiosis (Geurden et al., 2010a). However, in a previous study, ELISA was evaluated as the most sensitive (Se=89.0%) but IFA as the most specific (Sp=95.0%) diagnostic technique for the detection of *G. duodenalis* in dairy calves (Geurden et al., 2004).

Since IFA is a quite complicated technique and a time-consuming procedure which requires expensive equipment (fluorescence microscope), it is mainly recommended for research needs and not for routine diagnosis of giardiosis. For that reason, rapid tests (immunochromatographic tests) are widely used although they are usually reported to be less sensitive. For example, in dogs, their sensitivity has been evaluated in different studies to 48.0% and 67.0% (Geurden et al., 2008b; Costa et al., 2016). In calves rapid tests demonstrated even lower sensitivity estimated to 26.0% and 28.0% (Geurden et al., 2010a).

ELISA (enzyme-linked immunosorbent assay) is another commonly used assay which identifies *Giardia* antigens in faeces. In calves, ELISA has been evaluated as a more sensitive (Se=89.0%) but less specific (Sp=90.0%) technique compared to IFA (Geurden et al., 2004). Also in dogs, ELISA is regarded as a useful diagnostic tool (Se =88.9% and Sp=95.8%) for the diagnosis of giardiosis (Panini et al., 2013).

PCR assays are performed for the amplification of *Giardia* DNA in faeces (Koehler et al., 2014). However, due to the presence of multiple PCR inhibitors in faecal material, DNA amplification can be difficult, leading to false negative results (Tangtrongsup and Scorza 2010). Since there are several genes which can be targeted through PCR [β-giardin (bg), triose phosphate isomerase (tpi), glutamate dehydrogenase (gdh), the small subunit ribosomal RNA (SSU rRNA), elongation factor 1 alpha (ef1a) gene, variable surface protein (vsp), the *G. lamblia* open reading frame –C4 (GLORF-C4) and the internal transcribed spacers (ITS) of ribosomal DNA] (Koehler et al., 2014), the selection of the desirable genetic locus should be done based on the purpose of the use of the method. For example, molecular techniques for diagnostic reasons usually focus on multicopy genes like SSU-rDNA, so that high sensitivity is ensured, whereas for *Giardia*
assemblage determination more variable genes are used (Thompson and Ash, 2016). The genetic markers with the highest polymorphism are tpi and gdh followed by bg and C4 (Caccio and Ryan 2008). Besides, for more accurate differentiation and identification of the origin of the assemblages (zoonotic or not), Multilocus Genotyping (MLG) should follow (Tangtrongsup and Scorza, 2010; Feng and Xiao, 2011; Koehler et al., 2014).

1.12 Treatment

1.12.1 Dogs & Cats

Several drugs have been licensed for the treatment of giardiosis in companion animals. Of them, benzimidazoles (albendazole, fenbendazole, oxfendazole), nitroimidazoles (metronidazole, ronidazole), furazolidone and quinacrine have been used in dogs, whereas nitroimidazoles (metronidazole, secnidazole), quinacrine and furazolidone have been indicated for *Giardia* infections in cats (Thompson et al., 2008; Da Silva et al., 2011; Fiechter et al., 2012).

Together with chemotherapy, giardiosis can be effectively treated with the implementation of dietary and environmental control measures. These measures include the addition of fibre to the diet which can enrich the gut microbiota or inhibit the attachment of the parasite to microvilli (Tangtrongsup and Scorza, 2010), and also the cleaning and disinfection of the environment as well as the thorough shampooing of the animal in order to prevent re-infection through contaminated environment or fur (Zajac et al., 1998; Payne et al., 2002; Geurden and Olson, 2011).

1.12.2 Ruminants

Several compounds like fenbendazole (Geurden et al., 2006a), albendazole (O’Handley et al., 2000; Xiao et al., 1996), and paromomycin (Geurden et al., 2006b) can be used for treatment of giardiosis in calves.

Also in lambs, fenbendazole and secnidazole have been suggested as effective treatment options against *Giardia* (Geurden et al., 2011; Ural et al., 2014).

Because only a low number of *Giardia* cysts is needed for infection (Caccio et al., 2005) and a specific immune response against *Giardia* infection develops slowly in ruminants (Yanke et al.,
1998; O’Handley et al., 2003), it has been suggested that environmental disinfection in combination with animal treatment is necessary (Xiao et al., 1996; O’ Handley et al., 2000; Geurden et al., 2006a).

1.12.3 Horses

The administration of metronidazole suspension per os, is suggested for the treatment of giardiosis in horses (Kirkpatrick and Skand, 1985).

1.12.4 Humans

Treatment of giardiosis in humans is based on various compounds such as nitroimidazoles (metronidazole, tinidazole, secnidazole), benzimidazoles (albendazole, mebendazole), quinacrine, nitazoxanide, furazolidone and paromomycin (Escobedo et al., 2016a) implemented either alone or as a combined therapy (Escobedo et al., 2016b). However, the need for the development of more effective and less toxic drugs against this protozoan parasite has aroused. Recently, artemisinin and its derivatives have been suggested as an alternative treatment of giardiosis in humans (Ni Loo et al., 2016).

1.13 Epidemiology and zoonotic potential

As mentioned above Giardia spp is highly abundant, worldwide distributed and is among the most common intestinal parasitic infections of humans and animals. Since it has a direct life cycle, faecal-oral transmission seems to be very common especially in individual cases. However, regarding outbreaks, massive transmission occurs mainly through water and/or food (Adam et al., 2016).

Infected hosts may excrete very high numbers of cysts (up to billions) each day (Robertson, 2009; Adam et al., 2016). Cysts are robust, moderately resistant to chlorine disinfection (Adam et al., 2016) and can survive for weeks to months in the environment, especially in cool and damp areas. In cattle faeces they can remain infectious for a week (Olson et al., 1999). In soil, cyst infectivity was reduced by only 11.0% after 49 days at 4 °C and was non-infective after 7 days at 25 °C. In cold water Giardia cysts can survive for a long period of time, i.e. 56-84 days at 0 °C to 4 °C, while survival time is shorter at higher temperatures (Olson et al., 1999; Feng and Xiao, 2011).
Environmental contamination can lead to the contamination of drinking water and food. In fact, contaminated drinking water has been indicated as the vehicle of transmission in most outbreaks of giardiosis (Marshall et al., 1997; Ryan and Caccio, 2013). Also, treated (swimming pools) and untreated (lakes, rivers, streams) recreational water has been associated with *Giardia* outbreaks (Marshall et al., 1997; Adam et al., 2016). In fact, *Giardia* was the aetiological agent in 37.0% of the waterborne outbreaks that occurred between 2011 and 2016 worldwide (Efstratiou et al., 2017), whereas it was implicated in 35.2% of the waterborne outbreaks documented between 2004 and 2010 (Baldursson and Karanis, 2011). In the United States, 74.8% of the 242 *Giardia* outbreaks that have been reported from 1971 to 2011, affecting around 41,000 people, were associated with water. Of them 74.6% were linked to contaminated drinking water, whereas 18.2% were associated with recreational water (Adam et al., 2016). Again in the United States, between 2013 and 2014, 46.7% of the waterborne outbreaks reported were caused by *Giardia* spp (McClung et al., 2017). Also in Europe, waterborne outbreaks related to *Giardia* infection have been documented (Braeye et al., 2015; Hadjichristodoulou et al., 1998; Hardie et al., 1999; Nygard et al., 2006; Smith et al., 2006a; Guzman-Herrador et al., 2015). However, despite *Giardia* spp has been implicated in several outbreaks associated with water as described above, our knowledge regarding the sources of water contamination in the specific cases is with some exceptions (Robertson et al., 2006), inadequate and thus, further investigation should have been followed for the clarification and the detection of the primary origin of the contamination.

Also, important *Giardia* outbreaks associated with food have been reported globally, either as a result of water contamination or after direct contact with an infected individual (Osterholm et al., 1981; White et al., 1989; Espelage et al., 2010; Figgatt et al., 2017). For instance, in the United States, more than 20,000 laboratory confirmed cases were documented in 2006 (Scallan et al., 2011).

Animal contact has been also regarded as a risk factor of human giardiosis (Heyworth, 2016), however, the situation remains vague as it is not sure if transmission between animals and humans occurs via direct contact or if the presence of a common contaminated source initiates infection (Baneth et al., 2016). In general, the role of animals in the transmission of *Giardia* to humans is under debate (Caccio and Ryan, 2008; Sprong et al., 2009). According to some studies, exposure to farm animals and household pets was not associated with the occurrence of human
giardiosis (Espelage et al., 2010), but on the other hand, other reports implicate animals in the transmission cycle of the disease in humans either through direct contact or via an indirect mode of transmission e.g. contamination of food (Traub et al., 2003; Smith et al., 2006b; Feng and Xiao, 2011; Khan et al., 2011; Budu-Amoako et al., 2012; García-Cervantes et al., 2017; Murray et al., 2017).

Not only animal-to-human but also human-to-animal transmission is possible (Sprong et al., 2009). Apart from pets who share the same household with people, also wild animals can be infected from humans via contaminated environment, with the beaver’s case in Canada as the most wellknown example (Thompson et al., 2009, Prystajecky et al., 2015).

Undoubtedly, people also commonly infect each other (Baneth et al., 2016). Several outbreaks resulting from person-to-person transmission have been documented (Feng and Xiao, 2011). Foodborne outbreaks of giardiosis linked to infected food handlers (Figgatt et al., 2017) and food handlers who changed diapers of infected children prior to handling food have been reported (Osterholm et al., 1981; White et al., 1989). Outbreaks resulting from person-to-person transmission in child care centres are common (Enserink et al., 2015a, Enserink et al., 2015b, Pijnacker et al., 2016).

Community-wide outbreaks might be waterborne initially but might spread subsequently by person-to-person transmission (Feng and Xiao, 2011).

About the seasonality of giardiosis, most human cases are reported from June to October, probably due to increased exposure to recreational waters (Esch and Petersen, 2013).

Genotyping tools have helped us to evaluate the distribution of G. duodenalis strains in humans and in various animal species.

In humans, Giardia infections are characterized by the presence of assemblages A and B. Subtyping of assemblage A isolates revealed the existence of both sub-assemblages A1 and AII in human isolates, with AII being the most common (Feng and Xiao, 2011). Worldwide assemblage B is predominant in human giardiosis cases with around 58.0% prevalence compared to the 37.0% occurrence of the assemblage A, with no difference in distribution between developed and developing countries (Einarsson et al., 2016). However, the only difference that has been observed is the prevalence of mixed infections which are more frequent in developing
communities (5.2% prevalence compared to 3.2% detected in industrialized areas) (Ryan and Caccio, 2013; Einarsson et al., 2016).

Dogs are commonly infected with the host specific assemblages C and D; however, there are reports which have shown infection with the potentially zoonotic assemblage A, mainly the sub-assemblage Al, and less frequently the presence of assemblage B. In cats, the assemblages A and F seem to be predominant, with the cat-specific assemblage F being more frequently found. Of the assemblage A cat isolates, the sub-assemblage Al is the most frequent, however, AII and AIII were also reported (Feng and Xiao, 2011). Few studies have identified assemblage B or assemblage D in cats (Ryan and Caccio, 2013). The type of the Giardia assemblage that might dominate in dogs and cats could be determined by their living environment, that is the hosts and the genotypes that circulate and co-exist. For instance, household pets are mostly infected with assemblages A and B whereas shelter animals tend to be more commonly infected with the host restricted assemblages (Berrilli et al., 2004; Lalle et al., 2005; Leonhard et al., 2007; Szenasi et al., 2007; Claerebout et al., 2009; Ballweber et al., 2010; Upjohn et al., 2010). However, other studies on household dogs identified mainly assemblages C and D and shelter dogs were shown to be infected predominantly with assemblage A (Ryan and Caccio, 2013).

In livestock, assemblages A, B and E are more common either as mono-infection or as mixed infection, with E being the most frequently found, followed by A and B which is the less commonly reported assemblage (Geurden et al., 2008b; Robertson, 2009; Feng and Xiao, 2011; Abeywardena et al., 2015). Most of the assemblage A isolates belong to sub-assemblage Al (Feng and Xiao, 2011). Zoonotic transmission has been reported either via direct animal to human contact or through contamination of water or food (Ryan and Caccio, 2013).

In horses, molecular data is limited. However, some studies show the predominance of assemblages A, both sub-assemblages Al and AII, and B (Traversa et al., 2012; Ryan and Caccio, 2013; Santin et al., 2013; Qi et al., 2015) and some others the hoofed livestock-specific assemblage E (Veronesi et al., 2010).

Generally, among human or animal populations, mixed infections with more than one assemblage or sub-assemblage of G.duodenalis are possible (Sprong et al., 2009; Heyworth, 2016).

Through the evaluation of Giardia’s assemblages’ distribution in various hosts, we gain significant knowledge about the transmission patterns of this parasite, the sources of
contamination and the zoonotic risk that may arise. Until some years ago, genotyping of *Giardia* isolates was limited only to the use of 18 SSU-rRNA gene due to its multicopy nature and the high degree of sequence homology (Caccio and Sprong, 2010). But, the fact that 18 SSU-rRNA is a conserved gene and shows little variability, in combination with our increased need to clarify the transmission scenarios of *Giardia* and evaluate its zoonotic potential, led to the use of other markers with high genetic polymorphism which would let us proceed to the genetic characterization of the isolates up to sub-assemblage level. Thus, sub-assemblages AI, AII, BIII and BIV have been identified and regarded as potentially zoonotic, whereas sub-assemblage AIII was found exclusively in animals (Sprong et al., 2009).

However, since genetic variability has been observed within sub-assemblages, each sub-assemblage was further discriminated into subtypes (Sprong et al., 2009). Among assemblage A and B subtypes several genetic differences have been observed in human and animal isolates (Caccio and Ryan, 2008). In general, assemblage B demonstrates higher allelic sequence heterozygosity compared to assemblage A (Caccio et al., 2005; Caccio and Sprong, 2010). This high genetic diversity among *Giardia* assemblages led to the generation of multilocus genotypes (MLGs) originating from the combination of subtypes in different loci (Caccio and Ryan, 2008). Thus, through the use of multilocus genotyping, concrete results regarding the epidemiology and the potential zoonotic transmission patterns of *Giardia* spp could be obtained (Caccio et al., 2008).
CHAPTER I.2. Cryptosporidium spp

2.1 Introduction

Cryptosporidium spp is an intestinal protozoan which affects vertebrates including humans and which is responsible for several waterborne and foodborne outbreaks worldwide (Thompson et al., 2005; Chalmers and Katzer, 2013). Due to the severity of the symptoms that it can cause in the hosts, and subsequently to the economic losses that are related to Cryptosporidium infection, cryptosporidiosis has been included in the Neglected Diseases Initiative of the World Health Organization (WHO) (Chalmers and Katzer, 2013). Therefore, it has been estimated by WHO that in 2010 cryptosporidiosis resulted in 2,159,331 DALYs due to foodborne infections (Kirk et al., 2015).

2.2 Taxonomy

According to Ryan et al., 2016b, Cryptosporidium spp current taxonomy is as follows:

Phylum: Apicomplexa
Class: Gregarinomorphea
Subclass: Cryptogregaria

Thirty-one Cryptosporidium species and more than 70 genotypes have been recognized (Fayer, 2010; Certad et al., 2017) (Table 2).
<table>
<thead>
<tr>
<th>Species</th>
<th>Major hosts</th>
<th>Detected in humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. hominis (9 subtypes: Ia, Ib, Id, le ... lj)</td>
<td>Humans</td>
<td>Most common</td>
</tr>
<tr>
<td>C. viatorum</td>
<td>Humans</td>
<td>Yes</td>
</tr>
<tr>
<td>C. parvum (14 subtypes: Ila-llo)</td>
<td>Mammals, including humans</td>
<td>Common</td>
</tr>
<tr>
<td>C. meleagridis</td>
<td>Birds, mammals, humans</td>
<td>Common</td>
</tr>
<tr>
<td>C. canis</td>
<td>Dogs</td>
<td>Yes</td>
</tr>
<tr>
<td>C. felis</td>
<td>Cats</td>
<td>Yes</td>
</tr>
<tr>
<td>C. andersoni</td>
<td>Cattle</td>
<td>Yes</td>
</tr>
<tr>
<td>C. bovis</td>
<td>Cattle</td>
<td>Yes</td>
</tr>
<tr>
<td>C. ryanae</td>
<td>Cattle</td>
<td>No</td>
</tr>
<tr>
<td>C. xiaoi</td>
<td>Sheep, goats</td>
<td>Yes</td>
</tr>
<tr>
<td>C. ubiquitum</td>
<td>Ruminants, rodents, primates</td>
<td>Yes</td>
</tr>
<tr>
<td>C. suis</td>
<td>Pigs</td>
<td>Yes</td>
</tr>
<tr>
<td>C. scrofarum</td>
<td>Pigs</td>
<td>Yes</td>
</tr>
<tr>
<td>C. erinacei</td>
<td>Hedgehogs, horses</td>
<td>Yes</td>
</tr>
<tr>
<td>C. fayeri</td>
<td>Marsupials</td>
<td>Yes</td>
</tr>
<tr>
<td>C. macropodum</td>
<td>Marsupials</td>
<td>No</td>
</tr>
<tr>
<td>C. cuniculus</td>
<td>Rabbits</td>
<td>Yes</td>
</tr>
<tr>
<td>C. muris</td>
<td>Rodents</td>
<td>Yes</td>
</tr>
<tr>
<td>C. tyzzeri</td>
<td>Rodents</td>
<td>Yes</td>
</tr>
<tr>
<td>C. proliferans</td>
<td>Rodents</td>
<td>No</td>
</tr>
<tr>
<td>C. wrairi</td>
<td>Guinea pigs</td>
<td>No</td>
</tr>
<tr>
<td>C. rubeyi</td>
<td>Squirrels</td>
<td>No</td>
</tr>
<tr>
<td>C. baileyi</td>
<td>Poultry</td>
<td>No</td>
</tr>
<tr>
<td>C. avium</td>
<td>Birds</td>
<td>No</td>
</tr>
<tr>
<td>C. galli</td>
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</tr>
<tr>
<td>C. serpentis</td>
<td>Reptiles</td>
<td>No</td>
</tr>
<tr>
<td>C. varanii</td>
<td>Lizards</td>
<td>No</td>
</tr>
<tr>
<td>C. fragile</td>
<td>Toads</td>
<td>No</td>
</tr>
<tr>
<td>C. molnari</td>
<td>Fish</td>
<td>No</td>
</tr>
<tr>
<td>C. huwi</td>
<td>Fish</td>
<td>No</td>
</tr>
<tr>
<td>C. scophthalmi</td>
<td>Turbot</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 2. Cryptosporidium species classification (Ryan et al., 2014; Holubova et al., 2016; Kvac et al., 2016; Ryan et al., 2016b; Zahedi et al., 2016a; Khan et al., 2017)
2.3 Morphology

*Cryptosporidium* spp consists of various morphological features depending on the stage of the life cycle. These stages include sporozoites, trophozoites, merozoites, microgametocytes, macrogametocytes and oocysts which are released in the environment through faeces (Thompson et al., 2005). Since many species of *Cryptosporidium* exist, the oocysts of each species display morphological differences such as different size and shape, some are small and spherical whereas others are larger and more oval (Chalmers and Katzer, 2013; Zahedi et al., 2016a). However, regardless the species, although *Cryptosporidium* belongs to apicomplexan parasites which carry the apicoplast organelle, it is characterized by the absence of apicoplasts and mitochondria (Bouzid et al., 2013; Ryan et al., 2015).

2.4 Life cycle

The life cycle of *Cryptosporidium* is complicated, consisting of both sexual and asexual developmental stages. Infection begins with the ingestion by the host of the sporulated oocysts through contaminated water or food or directly via the faecal-oral route. Inhalation of the oocysts can also occur. Each oocyst contains four sporozoites which after excystation in the intestinal lumen or the respiratory tract, emerge and invade the epithelial cells and develop into trophozoites. Trophozoites undergo asexual division (merogony) and form Type I Meronts consisting of 8 merozoites. Some of these merozoites form Type II meronts which contain 4 merozoites and initiate the sexual phase of the life cycle. Macrogametocytes and microgametocytes are formed, fertilize and produce the zygote. Most of the zygotes develop into oocysts, the thick ones with a two-layered wall which are released to the environment, and the thin-walled oocysts which facilitate autoinfection. The prepatent period for *Cryptosporidium parvum* for example, ranges from 7 to 21 days (Thompson et al., 2005).
2.5 Pathogenesis

After invasion of Cryptosporidium spp into the intestinal epithelium various pathophysiological mechanisms are activated. Epithelial cells’ damage initiates and microvilli’s atrophy, crypts’ hyperplasia and cell apoptosis occur. Colonization of the parasite results in a shortened intestinal surface area leading to fluids’ and electrolytes’ transport disorder and malabsorption. Consequently, increased transepithelial permeability is observed and secretory diarrhoea is produced (Thompson et al., 2005; Certad et al., 2017).

2.6 Immunity

Both innate and adaptive host responses are important in the control of Cryptosporidium infection although the mechanisms are not completely understood particularly, in humans (Ryan et al., 2016a).

Studies based mainly on mouse models, have highlighted the fundamental role of the intestinal epithelial cells in both parasite multiplication and the protective immune response (Petry et al., 2010; Ryan et al., 2016a; Laurent and Lacroix-Lamande, 2017). Due to this response, several cytokines such as γ-interferon (IFN-γ) and also IL-12, IL-15 and IL-18, chemokines, antimicrobial peptides and nitric oxide are secreted from the infected epithelial cells, preventing or reducing
the severity of infection (Ryan et al., 2016a; Laurent and Lacroix-Lamande, 2017; Lemieux et al., 2017).

Also other mechanisms are implicated in the host immunity during Cryptosporidium infection. MicroRNA (miRNA), which are small RNA molecules of 23 nucleotides that result in gene silencing via translational suppression or mRNA degradation, target Toll-like receptor 4 (TLR4) and regulate TLR4-mediated anti-C. parvum defense as well as alter C. parvum infection burden in vitro (Ryan et al., 2016a).

