CHARACTERIZATION OF A NOVEL *CRYPTOSPORIDIUM* GENOTYPE IN RED-WINGED BLACK BIRDS

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
For the Degree of
MASTER OF SCIENCE

Major Department:
Veterinary and Microbiological Sciences

December 2014

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Cryptosporidium species cause cryptosporidiosis, characterized by acute gastroenteritis in humans and animals worldwide. Knowledge of the diversity of *Cryptosporidium* among mammals and birds is incomplete, especially in North American passerines. In this first molecular study of *Cryptosporidium* in a North American passerine, *C. parvum* and a novel genotype, called the red-winged black bird genotype were isolated. Genetic characteristics and phylogenetic analyses of the red-winged black bird genotype were described at the 18S rRNA, actin and HSP70 loci, and it was distinct from previously described species and genotypes. The novelty of this genotype was also supported by propagation studies in chickens, zebra finches and cockatiels that failed to produce patent infections. The study adds to our understanding of the co-evolution of the parasite with its hosts and potential sources of *C. parvum* transmission to susceptible human and animal hosts.

ACKNOWLEDGEMENTS

I would like to thank my Lord and Savior, Jesus Christ, for all He has done. I also thank the following people whose invaluable guidance, assistance, encouragement and prayers helped me complete this project.

I would like to thank my advisor, Dr. John McEvoy. Firstly, for helping me navigate the mires of sorrow in August 2013, then helping me understand the biology of *Cryptosporidium*, and encouraging me to think through the data critically. I also extend my sincere thanks to members of my thesis committee, Drs. Mark Clark, Eugene Berry, Nathan Fisher and Eakalak Khan for their support. I also thank Mrs. Catherine Giddings, Dr. Brianna Stenger and Adam Edwinson, for their encouragement and help with the research.

I also thank Dr. Martin Kváč for his excellent guidance during my stay at the Laboratory of Veterinary and Medical Protistology,Institute of Parasitology BC ASCR, České Budějovice, Czech Republic, and the members of his lab, Pavla Wagnerová, Werunka Prantlová , Dana Květonova, Jitka Prediger, Nikola Hromadová , Eva Myšková and Dr. Bohumil Sak for their friendship and hospitality.

I acknowledge the love, prayers, and encouragement of my parents, Mr. and Mrs. Jesudoss Chelladurai. Your prayers for me are a beautiful fragrance in the sight of our God.

I acknowledge the unending support of my friends from five continents, who make NDSU such a great place, Kylie Dale, Katie Kirschenmann, Tanner Post, Mayara Eler, Siri Sundara, David & Beth Lindahl, Jeremy & Trina Hamilton, Thiago deMenezes, Jade Sandbulte, Sunny Prashant, Katie Olson, Veronica Callez Torres, Collette Michal, Yong Swee Fong, Kathy Mok, Courage & Itai Mudzongo, Aceng Freda and many more. Wherever we may go from here, we will all one day meet on that beautiful shore in a land that is fairer than day.

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LITERATURE REVIEW

Introduction

Obligate protozoan parasites of the genus *Cryptosporidium* (phylum Apicomplexa) have been associated with opportunistic clinical cryptosporidiosis in healthy and immunocompromised human patients (Meisel et al., 1976; Nime et al., 1976).

Cryptosporidiosis is also seen in clinical and non-clinical settings worldwide in vertebrates of every class including mammals (Appelbee et al., 2005), birds, fish, amphibians (Ryan, 2010), and reptiles (Xiao et al., 2004b).

Ernest Tyzzer first described *Cryptosporidium* in the gastric glands (*Cryptosporidium muris*) (Tyzzer, 1907) and intestines (*Cryptosporidium parvum*) of the laboratory mouse (Tyzzer 1912). He subsequently described a parasite in the ceca of chickens that was morphologically similar to *Cryptosporidium parvum,* but he did not name it (Tyzzer, 1929). With the exception of descriptions of *Cryptosporidium meleagridis* in turkeys (Slavin, 1955) and *Cryptosporidium wrairi* in guinea pigs (Vetterling et al., 1971a), few studies on *Cryptosporidium* before the late 1970s have been conducted.

Cryptosporidium parvum was first isolated from a human with acute enterocolitis in 1976 (Nime, 1976). In the early 1980s, cryptosporidiosis emerged as a threat to the lives of immunocompromised patients diagnosed with AIDS (Pitlik et al., 1983). The focus on *Cryptosporidium* as a major human parasite intensified when it was identified as the cause of a waterborne outbreak affecting 516 people in Wiltshire and Oxfordshire, England, in 1991 (Richardson et al., 1991). The largest outbreak of waterborne cryptosporidiosis occurred in 1994, in Milwaukee, WI, with an estimated 403,000 people affected with gastrointestinal symptoms including acute watery diarrhea, abdominal cramps, vomiting and fever (Mac Kenzie et al., 1994). In the years since, about 20 different species of *Cryptosporidium* have been identified as human pathogens, causing infections in both

immunocompromised and immunocompetent patients (Ryan et al., 2014). However, more than 90% of human cases are caused by *C. parvum* and *C. hominis* (Ryan et al., 2014).

Taxonomy

Cryptosporidium is placed in the family Cryptosporidiidae, suborder Eimeriorina, order Eucoccdiorida, subclass Coccidiasina, class Sporozoasida, phylum Apicomplexa and Superkingdom Eukaryota (Current and Garcia, 1991; Egyed et al., 2003). However, this classification is not supported by phylogenies constructed from small subunit ribosomal RNA gene sequences (18S rRNA gene). Within the phylum Apicomplexa, the gregarines and *Cryptosporidium* occur as sister groups (Carreno et al., 1999).

Two major clades are evident in phylogenies constructed from sequences of *Cryptosporidium* rRNA and protein coding genes (Xiao et al., 2002), a gastric clade comprising *Cryptosporidium* spp. that infect the gastric epithelium, and an intestinal clade comprising *Cryptosporidium* that infect the intestinal epithelium (Upton and Current, 1985).

The conventional definition of a species is difficult to apply to *Cryptosporidium*; consequently, the type of evidence required to describe new *Cryptosporidium* species has been much debated *(Xiao et al., 2000).* Although morphology has traditionally been used to describe apicomplexan parasites, it is not particularly useful in the case of *Cryptosporidium*, which lacks variation in oocyst morphology (Xiao et al., 2004a). (Egyed et al., 2002)) proposed a polyphasic approach to taxonomy, where a valid species must have the following investigated in a standardized manner with precisely documented experimental conditions: (a) Morphology of oocysts, including the length, width, and oocyst index; (b) Infectivity of oocysts with adequate description of oocyst age, method of purification, storage, origin, and time of incubation for excystation; (c) Biological studies to determine host specificity, predilection site, virulence, oocyst shedding, and prepatent and patent periods (Cross transmission studies with appropriate positive and negative controls are deemed necessary. Importance is given to age of the experimental animal and its immune status); (d) Genotypic studies by direct sequencing or cloning of amplified genes that code for rRNA and

proteins, including 18S rRNA, Heat Shock Protein 70 (HSP70), and *Cryptosporidium* oocyst wall protein (COWP) (Egyed et al., 2003). This approach to *Cryptosporidium* taxonomy is generally followed in the description of new *Cryptosporidium* species and, depending on the source, twenty-six (Ryan et al., 2014) or thirty valid species (Šlapeta, 2013) are currently recognized and listed in Table 1.

Table 1: List of currently accepted valid species in the genus *Cryptosporidium*

Species	Type host	Reference
C. muris	Mouse	(Tyzzer, 1907, 1910)
C. parvum	Mouse	(Tyzzer 1912; Upton and Current, 1985)
C. meleagridis	Turkey	(Slavin, 1955)
C. wrairi	Guinea pig	(Vetterling et al., 1971a)
C. xiaoi	Sheep	(Barker and Carbonell, 1974; Fayer and Santín, 2009)
C. bovis	Calves	(Barker and Carbonell, 1974; Fayer et al., 2005)
C. cuniculus	Rabbit	(Inman and Takeuchi, 1979; Robinson et al., 2010)
C. felis	Domestic cat	(Iseki, 1979)
C. serpentis	Snakes	(Levine, 1980)
C. baileyi	Chicken	(Current et al., 1986)
C. varanii	Emerald monitor	(Pavlásek et al., 1995)
*(*synonym *C. saurophilum)*		
C. galli	Birds	(Ryan et al., 2003b)
C. andersoni	Cattle	(Lindsay et al., 2000)
C. canis	Dog	(Fayer et al., 2001)
C. hominis	Human	(Morgan-Ryan et al., 2002)
C. molnari	Fish	(Alvarez-Pellitero and Sitjà-Bobadilla, 2002)
C. suis	Pig	(Ryan et al., 2004)