Mannose-binding lectin (MBL), which is an evolutionarily conserved protein secreted by hepatocytes that functions in human innate immunity by binding to microbial surfaces and promoting opsonophagocytosis, has been shown to be important in the protection against cryptosporidiosis, as children and HIV-infected adults with mannose-binding lectin deficiency have increased susceptibility to cryptosporidiosis and more severe diseases (Ryan et al., 2016a).

Regarding cellular adaptive immunity, CD4+ T cells seem to be essential to the eradication of the parasite (Lemieux et al., 2017). Therefore, it has been shown that low counts of CD4+ T cells recorded in AIDS patients, made them more vulnerable to Cryptosporidium infection (Lemieux et al., 2017).

The role of humoral immunity in the protection from cryptosporidiosis is vague (Ryan et al., 2016a; Lemieux et al., 2017), however, the antibodies that have been linked to Cryptosporidium infection are serum IgM and IgG as well as serum and secretory IgA. Antibodies may support protection as it has been shown in the case of bovine cryptosporidiosis where hyperimmune bovine colostrum had prophylactic and therapeutic effects (Lemieux et al., 2017).

2.7 Cryptosporidiosis in dogs and cats

Cryptosporidiosis is a parasitosis that concerns dogs and cats worldwide, with prevalence rates reported between 0.0% and 29.4% in cats and 0.5% and 44.1% in dogs (Lucio-Forster et al., 2010). Although many of the infected animals are characterized by asymptomatic infection, severe manifestations can also be displayed. The most common symptoms include watery diarrhoea, anorexia and weight loss and are more frequent in young, mainly less than 6 months of age, and immunocompromised animals (Scorza and Tangtrongsup, 2010). Oocyst shedding
lasts for months in cats and for more than 80 days in dogs (Thompson et al., 2005; Hamnes et al., 2007; Santin, 2013).

2.8 Cryptosporidiosis in ruminants

_Cryptosporidium_ spp is widely endemic in ruminants and it is considered as one of the most frequently diagnosed enteropathogens in these animals.

Among _Cryptosporidium_ species that infect cattle, _C. parvum_ is linked to clinical disease (Thomson et al., 2017). Infection occurs in neonatal calves and oocyst shedding can initiate even in 2 days-old animals (Thompson et al., 2005). Clinical manifestations include profuse watery diarrhoea, abdominal pain, anorexia and weight loss and can lead to death as a result of severe dehydration (Santin, 2013). Infected animals can excrete a large number of oocysts per day (> $10^{10}$) and parasite's shedding can occur even during asymptomatic infection (Thomson et al., 2017).

_Cryptosporidium_ spp also infect young sheep and goats, mainly at ages between 1 to 3 weeks (Santin, 2013). The prepatent period is approximately 4 days (Thompson et al., 2005). Similar to bovine cryptosporidiosis, clinical symptoms in lambs and goat kids are characterized by diarrhoea which can be yellow pasty to watery, anorexia and illthrift. As in cattle, large numbers of oocysts ($10^8$-$10^{10}$) are excreted each day through faeces for a long time even from asymptomatic animals (Thompson et al., 2005; Santin, 2013).

2.9 Cryptosporidiosis in horses

Cryptosporidiosis is also prevalent in horses. Infected animals can be asymptomatic but also severe diarrhoea can occur in foals of a few weeks of age and in immunocompromised animals (Thompson et al., 2005; Veronesi et al., 2010; Santin, 2013).

2.10 Cryptosporidiosis in pigs

_Cryptosporidium_ has been detected in pigs of all ages worldwide (Farzan et al., 2011; Yui et al., 2014; Lin et al., 2015; Petersen et al., 2015; Schubnell et al., 2016), however, Petersen et al., 2015 have reported that piglets before weaning are more vulnerable to infection and more
intensely infected than older animals. The *Cryptosporidium* species/genotypes adapted to pigs are *C. scrofarum* and *C. suis*, but *C. parvum, C. muris, C. andersoni* and *C. tyzzeri* have been also isolated from these animals (Kvac et al., 2013; Santin, 2013; Yui et al., 2014). *Cryptosporidium suis* seems to be more common in young pigs, whereas *C. scrofarum* is more frequently found in older animals (>5 weeks) (Langkjaer et al., 2007; Johnson et al., 2008; Kváč et al., 2009b; Jeníkova et al., 2011; Kváč et al., 2014; Yui et al., 2014).

Although cryptosporidiosis in pigs is usually subclinical (Xiao, 2010), clinical manifestations can occur including diarrhoea, anorexia and vomiting. Severity of clinical signs seem to depend on the *Cryptosporidium* species that cause infection and co-infection with other enteropathogens (Santin, 2013), however, the pathogenicity of the disease in pigs has not been clarified yet (Yui et al., 2014).

### 2.11 Cryptosporidiosis in humans

*Cryptosporidium* is the most common diarrhoea-causing protozoan parasite worldwide, especially in developing countries, as it is less frequently observed in areas where hygiene, water quality and nutrition are adequate (Ryan et al., 2016a; Squire and Ryan, 2017). Nevertheless, *Cryptosporidium* is considered as the leading agent of waterborne disease outbreaks in the United States (Painter et al., 2016) and Europe (Semenza and Nichols, 2007; Hajdu et al., 2008; Guzman-Herrador et al., 2015; Utsi et al., 2016) causing high rates of morbidity and even mortality in children and immunocompromised individuals. The prevalence of cryptosporidiosis in HIV-infected patients with diarrhoea has been reported to range from 3.0% to 16.0% in developed countries (Putignani and Menichella, 2010).

Clinical signs appear between 2 and 14 days from the time of infection and can persist for 3 weeks (Thompson et al., 2005; Chalmers and Giles, 2010). However, severity and duration of symptoms vary with age and immune status of the host (Thompson et al., 2005). Young children and immunocompromised and malnourished individuals are more susceptible to cryptosporidiosis (Thompson et al., 2005; Bouzid et al., 2013; Ryan et al., 2016a; Caccio and Chalmers, 2016) and may excrete a high number of oocysts ranging between $5.0 \times 10^3$ to $9.2 \times 10^5$ oocysts/mL (Goodgame et al., 1993). On the contrary, disease in immunocompetent individuals is mainly characterized by self-limiting symptoms and even asymptomatic shedding of oocysts (Thompson et al., 2005; Bouzid et al., 2013; Caccio and Chalmers, 2016).
The most common clinical sign is diarrhoea which can be severe and watery to mild and intermittent (Thompson et al., 2005). Other clinical signs include general malaise, fever, fatigue, loss of appetite, nausea and vomiting. Infrequently, symptoms associated with cholecystitis, hepatitis, pancreatitis, reactive arthritis and respiratory problems are observed (Thompson et al., 2005).

Among the above species, *C. hominis* subtype Id, *C. parvum*, *C. canis* and *C. felis* have been associated with a more severe disease in HIV/AIDS patients. The risk of diarrhoea was higher in patients infected with *C. hominis* subtype Id compared to those infected with the subtype Ib whereas no diarrhoea was observed in patients infected with subtype Ia. Subjects infected with *C. meleagridis* showed no clinical signs and excreted a small number of oocysts. Long-term sequelae of infection, including ocular and articular pain, headache and fatigue, have been associated with *C. hominis* but not with *C. parvum* (Certad et al., 2017). Also, *C. hominis* has been responsible for more outbreaks than *C. parvum* in most regions (Ryan et al., 2016a).

### 2.12 Diagnosis

*Cryptosporidium* oocysts can be detected in faecal samples by a variety of diagnostics tests including direct microscopy, antigen detection methods and molecular techniques.

Several staining techniques have been used for the microscopic identification of *Cryptosporidium* spp with acid fast-modified Ziehl-Neelsen staining being the most common one. Other stains include Kinyoun (Lennette et al., 1985), negative malachite green staining (Elliott et al., 1999), negative carbol fuchsine staining (Casemore et al., 1985; Kuhnert-Paul et al., 2012), safranin methylene blue (Garcia, 2007, 2009) and trichrome (Garcia, 2007, 2009).

Recently, the TF-test (*Three Faecal Test* Coccidia parasitological technique has been validated for the detection of *Cryptosporidium* oocysts using a new dye, a combination of modified D’Antoni’s iodine solution and modified Masson’s trichome composition, with promising results (Inacio et al., 2016).

Above all microscopic techniques, the direct immunofluorescent antibody assay (IFA) that simultaneously detects *Giardia* cysts and *Cryptosporidium* oocysts is considered as the most sensitive and specific test. Specifically, it offers about 97.0% sensitivity compared to 75.0% sensitivity of acid-fast staining in human samples (Ryan et al., 2016a).
Apart from IFA, other immunoassays have been also evaluated and are widely used for the detection of Cryptosporidium spp. These include enzyme-linked immunosorbent assays (ELISAs), enzyme immunoassays (EIAs) and immunochromatographic (dipstick) assays (Ryan et al., 2016a). Similarly, compared to other antigen detection tests, IFA remains the most sensitive method and is also highly specific (Se: 97.4% & Sp: 94.8% in calves, Se: 97.4% & Sp: 100% in human samples) (Geurden et al., 2008a; Chalmers et al., 2011a). Biosensor chips, that detect and quantify C. parvum in real-time via anti-C. parvum IgM binding, have also been developed, however detection limits are relatively high (100 or more oocysts) and they have yet to be fully evaluated on water or faecal samples (Campbell et al., 2008; Kang et al., 2008).

Another major limitation of both conventional microscopy and antigen detection methods is that they cannot identify to species or subtype level, which is essential for understanding transmission dynamics and outbreaks, in particular for zoonotic species (Ryan et al., 2016a). PCR-based methods have also been developed for the diagnosis and the determination of Cryptosporidium species/genotypes and C. parvum and C. hominis subtypes. The SSU-rRNA is the most widely used marker since it is semi-conserved, and it has hyper-variable regions and a multi-copy nature. Other genes are also used such as 70kDa heat shock protein (HSP70) (Morgan et al., 2001), Cryptosporidium oocyst wall protein (COWP) (Xiao et al., 2000) and actin (Sulaiman et al., 2002). The 60-kDa glycoprotein (gp60) gene is a commonly used subtyping tool for the identification of C. parvum and C. hominis subtypes (Xiao, 2010).

### 2.13 Treatment

#### 2.13.1 Dogs and cats

Paromomycin, an aminoglycoside drug, is regarded the drug of choice for the treatment of cryptosporidiosis in dogs and cats (Barr et al., 1994; Scorza and Lappin, 2006; Shahiduzzaman and Daugschies, 2012). Apart from paromomycin, two macrolides, azithromycin and tylosin, have been also suggested with azithromycin being better tolerated by cats (Scorza and Lappin, 2006). Besides, nitazoxanide, previously approved for use in the treatment of giardiosis and cryptosporidiosis in humans has been administered to dogs and cats (Scorza and Lappin, 2006; Moron-Soto et al., 2017), however, it is not effective in immunocompromised animals (Shahiduzzaman and Daugschies, 2012). Together with the administration of drugs for the
effective elimination of the parasite, supportive therapy may also be needed for rehydration or the eradication of possible secondary infections that may co-exist (Thompson et al., 2008).

2.13.2 Ruminants

Several drugs have been tested for the treatment of cryptosporidiosis in ruminants. However, only a few have proved to control the parasite effectively and thus are widely used. Halofuginone lactate which has a cryptosporidiostatic activity on the sporozoite and merozoite stages of *C. parvum*, is quite effective against bovine cryptosporidiosis (Naciri et al., 1993; Thompson et al., 2008; De Waele et al., 2010) and also against *Cryptosporidium* infection in lambs and goat kids (Giadinis et al., 2007; Petermann et al., 2014). Paromomycin is suggested as an efficient drug in both prevention and treatment of cryptosporidiosis, mainly administered to small ruminants (Fayer and Ellis, 1993; Mancassola et al., 1995; Chartier et al., 1996; Viu et al., 2000; Shahiduzzaman and Daugschies, 2012).

2.13.3 Horses

Treatment of equine cryptosporidiosis is mainly based on supportive therapy since there is no licensed drug for this indication. However, the administration of nitazoxanide and paromomycin in combination with azithromycin has been documented (Shahiduzzaman and Daugschies, 2012).

2.13.4 Pigs

Control of cryptosporidiosis in pigs is quite complicated. The use of paromomycin seems to have promising results but not for severe cases. Besides, nitazoxanide can be partially effective to infected piglets but only in low doses.

2.13.5 Humans

Nitazoxanide is widely used for the treatment of human cryptosporidiosis, however, this compound is not efficient in HIV patients (Ryan et al., 2016b).

2.14 Epidemiology and zoonotic potential

Transmission of *Cryptosporidium* spp can be either direct by human-to-human or animal-to-human contact or indirectly via contaminated water or food (Ryan et al., 2016a). Also,
mechanical transmission is possible by vectors such as arthropods or birds (Thompson et al., 2005).

Infected hosts excrete large numbers of highly infectious and robust oocysts through their faeces (Ryan et al., 2016a; Certad et al., 2017). Oocysts proved to be viable for more than 12 weeks at -4 °C in water and cattle faeces and for 10 weeks in soil (Olson et al., 1999), however, it has been shown that temperature affects their viability and they become more susceptible as temperature raises (Olson et al. 1999; Peng et al., 2008).

Cryptosporidium is regarded as one of the most important causes of waterborne disease outbreaks worldwide. It has been responsible for around 60.0% of the reported waterborne disease outbreaks between 2004 and 2010 (Baldursson and Karanis, 2011). Waterborne infections involve drinking water, recreational waters such as swimming pools and waterparks and surface waters including water catchments and irrigation waters (Baldursson and Karanis, 2011; Ryan et al., 2017). Besides to prolonged survival in the environment, Cryptosporidium oocysts are also highly resistant to common disinfectants such as chlorine (Ryan et al., 2016a). Thus, the parasite cannot be inactivated with water treatments and transmission via drinking and recreational water is favoured (Ryan et al., 2016a). Surface waters can be contaminated by infected people or animals, particularly wildlife and livestock whose contaminated manure left can end up in rivers or lakes through run-off (Hofstra et al., 2013).

Foodborne outbreaks associated with Cryptosporidium infection have also been reported (Putignani and Menichella, 2010; Robertson and Chalmers, 2013). Food contamination can occur either from infected food handlers or from contaminated water during the preparation process (Ryan et al., 2016a). Vegetables are more likely to be contaminated as they might be fertilized with contaminated manure, watered with contaminated irrigation water or contaminated by infected animals’ faeces (Ryan et al., 2016a). Besides, marine molluscan bivalve shellfish could constitute a source of human infection (Robertson, 2007). Their capacity to filter water can lead to their contamination with Cryptosporidium oocysts and as a result they may pose a public health risk when consumed raw or lightly cooked (Robertson, 2007).

Human-to-human transmission can occur after direct contact with infected people in combination with inadequate sanitation. Apart from the human-to-human transmission, people can be infected by animals (Caccio and Chalmers, 2016). Direct contact with animals’ faeces and lack of hygiene measures can lead to infection (Ryan et al., 2016a).
Molecular diagnostic tools have helped us to better understand the transmission patterns of Cryptosporidium and evaluate its zoonotic or anthropotonic nature. Although more than 17 species have been identified in humans (Table 2), C. hominis and the zoonotic C. parvum are the most commonly detected species associated with human infections, followed by C. meleagris which is primarily found in birds (Chalmers and Giles, 2010; Zahedi et al., 2016a). Besides, C. hominis and C. parvum are responsible for the majority of the waterborne outbreaks reported worldwide with the exception of one outbreak occurred in UK for which C. cuniculus, the rabbit species, was incriminated (Chalmers and Giles, 2010; Zahedi et al., 2016a).

Sequence analysis of the 60kDa glycoprotein (gp60 or gp40/15) gene has revealed the presence of several subtypes of C. hominis and C. parvum. Specifically, nine subtypes of C. hominis, from Ia, Ib, Id to Ij, and 14 subtypes of C. parvum, from Ila to Ilo, have been detected (Ryan et al., 2014). Among those, C. parvum subtype Ila and Ild have been identified in both humans and animals (ruminants) and are considered zoonotic, whereas type IIC has only been found in humans (Xiao and Feng, 2008; Widmer and Lee, 2010; Xiao, 2010). On the contrary, C. hominis is considered a human pathogen (Xiao, 2010; Ryan et al., 2014; Khan et al., 2017). However, recently it has been reported in numerous wildlife hosts including a dugong and non-human primates and also its subtype IbA10G2 has been found in marsupials and cattle in Australia (Zahedi et al., 2016b).

The distribution of C. hominis and C. parvum in humans varies by geographic region. C. hominis tends to predominate in most parts of the world, especially in developing countries, while C. parvum is more frequent in the Middle East and both species are common in Europe (Shirley et al., 2012). C. hominis is more common in urban areas whereas C. parvum in rural areas with lower human population densities but high livestock density. The temporal distribution of both species is also different. Studies have seen more C. parvum in spring (lambing time) and more C. hominis in autumn (Chalmers et al., 2009; Pollock et al., 2010; The ANOFEL Cryptosporidium National Network, 2010).

The C. hominis subtypes Ia, Ib, Id and Ie, which are responsible for the majority of the human cases worldwide, have been all identified in developing countries. Subtype Ib is considered the main cause of diarrhoea in immunocompetent people in Europe and the USA (Khan et al., 2017). C. parvum subtype families Ila, Ilb, IIC and Ile have also been isolated from humans in developing
countries, although less frequently, due to the dominance of *C. hominis* in these areas (Akiyoshi et al., 2006; Muthusamy et al., 2006; Cama et al., 2007; Essid et al., 2018).

Ruminants are reported to be the major source of *C. parvum* transmission to humans. Cattle, sheep and goats have especially been implicated in human outbreaks (Shahiduzzaman and Daugschies, 2012). However, bovine cryptosporidiosis has been also linked to *C. andersoni*, which although not a human pathogen, has been documented in humans (Ryan et al., 2016a) as well as *C. bovis* and *C. ryanae* (Chako et al., 2010; Ryan et al., 2014) which mainly appear as age increases compared to infection with *C. parvum* which is most prevalent in neonatal animals (Chalmers and Giles, 2010).

In sheep, *C. parvum*, *C. xiaoi* and *C. ubiquitum* are mostly frequently identified although *C. hominis*, *C. andersoni*, *C. suis*, *C. fayeri* and *C. scrofarum* have been also reported (McLauchlin et al., 2000; Santin et al., 2007; Geurden et al., 2008b; Mueller-Doblies et al., 2008; Quilez et al., 2008; Fayer and Santin, 2009; Ryan et al., 2014; Tzanidakis et al., 2014).

In goats, mainly *C. parvum* and to a lesser extent *C. xiaoi* have been detected together with *C. hominis* and *C. ubiquitum* (Geurden et al., 2008b; Quilez et al., 2008; Fayer and Santin, 2009; Ryan et al., 2014; Tzanidakis et al., 2014). The presence of *C. hominis* and *C. parvum* in small ruminants in combination with the fact that *C. ubiquitum* has been detected in human cases of cryptosporidiosis (Ryan et al., 2016a), considers these animals a possible zoonotic reservoir for *Cryptosporidium*.

The zoonotic *C. parvum* and the equine-specific *Cryptosporidium* horse genotype have been mainly detected in horses (Smith et al., 2010; Caffara et al., 2013; Laatamna et al., 2015; Qi et al., 2015), but, occasionally, the human species *C. hominis* and *C. erinacei* have been also found (Kvac et al., 2014; Laatamna et al., 2015).

Although uncommon, the pig species *C. scrofarum* and *C. suis* have been documented in human cryptosporidiosis cases infecting both immunocompetent and immunocompromised individuals (Kvac et al. 2009a, Wang et al. 2013, Bodager et al. 2015). Among these reported cases, *C. scrofarum* has been detected in an immunocompetent man with diarrhoea who however, was co-infected with *Giardia duodenalis*, assemblage A. Thus, due to the co-presence of *Giardia*, *C. scrofarum* was not confirmed to be the primary cause of the gastroenteritis of that patient (Kvac et al., 2009a).
C. muris, mainly found in rodents, has been indicated as the cause of human infection in several cases (Ryan et al., 2016a).

The role of companion animals in the zoonotic transmission of Cryptosporidium seems to be limited. Dogs and cats are primarily infected with the host-specific species C. canis and C. felis respectively (Thompson et al., 2008; Abeywardena et al., 2015), however, other species have been also detected such as C. parvum in dogs (Abeywardena et al., 2015) and C. muris in cats (Santin et al., 2006; Pavlasek and Ryan, 2007). The canine- and feline- specific species have been implicated in human cases of clinical cryptosporidiosis mainly in developing countries but at a low rate, indicating that a minimal risk for public health exists (Thompson et al., 2008; Lucio-Forster et al., 2010).

Prevention of Giardia spp and Cryptosporidium spp infections

Since Giardia and Cryptosporidium are highly infectious organisms whose transmission can occur through many cycles, where humans, animals and the environment are involved, a holistic approach is needed for the prophylaxis and the control of these parasites.

In order to avoid person-to-person transmission, people should follow good hygiene practices. These include thorough hand washing before and during food preparation and eating, after using the toilet, after changing diapers or after caring for a patient who is suffering from diarrhoea. Besides, hygiene measures after contact with animals’ faeces would protect from possible zoonotic transmission (Baneth et al., 2016; Ryan et al., 2016a; Currie et al., 2017).

Recently, breast-feeding has been considered a protection weapon against clinical giardiosis. Specifically, the presence of high titers of anti-Giardia secretory immunoglobulin A (SIgA) in breast milk, seems to protects infants and young children from displaying symptoms. However, infection cannot be prevented (Muhsen and Levine 2012; Squire and Ryan, 2017). Clinical cryptosporidiosis could also be prevented via breast-feeding (Squire and Ryan, 2017). It has been reported that breast-feeding decreases intensity of parasite infection and it has been associated with low serum levels of IgE and TNF-α (Abdel-Hafeez et al., 2013).