Table 1: List of currently accepted valid species in the genus *Cryptosporidium* (Continued)

C. fayeri	Kangaroo	(Ryan et al., 2008)
C. ryanae	Cattle	(Fayer et al., 2008)
C. fragile	Toad	(Jirků et al., 2008)
C. macropodum	Kangaroo	(Power and Ryan, 2008)
C. ubiquitum	Cattle	(Fayer et al., 2010)
C. viatorum	Human	(Elwin et al., 2012)
C. tyzzeri	Mouse	(Ren et al., 2012; Tyzzer, 1912)
C. scrofarum	Pig	(Kváč et al., 2013)
C. erinacei	European hedgehog	(Kváč et al., 2014a)

In addition to those listed in Table 1, (Šlapeta, 2013)) recognizes *C. nasoris* (Hoover et al., 1981), *C. cichlidis* (Paperna and Vilenkin, 1996), *C. reichenbachklinlei* (Paperna and Vilenkin, 1996), and *C. scophthalmi* (Alvarez-Pellitero et al., 2004) as valid species, but these are not broadly accepted.

In addition to these named species, more than 70 genotypes have been named based on sequences of rRNA and protein coding genes. A genotype is a partial and temporary descriptor of a group of organisms, which are genetically unique (Plutzer and Karanis, 2009). Therefore, the described genotypes are awaiting full classification when sufficient morphological, biological, and genetic data are available.

Lifecycle of *Cryptosporidium parvum*

Tyzzer (1912; 1910) first described the lifecycles of *C. parvum* and *C. muris*, and electron-microscopic details of the *C. parvum* lifecycle were studied later in experimental infections of neonatal mice with isolates from an immunocompetent human with acute, self-limiting diarrhea, an immunosuppressed patient with chronic gastroenteritis, and a naturally infected calf (Current and Garcia, 1991). The six major events described in the lifecycle of *C. parvum* are excystation, merogony, gametogony, fertilization, oocyst formation, and

sporogony (Current and Garcia, 1991). Similar stages have been observed in *C. wrairi* derived from guinea pigs (Vetterling et al., 1971b).

Oocysts, the infective stage of the parasite, contain four naked sporozoites without a sporocyst. Following ingestion, the oocyst excysts to release motile sporozoites into the lumen of the duodenum and ileum (Current and Reese, 1986). Sporozoites invade intestinal epithelial cells where they occupy an intracellular but extracytoplasmic location and form a parasitophorous vacuole, enveloped by host membrane, at the apical surface. Sporozoites transform into round trophozoites, a feeding stage that obtains nutrients from the host cell (Marcial and Madara, 1986), before undergoing asexual replication, termed merogony, to produce eight merozoites in a type I meront. Merozoites released from type I meronts invade neighboring enterocytes in a manner similar to sporozoites, and develop into either type I meronts, repeating the cycle of asexual replication, or type II meronts containing four merozoites, which, upon release, develop into micro- and macro-gamonts. Sixteen bullet-shaped microgametes released by microgamonts seek out and penetrate macrogamonts and fuse with macrogametes to form a zygote (the sexual stage).

The zygote subsequently develops into a thick or thin walled oocyst. Eighty percent of oocysts are thick walled. These have a trilaminar wall (Harris and Petry, 1999), are shed fully sporulated, and are environmentally stable in the feces of the host (Current and Garcia, 1991). Thin walled oocysts rupture soon after their release from enterocytes, allowing the excysted sporozoites to infect cells and maintain an autoinfective cycle (Current and Reese, 1986).