Since water acts as a vehicle of transmission of giardiosis and cryptosporidiosis and as a result numerous waterborne outbreaks have been reported worldwide (Karanis et al., 2007; Baldursson and Karanis, 2011), preventive measures should also focus on this risk factor. Thus,
adequate treatment of drinking water should be provided (Peletz et al., 2013; Speich et al., 2016). Because *Giardia* cysts and *Cryptosporidium* oocysts are highly resistant to chlorination (Korich et al., 1990; Winiecka-Krusnell and Linder, 1998), the use of filters is recommended (Baneth et al., 2016; Ryan et al., 2017). Besides, untreated water from lakes, rivers, springs, ponds, streams or shallow wells should not be consumed unless it is boiled or filtered before. Also, raw vegetables and fruits should be washed thoroughly with uncontaminated water (Chalmers and Giles, 2010; Adam et al., 2016; Baneth et al., 2016; Caccio and Chalmers, 2016; Ryan et al., 2016a). Sanitation and hygiene measures should be also implemented in recreational water environments as globally many outbreaks are associated with exposure to recreational water activities (Causer et al., 2006; Jones et al., 2006; Wheeler et al., 2007; Hopkins et al., 2013; Adam et al., 2016; de Gooyer et al., 2017; Moreira and Bondelid, 2017; Ryan et al., 2017). Besides, healthy swimming behaviour is required. People with diarrhoea, especially children in diapers, should not swim at recreational water venues so that water contamination is prevented (Ryan et al., 2016a; Ryan et al., 2017).

Preventive measures should be also taken in order to reduce the risk of environmental contamination and therefore human infection, from animals. Since livestock constitutes a potential source of environmental contamination including surface water catchments (Wells, 2015), access of productive animals to these areas should be monitored (Castro-Hermida et al., 2009). Besides, manure should be properly managed by farmers, that is, stored for more than 12 weeks, decomposed and treated e.g. through anaerobic digestion (Garces et al., 2006), before use as a fertilizer for crops (Olson et al., 1999; Castro-Hermida et al., 2009; Grit et al., 2012; Vermeulen et al., 2017).

Preventive management measures should also include thorough cleaning and disinfection of the housing facilities (stables, farms, kennels, catteries) using products such as ammonia, chlorine dioxide and hydrogen dioxide or ozone, as well as the maintenance of a dry environment inside the buildings, a condition which would block the parasites development (Geurden et al., 2010b; Saleh et al., 2016).

Regarding prevention through vaccination, efficient vaccines against *Giardia* and *Cryptosporidium* are not available. Recently, a novel oral vaccine against *Giardia* for dogs and cats has been tested with promising results (Serradell et al., 2016).
Spatio-temporal patterns in *Giardia* spp and *Cryptosporidium* spp infection

Despite the abundance of *Giardia* spp and *Cryptosporidium* spp and their significant implication in human and veterinary medicine, little is known about the spatial and temporal patterns of these parasites. Understanding geographical and seasonal variability of risk factors which favour their prevalence, especially under the burden of the constant global environmental changes, would be a valuable knowledge about the locations and the time periods of increased risk and would contribute to the better prevention and control of the diseases caused by *Giardia* spp and *Cryptosporidium* spp (Jones et al., 2013). Besides, identifying the peaks in giardiosis and cryptosporidiosis incidence would be significant information that could be related to the occurrence of outbreaks (Valcour et al., 2016).

Globally, there are limited studies which have investigated the spatio-temporal clusters of *Giardia* spp cases (Lal and Hales, 2015; Asher et al., 2016; Valcour et al., 2016; Lal et al., 2018).

According to these studies, giardiosis tends to occur predominantly in urban areas indicating an anthropogenic pathway of transmission (Lal and Hales, 2015; Lal et al., 2018). Attendance at day care centres and changing diapers have been identified as important risk factors for giardiosis in metropolitan regions, supporting the above hypothesis (Hoque et al., 2003; Sagebiel et al., 2009; Pijnacker et al., 2016).

However, in cases where human populations live in close proximity to animals, the detection of the *Giardia* spp zoonotic assemblages have been reported in both populations (people and animals) suggesting potential for cross-species transmission (Delport et al., 2014; Ng et al., 2011; Asher et al., 2016).

Regarding seasonality, most human cases are reported from June to October, probably due to increased exposure to recreational waters (Esch and Petersen, 2013). However, spatio-temporal studies that were conducted in New Zealand (Lal and Hales, 2015; Lal et al., 2018), indicated that *Giardia* spp cases showed no seasonality. On the contrary, a similar study in Canada reported a spring peak for *Giardia* spp suggesting run-off from spring snow melt as a possible reason (Valcour et al., 2016).

Contrary to *Giardia*’s trend, *Cryptosporidium* spp infections seem to be more oriented to agricultural and livestock-dominated areas displaying a constant spring peak (Lal and Hales,
2015; Lal et al., 2018). Recurrent *Cryptosporidium* clusters in spring indicate a common environmental exposure and can be related to the high number of livestock births and as a result a high rate of *Cryptosporidium* spp oocysts shedding, occurring during this time period (Lal and Hales, 2015). Reported outbreaks associated with contact with livestock support this suggestion (Stefanogiannis et al., 2001; Grinberg et al., 2011). For that reason, *C. parvum* zoonotic genotype is more prevalent in spring, whereas the anthroponotic and more frequent in urban areas *C. hominis* is most commonly detected in autumn, possibly following the high contact of humans with recreational waters (Pollock et al., 2010; Chalmers et al., 2011b).
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Objectives
Both *Giardia* and *Cryptosporidium* are important parasites in human and veterinary medicine, causing diarrhoea in humans and in different animal species. Although outbreaks have been reported (Painter et al., 2016; Ryan et al., 2016; Utsi et al., 2016; Efstratiou et al., 2017; McClung et al., 2017), only limited information is available on the prevalence of *Giardia* and *Cryptosporidium* in humans and animals in Greece, about risk factors associated with infection and about their clinical importance.

Based on the official records, as presented by the Hellenic Centre for Disease Control and Prevention, there are limited reports of human parasitic diseases in Greece even when, in case of zoonoses, there are well documented data for the infections in animals. This for example is the case of cystic echinococcosis, for which Greece is considered highly endemic. However, although infection rates in livestock reach up to 39.3% for sheep and 14.7% for goats (Sotiraki and Chaligiannis, 2010), there are no official data about the disease prevalence in humans since 2009. This can be attributed either to underreporting (possibly due to misdiagnosed cases) or to the actual fact that indeed the number of infections is low because of proper control measures. Underreporting is unfortunately a fact in Greece as confirmed by Gibbons et al., 2014, providing salmonellosis and campylobacteriosis as an example. According to that study, Greece appears to be a country with high extent of underestimated (underreported and under-ascertained) infections, more than several other European countries. If this is the case when it comes to diseases like salmonellosis and campylobacteriosis with acute and severe clinical signs which force infected individuals to urgently seek for medical help, we can imagine the high rates of underestimation in case of giardiosis and cryptosporidiosis where clinical signs are often mild and self-limiting.

With animal infections being very common, *Cryptosporidium* and *Giardia* are considered to be zoonotic parasites, but both human-to-human and animal-to-human transmission cycles exist. However, and especially concerning *Giardia*, the importance of zoonotic transmission has been widely debated (Ryan and Caccio, 2013), mainly due to recent advances in genotyping. Therefore, identifying and understanding transmission cycles of those parasites between different host species is highly important to minimize disease burden and suggest appropriate preventive measures.

Crete island in Greece, has been selected for this study because it offers an interesting case to study transmission of *Cryptosporidium* and *Giardia*: Crete is a geographically restricted area,
densely populated (623,000 residents recorded in 2011), with a large tourist population, a high number of farm and companion animals (Hellenic Ministry of Rural Development and Food: http://minagric.gr), and proximity to both Europe and Africa. The presence of Cryptosporidium and Giardia has been studied in small ruminant farming systems on the island (Tzanidakis et al., 2014), prior to this thesis, using the same methodology. The results of that preliminary study, showing high infection rates of Cryptosporidium and Giardia in small ruminants, were an extra clue for us to expand these investigations to different hosts, as they confirmed the presence of the two protozoa in the area.

Specifically, the objectives of this Ph.D. thesis were:

- To estimate the occurrence of Giardia and Cryptosporidium in humans and different animal species such as horses and dogs and cats. Especially in the case of dogs and cats, we focused on household, shelter and shepherd (dogs) populations. In humans, we targeted different groups i.e. people with and without gastrointestinal symptoms as well as adults and children. Thus, we could evaluate and form a better view of which risk factors favour or not the prevalence of Giardia and Cryptosporidium in humans and companion animals.

- To identify, through the performance of the molecular methods, the Giardia assemblages and the Cryptosporidium species/genotypes/subtypes and therefore, detect the transmission cycles of the parasites (human-to-human, animal-to-animal, animal-to-human) to evaluate their zoonotic potential and investigate the sources of infection.
References


Based on:

The occurrence and genetic characterization of Cryptosporidium and Giardia species in foals in Belgium, The Netherlands, Germany and Greece


In: Veterinary Parasitology. 2015;211:170-174
Introduction

*Cryptosporidium* spp and *Giardia* spp are protozoan parasites that have been reported worldwide in a wide range of hosts, including horses.

In horses, excretion of *Cryptosporidium* oocysts has been reported in different geographical areas, with infection rates ranging between 0.75% (De Souza et al., 2009) and 25.0% (Smith et al., 2010). In some studies, the prevalence was higher in foals (Veronesi et al., 2010), while in other studies, the peak prevalence was observed in adult animals (Majewska et al., 1999, 2004). Part of this variation may be due to differences in study design, the limited number of animals and/or farms in the study, and the diagnostic technique that was used. Although an impact of *Cryptosporidium* infection on horse health has been reported (Netherwood et al., 1996; Majewska et al., 2004; Grinberg et al., 2008; Frederick et al., 2009), it seems to be less important compared to ruminants and especially intensively reared calves, in which infection with *Cryptosporidium parvum* is an important cause of neonatal diarrhoea (de Graaf et al., 1999).

Similarly, the prevalence of *Giardia duodenalis* has been reported in horses from various locations with considerable variation (0.5–35.0%) (Pavlásek et al., 1995; Olson et al., 1997; Atwill et al., 2000; De Souza et al., 2009; Veronesi et al., 2010; Traversa et al., 2012; Santin et al., 2013), but the number of studies on the prevalence of *G. duodenalis* in foals is more limited compared to studies on *Cryptosporidium*. Moreover, the impact of *Giardia* infection on equine health remains undefined, in contrast to calves and lambs, where *Giardia* infections have been associated with a decreased growth and diarrhoea (Olson et al., 1995; Geurden et al., 2010).

The relevance of animal infections is not only limited to the impact on animal health or production, but should also be considered from a public health point of view as they may also be a source of infection, either by infecting people by direct contact or by contaminating water supplies, since many outbreaks of infections by both parasites are waterborne (Balduresson and Karanis, 2011). An increasing number of studies seems to indicate a public health relevance of equine *Cryptosporidium* infections. Horses were frequently infected with the zoonotic *C. parvum* (Ryan et al., 2003; Grinberg et al., 2008; Imhasly et al., 2009; Smith et al., 2010; Laatamna et al., 2015) and occasionally the human species *Cryptosporidium hominis* was detected in horses (Laatamna et al., 2015). Similarly for *G. duodenalis*, the assemblages A
and B have been identified, although the zoonotic potential of *G. duodenalis* in horses remains largely unexplored (Traub et al., 2005; Traversa et al., 2012; Santin et al., 2013).

In Europe, with the exception of Italy – where extensive studies have been performed (Veronesi et al., 2010; Traversa et al., 2012; Caffara et al., 2013) – there are only limited data on the prevalence of both protozoal infections in horses as well as their zoonotic importance. The objective of the present study was to acquire additional data on the occurrence and genotypes of *Cryptosporidium* and *Giardia* in foals in regions of Europe where no records were available.

**Materials and methods**

**Sampling**

Convenience samples were collected from animals that belonged either to individual owners or to larger farms/properties in four different countries, 3 countries situated in Western Europe (i.e. Belgium, The Netherlands and Germany) and one Mediterranean country, i.e. Greece. The samples were collected from 51 sites in Belgium, 30 in The Netherlands, 2 sites in Germany and 82 different sites from all over Greece. Sampling was performed in spring and summer, i.e. shortly after the foaling season. Individual faecal samples were collected from foals between 1 week and 6 months of age. The faecal samples were collected directly from the rectum and immediately transported to the participating lab in each country where they were stored at 4°C and examined within 4 days of sampling. When a sample was found to be positive by coproscopic analysis for one of the two parasites, the sample was withheld for DNA extraction and molecular genotyping.

For every horse/sample, a data form was completed by interviewing the owner, providing information on age, breed, sex and presence or absence of diarrhoea (up to a maximum of 15 days before sampling).

**Detection of Cryptosporidium and Giardia**

A quantitative direct immunofluorescence assay (IFA) based on the commercial MERIFLUOR *Cryptosporidium/Giardia* kit (Meridian Diagnostics Inc., Cincinnati, Ohio) was performed. Briefly, one gram of the faecal material was suspended in 100 ml of distilled water and strained through a layer of surgical gauze. After sedimentation for 1 h and centrifugation at 3000 × *g* for 5 min, the sediment was resuspended in distilled water up to a volume of 1 ml. After thorough vortexing, an aliquot of 20 μl was pippeted onto a treated IFA-slide. After staining of the slide, as instructed by the manufacturer, the
entire smear was examined at a 400× magnification under a fluorescence microscope. The number of Cryptosporidium oocysts per gram of faeces (OPG) and Giardia cysts per gram of faeces (CPG) was obtained by multiplying the total number of cysts on the smear by 50.

**Molecular identification**

DNA was extracted from the 1 ml faecal sample using the QIAamp® Stool Mini Kit (Qiagen) according to the manufacturer’s instructions, incorporating an initial step of 3 freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95°C for 5 min) in the protocol to maximise disruption of (oo) cysts. For the amplification of the Cryptosporidium 18S ribosomal DNA gene (18S rDNA) and HSP70 gene, previously described PCR protocols were used (Morgan et al., 2001; Xiao et al., 2001). For the identification of Giardia DNA, the β-giardin gene (Lalle et al., 2005) and the triose phosphate isomerase (TPI) gene (Geurden et al., 2008) were used. In all above-mentioned PCR reactions, bovine serum albumin (BSA) was added to a final concentration of 0.1 μg BSA/μl reaction mixture. Amplification products were visualised on 1.5% agarose gels with ethidium bromide. A positive (genomic DNA from a positive faecal sample) and negative (PCR water) control sample were included in each PCR reaction. PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and fully sequenced using the Big Dye Terminator v.3.1 Cycle sequencing Kit (Applied Biosystems). Sequencing reactions were analysed on a 3100 Genetic Analyzer (Applied Biosystems) and assembled with the program Seqman II (DNASTAR, Madison WI, USA). To determine the genotypes and subgenotypes, the fragments were aligned with the homologous sequences available in the GenBank database, using MegAlign (DNASTAR, Madison WI, USA). The β-giardin, TPI, 18S rDNA and HSP70 nucleotide sequences obtained in this study were deposited in GenBank under accession numbers KM926502–KM926526, KM926527–KM926548, KM926549 and KM926550–KM926551, respectively.

**Statistical analysis**

Univariate analysis was carried out by descriptive statistics and results were expressed as mean (M), standard deviation (SD), median (Mdn) and minimum (min) and maximum (max) values. Within the Giardia infected animals, the association between age and CPG values was analysed by the non-parametric Spearman’s rho correlation coefficient. In addition, the non-parametric Mann–Whitney test was utilised to compare the age of foals with and without diarrhoea. Finally, a multivariable logistic regression model was constructed to assess the main effects of age and CPG values and their interaction
on the status (presence/absence of diarrhoea) of foals. Data were analysed using the SPSS statistics software (version 19.0).

Results

Occurrence of Cryptosporidium and Giardia

In total, 398 foals from 4 different countries were examined in the present study, i.e. 134 in Belgium, 44 in The Netherlands, 30 in Germany and 190 in Greece. The mean age was 64 days (SD ± 56.24), the median age was 31 days with a range of 5–180 days. Of the foals, 163 were male and 191 were female. For 44 foals, gender was not recorded (Table 1).

Eight foals were found positive for Cryptosporidium (2.0%) and the oocyst excretion ranged from 50 to 2450 OPG with a median excretion of 750 OPG. For Giardia, 49 foals (12.3%) were found to excrete cysts, with a range of 50–4,000,000 CPG, and a median excretion of 450 CPG. Four of these foals were co-infected with Giardia and Cryptosporidium, all less than 1 month of age.

The age distribution of Giardia infected animals was: 55.6% (25/45) for animals aged 1–30 days, 15.6% (7/45) for animals aged 31–60 days, 13.3% (6/45) for animals aged 61–90 days, 6.7% (3/45) for animals aged 91–120 days and 8.9% (4/45) for animals aged 121–180 days. The Spearman’s correlation coefficient revealed a statistically significant negative association between “the age of the foal” (in days) and CPG values ($r(42)=-0.306$, $p=0.049$). The parasitic burden (expressed as median value of CPG per age group) was also higher in younger animals, i.e. 1700 CPG for foals aged up to 60 days, 550 CPG for foals between 61 and 120 days old and 150 CPG for 121–180 days old foals.

Eleven out of 88 foals (12.5%) that showed diarrhoea at or shortly before sampling were positive for Giardia and only 1 (1.1%) was positive for Cryptosporidium. However, no significant correlation was found between “history of diarrhoea” and infection intensity (expressed as CPG or OPG values). Similar results were obtained when intensity of infection was collated to “the age of the foals”. In the above mentioned correlation, the samples from Germany were not included because the factor “history of diarrhoea” was not recorded. Within the Giardia infected animal population, the 42 animals/cases were split, 31 without “history of diarrhoea” and 11 with “history of diarrhoea”. There was a statistically significant difference in the distributions of “age of the foal” between subjects “without history of diarrhoea” (Mdn=60; range 7–180) and subjects “with history of diarrhoea” (Mdn=21; range 10–30) ($Z=-3.116$, $p=0.002$). Finally, in the multivariable binary logistic regression model, there was no statistically significant main effect of age (in days) ($\chi^2=2.582$, $p=0.108$ with df=1) and CPG values
\( \chi^2 = 1.007, \ p = 0.316 \) \text{ with } \text{df}=1 \) on the outcome of the dependent variable “history of diarrhoea”. In addition, there was no statistically significant interaction effect of age and CPG values on the outcome of the dependent variable \( \chi^2 = 1.132, \ p = 0.287 \) \text{ with } \text{df}=1 \).

**Genotyping**

Six out of the 8 *Cryptosporidium*, positive samples did not amplify or yielded poor sequences. The remaining 2 samples belonged to *Cryptosporidium* horse genotype (Table 1). Most *Giardia* positive samples were identified as assemblage Al \( (n=9) \) or BIV (-like) \( (n=12) \). Additional genotypes were assemblage A \( (n=1) \), AII \( (n=4) \), B \( (n=2) \) and E \( (n=3) \). Mixed infections of assemblages A and B were also detected in 8 samples (2 from Belgium and 6 from Greece).
Table 1. Number of positive foals and prevalence of *Giardia* spp. and *Cryptosporidium* spp. in faecal samples from foals in Belgium (BE), Germany (GE), Greece (GR) and The Netherlands (NL). The total number of foals in each country is provided, as well as the number of male and female foals (from those foals the information was collected).

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<th>Recent diarrhoea (questionnaire data)</th>
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<tr>
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<td>163</td>
<td>191</td>
<td>49 (12.3)</td>
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</table>
Discussion

Infections with *Giardia* spp and *Cryptosporidium* spp were identified in foals aged up to 6 months, from Belgium, Germany, Greece and The Netherlands. The overall detection rate of *Cryptosporidium* spp was 2.0%. The infection rates for *Cryptosporidium* spp were low in Belgium (4.5%) and Greece (1.1%), whereas no positive samples were found in Germany and The Netherlands. The latter, taking also into account the lower number of foals sampled in these countries, may suggest an overall low prevalence in those countries. Excretion of *Cryptosporidium* oocysts was previously reported to range between 0.75% (De Souza et al., 2009) and 25.0% (Smith et al., 2010). The low occurrence and level of oocyst excretion may be indicative for a low infection pressure at the different sites participating in this study, as horses usually are not as intensively kept as livestock. Similarly, *Cryptosporidium* was also reported to occur less frequently in calves kept under extensive farming conditions compared to intensive farming (Geurden et al., 2006). In ruminants, the prevalence of *Cryptosporidium* most often peaks within the first few weeks after birth (Geurden et al., 2008). Similar to what has been found in ruminants, in the present study, all *Cryptosporidium* positive foals were aged less than 37 days. This is in agreement with a higher prevalence in foals as observed by Veronesi et al. 2010, while in other studies, the prevalence peaked in adult horses (Majewska et al., 1999, 2004).

The public health and the veterinary importance of equine *Cryptosporidium* infections have been discussed in previous studies (Grinberg et al., 2008, 2009; Robinson et al., 2008; Imhasly et al., 2009; Xiao et al., 2009; Smith et al., 2010; Perrucci et al., 2011; Santin et al., 2013), however, morphological and biological data are not yet sufficient available for horse genotypes (Ryan et al., 2014). In the present study, the zoonotic potential of *Cryptosporidium* infections (e.g. through contamination of surface water) could not be evaluated, due to the limited number of isolates and the poor amplification and sequencing results, presumably as a consequence of the low oocyst excretion found in the samples examined.

*G. duodenalis* infection rates were comparable in all countries (Table 1). The overall infection rate of *G. duodenalis* found in the present study (12.3%) is in line with previous reports (0.50–35.0%) in horses (Pavláseket et al., 1995; Olson et al., 1997; Atwill et al., 2000; De Souza et al., 2009; Veronesi et al., 2010; Santin et al., 2013). Despite the extensive rearing described above, high cyst excretion was sometimes observed, especially in younger animals. Co-infections with *Giardia* and *Cryptosporidium* were detected only in foals aged less than 30 days, since *Cryptosporidium* infection was only recorded in animals of that age. No association was found between *G. duodenalis* cyst counts and diarrhoea. This agrees with
previous findings where *Giardia* infection was not correlated with the presence of diarrhoea in horses (Xiao and Herd, 1994; Veronesi et al., 2010; Santin et al., 2013). The finding of the apparent lack of any correlation between *Giardia* and diarrhoea could also be due to the experimental design, since the animals were only sampled once, and *Giardia* cyst shedding can be intermittent (Patton, 2013). Within the *Giardia* infected animals, the presence of diarrhoea was significantly correlated with the age of the foal, as diarrhoea was present only in animals less than one month old.