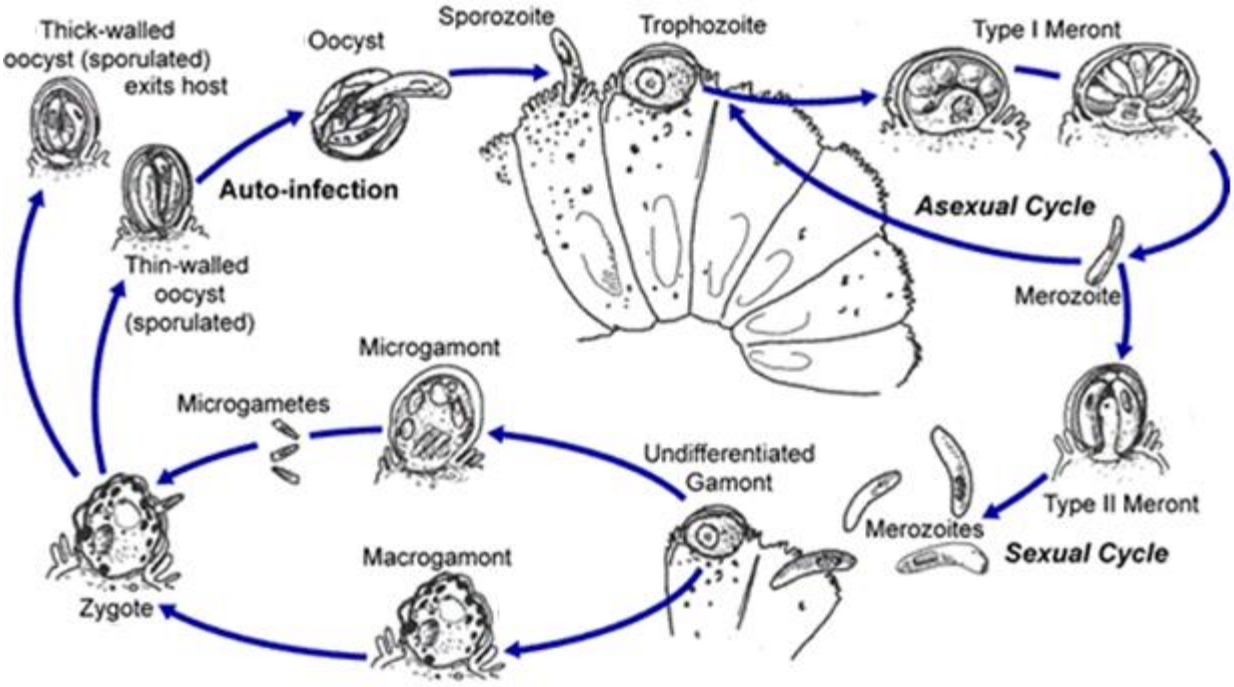

Figure 1. Lifecycle of *Cryptosporidium* derived from studies of *C. parvum* (CDC/Alexander J. da Silva, 2002; Current and Garcia, 1991)

Pathogenesis of human *Cryptosporidium* infections

Cryptosporidium parvum is directly or indirectly transmitted between animals and humans, and as few as 10 oocysts can cause disease in humans (Okhuysen et al., 1999). The parasite does not generally cause a systemic infection, but is localized at an intracellular but extracytoplasmic location in parasitophorous vacuoles, on the apical surface of the epithelial cells (Okhuysen and Chappell, 2002). Many putative virulence factors have been identified that function in excystation, adhesion, locomotion, invasion, and membrane lysis (Bouzid et al., 2013). The process of attachment, invasion, and replication causes disintegration of the microvilli at the sites of attachment (Pohlenz et al., 1978), which disrupts epithelial barrier function (Adams et al., 1994).

In addition, the innate immune system, including intestinal epithelial cells, dendritic cells (Auray et al., 2007), interferon gamma, natural killer cells (Chen et al., 1993), phagocytes (Takeuchi et al., 2008), nitric oxide (Gookin et al., 2004), and the complement

system (Kelly et al., 2000) play a role in immunity against the parasite. The adaptive immune system responds primarily through helper T cells (Aguirre et al., 1994), with Th1 cytokines like IL-12 (Urban et al., 1996) and IL-18 (Ehigiator et al., 2005) predominating.