Genetic characterization of the *Giardia* isolates found in this study indicated that *G. duodenalis* A and B assemblages were the predominant genotypes. These assemblages with zoonotic potential are common in humans in Europe (e.g. Geurden et al., 2009; Lebbad et al., 2011; Alexander et al., 2014; Mateo et al., 2014) and have been identified in foals in Italy, The United States and Australia (Ey et al., 1997; Traub et al., 2005; Traversa et al., 2012; Santin et al., 2013).

Assemblage B was found to be the most prevalent genotype. Genotyping revealed high sequence heterogeneity in assemblage B, with many samples identified as (or closely related to) BIV or BIV-like sub-assemblages. Genetic heterogeneity within assemblage B is a well documented phenomenon but it is still not known if this genetic heterogeneity is due to high allelic sequence heterozygosity in such parasites and/or due to common mixed infections with different sub-groups of isolates (Ankarklev et al., 2012). Accordingly, it remains difficult to assign specific assemblage B isolates to different sub-genotypes and no clear distinction between zoonotic and host-adapted genotypes can be made for assemblage B (Sprong et al., 2009). Within assemblage A, sub-assemblages AI and AII were identified. Although both of them have been found in humans and animals, sub-assemblage AI is mainly seen in livestock and pets, whereas sub-assemblage AII is predominantly seen in humans. According to Sprong et al. 2009, isolates found in both humans and animals are not always epidemiologically linked, and finding similar sub-assemblages simply highlights the global distribution of those genotypes, providing little evidence for zoonotic transmission. Assemblage E was also found in two samples from Greece. Assemblage E is a non-zoonotic genotype and is usually detected in hoofed livestock (Thompson, 2004; Geurden et al., 2008; Tzanidakis et al., 2014). Assemblage E has been reported in horses in previous studies in Italy (Veronesi et al., 2010; Traversa et al., 2012).

To conclude, in the present study, a low *Cryptosporidium* and a moderate occurrence of *G. duodenalis* in foals in all 4 countries involved were detected. This to an extent correlates to the detection method; i.e. prevalence of both parasites could have been higher if real-time PCR was used for the initial detection (Boadi et al., 2014; Operario et al., 2015). The low infection rates of these two protozoa could be due to the living and management conditions of the horses which are in general less intensive than in farm
animals. Most of the *G. duodenalis* isolates were assemblage AI and BIV (like), which are found predominantly in animals, or the hoofed livestock-specific assemblage E. This, combined with the presence of *Cryptosporidium* horse genotype, suggests a low risk for zoonotic transmission concerning both parasites in the context of horses. At the same time, it illustrates the difficulty of evaluating a potential public health threat based solely on genetic data without considering the epidemiological background of human clinical infections.

**Acknowledgements**

The authors would like to thank the Hellenic Scholarship Foundation for supporting Mrs. Kostopoulou’s PhD thesis. Moreover, we are grateful to all the vets that helped during sampling and especially Dr. A. Stefanakis, Dr. E. Fragkiadaki, Mrs. A. Mitsoura and Mr. A. Malamas.

**Appendix A. Supplementary data**

**Additional file 1. Table S1.** Number of *Giardia* cysts and *Cryptosporidium* oocysts detected by IFA and *Giardia duodenalis* assemblages identified by sequence analysis from positive foals examined in Belgium (BE), Germany (GE), Greece (GR) and the Netherlands (NL). (TPI: Triosephosphate isomerase gene; ND: no data).
References


De Souza P.N., Bomfim T.C., Huber F., Abboud L.C., Gomes R.S. Natural infection by *Cryptosporidium* sp., *Giardia* sp. and *Eimeria leuckarti* in three groups of equines with different handlings in Rio de Janeiro, Brazil. Vet Parasitol. 2009;160:327-333.


### Supplementary data

**Additional file 1. Table S1.** Number of *Giardia* cysts and *Cryptosporidium* oocysts detected by IFAT and *Giardia duodenalis* assemblages identified by sequence analysis from positive foals examined in Belgium (BE), Germany (GE), Greece (GR) and the Netherlands (NL). (TPI: Triosephosphate isomerase gene; ND: no data)

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<th>Foal ID</th>
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<th><em>Giardia</em> cysts/g</th>
<th><em>Cryptosporidium</em> oocysts/g</th>
<th>beta-giardin</th>
<th>TPI GEN</th>
<th>tpi A</th>
<th>tpi B</th>
<th>tpi E</th>
<th>18S Crypto</th>
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Chapter III

Based on:

**Abundance, zoonotic potential and risk factors of intestinal parasitism amongst dog and cat populations: The scenario of Crete, Greece**

D. Kostopoulou, E. Claerebout, D. Arvanitis, P. Ligda, N. Voutzourakis, S. Casaert, S. Sotiraki

In: Parasites & Vectors. 2017;10:43
Introduction

Intestinal parasite infections are still abundant in companion animals, despite all the highly efficient drug formulations available and the control measures taken by owners and veterinarians (Capelli et al., 2003; Palmer et al., 2008; Claerebout et al., 2009; Joffe et al., 2011; Ortuno and Castella, 2011; Riggio et al., 2013; Itoh et al., 2015; Nijsse et al., 2016). Moreover, parasites are responsible for some of the most important and well-recognized zoonoses transmitted from companion animals to man globally such as *Giardia* spp, *Cryptosporidium* spp, *Toxocara* spp, hookworms and *Echinococcus granulosus* (Lee et al., 2010; Deplazes et al., 2011; Chen et al., 2012; Mcpherson et al., 2013; Pereira et al., 2016).

Nowadays, changes due to climate alterations and social behaviour that affect humans’ lives and consequently the lives of the animals which live close to them (Sutherst et al., 2004; Tabachnick, 2010), alter the interactions between humans and pathogens leading to (re)emergence of several diseases, including zoonotic ones (Jones et al., 2008; Otranto and Eberhard, 2011).

The distribution of zoonoses associated with companion animals is highly affected by animals’ movements (between regions, countries and continents) which in fact are the means to relocate pathogens and vectors they harbour. The above is becoming more and more important since human travel continues to increase in parallel with the population and financial status increase, and when humans travel, they often take their companion animals, particularly dogs.

All the above is in fact unfolding the reasons why it is crucial to fill the gaps on the current distribution of these diseases in a constantly changing environment and to describe the risks associated with pet infection in order to assure their well-being and to prevent the free movement of zoonotic pathogens.

The aim of our study was to investigate the presence and infection intensity of intestinal parasites in dogs and cats, the risk factors (such as lifestyle, veterinary care, etc.) that influence those infections and their zoonotic potential. This was done by performing a cross-sectional epidemiological study within a defined animal/human community, i.e. the island of Crete, as a case scenario.
Materials and methods

Populations studied

Faecal samples were collected from different dog populations (shelter, household and shepherd) as well as shelter and household cats in Crete Island in Southern Greece (Figure 1), from October 2011 to January 2015.

Crete is the largest and most densely populated island of Greece (623,000 residents recorded in 2011) with a population well distributed in urban and rural areas. The island is also a highly popular tourist destination (approximately 3.5 million international tourist passengers’ arrivals in 2013) (Region of Crete: www.crete.gov.gr). Moreover in Crete, in addition to the high number of companion animals, there is a significant livestock and wildlife population (Hellenic Ministry of Rural Development and Food: http://minagric.gr).

Since data on the precise population of pets in the location were not available, the sample size was determined estimating the dog and cat population size as “infinite”. The prevalence of intestinal parasitism in different dog and cat studies in Europe varies enormously depending on the sampled animal population and the diagnostic techniques that were used (Dubna et al., 2007; Martinez-Moreno et al., 2007; Claerebout et al., 2009; Overgaauw et al., 2009; Riggio et al., 2013; Beugnet et al., 2014; Ortuno et al., 2014; Zanzani et al., 2014; Simonato et al., 2015). In this study, in order to calculate the sample size (with a precision of 5% and a 95% confidence interval) we selected to relate our “expected prevalence” values to recent reports of Giardia prevalence in Europe. Therefore, the targeted sample size was defined as follows: for household dogs up to 200 dogs (reported prevalence 10–20%); for shelter dogs up to 400 dogs (reported prevalence 20–50%); for household cats 138 cats (reported prevalence < 10%) and for shelter cats 385 cats (reported prevalence 10–50%) (Dubna et al., 2007; Hamnes et al., 2007; Leonhard et al., 2007; Martinez-Moreno et al., 2007; Papazahariadou et al., 2007; Claerebout et al., 2009; Barutzki et al., 2011; Ortuno and Castella, 2011). For shepherd dogs there is little information available and given the difficulties in approaching and handling such dogs we aimed at collecting the maximum feasible number of samples. In order to achieve the most accurate coverage of the whole island, the animals enrolled in our study were allocated proportionally to the four different counties of the island according to the inhabitant’s population density (Figure 1).

Individual rectal faecal samples were randomly collected from dogs and cats of all ages with or without intestinal symptoms from 561 households, 11 shelters and 29 sheep and goat farms. After collection,
the samples were immediately transported under vacuum (Rinaldi et al., 2011) to the laboratory where they were stored at 4 °C and examined within 2 days. When a sample was found to be positive by coproscopic analysis for *Giardia* spp or *Cryptosporidium* spp, it was stored at -20°C until DNA extraction was performed and molecular genotyping followed.

For every animal/sample, a data-form was completed by interviewing the owner or in case of shelters the person who was responsible for the animals, providing information on age, sex, breed, living conditions (indoors or outdoors), presence of other animals, the presence or absence of diarrhoea (up to maximum 1 month before sampling), if the animal had travelled recently and the antiparasitic treatment plan followed (including time of last treatment). Faecal consistency was recorded for all faecal samples. The consistency of individual faecal samples was scored using the following scale: 1, formed; 2, soft; 3, diarrhoea; 4, haemorrhagic diarrhoea.

**Parasitological techniques**

The presence of worm eggs and protozoan oocysts was determined by applying two different methods, i.e. a sedimentation (acid/ether) and a sedimentation/flotation technique (using a saturated sugar salt solution as a flotation fluid with 1.28 specific gravity) (MAFF, 1986). For the detection of *Giardia* spp and *Cryptosporidium* spp (oo)cysts a quantitative direct immunofluorescence assay (IFA) based on the commercial MERIFLUOR *Cryptosporidium/Giardia* kit (Meridian Diagnostics Inc., Cincinnati, Ohio) was used (Geurden et al., 2008a; Kostopoulou et al., 2015).

**Molecular analyses**

DNA was extracted from the positive *Giardia* spp and *Cryptosporidium* spp faecal samples using the QIAamp® Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. For the amplification of the *Cryptosporidium* 18S ribosomal RNA gene (rDNA18S) and HSP70 gene, previously described PCR protocols were used (Morgan et al., 2001; Xiao et al., 2001). For the identification of *Giardia* DNA, the *Giardia* rRNA 18S gene (rDNA 18S) (Hopkins et al., 1997), the β-giardin gene (Lalle et al., 2005), the triose phosphate isomerase (TPI) gene (Geurden et al., 2008b) and the glutamate dehydrogenase (GDH) gene (Read et al., 2004) were used. Amplification products were visualised on 1.5% agarose gels with ethidium bromide. A positive (genomic DNA from a positive faecal sample) and negative (PCR water) control sample were included in each PCR reaction.
PCR products were purified and sequenced from both strands. PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and fully sequenced using the Big Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems, California, USA). Sequencing was performed by an external company (GATC Biotech) using the Big dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems) and the reactions were analyzed using a 3730xl DNA Analyzer (ThermoFisher Scientific). Sequences were assembled using Seqman 5.0 Software (Lasergene DNASTAR) and were aligned using the Basic Local Alignment Search Tool (BLAST) as well as compared with reference sequences using MegAlign (Lasergene DNASTAR). For multilocus genotyping Clustal X, 2.0.11 software was used and reference sequences were selected according to Caccio et al. 2008.

**Statistical analysis**

Descriptive statistical analyses and multivariate methodologies were performed using the statistical language R (R Core Team, 2013) and the pscl package (Jackman, 2008). Two approaches were applied as follows.

**Multivariate binary logistic models**

The effect of the independent variables (age in months, gender, food, travel, neutering, living conditions, living with other animals, antiparasitic treatment, time between treatment and sampling date, diarrhoea during the last month, faecal score and type) on a sample being or not infected by a parasite was studied through the utilization of multivariate logistic models with forward LR selection. Initially, a test of the full model against a constant only model was performed in order to assess whether there was a statistically significant effect of the examined independent predictors on the response variable through the utilization of the Omnibus Tests of Model Coefficients, which uses the Chi-square test to see if there is a significant difference between the log-likelihood (-2LL) of the baseline model (constant model) and the model with the predictors. In addition, the Hosmer & Lemeshow (H-L) test was performed to test whether the model provides a good fit to the data. (Additional file 2: Table S1).

**Multivariate zero-inflated models**

The effect of the independent variables on the parasitic infection intensity (egg/(oo)cyst counts per gram) was studied through the utilization of a zero-inflated negative binomial model (Lambert, 1992) due to the excess of zero counts and over dispersion of the data. In this analysis the group of shepherd dogs were not included due to the limited number of samples examined. (Additional file 2: Table S2).
**Fig. 1.** Map of Crete demonstrating the locations of different sample points per animal population category. *Key:* triangles: shelter dogs/cats; rhombi: shepherd dogs; ellipses: household dogs/cats with the number of animals sampled.
**Results**

**Dogs**

A total of 879 faecal samples from dogs were investigated for the presence of intestinal parasites. Of these samples, 278 were derived from shelter dogs, 529 from household dogs and 72 from shepherd dogs (Table 1). In total, 38.3% of dogs were found harbouring at least one intestinal parasite. Precisely, 25.5% were harbouring one parasite, 8.9% two and the rest 3-6 different species. The overall infection rate was 25.2% (CI: 22.4–28.1) for *Giardia* spp; 9.2% (CI: 7.3-11.1) for *Ancylostoma/Uncinaria* spp; 7.6% (CI: 5.9-9.4) for *Toxocara* spp; 5.9% (CI: 4.4-7.5) for *Cryptosporidium* spp; 4.6% (CI: 3.2-5.9) for *Cystoisospora* spp; 2.7% (1.7-3.8) for *Toxascaris leonina*; 1.7% (CI: 0.9-2.6) for *Capillaria* spp; 0.8% (CI: 0.2-1.4) for taenid eggs, 0.2% (CI: 0-0.5) for *Dipylidium caninum*; and 0.1% (CI: 0-0.3) for *Strongyloides stercoralis*. The results for the different dog populations are shown in Table 1.

Among the different canine populations studied, shelter dogs had the highest infection rates. In particular, 62.9% of the shelter dogs were infected with at least one species of endoparasite compared to 51.4% of the shepherd dogs and 23.8% of the household dogs. According to the multivariate binary logistic model analysis, the odds ratio (OR) of *Giardia* infection was higher in shelter dogs than household dogs (11.24 times higher) and shepherd dogs (15.63 times higher). However, based on the multivariate zero-inflated model, among *Giardia*-infected individuals, household dogs had generally higher cyst counts than shelter dogs (OR = 1.602). Regarding *Cryptosporidium*, and according to the multivariate zero-inflated model, the odds ratio in favour of zero Cryptosporidium OPG for household dogs was 8.248 times higher than that for shelter dogs, suggesting that household dogs were less prone to *Cryptosporidium* infection than shelter dogs. However, *Cryptosporidium*-positive household dogs shed more oocysts than infected shelter dogs (OR = 12.182). No statistically significant correlations between infection with the other parasites and their living conditions were detected in both models (Table 2).
The mean age of the sampled dogs was approximately 3 years (39.5 months ± 41.8, SD). The majority of the dogs were adults (≥ 12 months, n = 642), while 229 of them were younger than 12 months and 8 were of unspecified age. There was a significant correlation between age and *Giardia* infection (Figure 2) and between age and *T. leonina* infection intensity. According to the multivariate binary logistic model analysis, as age increased by one month, the odds of detecting *Giardia* cysts decreased by 1.9% = [(0.981–1) × 100] which is also confirmed by the multivariate zero-inflated model, according which the odds of absence of *Giardia* cysts are increased by one unit increase of age. Similarly, according to the multivariate zero-inflated model, as age increased by one month, the odds of detecting *T. leonina* eggs decreases by 7% = [(0.93–1) × 100]. Regarding the other parasites studied, their correlation with age was not statistically significant.

Of the dogs which had a history of recent diarrhoea, 43.1% were positive for at least one intestinal parasite. However, faecal consistency was not significantly associated with parasitic infection. The statistical analyses showed that signs of diarrhoea (based on faeces consistency) were significantly more often present in younger animals (*U* = 100,667, *P* = < 0.001). Moreover, there was a statistically significant association between the factors “recent record of diarrhoea” and “live with other animals”, \( \chi^2 \) (6, *N* = 1138) = 29.495, *P* = <0.001.

On average, all of the dogs sampled received 2.1 anthelmintic treatments/year (range 0–6). The arithmetic mean of anthelmintic treatments/year was 2.3 for household dogs, 2.2 for shelter dogs and 0.5 for shepherd dogs. Information about anthelmintic treatments was not defined in 48 cases (5.5%). The frequency of antiparasitic treatment was also associated with diarrhoea and more specifically, the effect of the odds of one treatment per year increase resulted in a decrease by 0.828 times in the trace of “recent record of diarrhoea”, implying diarrhoea to be caused by parasite infestation. However, the number of antiparasitic treatments/year received was not statistically associated with parasitic infection.

The risk analyses of all the other factors which were evaluated in this study, such as the gender of the animals, their living conditions (indoors/outdoors), the type of food, and recent travelling, showed no statistically significant correlation with parasitic infection. Since almost all shelter dogs had access to the external environment and the shepherd dogs were also living outside, the risk factor “living indoors/outdoors” was assessed only for household dogs. The risk factor “recent travelling” was also not analysed since only 4.2% of the dogs had been travelling during the last months before sampling, including within counties. The same applied for the “type of food” factor, since the majority of the dogs
were eating industrial/cooked food and only 28 were fed with raw meat/offal, 64% of these being shepherd dogs.

*Giardia* spp was the most prevalent parasite in all dogs (25.2%) and also in shelter (54.3%) and household (12.9%) dogs in particular. The range of the cysts being shed by the infected animals varied from 100 to 275,800 cysts per gram of faeces with 6,855 cysts shed on average. In the samples derived from shelter and household dogs, the dog-specific assemblages C and D were dominating, either alone \(^{n=72}\) or in mixed infections \(^{n=15}\). A limited number of dogs were infected with assemblage A \(^{n=2}\), assemblage Al \(^{n=1}\), assemblage All \(^{n=1}\) or a mixture of A with C or D \(^{n=5}\) or BIV-like and C \(^{n=1}\) (Table 3). Regarding shepherd dogs, no positive PCR products were sequenced successfully. Multilocus genotyping was performed from one dog sample which was classified as sub-assemblage Al using 3 genetic loci (bg, TPIGEN and GDH). Alignment analysis of the isolate showed 100% homology when compared to reference sequences A5 for bg; A1 for TPIGEN and A1 for GDH (Caccio et al., 2008), resulting in multilocus genotype MLGA1 (Wang et al., 2016).

The PCR results for *Cryptosporidium* positive samples showed that the HSP70 gene amplified 23.6% of the samples, whereas the 18S rDNA gene amplified 5.6%. Sequencing revealed the presence of *Cryptosporidium canis* in 2 household dogs and *C. scrofarum* in a shelter dog.

**Cats**

In total, 264 faecal samples from cats were collected; 59 samples from shelters and 205 from owned cats. Unfortunately, it was not possible to reach the target of 385 shelter cats. Overall, 38.1% of the cats were harbouring at least one intestinal parasite. Precisely 26.4% were harbouring one parasite, 8.3% two and the rest 3–4 different species. The prevalence was 20.5% (CI: 15.6–25.3) for *Giardia* spp; 9.5% (CI: 5.9–13.0) for *Cystoisospora* spp; 8.3% (CI: 5.0–11.7) for *Toxocara* spp; 7.6% (CI: 4.4–10.8) for *Ancylostoma/Uncinaria* spp; 6.8% (CI: 3.8–9.9) for *Cryptosporidium* spp; 4.2% (CI: 1.8–6.6) for *Capillaria* spp; 0.8% (CI: 0.0–1.8) for taeniid eggs; and 0.4% (CI: 0–1.1) for *Hammondia/Toxoplasma*. The results among different feline populations are shown in Table 4.

The mean age of the sampled cats was 3.4 years (40.8 months ± 48.9, SD). The majority of the cats were adults (≥ 12 months, \(^{n=161}\)), while 97 of them were younger than 12 months and 6 were of unspecified age.
Among the different feline populations studied, shelter cats had the highest infection rates. Specifically, 55.9% of the shelter cats were infected with at least one species of intestinal parasite compared to 33.2% of the household cats. However, infection rates of the different parasites were not statistically different between different cat populations.

Of the cats which had a history of diarrhoea (30.9%), 32.9% were infected with at least one parasite. On average, all cats sampled received 2.3 anthelmintic treatments/year (range 0–6). The mean number of anthelmintic treatments/year was 1.9 for household cats and 2.7 for shelter cats. Information about anthelmintic treatments was unknown in one case. Only 1.5% of the cats had been travelling during the last months including within counties. No significant associations were found between parasite infections and risk factors or between parasite infections and diarrhoea.

*Giardia* spp was the most prevalent parasite (20.5%), both in shelter cats (39.0%) and household cats (15.6%). When targeting the 18S rRNA gene, assemblage A was identified in 10 cat samples. In 6 of these samples, no amplification was obtained with the other genes, while in 4 samples only assemblage F was detected in at least one of the other loci. Assemblage F was also found alone in 2 samples. Also, in two different cases, the typing revealed the presence of assemblage BIV-like (*n* = 1) or the dog specific assemblage C (*n* = 1) (Table 4).

Genotyping of *Cryptosporidium* positive samples showed the presence of the feline specific species *Cryptosporidium felis* (*n* = 4).
Fig. 2. Prevalence of *Giardia* spp in different dog populations and different age groups.
Table 1. Prevalence of intestinal parasites and factors associated with this prevalence in different dog populations. Percentages given for specific parasites refer to percentage of dogs that were found positive for an infection within a category of risk factor.

<table>
<thead>
<tr>
<th>Dog population</th>
<th>Prevalence (95% CI)</th>
<th>Median(^\text{a}) (Range)</th>
<th>History of diarrhoea</th>
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<tbody>
<tr>
<td></td>
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<td>With diarrhoea</td>
<td>Without diarrhoea</td>
</tr>
<tr>
<td>Shelter</td>
<td>Giardia</td>
<td>54.3 (48.5-60.2)</td>
<td>4450 (100-222800)</td>
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<tr>
<td></td>
<td>Cryptosporidium</td>
<td>14.7 (10.6-18.9)</td>
<td>200 (100-1400)</td>
</tr>
<tr>
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<td>Toxocara spp</td>
<td>12.2 (8.4-16.1)</td>
<td>79 (1-12000)</td>
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<td></td>
<td>T. leonina</td>
<td>6.1 (3.3-8.9)</td>
<td>153 (2-3330)</td>
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<td>Hookworms</td>
<td>9.7 (6.3-13.2)</td>
<td>31 (3-588)</td>
</tr>
<tr>
<td></td>
<td>Capillaridae</td>
<td>0.7 (0.0-1.7)</td>
<td>1.5 (1 &amp; 2)</td>
</tr>
<tr>
<td></td>
<td>Cystoisospora</td>
<td>7.6 (4.4-10.7)</td>
<td>26 (1 – 1800)</td>
</tr>
<tr>
<td>Household</td>
<td>All</td>
<td>23.8 (20.2 – 27.4)</td>
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</tr>
<tr>
<td></td>
<td>Giardia</td>
<td>12.9 (10.0-15.7)</td>
<td>10400 (100 – 275800)</td>
</tr>
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<td>Cryptosporidium</td>
<td>1.9 (0.7-3.1)</td>
<td>300 (100 – 40300)</td>
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</tr>
<tr>
<td></td>
<td>T. leonina</td>
<td>0.9 (0.1-1.8)</td>
<td>51 (15 – 223)</td>
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<tr>
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<td>Hookworms</td>
<td>5.3 (3.4-7.2)</td>
<td>9 (1 – 423)</td>
</tr>
<tr>
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<td>Capillaridae</td>
<td>1.9 (0.7-3.1)</td>
<td>4.5 (1 – 1246)</td>
</tr>
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<td>Cystoisospora</td>
<td>2.5 (1.1-3.8)</td>
<td>7 (1 – 8400)</td>
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<td>Shepherd</td>
<td>All</td>
<td>51.4 (39.8 – 62.9)</td>
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<td>4.2 (0.0-8.8)</td>
<td>11800 (3000 – 122700)</td>
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<td>Cryptosporidium</td>
<td>1.4 (0.0-4.1)</td>
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<td>Toxocara spp</td>
<td>8.3 (1.9-14.7)</td>
<td>118.5 (27-2543)</td>
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<td>2.8 (0.0-6.6)</td>
<td>282.5 (109 &amp; 456)</td>
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<td>33.3 (22.4 – 44.2)</td>
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<td>8.3 (1.9 -14.7)</td>
<td>3.5 (1 – 41)</td>
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<tr>
<td></td>
<td>taenid</td>
<td>6.9 (1.1 – 12.8)</td>
<td>16 (3 – 72)</td>
</tr>
</tbody>
</table>

\(^\text{a}\)Median number of cysts/oocysts/eggs per gram of faeces
Table 2. Prevalence of intestinal parasites and factors associated with this prevalence in different cat populations. Percentages given for specific parasites refer to percentage of cats that were found positive for an infection within a category of risk factor

<table>
<thead>
<tr>
<th>Cat population</th>
<th>Parasitic species</th>
<th>Infection intensity</th>
<th>History of diarrhoea</th>
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<td>Prevalence (%)</td>
<td>Median† (Range)</td>
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<td></td>
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<td>(95% CI)</td>
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<td>Shelter</td>
<td>All Giardia</td>
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<td>Cryptosporidium</td>
<td>39.0 (26.5 – 51.4)</td>
<td>100 (100 – 700)</td>
</tr>
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<td>Toxocara spp</td>
<td>11.9 (3.6 – 20.1)</td>
<td>3 (1 – 63)</td>
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<tr>
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<td>T. leonina</td>
<td>10.2 (2.5 – 7.9)</td>
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<tr>
<td></td>
<td>Hookworms</td>
<td>5.1 (-0.5 – 10.7)</td>
<td>7 (5-22)</td>
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<td>Capillaridae</td>
<td>5.1 (0.0 – 10.7)</td>
<td>38 (17 – 84)</td>
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<td>Cystoisospora</td>
<td>8.5 (1.4 – 15.6)</td>
<td>42 (2 – 2330)</td>
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<tr>
<td>Household</td>
<td>All Giardia</td>
<td>33.2 (26.7 – 39.6)</td>
<td>5800 (100 – 248100)</td>
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<td>Cryptosporidium</td>
<td>15.6 (10.6 – 20.6)</td>
<td>400 (100 – 1800)</td>
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<td>Toxocara spp</td>
<td>5.4 (2.3 – 8.5)</td>
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<td>3.9 (1.3 – 6.6)</td>
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<td>Cystoisospora</td>
<td>9.8 (5.7-13.8)</td>
<td>84.5 (1-6114)</td>
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Table 3. Genotyping results of samples from dogs infected by *Giardia duodenalis* (at all different loci)

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<td>Shelter Dog</td>
<td>C</td>
</tr>
<tr>
<td>87</td>
<td>Household Dog</td>
<td>C</td>
</tr>
<tr>
<td>88</td>
<td>Household Dog</td>
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</tr>
<tr>
<td>89</td>
<td>Household Dog</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>Household Dog</td>
<td>D</td>
</tr>
<tr>
<td>91</td>
<td>Household Dog</td>
<td>D</td>
</tr>
<tr>
<td>92</td>
<td>Shelter Dog</td>
<td>AI</td>
</tr>
<tr>
<td>93</td>
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<tr>
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<td>96</td>
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<tr>
<td>105</td>
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<tr>
<td>106</td>
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Table 4. Genotyping results of samples from cats infected by *Giardia duodenalis* (at all different loci)

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Discussion

The infection rates of intestinal parasites detected in this study, revealed a high prevalence of parasitic infections (38.2%) and the presence of different species of endoparasites in both dogs and cats. These infection rates were equally distributed within animal species (38.3% for dogs and 38.1% for cats) involved in the study. With the exception of shepherd dogs, *Giardia* spp was the most prevalent parasite detected in the dog and cat populations followed by significant prevalences of ascarids, hookworms and taeniid infections. These results are also reported in other studies which consider *Giardia* the most common enteric parasite of dogs and cats in developed countries (Palmer et al., 2008; Thompson et al., 2008; Claerebout et al., 2009; Scaramozzino et al., 2009; Ballweber et al., 2010; Barutzki et al., 2011; Polak et al., 2014; Zanzani et al., 2014; Osman et al., 2015). In shepherd dogs, hookworms were the most prevalent parasite species detected.

Among the targets of this study was to investigate the potential effect of animal lifestyle to parasitism so animals living in households, shelters or farms were included. The results showed that more than half of the shelter dogs and cats were infected with at least one species of endoparasite, which was more or less expected, taking into consideration the less hygienic conditions that those animals are living in combined with a high population density that usually exists in shelters. A high level of parasitism has been previously reported in shelter dogs (Claerebout et al., 2009; Ortuno and Castella, 2011; Ortuno et al., 2014; Simonato et al., 2015) while in shelter cats the prevalence observed in other studies was lower (Becker et al., 2012; Ito et al., 2016).

More than half of the shepherd dogs (51.4%) were positive for at least one species of intestinal parasite. The infection rate of intestinal parasites estimated in shepherd dogs in this study was higher than in a previous record from Greece (26.0%) (Papazahariadou et al., 2007). Such differences are expected in cross-sectional studies especially given time and region differences. However, our results were in agreement with a study conducted in farm dogs in Portugal (57.4%) (Mateus et al., 2014). Shepherd/farm dogs often receive less veterinary care and preventive treatments. Compared to a general average of more than 2 anthelmintic treatments per year, shepherd dogs in our study received only 0.5 treatments per year.

Although the prevalence of intestinal parasites in household dogs was lower than in shelters, although not statistically significant for many species, the percentage of individuals infected was still noteworthy
In similar studies conducted in Italy the prevalence of intestinal parasites in household dogs was even higher, reaching 57.0% of the animals (Riggio et al., 2013; Zanzani et al., 2014). In our study there was no difference in the risk of infection between dogs living in an apartment with no access to a yard or a garden and dogs living in a house with access to outdoors. A reason for that could be that even dogs that are kept permanently indoors are regularly being walked by their owners in public places getting in close contact with other dogs (including stray ones) or their contaminated faeces. Similar results were recorded in the household cats studied, but this was probably due to the fact that the majority of them also live partially outdoors. The parasitism reported in household cats in this study is in agreement with the infection rates reported in Austria, Belgium, the Netherlands, France, Hungary, Italy, Romania and Spain (Riggio et al., 2013; Beugnet et al., 2014; Nijsse et al., 2016).

Although both household and shelter dogs received regular anthelmintic treatments (i.e. an average of 2.3 for household dogs and 2.2 for shelter dogs per year), this seemed not to control parasitism efficiently. This is in agreement with the general recommendation by ESCCAP for roundworms in which it is suggested that annual or twice yearly treatments do not have a significant impact on the prevalence of patent infections within a population, and therefore a treatment frequency of at least 4 times per year is recommended (Worm Control in Dogs and Cats - ESCCAP, www.esccap.org). Recent modeling indicated that the environmental Toxocara contamination by dogs can only be reduced significantly if compliance to the four times a year treatment advice is sufficiently high (90.0%) or if at least half of the dog owners consistently remove their dog’s faeces (Nijsse et al., 2015). In cats, the frequency of anthelmintic treatment differed between categories, with shelter cats being more frequently treated (i.e. an average of 1.9 for household cats and 2.7 for shelter cats per year). This could be explained by a misconception of the cat owners that indoor cats do not need preventive treatments (Matos et al., 2015).

Despite the high prevalence of parasitic infections, most animals were healthy with no obvious signs of suffering probably due to the low parasitic burden, as at least suggested by the low number of egg/(oo)cyst output recorded in most cases (even if usually there is not a clear correlation between numbers of eggs/(oo)-cysts and clinical signs). It was not statistically proven that recent records of diarrhoea were correlated to parasitism as also shown previously (Hill et al., 2000; Hackett et al., 2003) although there was evidence that anthelmintic treatment had a positive effect on reducing such records. A supporting argument for the absence of clinical disease could be that the majority of animals were adults at the time of sampling. Young animals are more sensitive to parasitism (Gates et al., 2009) but
although in this study there was a tendency of older animals (>2 year-old dogs and >1 year-old cats) to be less infected, this was not statistically significant for most parasites. The only statistically proven facts were that the chance to get infected by *Giardia* spp and the infection intensity of *T. leonina* was negatively correlated to age in dogs.

Given the high prevalence and the potential zoonotic importance, *Giardia* and *Cryptosporidium* positive samples were further investigated by PCR and sequencing of the positive PCR products. In dogs, the host-specific assemblages C and D dominated, which has been described before in various studies (Sulaiman et al., 2003; Berilli et al., 2004; Lalle et al., 2005; Barutzki et al., 2007; Souza et al., 2007; Scorza et al., 2012; Zanzani et al., 2014; Pallant et al., 2015; Simonato et al., 2015). Few dogs were (co)-infected with assemblage A, and the majority of these were identified as sub-assemblage AI. Sub-assemblage AI is frequently found in animals, while humans are most frequently infected with sub-assemblage AII (Xiao and Fayer, 2008; Minetti et al., 2015). The sequence analysis in one *G. duodenalis* sample further revealed a multilocus genotype (MLG) which was previously described in calves in China (Wang et al., 2016). Together, these results suggest that there is no significant risk for zoonotic transmission of *Giardia* infections from dogs in Crete.

In cats, the genotyping results seemed to indicate the dominance of the potentially zoonotic assemblage A in shelter animals and the co-infection of assemblages A and the feline specific assemblage F in household cats. However, the zoonotic assemblage A was identified only at the 18S rDNA locus, while only assemblage F was identified at the other loci. Since no distinction could be made between assemblages A and F in the amplified region of the conserved rRNA 18S gene, it cannot be excluded that (some of) the samples that were amplified with rDNA 18S gene were assemblage F instead of A. Therefore, no conclusion can be drawn on the zoonotic risk associated with *Giardia* infections in cats.

Regarding *Cryptosporidium*, the dog specific *C. canis* was identified in only two household dogs and the pig specific *C. scrofarum* in one shelter dog. *Cryptosporidium canis* has been also detected in household dogs in other studies (Lucio-Forster et al., 2010; Ryan et al., 2014; Osman et al., 2015) and isolated in humans, mainly children and immunocompromised individuals in developing countries (Xiao et al., 2007; Bowman et al., 2010), suggesting its potential public health impact. To our knowledge, this is the first case of *C. scrofarum* infection reported in a dog. Since keeping backyard pigs is quite a common practice in the area, it is possible that this dog ingested the oocysts before being transferred to the shelter. In such a scenario this could be a case of pseudoparasitism, given that this dog was 2.5 month-old and only recently introduced to the shelter. In cats, sequencing was not efficient; nevertheless, it revealed the
presence of the feline-specific *C. felis*. Since our genotyping results revealed the presence of host-specific *Cryptosporidium* species in both dogs and cats which have been implicated in very few human infections and mainly in developing countries, we could suggest that the zoonotic potential of *Cryptosporidium* from dogs and cats in the study area is low.

Apart from *Giardia* and *Cryptosporidium*, ascarids, hookworms and taeniids are also considered to be zoonotic (Dakkak, 1992; Caccio and Ryan, 2008; Thompson and Smith, 2011; Traversa, 2012; Macpherson, 2013). The two major ascarid species *T. canis* and *T. cati* (to a lesser extent) are responsible for human infections (Traversa, 2012; Macpherson, 2013). In our study the prevalence of *Toxocara* spp in dogs and cats was 7.6 and 8.3%, respectively. In dogs, we characterised all *Toxocara* eggs found as *Toxocara* spp since those infections were only microscopically diagnosed and as previously suggested they could either belong to *T. canis* or *T. cati* since coprophagy is not unusual for dogs and the presence of *T. cati* eggs in dog faeces might in fact relate to pseudoparasitism (Fahrion et al., 2011; Nijsse et al., 2014). The infection rates found in the present study are similar to those reported in Europe which vary from 3.5 to 34.0% for *T. canis* in dogs from different epidemiological environments and from 7.2 to 76.0% for *T. cati* in cats (Parsons, 1987; Fok et al., 2001; Habluetzel et al., 2003; Le Nobel et al., 2004; Dubna et al., 2007; Martinez-Carrasco et al., 2007; Lee et al., 2010; Nijsse et al., 2015; Nijsse et al., 2016). The *Toxocara* infection was high, especially in shelter dogs and cats, as also reported before (Haralambidis, 1993; Simonato et al., 2015; Villeneuve et al., 2015). Although mainly *T. canis* is considered responsible for human toxocarosis (Fisher, 2003), the role of *T. cati* in human toxocarosis should not be underestimated (Overgaauw, 1997; Fisher, 2003; Smith et al., 2006). In Greece, toxocarosis in humans has not been studied extensively since published data are restricted only to some sporadic cases (Xinou et al., 2003; Haralambidou et al., 2005) and one study regarding the seroprevalence of *T. canis* in children (Theodoridis et al., 2001). Our results combined to all European studies presented above strongly suggest that more information is needed.

Hookworm infection rates were 9.2% in dogs and 7.6% in cats. The highest infection rates of hookworms were identified in shepherd dogs (33.3%) similar to the study of Mateus et al. 2014 in Portugal (31.0%). Since different hookworm species were not differentiated, the zoonotic risk associated with hookworm infections could not be determined.

The detection of taeniid eggs in shepherd dogs is worth mentioning. Unlike shelter and household dogs, shepherd dogs seem to be more prone to taeniid infection, which possibly is due to the frequent consumption of raw meat and carcasses (Jenkins et al., 2006; Palmer et al., 2008). Echinococciosis is still
endemic in Greece with a high prevalence reported in livestock (Sotiraki and Chaligiannis, 2010; Katzoura et al., 2013; Chaligiannis et al., 2015). However, there are no recent reports regarding the prevalence of taeniids in dogs. Taking into consideration our results in combination with the high prevalence of *E. granulosus* in livestock, which is transmitted through dogs, we could assume that shepherd dogs in Greece could be a reservoir for human infections.

**Conclusions**

In conclusion, we have recorded high levels of (multi)-parasitism in both dogs and cats in the study area. Most of the animals were harbouring different species of parasites sometimes in high numbers according to the egg/(oo)cyst counts. This is proof that those parasites are greatly abundant within animal populations regardless of lifestyle. Thus, the results of our study stress the need for better anthelmintic control schemes and control of intestinal protozoa in dogs and cats, tailored to their individual needs, in order to safeguard animal and public health. Thus, a holistic approach for the management of endoparasites in companion animals should be implemented including for instance, proper administration of the drugs e.g. dose and frequency of administration.

**Acknowledgements**

This work is a part of a PhD scholarship funded by the Greek State Scholarships Foundation. The authors will also like to thank all the veterinarians, the shelter owners, Dr Alexandros Stefanakis, Mr Anastasios Anastasiades and other colleagues in Crete who contributed in this study.

**Funding**

This work is a part of a PhD scholarship funded by the Greek State Scholarships Foundation.

**Authors’ contributions**

This study was conducted by DK as part of her PhD thesis. Also, DK participated in the data collection and analysis and developed the first draft of the manuscript. All other authors played a role in data collection and analysis, and interpretation of findings (EC, DA, PL, NV, SC and SS). All authors read and approved the final manuscript.
Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethical considerations

The study was carried out in compliance with the national animal welfare regulations. Diagnostic veterinary procedures are not within the context of relevant EU legislation for animal experimentations (Directive 86/609/EC) and may be performed in order to diagnose animal diseases and improve animal welfare. Samples were collected by registered veterinarians who ensured owners consent and caused no suffering.

Appendix A. Supplementary data

Additional file 1: Table S1: Binary logistic model for Giardia spp infection rate in dogs.

Table S2: Zero-inflation negative binomial model for parasite infection intensity in dog samples.
References


Thompson R.C., Palmer C.S., O’Handley R. The public health and clinical significance of *Giardia* and *Cryptosporidium* in domestic animals. Vet J. 2008;177;18-25.


Additional file 1. Table S1. Binary logistic model for *Giardia* spp infection rate in dogs

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Notes⁹ Set to zero because it was redundant

R² (Nagelkerke): 34.4%

Omnibus Tests of Model Coefficients: $\chi^2(6) = 201.586, p < 0.001$

H-L test: $\chi^2(8) = 4.309, p = 0.828$
Additional file 1. Table S2. Zero-inflation negative binomial model for parasite infection intensity in dog samples

i) *Giardia* spp cysts per gram

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**Notes**

Log-likelihood: -2599 on 6 df

The logit link function was used in the zero-inflation component

ii) *Cryptosporidium* spp oocysts per gram

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iii)  *T. leonina* eggs per gram

### Count Component

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<td>0.930</td>
</tr>
<tr>
<td>log(theta)</td>
<td>-0.949</td>
<td>0.413</td>
<td>-2.297</td>
<td>0.022</td>
<td>0.387</td>
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</table>

### Zero-inflation Component

<table>
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<tr>
<th>Variable</th>
<th>B</th>
<th>SE</th>
<th>z</th>
<th>p</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.998</td>
<td>0.095</td>
<td>10.532</td>
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<tr>
<td>type: Household</td>
<td>0.465</td>
<td>0.137</td>
<td>3.398</td>
<td>0.001</td>
<td>1.592</td>
</tr>
</tbody>
</table>

### Notes

Log-likelihood: -549.2 on 5 df

The clog-log link function was used in the zero-inflation component
Chapter IV

Based on:

Human enteric infections by parasites in Greece, with focus on Giardia and Cryptosporidium

D. Kostopoulou, E. Claerebout, D. Arvanitis, P. Ligda, S. Casaert, S. Sotiraki

Submitted in Hippokratia Medical Journal
Introduction

Pieces of evidence of the presence of parasites in humans have been recorded even in prehistoric times. Through history, people have been infected with a variety of parasitic species and in different intensity, depending on the lifestyle during each time period (Mitchell, 2015). In the modern world, parasites are most abundant in developing countries, but are also present in developed communities, having significant consequences on public health and the economy. According to World Health Organization estimates, foodborne parasitic diseases resulted in 48.4 million cases and 59,724 deaths annually resulting in 8.78 million Disability Adjusted Life Years (DALYs), from 2010 to 2015 globally (Torgerson et al., 2015).

Poor hygiene and sanitation conditions and exposure to animals are factors thought to affect parasite transmission (Fletcher et al., 2012). This may be explained a) by the fact that parasite life cycles often involve several hosts like people, animals, vectors, and transmission routes like food, water, surfaces, air and b) by the ability of parasite’s life stages to survive adverse environmental conditions. Research interest in health issues shared among people, animals and the environment has increased rapidly in recent years, as emerging zoonotic infections like giardiosis and cryptosporidiosis have been recognized as a global public health concern being responsible for several outbreaks worldwide. Cryptosporidium is being considered as the leading agent of waterborne disease outbreaks in the United States (Painter et al., 2016) and Europe (Efstratiou et al., 2017), causing high rates of morbidity and even mortality in children or immunocompromised individuals. Infection by Giardia duodenalis on the other hand has been responsible for a significant number of outbreaks within the US (Adam et al., 2016) and Europe (Hadjichristodoulou et al., 1998; Braeye et al., 2015; Enserik et al., 2015; Guzman-Herrador et al. 2015).

Although several studies on parasitic infections in Greece have been published, most of these papers are case reports and/or refer to a single parasite species (Kouklakis et al., 2007; Vassalos et al., 2009; Panidis et al., 2011; Arkoulis et al., 2012; Gialamas et al., 2012; Printza et al., 2013; Mentessidou et al., 2016; Patsantara et al., 2016). Available information referring to gastrointestinal parasitic infection rates is limited (Papazahariadou et al., 2004), stressing the need for updated information on parasite abundance.

Data on the abundance of intestinal parasites is considered critical for public health protection, showing more than local interest, since Greece is an important first country of arrival in Europe for migrants and
asylum seekers, serving as a transit country in the migratory journey to Northern Europe and large numbers of tourists visit Greece annually from all over the world.