Symptoms of clinical cryptosporidiosis include non-pathognomonic signs of acute watery diarrhea, nausea, vomiting, and a low grade fever (Current and Garcia, 1991). In healthy, immunocompetent hosts, infection is self-limiting. In immunosuppressed hosts, like patients suffering from AIDS, a chronic and frequently fatal infection is seen, which may involve body systems other than the gastrointestinal tract, such as the biliary system and respiratory system (Hunter and Nichols, 2002; López-Vélez et al., 1995).

Epidemiology of human infections

Human cryptosporidiosis occurs world-wide and pathogenic *Cryptosporidium* species, that cause clinically relevant disease, can be found and transmitted from many different hosts (Meinhardt et al., 1996). *Cryptosporidium parvum* and *C. hominis*, are the most common species that affect humans, while *C. meleagridis, C. felis*, and *C. canis* are the others that are found in order of importance; although more than 20 species have been known to cause clinical cryptosporidiosis in humans (Ryan et al., 2014; Xiao and Feng, 2008).

Diarrhea caused by *Cryptosporidium* is especially severe in neonates in the age group of 12-23 months in low-income countries (Kotloff et al., 2013). The immunocompromised, particularly those with AIDS, primary immunodeficiency disorders, malignant diseases, organ transplants, and those who are malnourished are at the highest risk (Hunter and Nichols, 2002).

Children and immunocompromised patients show acute enterocolitis (Nime et al., 1976) and severe, debilitating diarrhea (Meisel et al., 1976) that is often fatal. The disease is the second most frequent cause of fatal diarrhea in children younger than 5 years in low-income countries in Sub Saharan Africa and Asia (Kotloff et al., 2013). Because of the link

to poverty and childhood mortality, there has been a call for urgent investment in research on cryptosporidiosis (Striepen, 2013).

CryptoNet, a CDC initiative, is a network used to track outbreaks in the United States using molecular data (CDC, 2013). It is based on the Bionumerics platform and works by providing expertise for the differentiation and tracking of outbreaks of *Cryptosporidium* by sequencing the18S rRNA and gp60 genes (CDC, 2013).

There is no vaccine or consistently effective drug treatment for cryptosporidiosis. Pharmaceutical control options in humans are limited to nitazoxanide, paromomycin, and azithromycin, which have limited effectiveness, particularly in the immunocompromised, so the identification of new drug targets is a priority (Gargala, 2008). Some vaccines targets have been identified using reverse vaccinology from genome sequences of *C. hominis* and *C. parvum*, and vaccines made using three of these recombinant surface antigens have been shown to generate a strong immune response in mice (Manque et al., 2011). No vaccines have reached the human trial stage so far.

Public health significance of *Cryptosporidium* dissemination by birds

Although *C. parvum* typically infects mammals, viable oocysts can be disseminated by birds, particularly free ranging aquatic birds (Jellison et al., 2007; Majewska et al., 2009). In Maryland, *C. parvum* oocysts from Canada Geese were infective for neonatal mice (Graczyk et al., 1998). The shedding of infective *C. parvum* oocysts by birds could be the result of an active infection or may be a consequence of mechanical transmission. It has been shown that while Canada geese and Peking ducks are refractory to *C. parvum* infection under experimental conditions, oocysts can retain their infectivity following passage (Graczyk et al., 1996; Graczyk et al., 1997).

Non-aquatic birds, such as domestic chickens, ducks, and quails also have been identified as mechanical transporters of *Cryptosporidium parvum*, although they are not the only major sources (Bomfim et al., 2013)

Species and genotypes that parasitize birds

Three species of *Cryptosporidium* have been described in birds. Many avian genotypes have also been described, that may eventually be recognized as separate species when sufficient morphological and biological data are obtained (Ryan, 2010).