The aim of this study was to update and compile the current knowledge on the abundance of intestinal parasites in Greece in order to provide useful estimates for surveillance and control activities as well as to advance the knowledge of the zoonotic relevance of *Giardia* and *Cryptosporidium* species, since they are among the most commonly detected pathogens associated with diarrhoea in humans (Baldursson and Karanis, 2011).

The data presented in this paper are a component of a larger coordinated endeavour undertaken by the authors to study the occurrence of *Giardia* and *Cryptosporidium* in humans (current study) as well as in livestock and companion animals (Tzanidakis et al., 2014; Kostopoulou et al., 2015; Kostopoulou et al., 2017), in an effort to uncover parasite transmission pathways and provide clues for holistic parasite control strategies, in a ‘One Health’ approach. The latter also justifies the stronger focus given in the sample analyses for those parasites.

**Materials and Methods**

Stool samples were randomly collected from humans [children (<18 years old) and adults] in selected areas in Greece, i.e. the Regions of West Macedonia, Central Macedonia and East Macedonia and Thrace (referred to as Northern Greece and neighbouring the Balkans) and Region of Crete in the south (neighbouring the Middle East and North Africa) over a period of almost 3 years. Samples were collected from a State General Hospital and 2 private practitioners located in Northern Greece and 1 State General Hospital, 4 Private Clinics and 10 private practitioners in Crete.

After collection, the samples were immediately transported under vacuum to the laboratory, where they were stored at 4 °C and examined within 2 days.

The study was carried out in compliance with the National and Institutional ethical regulations. For every individual, a data-form was completed after their consent or in case of children after the consent of their parents, providing information on age, sex, reason of consulting a doctor, absence or presence of gastrointestinal symptoms and specification of the symptoms, general health status (focusing on possible immunodeficiency signs), any recent contact with animals and which species and travel history.
Parasitological techniques

Faecal consistency was recorded for all stool samples. The consistency of individual faecal samples was scored using the following scale: 1=formed; 2=soft; 3=diarrhoea; 4=haemorrhagic diarrhoea.

The presence of worm eggs and protozoal (oo)cysts was determined by applying 2 different concentration methods, i.e. a sedimentation (acid/ether) and a sedimentation/flotation technique (using a saturated sugar salt solution as a flotation fluid with 1.28 specific gravity) (MAFF, 1986). For the detection of Giardia spp and Cryptosporidium spp (oo)cysts, a quantitative direct immunofluorescence assay (IFA) based on the commercial MERIFLUOR Cryptosporidium/Giardia kit (Meridian Diagnostics Inc., Cincinnati, Ohio) was used (Panidis et al., 2011).

Statistical analysis

Statistical analysis was performed using SPSS ver. 21.0. Binary and/or ordinal logistic regression analyses were used to estimate the effects of parasitism and parasitic burden on the occurrence and severity of diarrhoea and to investigate correlations between risk factors and parasitism. Values of P < 0.05 were considered significant.

Molecular analyses

DNA was extracted from the positive Giardia spp and Cryptosporidium spp stool samples using the QIAamp® Stool Mini Kit (Qiagen) according to the manufacturer’s instructions. For the amplification of the Cryptosporidium 18S ribosomal DNA gene (18S rDNA) and HSP70 gene, previously described PCR protocols were used (Kostopoulou et al., 2017). For the identification of Giardia DNA, the Giardia 18S ribosomal DNA gene (18S rDNA), the β-giardin gene the triose phosphate isomerase (TPI) gene and the glutamate dehydrogenase (GDH) gene were used (Kostopoulou et al., 2017). Amplification products were visualized on 1.5% agarose gels with ethidium bromide. A positive (genomic DNA from a positive faecal sample) and negative (PCR water) control sample were included in each PCR reaction.

PCR products were purified and sequenced from both strands. PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and fully sequenced by an external company (GATC Biotech) using the Big dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems) whereas the reactions were analysed using a 3730xl DNA Analyzer (ThermoFisher Scientific). Sequences were assembled using
Seqman 5.0 Software (Lasergene DNASTAR) and aligned using the Basic Local Alignment Search Tool (BLAST) as well as compared with reference sequences using MegAlign (Lasergene DNASTAR). For multilocus genotyping Clustal X, 2.0.11 Software was used using reference sequences according to Caccio et al., 2008 (Caccio et al., 2008).

Results

Overall, 876 stool samples were collected in Greece. Specifically, the samples were originated from 822 adults and 54 children from Northern Greece (n=436) and Crete region (n=440), and in total samples coming from males (n=586) were twice the number of the ones from females (n=290). Among the sampled adults 156 had a record of gastrointestinal symptoms, but the majority (n=666) did not have any clinical sign. The healthy adults were mostly professionals in the food sector obliged by the Greek State to undergo regular parasitological stool examinations, in order to acquire a health certificate. Of the children examined, 20 had a recent history of clinical signs from the gastrointestinal tract.

Thirty-six (4.1%) of the individuals examined harboured at least one intestinal parasite, 3 were children and the other 33 were adults. In particular, 33 (3.8%) were found positive for protozoa. The protozoa recorded were Blastocystis hominis (1.8%), Giardia duodenalis (1.3%), Cryptosporidium spp (0.6%), Entamoeba coli (0.2%) and Entamoeba spp (0.1%). Among the above infections, the following co-infections were detected: two cases of G. duodenalis and Cryptosporidium spp co-existence, one case of G. duodenalis and B. hominis and one of E. coli plus Entamoeba spp. Four people (0.5%) were infected with helminths, 2 of which were positive for Enterobius vermicularis, 1 for Taenia sp and 1 for hookworms.

Of the individuals examined, 20.0% (n=175) displayed gastrointestinal symptoms according to the questionnaire. Of them, 8.0% (n=14) were positive for parasites. No significant correlation was observed between gastrointestinal symptoms and parasite infection (P > 0.05). However, for some parasites, most infected people did show clinical disease. Both patients with enterobiosis, aged 9 and 15 years, suffered from abdominal pain. Five people were infected with Cryptosporidium spp, two of whom were also positive for G. duodenalis. One of the Cryptosporidium infected who had symptoms of severe diarrhoea was immune-suppressed since he had recently undergone kidney transplantation. No significant correlation was observed between Cryptosporidium infection levels and diarrhoea (P > 0.05). Five out of
11 (45.5%) *G. duodenalis* positive individuals displayed gastrointestinal symptoms. Three of them, all adults, had diarrhoea, whereas a 9-year-old boy, also co-infected with *Cryptosporidium* spp, complained about abdominal pain. No significant correlation was observed between *G. duodenalis* cyst counts and diarrhoea (P > 0.05). Details about *G. duodenalis* and *Cryptosporidium* spp infections are presented in Table 1.

Most of the *Blastocystis* infected people were asymptomatic, except for a 9 year-old girl who had a history of eosinophilia in a blood examination a week before the faecal sample was taken.

A patient infected with both *Entamoeba* spp and *E. coli* had severe diarrhoea which did not respond to antibiotics treatment. People infected with *E. coli* alone, taeniid and hookworm eggs did not report any gastrointestinal symptoms.

No association between parasitism and age, occupation or pet ownership was found (P > 0.05).

**Genotyping results**

Seven *G. duodenalis* positive samples were successfully genotyped. Sequencing results are presented in Table 2. Multilocus genotyping was performed for two samples which were classified as sub-assemblages AII using 3 genetic loci (bg, TPIGEN and GDH). Alignment analysis of the isolates showed 100% homology when compared to reference sequences A2 for bg and A2 for TPIGEN , however, there was one SNP at the gdh locus (a “T” instead of a “C” in position 237), resulting in a new MLG. As for *Cryptosporidium*, the HSP70 gene was amplified for one sample, however sequencing was not successful.

**Discussion**

Intestinal parasitic infections are distributed throughout the world; however, their prevalence differs from region to region due to different environmental, social and geographical factors. The results of the current study confirmed the presence of different intestinal parasitic species in humans in Greece, though in low abundance, which is more or less expected in developed countries where hygiene and sanitation standards are high. The prevalence of intestinal parasites in humans in Greece has been evaluated previously showing infection in 11.4% (Papazahariadou et al., 2004) of the studied population.
Of the parasitic infections detected, protozoans (3.8%) were more common than helminths (0.5%). This has been also reported before in other studies worldwide (Dagnew et al., 2012; Colli et al., 2014; Kiani et al., 2016), including Greece (Papazahariadou et al., 2004).

*Blastocystis hominis* (1.8%) was the most common parasite found which is in line with other studies (Masucci et al., 2011). Our findings were closer to the lower level (1.8%), possibly due to the fact that 80.0% of the people who participated in the study were clinically healthy, which is in accordance with previous findings in Greece (Vassalos et al., 2009; Papazahariadou et al., 2004).

*G. duodenalis* was the second most common parasite (1.3%). This result is in line with other European studies where however, the prevalence of *Giardia* infection varies depending on the study population and the diagnostic techniques used (Okulewicz et al., 1998; Werner et al., 2001; Crotti et al., 2005; Giangaspero et al., 2007; Davies et al., 2009; Sagebiel et al., 2009). In Northern Greece, an infection rate of 2.3% was previously reported (Papazahariadou et al., 2004). Giardiosis was previously confirmed in 0.5% (Kafetzis et al., 2001) and 3.0% (Maltezou et al., 2001) of children with acute diarrhoea in Greece. Although there was also an obvious trend towards the presence of diarrhoea in *Giardia* infected individuals in the present study, the number of *Giardia* cysts in faeces was not significantly different between healthy individuals harbouring the parasite and individuals with diarrhoea. Asymptomatic carriers having high parasitic burden is not an unusual finding (Feng and Xiao, 2011). Travel history and close contact with animals (pets/farm animals) were not associated with infection in this study. All *Giardia* positive samples were identified as sub-assemblage AII, which predominantly infects humans (Feng and Xiao, 2011). Although sub-assemblage AII can also be found in different animal species, it is predominantly found in humans (Sprong et al., 2009). Moreover, previous studies in Greece indicated that mainly assemblage E is present in ruminants (Tzanidakis et al., 2014) sub-assemblages Al and BIV-like in horses (Kostopoulou et al., 2015) assemblage C and D in dogs and assemblage F in cats (Kostopoulou et al., 2017), which suggests that human-to-human transmission of *Giardia* was more likely to be the source of infection in the present study. Similar conclusions (as regards travel and contact with animals) were also reported from a previous study in Germany referring to symptomatic patients (Espelage et al., 2010), whereas travel was characterized as a risk factor for giardiosis in returning UK travelers but only returning from long trips from South/South East Asia and Africa (Takaoka et al., 2016).

*Cryptosporidium* spp were found in 0.6% of the samples. In Greece, *Cryptosporidium* was detected before in 2.7% of the study population (Papazahariadou et al., 2004) and was also implicated together
with *Giardia*, in an outbreak of diarrhoea in Crete (Hadjichristodoulou et al., 1998). Besides, a few case reports of cryptosporidiosis have been published in Greece (Printza et al., 2013; Megremis et al., 2004). The results of the current study showed an evident clinical appearance in the majority of *Cryptosporidium* infected cases.

Only two individuals (0.2%) were found harbouring pinworm eggs and both of them were children. A low infection rate for *E. vermicularis* could be due to the fact that the worm’s eggs are sticky and adhere to the perianal skin or clothing so that only few of them pass to the faeces and consequently are not recorded in coproscopic analysis. In Greece, 1.1% of the population was found infected (Papazahariadou et al., 2004) while in another study, a prevalence of 48.8% in children was found using haematological and serological parameters (Patsantara et al., 2016). Most studies regarding *E. vermicularis* in Greece are case reports where the parasite is commonly associated with acute appendicitis and less often with other pathological or clinical findings (Kollias et al., 1992; Batistatou et al., 2002; Tsibouris et al., 2005; Kouklakis et al., 2007; Zahariou et al., 2007; Panidis et al., 2011; Arakoulis et al., 2012; Gialamas et al., 2012; Mentessidou et al., 2016). Similarly, in our study, both infected children suffered from abdominal pain.

*Taeniid* and Hookworm eggs were detected once each in stool samples from an immigrant from India and a native woman, both originating from Crete. As regards human hookworm infection, there are no data recording such infection in Greece whereas human taeniosis has been previously reported although only in sporadic cases due to *Taenia saginata* infection (Papazahariadou et al., 2004; Paterakis et al., 2007; Papageorgiou et al., 2009). Unfortunately, in the present case we did not succeed to characterise the species of the parasite.

**Conclusions**

Overall, the results of the current study indicate that there is not an increased infection rate of parasitism compared to previous published data. Since in the current study participants consisted mainly of healthy adults, the real prevalence of intestinal parasitism in Greece was maybe underestimated. Although the results cannot be extrapolated to the general population in Greece, they suggest that increased migration and tourism had no major impact on parasitism in the studied areas. Besides, in the majority of the cases, both parasitism and symptoms were not related to travel but acquired indigenously and individuals were just visiting a doctor as part of a routine health screen. The above
confirms the status of Greece as a “low-risk” country concerning intestinal parasitic infections (http://www.who.int/ith).

There were no indications of significant zoonotic parasite transmission. Specifically referring to Giardia infections, genotyping results demonstrated the dominance of sub-assemblage All in infected individuals, indicating a human to human transmission cycle. A novel multi-locus genotype was identified, which has not been described in humans or animals previously.

The absence of a significant association between parasitism and clinical disease was probably due to the low number of positive samples. However, we should not underrate the clinical importance of such infections, given the high proportion of gastrointestinal signs for some parasitic infections, such as Giardia (36% diarrhoea in infected people), Cryptosporidium (80% diarrhoea) and pinworms (100% abdominal pain).

Acknowledgements

The authors would like to thank the Hellenic Scholarship Foundation for supporting Mrs. Kostopoulou thesis. We would also like to thank all the medical doctors who participated in the study as well as Dr A. Gelasaki, Mr. N. Voutzouraki, Mrs. K. Saratsi and Mrs K. Antoniadou who contributed to the completion of this work.

Funding

This work is a part of a PhD scholarship funded by the Greek State Scholarships Foundation.

Authors’ contributions

This study was conducted by DK as part of her PhD thesis. Also, DK participated in the data collection and analysis and developed first draft manuscript. All other authors played a role in data collection and analysis and interpretation of findings (EC, DA, PL, CS, SS). All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.
Consent for publication

Not applicable.

Ethical considerations

This study was carried out in compliance with the National and Institutional ethical regulations.
References


Table 1. Data about *Giardia* spp and *Cryptosporidium* spp positive samples. CPG = *Giardia* cysts/gram faeces. OPG = *Cryptosporidium* oocysts/gram faeces. Fsc = faecal score.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Parasite</th>
<th>CPG/OPG</th>
<th>Fsc</th>
<th>Clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Location</strong></td>
<td><strong>Region of Crete</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>31 yrs</td>
<td><em>Giardia</em></td>
<td>300,000</td>
<td>1</td>
<td>No signs</td>
</tr>
<tr>
<td>F</td>
<td>no data</td>
<td><em>Giardia</em></td>
<td>418,600</td>
<td>2</td>
<td>No signs</td>
</tr>
<tr>
<td>F</td>
<td>32 yrs</td>
<td><em>Giardia</em></td>
<td>400,000</td>
<td>2</td>
<td>No signs</td>
</tr>
<tr>
<td>F</td>
<td>no data</td>
<td><em>Giardia</em></td>
<td>105,200</td>
<td>2</td>
<td>Diarrhoea</td>
</tr>
<tr>
<td>M</td>
<td>2.5 yrs</td>
<td><em>Giardia</em> &amp; <em>Cryptosporidium</em></td>
<td>27,300 CPG &amp; 300 OPG</td>
<td>2</td>
<td>Abdominal pain</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td><strong>North/North East Greece</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>no data</td>
<td><em>Giardia</em></td>
<td>28,000</td>
<td>2</td>
<td>No signs</td>
</tr>
<tr>
<td>M</td>
<td>22 yrs</td>
<td><em>Giardia</em></td>
<td>500</td>
<td>3</td>
<td>Diarrhoea</td>
</tr>
<tr>
<td>M</td>
<td>34 yrs</td>
<td><em>Giardia</em></td>
<td>2,300,000</td>
<td>2</td>
<td>No signs</td>
</tr>
<tr>
<td>M</td>
<td>27 yrs</td>
<td><em>Giardia</em></td>
<td>65,300</td>
<td>3</td>
<td>Diarrhoea</td>
</tr>
<tr>
<td>M</td>
<td>29 yrs</td>
<td><em>Giardia</em></td>
<td>1,300</td>
<td>2</td>
<td>Diarrhoea</td>
</tr>
<tr>
<td>M</td>
<td>no data</td>
<td><em>Cryptosporidium</em></td>
<td>600</td>
<td>3</td>
<td>Diarrhoea</td>
</tr>
<tr>
<td>F</td>
<td>25 yrs</td>
<td><em>Cryptosporidium</em></td>
<td>1,100</td>
<td>3</td>
<td>Diarrhoea</td>
</tr>
<tr>
<td>F</td>
<td>46 yrs</td>
<td><em>Cryptosporidium</em></td>
<td>500</td>
<td>3</td>
<td>Diarrhoea</td>
</tr>
<tr>
<td>M</td>
<td>no data</td>
<td><em>Giardia</em> &amp; <em>Cryptosporidium</em></td>
<td>76,800 CPG &amp; 200 OPG</td>
<td>2</td>
<td>No signs</td>
</tr>
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</table>
### Table 2. *Giardia* spp genotyping results

<table>
<thead>
<tr>
<th>No of sample</th>
<th>18S</th>
<th>bg</th>
<th>TPI GEN</th>
<th>TPI A</th>
<th>gdh</th>
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<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>All</td>
<td>All</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>All</td>
<td>All</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>All</td>
<td>All</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>All</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
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<td>All</td>
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</tbody>
</table>
Chapter V

General discussion
Introduction

*Giardia* and *Cryptosporidium* have received much attention not only for their prevalence and virulence in various animal species, but also in humans. Several recent epidemics (Wang et al., 2013; Guzman-Herradore et al., 2015; Painter et al., 2016; Efstratiou et al., 2017) caused by these two protozoa have been reported worldwide, linked mainly to water contamination in both developing and developed communities.

During the last decades numerous studies have been conducted, which led to a greater understanding of their worldwide distribution and disease burden caused. According to WHO estimates, in 2010, giardiosis resulted in 171,100 disability-adjusted life years (DALYs) and cryptosporidiosis in 2,159,331 DALYs (Kirk et al., 2015).

Moreover, by the application of modern biological techniques, several diagnostic assays have been developed and validated with different sensitivity and specificity, which are performed either as a part of routine diagnostic services or at a research level. Despite these advancements, still, there is a big debate related to their transmission pathways within and between host species and their interactions, especially concerning *Giardia*. What has being discussed is whether *Giardia* can be potentially zoonotic and subsequently, which transmission cycles are implicated in human infections and in water or food sources’ contamination.

We have tried to fill in these “gaps” of knowledge by studying the abundance of both parasites in Greece and by characterizing them at a subspecies/assemblage level within major host species (humans, dogs, cats, horses, small ruminants). Despite the wide distribution of *Giardia* and *Cryptosporidium* in both animals and humans globally, little is known about the presence of these protozoa in animal and human populations in Greece. There were 456 outbreaks of waterborne and foodborne diseases reported by the Hellenic Center for Disease Control and Prevention between 2004 and 2017 (http://www.keelpno.gr). For 64.5% of the above outbreaks (n=294), *Salmonella* spp were considered as the primary aetiological cause, followed by other bacteria or viruses. On the contrary, parasites were implicated in only one outbreak during this time period but any further information about the responsible parasitic species is not provided. Our interest to conduct this study in Greece arised largely by the geographical location of the country (at the South end of Europe bordering the Balkans, Africa and the Middle-East), which attracts immigrants and refugees especially during the last decades. This geographical location and the climatic conditions are also responsible for the very intense touristic
activity of the country which translates to millions of tourists visiting various Greek destinations annually from all over the world, often together with their pets. All the above favours the invasion of new parasitic species with potential public health risk not necessary restricted to the area. Therefore, our target was to better understand the routes of transmission of zoonotic parasites focusing on *Giardia* and *Cryptosporidium* among various host populations living in close proximity and the possible existence of a zoonotic risk.

In an effort to reduce as much as possible environmental factors affecting our results i.e surface waters (rivers) originating from other regions or neighbouring countries, we focused our studies on a controlled (restricted) area such as the island of Crete. Crete was also the study area for a preliminary study that we had carried out in order to evaluate the prevalence and the zoonotic potential of *Giardia* spp and *Cryptosporidium* spp in small ruminants (Tzanidakis et al., 2014). Following this study and in order to have a complete view of the prevalence of these two protozoa also in other animal species, we proceeded (as part of this thesis) to the estimation of the parasitism in companion animals (dogs and cats) and foals of the island. In a “One Health” approach we continued our survey in the human populations of Crete.

However, due to the difficulties we met during our sampling such as the low number of foals in the island and the low correspondence of individuals who were willing to participate in the study, we expanded our sampling area to various parts of Greece regarding foals, and to Northern Greece regarding humans. The number of samples collected from Crete is mentioned in Table 1.
<table>
<thead>
<tr>
<th>Host</th>
<th>No of samples</th>
<th>Prevalence of Giardia</th>
<th>Prevalence of Cryptosporidium</th>
<th>Genotyping Giardia</th>
<th>Genotyping Cryptosporidium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans-Adults</td>
<td>389</td>
<td>1.03%</td>
<td>0</td>
<td>All</td>
<td>Not successful</td>
</tr>
<tr>
<td>Humans &lt;18 yrs</td>
<td>51</td>
<td>1.96%</td>
<td>1.96%</td>
<td>All</td>
<td>Not successful</td>
</tr>
<tr>
<td>Dogs</td>
<td>879</td>
<td>25.2%</td>
<td>5.9%</td>
<td>C/D (n=72), C+D (n=15), A (n=2), Al (n=1), All (n=1), A+C+D (n=5), BIV-like + C (n=1)</td>
<td>C. canis (n=2) C. scrofarum (n=1)</td>
</tr>
<tr>
<td>Cats</td>
<td>264</td>
<td>20.5%</td>
<td>6.8%</td>
<td>A (n=6), A+ F (n=4), F (n=2), BIV-like (n=1), C (n=1)</td>
<td>C. felis (n=4)</td>
</tr>
<tr>
<td>Foals</td>
<td>50</td>
<td>6%</td>
<td>0</td>
<td>A + B (n=3), All (n=1)</td>
<td>Not successful</td>
</tr>
<tr>
<td>Lambs</td>
<td>429</td>
<td>37.3%</td>
<td>5.1%</td>
<td>E, A + E</td>
<td>C. parvum (Ild), C. ubiquitum, C. xiaoi</td>
</tr>
<tr>
<td>Goat Kids</td>
<td>255</td>
<td>40.4%</td>
<td>7.1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Samples collected from different hosts from Crete Island

**Giardia and Cryptosporidium in animals**

Our results revealed the presence of *Giardia duodenalis* and *Cryptosporidium* spp in foals, dogs, cats and humans in Greece. Overall, according to all generated results, including those of our similar recent study in small ruminants of Crete, for *Giardia*, the infection rates were quite high, especially in lambs (37.3%) and goat kids (40.4%), as well as in shelter populations of dogs (54.3%) and cats (39.0%). *Cryptosporidium* prevalence was below 10% in all animal groups apart from shelter dogs (14.7%) and cats (11.9%).

Few studies have been conducted in Greece evaluating the presence of *Giardia* and *Cryptosporidium* in companion animals, focusing mainly on dog populations (Haralabidis et al., 1988; Papazahariadou et al.,
2007; Symeonidou et al., 2017; Symeonidou et al., 2018), with estimated infection rates similar to our findings in most cases, although different diagnostic tools were applied.

*Giardia* and *Cryptosporidium* have been also found in dogs and cats in several other countries in Europe including those located in the Mediterranean basin (Causape et al., 1996; Dubna et al., 2007; Claerebout et al., 2009; Overgaauw et al., 2009; Epe et al., 2010; Upjohn et al., 2010; Dupont et al., 2013; Zanzani et al., 2014; Osman et al., 2015; Paoletti et al., 2015; Piekarska et al., 2016; de Lucio et al., 2017; Ferreira et al., 2017; Hinney et al., 2017; Adell-Aledon et al., 2018). Our results (Chapter 3) are in agreement with the published data, however, variations in infection rates have been observed and maybe attributed to the differences in the type of studies (cross-sectional vs longitudinal studies), the sample size and the diagnostic methods used.

As expected due to their living conditions, shelter dogs and cats tend to be highly infected with both *Giardia* and *Cryptosporidium*, not only in Greece but also in other European countries (Causape et al., 1996; Claerebout et al., 2009; Scaramozzino et al., 2009; Ferreira et al., 2011; Dado et al., 2012; Ortuno et al., 2014; Simonato et al., 2015; Gil et al., 2017). However, especially in the case of catteries, the observed infection rates were significantly higher than those reported in other studies (Robben et al., 2004; Ferreira et al., 2011; Gil et al., 2017).

Regarding production animals, investigations have been previously conducted in Greece with focus on cryptosporidiosis in ruminants. Since the herds included in those studies had a history of diarrhoea or the animals were diarrhoeic at the time of sampling, the infection rates reported in both cattle (Panousis et al., 2007; Arsenopoulos et al., 2017) and small ruminants (Panousis et al., 2008; Giadinis et al., 2015) were much higher than our records (Tzaniidakis et al., 2014). Comparing our results with other studies in Europe, it seems that the high prevalence of *Giardia* reported in lambs and goat kids in Crete is in line with these surveys (Gomez-Mucoz et al., 2009; Geurden et al., 2010; Castro-Hermida et al., 2011). However, regarding *Cryptosporidium*, in our case, by testing animals of a similar age results showed lower levels of infection compared to other European studies (Geurden et al., 2008; Robertson et al., 2010; Kaupke et al., 2017a), probably due to differences in breed or management and husbandry practices (e.g. less intensified systems prevailing in Crete) (Mahfouz et al., 2014).

Since as already mentioned, a significant part of our studies has been focused on studying the epidemiological scenario on Crete island, we decided not to proceed with studies investigating the occurrence of *Giardia* and *Cryptosporidium* in bovines, because contrary to the large numbers of sheep
and goats living all over the island (according to Official National Statistical data they are a population of over 1,500,000 individuals), cattle herds are almost absent.

Especially for foals, our studies included horse samples not only from Greece (the majority of the samples) but also from Belgium, Germany and the Netherlands as part of a larger survey. The infection rates found, were similar among countries but lower than in other studies conducted in Italy in which a higher prevalence of both *Giardia* and *Cryptosporidium*, has been found (Veronesi et al., 2010).

In general, taking into consideration our results in animals and comparing them with similar studies in Europe (Geurden et al., 2008; Paoletti et al., 2008; Claerebout et al., 2009; Geurden et al., 2010; Tysnes et al., 2011; de Lucio et al., 2017; Gil et al., 2017), we could assume that high infection rates of *Giardia* spp exist in different animal populations (companion animals and livestock) in Greece. On the contrary, in the case of *Cryptosporidium* spp, among the different animal species that were tested in the current study, only shelter dogs and cats seem to be highly infected.

The above protozoal infections in all animal species have detained us mainly for their public health significance and to a lesser extent for their impact on animals’ health. However, although in most cases the infections are asymptomatic or the symptoms are self-limited in both companion and production animals, they may have severe economic consequences especially in livestock (Tangtrongsup and Scorza, 2010; Xiao, 2010; Feng and Xiao, 2011; Santin, 2013; Abeywardena et al., 2015). That includes economic losses due to animals’ death, treatment costs and reduced productivity (weigh losses and reduced carcass quality) (Geurden et al., 2010; Sweeny et al., 2011; Thomson et al., 2017).

**Giardia and Cryptosporidium in humans**

Apart from a previously reported outbreak of giardiosis in a hotel in Crete (Hadjichristodoulou et al., 1998; Hardie et al., 1999), there were no epidemiological data available for the human population in Greece. Despite the high infection rates in the studied animal species we have recorded, which are indicative of the presence and the circulation of *Giardia* and *Cryptosporidium* in the environment, the occurrence of parasitism in humans was low (1.3% *Giardia* & 0.6% *Cryptosporidium*). This is in agreement with the absence of any report of outbreaks caused by *Giardia* spp and/or *Cryptosporidium* spp by the Hellenic Center for Disease Control and Prevention between 2004 and 2017 (http://www.keelpno.gr). As previously mentioned, only one waterborne/foodborne outbreak of parasitic origin was recorded in 2010 and without any clarification of the responsible parasitic species.
*Giardia* spp and *Cryptosporidium* spp infections are commonly underreported by the surveillance systems possibly because they are misdiagnosed by the medical doctors in Greece either because of lack of background epidemiological knowledge of the presence of those parasites (which most often co-exist with other enteropathogens), or because the diagnostic techniques routinely performed are of limited sensitivity failing to detect the responsible parasitic agent.

In the current study, only 11 people out of 876 were found infected with *Giardia*, two of whom were co-infected with *Cryptosporidium*. Since it is obvious from our results that the two protozoa do exist in the environment and are present in animal populations, a reason for the low prevalence in humans could be that *Giardia* and *Cryptosporidium* infections may be underestimated, since many of the individuals participating in our study were immunocompetent and clinically healthy people (80.1%), and most of them were adults (93.8%). According to the literature, children and immunocompromised individuals are more vulnerable to *Giardia* and *Cryptosporidium* infection (Thompson et al., 2005; Haliez and Buret, 2013; Ryan et al., 2016; Soares and Tasca, 2016). In our study we had chosen not to focus on immunocompromised or clinically ill individuals because this was not the main objective of the study. Our main interest was to investigate the presence of those parasites amongs the general human population and to genetically characterise the strains found in order to trace the route of infection.

However, although we managed to follow a predefined sampling strategy in the case of dogs and cats, this was not possible with foals’ and humans’ sampling as previously mentioned. Thus, especially in the case of humans, we were more restricted to samples obtained through medical doctors, though mostly from clinically healthy individuals (food workers). Our study population was randomly selected since we could not include a predefined target group. This approach could influence our results although there was an equal geographical distribution of the samples through Crete and also in Northern Greece.

Of course contact with animals could have been an important risk factor, which could have led to the transmission of *Giardia* spp and *Cryptosporidium* spp especially to pet owners. However none of the infected participants reported any contact with animals, but then again, the low number of infected individuals is restricting further analyses and risk factor characterization.

Greece is a country that sufficient sanitary conditions are ensured. This is confirmed by the low infection rates of the parasites that were detected in humans in the current study, contrary to what has been found in animal populations, including zoonotic assemblages/genotypes. Thus, the risk factor “hygiene measures” seems not to exist in our case. Similarly, another possible risk factor could have been contaminated water through which *Giardia* spp and *Cryptosporidium* spp are frequently transmitted.
However, it seems that adequate treatment to water supplies is provided following the European Union Regulation 2015/1787 (L260, 7.10.2015) and under this condition any possible transmission is prevented. This is reinforced by the limited (only one) unofficial record of any waterborne/foodborne outbreak caused by *Giardia* and/or *Cryptosporidium* in Greece.

**Zoonotic transmission of *Giardia***?

Overall, despite animal infections being very common, the importance of zoonotic transmission of *Giardia* has been widely debated. The most popular hypothesis is that animals tend to be largely, but not exclusively, infected with their ‘own’ animal-specific *Giardia* isolates that have no, or very limited, impact on human health (Tysnes et al., 2014; Caccio et al., 2017) and this hypothesis is supported by the genotyping results of our studies. Humans are mainly infected with the anthroponotic assemblages A and B (Caccio et al. 2017), which is also the case in our survey. Although assemblages A and B have also been isolated from animals, especially dogs and cats, we cannot fully justify that these isolates are responsible for transmission to humans (Thompson and Ash, 2016). However, there are reports suggesting that assemblage A is mainly associated with human infections caused by dogs and cats, and that assemblage B is mostly related to anthroponotic transmission (Anuar et al., 2014; Minetti et al., 2015; Pijnacker et al., 2016; Caccio et al., 2017). In contrast, some studies report the presence of animal specific assemblages in human isolates and the detection of non-adapted assemblages among different animal species, such as for instance, the presence of feline specific assemblage/genotype of *Giardia* and *Cryptosporidium* in cattle in Spain (Cardona et al., 2015). However, the possibility of passive carriage of cysts instead of real infection cannot be excluded (Caccio et al., 2017).

In the present study, in agreement with several published data from Europe, the canine and feline assemblages were the dominant ones in companion animals (Sprong et al., 2009; Feng and Xiao, 2011; Beck et al., 2012; Ortuno et al., 2014; Pallant et al., 2015; Sommer et al., 2015; Piekarska et al., 2016; de Lucio et al., 2017; Adell-Aledon et al., 2018; Sommer et al., 2018). However, *Giardia* zoonotic assemblages were also isolated from dogs and cats (AI, AII, BIV-like). Similar recent studies conducted in Spain support that such isolates are not epidemiologically linked to human derived sequences and this because they have recorded a high genetic diversity in AII sequences derived from canine isolates but not from human origin ones (Adell-Aledon et al., 2018). Unfortunately, in our study, a multi-locus genotyping approach was achieved only for one dog sample where assemblage A was assigned in three
markers and classified as multilocus genotype MLGA1, previously described only in calves in China (Wang et al., 2016). Altogether, data from both our work and other studies reinforce the suggestion that dogs are not a significant reservoir of zoonotic *Giardia* assemblages.

In cats, the zoonotic assemblage A was identified exclusively at the 18 SSU-rRNA locus while the feline assemblage F was detected at other loci even in the same samples. Since 18 SSU-rRNA is a conserved gene, we could not make a distinction between the above assemblages and we cannot exclude the possibility that the isolated assemblage A is in fact assemblage F. Although according to the literature, cats are known carriers of assemblage A and F, but not of assemblage B (Xiao and Fayer, 2008; Gil et al., 2017), we detected assemblage BIV-like from a shelter cat. The inability to distinguish the two assemblages in cats through 18 SSU-rRNA locus has been also reported in another survey (Sommer et al., 2018). Thus, the potential zoonotic risk from *Giardia* infected cats cannot be evaluated in the present study. Cats have been implicated in the animal to human transmission cycle of *Giardia* as subtypes of assemblages F and MLG belonging to sub-assemblage AII have been also found in human isolates (Sprong et al., 2009).

In foals, apart from the detection of the hoofed livestock-specific assemblage E, we also identified assemblage B, which in fact was the most prevalent assemblage, and sub-assemblage AI. Both of the latter two (sub)assemblages have proven zoonotic relevance and this was not a completely new finding since similar results have been also reported from other researchers (Veronesi et al., 2010; Traversa et al., 2012; Ryan and Caccio, 2013; Santin et al., 2013). However, since multilocus genotyping was not possible in our studies, similarly as with other species, the role of equidae in the transmission of *Giardia* in humans remains unclear and needs to be further investigated.

As regards small ruminants, assemblage A (no further characterised into sub-assemblages) was isolated in a limited number of animals in our earlier study in Crete, which again was more or less expected since it has been previously isolated from livestock in Europe (Gomez-Munoz et al., 2012; Minetti et al., 2014). Although such findings suggest that there is a potential for *Giardia* spp to be transmitted from small ruminants to humans in the island of Crete, evidence remains weak and has to be further clarified.

In the human *Giardia* positive samples, PCR and sequencing revealed only the presence of assemblage AII, which is primarily a human-adapted assemblage, although it has been also isolated from animals (Wang et al., 2014; Pallant et al., 2015; Thompson and Ash, 2016; Xu et al., 2016). Further investigation, through multi-locus genotyping (MLG), confirmed these results and provided no evidence for *Giardia* transmission from animals to humans or vice versa. However, MLG was performed only for a very low
number of samples (n=2), so these results cannot be indicative of the whole study. Contrary to what has been documented at the literature referring to the dominance of assemblage B (~58%) over assemblage A (~37%) in human isolates from various regions in the world (Ryan and Caccio, 2013), in our study we have no evidence of the presence of assemblage B in humans. This could be a consequence of the low prevalence of the parasite in the participating individuals. However, there are reports worldwide, which are in line with our findings and mention the mere existence or predominance of assemblage A in human samples (Babaei et al., 2008; Yong et al., 2000; Wang et al., 2011; Wang et al., 2013; Azcona-Gutiérrez et al., 2017).

**Zoonotic transmission of Cryptosporidium?**

Similarly to *Giardia*, the presence of animal specific *Cryptosporidium* species/genotypes e.g. *C. canis*, *C. felis* and *Cryptosporidium* horse genotype in dogs, cats and foals respectively, illustrates a low risk for zoonotic infections. These results are in line with other studies in Europe (Caffara et al., 2013; de Lucio et al., 2017; Gil et al., 2017; Kvac et al., 2017). However, our previous findings in small ruminants in the area, with the detection of *C. parvum* (subtype IId), *C. ubiquitum* and *C. xiaoi*, suggest an existing, although limited, zoonotic risk. These results are similar to a recent study in Spain (Diaz et al., 2018) and correspond to what has been previously published in the literature, i.e. *Cryptosporidium* infections in humans can be linked to infected livestock (Fayer et al., 2000; Stantic-Pavlinic et al., 2003; Thompson et al., 2008; Ibrahim et al., 2016; Conrad et al., 2017; Vermeulen et al., 2017).

Nevertheless, although as shown above, our genotyping results were rather anticipated, there were cases of contradictory findings such as the isolation of the canine assemblage C from a household cat which however, had unlimited access to the external environment, and the pig-specific *Cryptosporidium scrofarum* genotype from a shelter dog, which may have occurred due to accidental ingestion of cysts and oocysts respectively and these animals could act as passive carriers of the parasites.

The amplification and sequencing of the human *Cryptosporidium* positive sample was not successful. The presence of PCR inhibitors in stool specimens in combination with the robust cell walls of the *Cryptosporidium* oocysts and the low infection intensity (300 oocysts per gram of faeces) detected with the immunofluorescence assay, could influence in a negative way the efficacy of the molecular techniques performed (Oikarinen et al., 2009; Surl et al., 2011; Schrader et al., 2012). Thus, in combination to the fact that we have only one positive sample, we cannot draw any conclusions about zoonotic transmission of *Cryptosporidium* in humans. Overall, we could conclude that there is minimal
zoonotic risk taking also into account the results drawn from the animal studies, which showed the presence of mainly host-specific *Cryptosporidium* species/genotypes. However, although unusual, globally, species/genotypes such as *C. meleagris*, *C. felis*, *C. canis*, *Cryptosporidium* horse, skunk and rabbit genotype as well as cervine genotypes, have been implicated in human cases of clinical cryptosporidiosis, even in immunocompetent individuals, but at a low rate and mainly in developing countries (Robinson et al., 2008; Thompson et al., 2008; Xiao et al., 2009; Lucio-Forster et al., 2010; Beser et al., 2015; González-Díaz et al., 2016). Unlike small ruminants and foals, where we met no difficulties in genotyping either *Giardia* or *Cryptosporidium* positive faecal samples, our amplification and sequencing results in dog and cat samples did not have the expected success rates, especially in the case of *Cryptosporidium* (10% success for 18 SSU-rRNA and 13% success for HSP70 gene). This phenomenon has been documented before mainly in isolates of canine and feline origin and could be interpreted as a result of the presence of PCR inhibitors in dog and cat faeces and the low number of *Giardia* and *Cryptosporidium* (oo)cysts detected by immunofluorescence (Tangtrongsup and Scorza, 2010; de Lucio et al., 2016; Cardona et al., 2015; Gil et al., 2017; Adell-Aledon et al., 2018). In our case the parasitic burden in dogs ranged from 100 to 275,800 CPG, but the genotyping results were inconsistent showing often positive outcome when CPG was low and vice-versa.

**Conclusions**

In general, our results, in an effort to evaluate the epidemiologic scenario of *Giardia* and *Cryptosporidium* among people and various animal species who co-exist and interact in the same environment, support the fact that transmission cycles between animals and humans remain separated especially to what *Giardia* infections concerns (Figure 1).

The high infection rates of *Giardia* and *Cryptosporidium* in animals and therefore the high rates of environmental contamination that may exist, in combination with the low parasitism which was detected in humans who share this contaminated habitat, support that conclusion. The predominance of non-zoonotic genotypes of both *Giardia* and *Cryptosporidium* makes the transmission scenario of these protozoa from animals to humans rather faint. Thus, animals seem to share their own animal-specific assemblages/genotypes and humans the anthropoontic ones.

Despite the isolation of zoonotic assemblages from *Giardia* infected companion and productive animals, overall this evidence was rather weak allowing us to conclude that animals were not actively involved in the spread of the two parasites to humans. Therefore, we are more oriented to the human-to-human cycle as the most possible and predominant mode of transmission in humans in the present study, while
we cannot 100% rule out the possibility of zoonotic transmission. Although our MLG results support the same conclusion unfortunately we should have the chance to perform multi-locus genotyping in a higher number of samples for those conclusions to be solid. Likewise, our data do not confirm any transmission from humans to animals’ pathway of the parasites.

Fig. 1. *Giardia* transmission cycles and the infection rates detected in our study (As shown by Caccio et al., 2017)
Opportunities for future studies

Our work was based on cross-sectional studies. Ideally, the transmission dynamics of *Giardia* and *Cryptosporidium* could have been better clarified if we could perform a longitudinal surveillance, which would allow identification of temporal-spatial peaks of prevalence and the infection dynamics’ seasonality of the parasites. Based on our results in humans and the different animal populations, a longitudinal survey could be supported by a further investigation of the parasites’ prevalence in sheep and goats in Crete. In our preliminary study in small ruminants, high prevalence of *Giardia* was detected including the zoonotic assemblage A, which therefore was not further characterized. Besides, sheep and goats graze the pastures which surround the drinking water basins of the island so a simultaneous investigation of the natural water sources of the area would provide valuable knowledge in an effort to investigate the epidemiological patterns of *Giardia* infection and assess the potential risk for public health. Inclusion of humans in the longitudinal study would be ideal, however, the significant difficulties we met during sampling process remains a discouraging scenario.

Infections in humans could have occurred after direct contact with already infected individuals or via contaminated water or food e.g. raw vegetables and fruits. In an attempt to obtain evidence of zoonotic transmission, we designed a simultaneous sampling of humans and domestic animals living in localised foci, although we did not preclude the investigation of the occurrence of *Giardia* and *Cryptosporidium* in water sources (treated or untreated) or in drinking water or vegetables in the area, but this is something that should be taken into consideration and probably performed in future studies. Specifically for the Crete island and due to the constant tourism growth, the evaluation of the water contamination of the swimming pools in the tourists’ accommodation spots would constitute significant data about the possible “import” and “export” of *Giardia* and *Cryptosporidium* in the island.

Similarly, as Crete receives refugees continuously, a surveillance of *Giardia* and *Cryptosporidium* infection level in these individuals would be also valuable.

In addition, our survey could have expanded via the investigation of environmental contamination, to the possible infection of wildlife especially in the case of *Giardia* where zoonotic species have been implicated in cases of giardiosis of wildlife species (Thompson and Ash, 2016). Crete is characterized by a wide variety of wildlife. This wildlife includes many mammals some of which are the Cretan Agrimi Wild Goat also known as “Kri-Kri”, the Cretan Wild Cat, the Cretan White-toothed Shrew, the Cretan Spiny Mouse, high numbers of weasels, hedgehogs, Brown Hares and European rabbits. Also, many
flocks of free roaming wild sheep and goats exist in the island. Through the evaluation of the parasitism of wildlife species we can have a more complete view of the transmission patterns of *Giardia* and *Cryptosporidium* in the area and reach more precise conclusions.

Reffering back to the difficulties we met during sample processing procedures specifically in acquiring a satisfactory DNA quality to run the PCR protocols it would be important to perform studies aiming to overcome such problems. During our studies and in order to increase our success rates we proceeded to some modifications of the selected protocols like for example altering the performance of the PCR mix by adding Bovine Serum Albumine (BSA) and by replacing BSA with Dimethyl Sulfoxide (DMSO). During this effort we performed multiple reactions trying different concentrations of BSA and DMSO each time. Both BSA and DMSO boost PCR and increase PCR robustness and are recommended for difficult (low purity) templates (Abu Al-Soud and Rådström, 2000). In addition, we tried to alter the concentration of MgCl₂. For instance, by increasing the MgCl₂ concentration Taq DNA polymerase’s activity is increased and sensitivity is higher, whereas lower MgCl₂ concentration lowers Taq’s activity but increases specificity (Roux, 1995). Moreover we changed the annealing temperature of the reactions by decreasing the temperature in order to increase sensitivity (Roux, 1995) and tried to amplify all the genes that are recommended by publications for the detection of *Giardia* and *Cryptosporidium*.