Cryptosporidium meleagridis

Cryptosporidium meleagridis was first described in turkey poults with clinical enteritis (Slavin, 1955). Natural infections have subsequently been found in the Indian ring necked parakeet (*Psittacula krameri*) (Morgan et al., 2000), cockatiels (*Nymphus hollandicus*) (Abe and Iseki, 2004), chickens (*Gallus gallus*) (Darabus and Olariu, 2003), and humans (Matos et al., 2004; Pedraza-Diaz et al., 2001; Pedraza-Díaz et al., 2000). *Cryptosporidium meleagridis* is unusual in being infective for mammalian and avian species and it is the third most frequent cause of human cryptosporidiosis after *C. parvum* and *C. hominis* (Chalmers et al., 2009). Infectivity of *C. meleagridis* for humans was recently demonstrated experimentally, and chicken-derived *C. meleagridis* was shown to be infective for mice, calves, pigs, rabbits, and rats, demonstrating that direct transmission from birds to mammals can occur (Chappell et al., 2011; Darabus and Olariu, 2003). In addition, a human derived *C. meleagridis* was shown to be infective for chickens, mice, piglets, and calves, demonstrating that transmission can occur in both directions (Akiyoshi et al., 2003). In further support of this conclusion, a sequential cross transmission study successfully demonstrated the transmission and passage of oocysts of a Hungarian turkey-derived *C. meleagridis* isolate through one-week-old turkey poults, immunosuppressed mice, and seven-day-old chickens (Sréter et al., 2000).

Oocysts of *C. meleagridis* are fully sporulated when they are excreted in feces, and measure of 5.2 µm by 4.6 µm with sporozoites measuring 5.2 µm by 1.4 µm. The oocyst length to width ratio is 1.13 (Lindsay et al., 1989a) (Table 2).

The lifecycle of *C. meleagridis* has been described and is the same as that of *C. parvum* (Akiyoshi et al., 2003).

Cryptosporidium baileyi

Cryptosporidium baileyi was first described in broiler chickens (Current 1986), and was subsequently found to naturally and experimentally infect ostriches (*Struthio camelus*) (Ryan et al., 2003a), ducks (*Anas platyrhynchos*) (Lindsay et al., 1989b), geese (*Branta canadensis*) (Chvala et al., 2006), cormorants (*Phalacrocorax* sps.) (Jellison et al., 2004), otus owls (*Otus scops*) (Molina-Lopez et al., 2010), mixed breed falcons (*Falco rusticolus* x *Falco cherrug*) (van Zeeland et al., 2008), black headed gulls (*Larus ridibundus*) (Pavlásek, 1993), whooping cranes (*Grus vipio*), eastern golden backed weavers (*Ploceus jacksoni*), gray bellied bulbuls (*Pycnonotus* sps.) (Ng et al., 2006), cockatiels (*Nymphicus hollandicus*) (Lindsay et al., 1990), budgerigars (*Melopsittacus undulatus*) (Goodwin and Krabill, 1989), red crowned amazons (*Amazona dufresniana*), channel billed toucans (*Phamphastus vitellinus*) (Ryan et al., 2003a), crested lark (*Galerida cristata*), black billed magpie (*Pica pica*), gouldian finch (*Chloebia gouldiae*), white java sparrow (*Padda oryzivora*), common myna (*Acridotheres tristis*), red billed leiothrix (*Leiothrix lutea*), zebra finch (*Taenipygia guttata*) (Qi et al., 2011) and saffron finch (*Sicalis flaveola*) (Nakamura et al., 2009).

It primarily causes a respiratory infection with high morbidity and mortality. The bursa and cloaca are also infected. Other sites of infection include the conjunctiva, trachea, air sac, intestines, ceca, bursa, and kidneys (Lindsay et al., 1989b).

Oral and intratracheal inoculations of *C. baileyi* oocysts caused infection in the cloaca, bursa of Fabricius, terminal colon, and cecum of chicks. Intratracheal inoculation caused clinical respiratory cryptosporidiosis (Lindsay et al., 1986). Intraocular inoculation of oocysts caused infection of the conjunctival epithelium in chicks but not in turkeys, which developed cloacal infections without clinical signs (Lindsay et al., 1987a). Intraabdominal inoculation produced clinical infection in the airsacs, bursa of Fabricius, and cloaca. Intravenous inoculation did not produce infection (Lindsay et al., 1987b). Per rectal, ingluval

inoculation caused parasite localization in the bursa of Fabricius and cloaca, with marked histological changes in the bursa, and shedding of large numbers of oocysts, but no infection was found in the respiratory tract (Yuan et al., 2014).

Oocysts of *C. baileyi* are excreted fully sporulated and measure 6.6 by 5.0 µm with a shape index of 1.33 (Lindsay et al., 1989a) (Table 2).

Cryptosporidium galli

Cryptosporidium galli primarily infects the proventricular epithelium (Ryan et al., 2003b).

Natural infections of *C. galli* have been detected in chickens (*Gallus domesticus*), grouse (*Tetrastes bonasia rupestris*), capercaillies (*Tetrao urogallus*), Cuban flamingo *Phoenicopterus ruber*), turquoise parrot (*Neophema pulchella*), chestnut finch (*Lonchura castaneothorax*), painted firetail finch (*Emblema pictum*), Parson's finch (*Poephila cincta*) zebra finch (*Taeniopygia guttata*) pine grosbeak (*Pinicola enucleator*) red-cowled cardinal (*Paroaria dominicana*) (Ng et al., 2006; Ryan et al., 2003b), chestnut finch (*Lonchura castaneothorax*) (Morgan et al., 2001), canaries (*Serinus canaria*), cockatiel (*Nymphicus hollandicus*), lesser seed-finches (*Oryzoborus angolensis*)(Antunes et al., 2008), Bohemian waxwing (*Bombycilla garrulous*), silver-eared mesia (*Leiothrix argentauris*)(Qi et al., 2011), green-winged saltator (*Saltator similis*), goldfinch (*Carduelis tristis*), saffron finch (*Sicalis flaveola*), slate-coloured seedeater (*Sporophila schistacea*) and rufous-bellied thrush (*Turdus rufiventris*) (Sevá et al., 2011). Juvenile 9-day-old chickens are susceptible to experimental infection with *C. galli*, with a prepatent and patent period of 25 and 6 days, respectively. In contrast, 40-day-old chickens are not susceptible to experimental infection (Ryan et al., 2003b).

Oocysts of C. *galli* measure 8.0-8.5 µm by 6.2-6.4 µm with a shape index of 1.30, and they are fully sporulated when excreted (Ryan et al., 2003a) (Table 2).

Cryptosporidium avian genotype I

Cryptosporidium avian genotype I was first isolated from a red factory canary (*Serinus canaria*) with no clinical signs in Western Australia (Ng et al., 2006) . It was subsequently reported in 3.8% and 4.1% of captive Indian peafowl (*Pavo cristatus*) and canaries (*Serinus canaria*) in Brazil respectively (Nakamura et al., 2009).

This genotype shares 99.5% and 95.7% sequence similarity with *C. baileyi* at the 18S rRNA and actin loci, respectively (Ng et al., 2006).

Cryptosporidium avian genotype II

Cryptosporidium avian genotype II has been identified only in psittacines like major mitchell cockatoos (*Cacatua leadbeateri*), eclectus (*Eclectus roratus*), cockatiels (*Nymphicus hollandicus*), sun conures (*Aratinga solstitialis*), princess parrots (*Polytelis alexandrae*), galahs (*Eolophus roseicapilla*), alexandrine parrots (*Psittacula eupatria*) (Ng et al., 2006), white-eyed parakeet (*Aratinga leucophthalma*) (Sevá et al., 2011) and ostriches (*Struthio camelus*) (Meireles et al., 2006), suggesting that the host range may be restricted to these birds. None of the infected birds showed clinical signs of cryptosporidiosis.

The prevalence of avian genotype II in ostriches in central Vietnam was 23.7%. The highest prevalence was found in juvenile birds of the age group 61 – 90 days. Prevalence declined sharply to 0% and 5.8% in the 3–12 month and >12 month age categories, respectively (Nguyen et al., 2013). Avian genotype II shares 97.4% and 88.3% identity with *C. baileyi* at the 18S rRNA and actin genes, respectively (Ng et al., 2006).

Cryptosporidium avian genotype III

Cryptosporidium avian genotype III has been identified in cockatiels (*Nymphicus hollandicus*), galahs (*Eolophus roseicapilla*), and sun conures (*Aratinga solstitialis*) in Australia (Ng et al., 2006); cockatiels (*Nymphicus hollandicus*) and red billed magpies (*Urocissa erythrorycha*) in China (Qi et al., 2011); and peach faced lovebirds (*Agapornis roseicollis*) in Japan (Makino et al., 2010).