However, and in spite all our efforts, there is still need for improvement and it would be crucial for similar future studies to introduce an improved sample treatment in order to acquire better purified DNA product and therefore better-quality PCR results. Purification could be done after floatation of the *Giardia* and/or *Cryptosporidium* positive faecal samples and selection of the concentrated cysts and/or oocysts. DNA could be then extracted by these purified cysts and/or oocysts. Another helpful tool that we could use in the future and could increase PCR success is DNA cloning.

Further investigations on the *Giardia* and *Cryptosporidium* genome would enable us to better understand their transmission dynamics. The use of a more recently introduced molecular technique (like for example Next Generation Sequencing) could lead to a better identification and clarification of the parasites’ subtypes. This could also help to the better detection of *Giardia* and *Cryptosporidium* species in case of mixed infections where dominant strains might competitively exclude others. Besides, as already done in human and pig *Cryptosporidium* isolates (Paparini et al., 2015; Kaupke et al., 2017b), NGS could allow species identification in samples with low abundance of parasite DNA. Regarding *Giardia*, as the whole genome of assemblages A, B and E has been analysed showing similarities
between A and E (Caccio et al., 2017), it would be of great interest to establish genome sequences of the other animal-specific *Giardia* assemblages (Caccio et al., 2017).

In summary, our study indicates that companion animals and livestock can act as significant contributors to *Giardia* cyst and *Cryptosporidium* oocyst environmental contamination in Greece. Infections are also present in people although in low rates. Zoonotic risk of *Giardia* spp and *Cryptosporidium* spp seems to be limited, however, it should not be neglected. Further investigations of water sources in the area would be valuable and could also provide interesting data about the occurrence and the epidemiology of the parasites in the rich wildlife of the island.
References


Summary
*Giardia* spp and *Cryptosporidium* spp are two important parasitic protozoa which affect various hosts, including humans. Globally, about 280,000 human cases of giardiosis and 748,000 cases of cryptosporidiosis are reported every year. The fact that these two protozoa are widespread and abundant not only in the developing world but also in developed countries, together with their implication in several disease outbreaks and their zoonotic potential, urged us to study their prevalence and transmission scenarios in Greece, and specifically in a region that has not previously been investigated. Selection of Crete island as the study area was based on its high human population density and the large numbers of companion animals and livestock who share the same restricted environment and thus would enable us to better investigate the transmission dynamics of the two parasites.

**Chapter 1** provides a review of the published literature on some important points and current data regarding *Giardia* and *Cryptosporidium*. This chapter describes the biology of the parasites, their impact on human and animal health (horses, dogs, cats, livestock), their epidemiology and their zoonotic character, as well as ways of prevention and control of their transmission.

In **Chapter 2**, we evaluated the presence of *Giardia* and *Cryptosporidium* in foals between the age of 1 week and 6 months in Belgium, The Netherlands, Germany and Greece using a quantitative direct immunofluorescence assay (IFA). Positive samples were genotyped, based on the 18S ribosomal DNA gene and the heat shock protein (HSP70) gene for *Cryptosporidium* and on the β-giardin gene and the triose phosphate isomerase (TPI) gene for *Giardia*. In total, 134 foals from Belgium, 44 foals from The Netherlands, 30 foals from Germany and 190 foals from Greece were examined. No *Cryptosporidium* oocysts were identified in faecal samples from foals in Germany and The Netherlands. In Belgium and Greece, 4.5% and 1.1% of the foals examined were *Cryptosporidium* positive, respectively, all with a low oocyst excretion ranging from 100 to 2450 oocysts per gram of faeces. For *Giardia*, 14.2%, 11.4%, 10.0% and 11.6% of the foals in Belgium, The Netherlands, Germany and Greece, respectively, were found to excrete cysts, with a range of 50 up to 4,000,000 cysts per gram of faeces. Younger animals secreted significantly more *Giardia* cysts than older horses (p<0.05), but no significant correlation between *Giardia* infection and diarrhoea was observed. Most *Giardia* positive samples belonged to assemblage AI and/or BIV, but also assemblage E was detected in two samples. Together with the identification of *Cryptosporidium* horse genotype, this suggests only a low risk for zoonotic transmission.

**Chapter 3** focused on the occurrence of intestinal parasites in different dog and cat populations in Crete, Greece. Also, our objectives were to estimate the zoonotic potential and to identify risk factors associated with parasite infections. Faecal samples were collected from 879 shelter, household and
shepherd dogs as well as 264 shelter and household cats. The samples were analysed using sedimentation/flotation techniques for helminth eggs and coccidian oocysts, whereas *Giardia* and *Cryptosporidium* were detected by IFA. Besides, PCR and sequencing were performed to evaluate the zoonotic potential of *Giardia* and *Cryptosporidium* positive samples. High levels of parasitism in both dogs and cats were recorded. *Giardia* was the most prevalent parasite in all dog and cat populations except for shepherd dogs. In dogs, the overall prevalence was 25.2% (CI: 22.4-28.1) for *Giardia* spp; 9.2% (CI: 7.3-11.1) for *Ancylostoma/Uncinaria* spp; 7.6% (CI: 5.9-9.4) for *Toxocara* spp; 5.9% (CI: 4.4-7.5) for *Cryptosporidium* spp; 4.6% (CI: 3.2-5.9) for *Cystoisospora* spp; 2.7% (CI: 1.7-3.8) for *Toxascaris leonina*; 1.7% (CI: 0.9-2.6) for *Capillaria* spp; 0.8% (CI: 0.2-1.4) for taeniid eggs; 0.2% (CI: 0-0.5) for *Dipylidium caninum* and 0.1% (CI: 0-0.3) for *Strongyloides stercoralis*. In cats, the prevalence was 20.5% (CI: 15.6-25.3) for *Giardia* spp; 9.5% (CI: 5.9-13.0) for *Cystoisospora* spp; 8.3% (CI: 5.0-11.7) for *Toxocara* spp; 7.6% (CI: 4.4-10.8) for *Ancylostoma/Uncinaria* spp; 6.8% (CI: 3.8-9.9) for *Cryptosporidium* spp.; 4.2% (CI: 1.8-6.6) for *Capillaria* spp; 0.8% (CI: 0-1.8) for taeniid eggs; and 0.4% (CI: 0-1.1) for *Hammondia/Toxoplasma*. Concerning the risk factors evaluated, there was a negative association between age and *Giardia* infection and between age and *T. leonina* infection intensity for dogs. Sequencing results revealed the presence of mainly animal-specific *G. duodenalis* assemblages C and D in dogs and assemblages F, C and BIV-like in cats, with only a limited number of (co-)infections with assemblage A. However, regarding cats, since the zoonotic assemblage A was identified exclusively at the conserved 18 SSU-rRNA locus while assemblage F was detected only at other loci even in the same samples, we cannot exclude the possibility that the isolated assemblage A is in fact assemblage F. As for *Cryptosporidium*, the dog-specific *C. canis* and the pig-specific *C. scrofarum* were detected in dogs and the cat-specific *C. felis* was detected in cats. Genotyping results suggest a limited zoonotic risk of *Giardia* and *Cryptosporidium* infections from dogs and cats in Crete. Taeniid eggs were more prevalent in shepherd dogs, suggesting access to offal and posing a threat for cystic echinococcosis transmission. Infection rates of *Toxocara* spp in both dogs and cats show that companion animals could be a significant source of roundworm infection to humans.

In Chapter 4, our aim was to study the presence of intestinal human parasitic infections and to identify the potential source of contamination in two different areas of Greece (Makedonia and Crete). In total, 876 stool samples were collected from 822 adults and 54 children. Immunofluorescence was used for the detection of *Giardia* and *Cryptosporidium*, whereas sedimentation (acid/ether) and concentration/flotation techniques were performed to detect other intestinal parasites. PCR followed by sequencing was applied to genotype *Giardia* and *Cryptosporidium* positive samples. Thirty-seven (4.5%)...
of the individuals examined harboured at least one species of intestinal parasite, the majority of which were protozoa (3.8%). The specific species found were Blastocystis hominis (1.8%), G. duodenalis (1.3%), Cryptosporidium spp (0.6%), Entamoeba coli (0.2%) and E. histolytica (0.1%). As helminths concerns, 2 people were positive for Enterobius vermicularis, 1 for Taenia spp and 1 for Trichostrongylidae. Genotyping results revealed the presence of G. duodenalis sub-assemblage AII, whereas sequencing was not successful for Cryptosporidium positive samples. A novel multi-locus genotype of G. duodenalis was identified, which has not been described in humans or animals previously. Overall, occurrence of intestinal parasites in humans was low and similar to previously published data, suggesting that current socioeconomic changes (increased migration and tourism) had no major impact on parasitism in the studied area. As regards clinical relevance of all parasitic species detected in this study as well as any indication of potential sources of infection and risk factors no safe associations to those infections could be made since the limited number of positive samples did now allow us to draw any statistically significant conclusion. Based on the parasite species and genotypes detected, there was no indication that animals were an important source of infection.

In Chapter 5, data presented in this thesis are discussed and compared with similar recently published studies in the study area and beyond. Overall, prevalence data seem to be consistent with a common epidemiological scenario in which G. duodenalis and Cryptosporidium spp are abundant in a wide range of domesticated animals including horses, ruminants, dogs and cats. Prevalence rates of infection in dogs and cats are high but vary depending on the population, with kennelled animals presenting with higher infection rates. Humans are also infected but in much lower rates. The potential of a zoonotic transmission of all animal’s species to humans, especially to what Giardia regards, seems to be weak since in most of animals and human cases genotype analyses showed infection by the host-specific isolates. However, since there were cases where animals harboured potentially zoonotic assemblages/sub-assemblages of Giardia, the above statement (although resonates with and coheres to current facts) should be further validated in the future by using novel approaches and new generation molecular tools.
Samenvatting
*Giardia* spp en *Cryptosporidium* spp zijn twee belangrijke parasitaire protozoa, die een groot aantal verschillende diersoorten kunnen infecteren, waaronder ook de mens. Wereldwijd worden jaarlijks ongeveer 280.000 gevallen van humane giardiose en 748.000 gevallen van cryptosporidiose gerapporteerd. De wijd verspreiding van deze twee parasieten in zowel ontwikkelingslanden als ontwikkelde landen, hun regelmatige betrokkenheid bij ziekte-uitbraken en hun zoönotisch potentieel waren de aanleiding om het voorkomen en de transmissie van *Giardia* en *Cryptosporidium* te bestuderen in Griekenland, een land waar weinig data over de transmissie van *Giardia* en *Cryptosporidium* beschikbaar waren. Het eiland Kreta werd uitgekozen als studiegebied, omdat de hoge bevolkingsdichtheid, de grote mobiliteit (toeristen en migranten) en het grote aantal gezelschapsdieren en landbouwdieren in een afgebakend gebied, een geschikte situatie creëert om de transmissie van *Giardia* en *Cryptosporidium* te bestuderen.

**Hoofdstuk 1** geeft een overzicht van de wetenschappelijke literatuur in verband met enkele belangrijke aspecten van *Giardia* en *Cryptosporidium* infecties. Achtereenvolgens wordt de biologie van de parasieten, hun medisch en veterinair belang, de epidemiologie en hun zoönotisch karakter besproken, gevolgd door de behandeling en mogelijke preventieve maatregelen om de verspreiding ervan tegen te gaan.

In **hoofdstuk 2** werd het voorkomen van *Giardia* en *Cryptosporidium* onderzocht bij veulens van 1 week tot 6 maanden oud in België, Nederland, Duitsland en Griekenland, met behulp van een kwantitatieve directe immunofluorescentietest (IFT). Positieve mest monsters werden gegenotypeerd met PCR, waarbij het 18S rRNA genen het ‘heat shock protein’ (HSP70) gen werden gebruikt voor *Cryptosporidium* en het β-giardine genen het triose phosphate isomerase (TPI) gen voor *Giardia*. In totaal, werden 134 veulens bemonsterd in België, 44 in Nederland, 30 in Duitsland en 190 in Griekenland, waarvan 45 in Kreta. In mest monsters uit Duitsland en Nederland werd geen *Cryptosporidium* gevonden. In België en Griekenland waren respectievelijk 4,5% en 1,1% van de veulens positief voor *Cryptosporidium*, zij het met een lage oöcysten-uitscheiding, tussen 100 en 2450 oöcysten per gram feces (OPG). *Giardia* cysten werden teruggevonden bij 14,2%, 11,4%, 10,0% en 11,6% van de veulens in respectievelijk België, Nederland, Duitsland en Griekenland, waarbij tussen de 50 en 4.000.000 cysten per gram feces (CPG) werden geteld. Jongere dieren scheidden significant meer *Giardia* cysten uit dan oudere veulens (p<0.05), maar er werd geen correlatie gevonden tussen *Giardia* infectie en de aanwezigheid van diarree. De meeste *Giardia* isolaten behoorden tot assemblage A1 en/of BIV, maar ook het assemblage E, dat specifiek is voor landbouwdieren, werd in twee monsters aangetroffen. Samen met de identificatie
van het Cryptosporidium 'horse genotype' wijzen deze resultaten op een beperkt risico voor zoönotische transmissie van paard naar mens.

**Hoofdstuk 3** focust op de aanwezigheid van darmparasieten in verschillende honden- en kattenpopulaties in Kreta. Naast het inschatten van de prevalentie werd ook getracht van het zoönotisch potentieel van deze infecties te identificeren die met de infecties geassocieerd zijn. Stoelgang werd verzameld van 879 honden (asielhonden, huishonden en herdershonden) en 264 katten (asiel- en huiskatten). De monsters werden onderzocht op aanwezigheid van wormeieren en oöcysten van coccidia met behulp van sedimentatie-flotatie technieken en met de IFA voor *Giardia* en *Cryptosporidium*. Het zoönotisch potentieel van *Giardia*- en *Cryptosporidium*-positieve monsters werd onderzocht met PCR en sequenceren, waarbij dezelfde genen werden gebruikt als in hoofdstuk 2. Er werd een hoge besmettingsgraad met darmparasieten gevonden bij zowel honden als katten. *Giardia* was de meest voorkomende parasiet bij alle honden- en kattenpopulaties, behalve bij herdershonden. In honden werden de volgende prevalenties gevonden: 25.2% (CI: 22.4-28.1) voor *Giardia* spp; 9.2% (CI: 7.3-11.1) voor *Ancylostoma/Uncinaria* spp; 7.6% (CI: 5.9-9.4) voor *Toxocara* spp; 5.9% (CI: 4.4-7.5) voor *Cryptosporidium* spp; 4.6% (CI: 3.2-5.9) voor *Cystoisospora* spp; 2.7% (CI: 1.7-3.8) voor *Toxascaris leonina*; 1.7% (CI: 0.9-2.6) voor *Capillaria* spp; 0.8% (CI: 0.2-1.4) voor *Taenia*-type eieren; 0.2% (CI: 0-0.5) voor *Dipylidium caninum*; 0.1% (CI: 0-0.3) voor *Strongyloides stercoralis*. In katten was de prevalentie 20.5% (CI: 15.6-25.3) voor *Giardia* spp; 9.5% (CI: 5.9-13.0) voor *Cystoisospora* spp; 8.3% (CI: 5.0-11.7) voor *Toxocara* spp; 7.6% (CI: 4.4-10.8) voor *Ancylostoma/Uncinaria* spp; 6.8% (CI: 3.8-9.9) voor *Cryptosporidium* spp; 4.2% (CI: 1.8-6.6) voor *Capillaria* spp; 0.8% (CI: 0-1.8) voor *Taenia*-type eieren en 0.4% (CI: 0-1.1) voor *Hammondia/Toxoplasma*. Betreffende de risicofactoren, werd een negatieve associatie gevonden tussen leeftijd en *Giardia* infectie en tussen leeftijd en *T. leonina* infectiegraad bij honden. PCR en sequenceren toonde voornamelijk de diersoort-specifieke *G. duodenalis* assemblages C en D aan bij honden en assemblages F, C en BIV-like bij katten, met slechts een beperkt aantal (meng)infecties met het potentieel zoönotische assemblage A. Omdat assemblage A bij de katten enkel werd gevonden met het geconserveerde 18 SSU-rRNA locus, terwijl in dezelfde mestmonster alleen assemblage F werd gevonden m.b.v. de andere loci, kan niet uitgesloten worden dat de assemblage A isolaten in feite tot assemblage F behoorden. Voor *Cryptosporidium* werden bij honden enkel de diersoort-specifieke species *C. canis* en *C. scrofarum* geïdentificeerd en bij katten de kat-specifieke *C. felis*. Deze resultaten wijzen erop dat *Giardia* en *Cryptosporidium* infecties bij honden en katten in Kreta slechts een heel beperkt zoönotisch risico met zich meebrengen. *Taenia*-type eieren werden veel teruggevonden bij herdershonden, hetgeen aangeeft dat het voeren van slachtafval bij deze
hondenpopulatie een risico inhoudt voor transmissie van *Echinococcus granulosus*. De vrij hoge prevalentie van *Toxocara* spp. bij honden en katten toont aan dat gezelschapsdieren een belangrijke besmettingsbron kunnen zijn van spoelworminfecties voor de mens.

Het doel van *hoofdstuk 4* was te onderzoeken welke darmparasieten voorkomen bij mensen in twee verschillende gebieden in Griekenland (Macedonië en Kreta) en mogelijke besmettingsbronnen te identificeren. In totaal werden 876 stoelgangmonsters verzameld van 822 volwassenen en 54 kinderen. IFT werd gebruikt voor de detectie van *Giardia* en *Cryptosporidium*, terwijl een zuur/ether sedimentatiotechniek en een concentratie-flotatie techniek werden gebruikt om andere darmparasieten aan te tonen. PCR en sequenceren werden gebruikt om *Giardia*- en *Cryptosporidium*-positieve monsters te genotyperen. Zevenendertig individuen (4,5%) waren besmet met minstens één soort darmparasieten, waarvan de meerderheid (3,8%) protozoa waren: *Blastocystis hominis* (1.8%), *G. duodenalis* (1.3%), *Cryptosporidium* spp (0.6%), *Entamoeba coli* (0.2%) en *E. histolytica* (0.1%). Genotypering van positieve monsters bevestigde de aanwezigheid van *G. duodenalis* sub-assemblage AII. Een nieuw multi-locus genotype van *G. duodenalis* werd geïdentificeerd, dat nog niet eerder werd beschreven in mensen of dieren. Genotypering was niet succesvol voor *Cryptosporidium*. Wat worminfecties betreft, waren twee personen besmet met *Enterobius vermicularis*, 1 met *Taenia* spp en 1 met Trichostrongylidae. Over het algemeen was de prevalentie van darmparasieten laag, en lijkt deze niet gestegen te zijn in vergelijking met eerdere studies, wat erop wijst dat de gewijzigde socio-economische situatie (toegenomen toerisme en migratie) geen grote impact heeft gehad op het risico voor besmetting met parasieten. Door het kleine aantal positieve monsters konden geen significante banden aangetoond worden tussen de aanwezigheid van parasieten en klinische symptomen of risicofactoren. Op basis van de geïdentificeerde parasieten-species en genotypes is er geen indicatie dat dieren een belangrijke besmettingsbron waren.

In *hoofdstuk 5* worden de resultaten van de vorige hoofdstukken besproken in de context van de recente literatuur. De bekomen prevalentiedata stemmen vrij goed overeen met andere studies die aangeven dat *Giardia* en *Cryptosporidium* wijdverspreid zijn bij verschillende diersoorten. De prevalentie bij gezelschapsdieren was vrij hoog, vooral bij dieren uit asiëlen. De lage prevalentie van *Giardia* en *Cryptosporidium* bij mensen en de kleine proportie van zoönotische species/genotypes bij dieren, vooral voor *Giardia*, wijzen echter op een beperkt belang van zoönotische transmissie van deze parasieten in Kreta. Voor een volledige opheldering van de transmissiecyclen is verder onderzoek nodig, waarbij gebruik kan gemaakt worden van een nieuwe generatie moleculaire tools, zoals next generation sequencing, en
waarbij de rol van direct contact en voedings- of water-gerelateerde transmissie kan onderzocht worden.
Acknowledgements
All this work would have not been successfully completed without the precious help of a number of people.

First, I would like to thank from the bottom of my heart, both my promoters Prof. Edwin Claerebout and Dr. Smaragda Sotiraki. Although thousands of miles far away, Prof. Edwin Claerebout’s presence was active and constant all over the years. He was always next to me supporting me with patience and kindness and encouraging me to accomplish my goals. Dr Sotiraki, was the reason why I started this journey and 100% supported me at every difficulty I met. She has been tough on me when things were easy and more importantly she was going easy on me when things were tough. Edwin and Smaro, you have been my mentors and you will continue to be for the rest of my professional life. I will never forget how many times you were there for me.

My sincere gratitude goes also to the members of the examination committee: Dr. Alexandros Stefanakis, Dr. Thomas Geurden, Prof. Sarah Gabriel and Prof. Pierre Dorny for their valuable and very beneficial suggestions that helped improving the present dissertation.

I would also like to thank my colleagues from UGENT and especially Stijn Casaert for his constant help and support.

My thanks also go to all the colleagues from the Veterinary Research Institute for providing me the best work environment and support with special thanks to our parasitology group: Panagiota Ligda, Dimitrios Arvanitis, Nikolaos Voutzourakis, Katerina Saratsi and Anastasios Saratsis. Thank you for being great colleagues but mainly for being adorable friends.

Special thanks to Konstantina Antoniadou, Anastasios Anastasiadis, Dr. Ioannis Ignatiadis and all the veterinarians and medical doctors for their precious help and collaboration. Also, thanks to Dr. Theodosios Theodosiou and Dr. Nikolaos Mittas for their statistical support.

Of course, I attribute this thesis to my parents who were the reason why I become the person I am now. I thank them for all of the sacrifices they have done for me. Also, I thank my sister, my brother, and my grandparents for their support.