Birds from Australia infected with avian genotype III showed no clinical signs. However, avian genotype III infection of the proventricular epithelium of peach faced lovebirds (*Agapornis roseicollis*) in Japan was associated with chronic vomiting, weight loss, and thickened proventriculus (Makino et al., 2010).

Avian genotype III shares 98.7%, 98.3% and 95.5% identity with the Eurasian woodcock genotype, *C. serpentis*, and *C. galli*, respectively, at the 18S rRNA gene locus, and 98.5%, 94.6%, and 96.6% identity, respectively, at the actin locus (Ng et al., 2006).

Cryptosporidium avian genotype IV

Cryptosporidium avian genotype IV has been identified in a Japanese white eye (*Zosterops japonica*) with anorexia and diarrhea (Ng et al., 2006). This genotype shares 96.5% sequence similarity with *C. galli* at the 18S rRNA gene. No other genes have been sequenced from this isolate and no other isolates have been reported.

Cryptosporidium avian genotype V

Cryptosporidium avian genotype V was first identified in cockatiels in Japan (Abe and Makino, 2010) and was later described in blue fronted amazons (*Amazona aestiva*) in Brazil (Nakamura et al., 2014). Japanese isolates shared 99.7%, 98.3%, and 97.8% similarity, respectively, with avian genotype II, avian genotype I, and *C. baileyi* at the 18S rRNA gene. (Abe and Makino, 2010).

Oocysts of avian genotype V measure 5.8 µm (range: 5.0 – 6.6 µm) by 4.5 µm (range: 4.1 – 5.2 µm) with a shape index of 1.37 (Qi et al., 2011) (Table 2).

Cryptosporidium Eurasian woodcock genotype

Cryptosporidium Eurasian woodcock genotype has been identified in a wild caught Eurasian woodcock (*Scolopax rusticola*) housed at the Prague Zoo. The bird died during a quarantine period and endogenous stages were detected in the proventriculus at necropsy (Ryan et al., 2003a).

The Eurasian woodcock genotype genotype shares 95.5% sequence similarity with *Cryptosporidium galli* at the 18S rRNA locus (Ryan et al., 2003a).

Oocysts of the Eurasian woodcock genotype measure were 8.5 µm by 6.4 µm , with a shape index 1.32 (Ryan et al., 2003a) (Table 2).

Cryptosporidium duck genotype

The *Cryptosporidium* duck genotype was first isolated from a black duck (*Anas superciliosa*) in Australia (Morgan et al., 2001), and was later found in Canada geese (*Branta canadensis)* in Ohio, USA (Zhou et al., 2004). This genotype is 96% similar to *Cryptosporidium baileyi* at the 18S rRNA locus (Zhou et al., 2004).

Goose genotypes

Goose genotypes I and II were identified in 17.2% and 4.3% of Canada geese, respectively in the USA (Zhou et al., 2004).

Goose genotypes III and IV have been identified in Canada geese from New York and Illinois, USA (Jellison et al., 2004).

Lifecycle of avian *Cryptosporidium*

Although the first avian *Cryptosporidium* species, *C. meleagridis*, was reported by (Tyzzer, 1929), it was neither named nor was its lifecycle described; it was finally recognized as an unique species from infected turkeys in 1955, but the localization of its endogenous stages were not described until 1994 (Pavlasek, 1994; Slavin, 1955).

Current et al., (1986) described the lifecycle of *C. baileyi* from infected broiler chickens. Although similar to *C. parvum*, *C. baileyi* additionally produces type III meronts with eight merozoites, and develops in the ileum and large intestine before spreading to the cloaca and bursa of Fabricius by day four post infection.

Over the course of the last two decades, several other ultrastructural studies of the lifecycle stages of avian *Cryptosporidium* have been published, but the species studied were not described (Itakura et al., 1985; Tadeja-Simborio and Itakura, 1993; Tadeja-Simborio et al., 1993).

Table 2: Oocyst morphology of *Cryptosporidium* species and genotypes infecting birds

		Oocyst morphology			
Species / Genotype	**Site of infection**	**Length (**µm)	**Width (**µm)	**Shape index**	**References**
C. meleagridis	Small intestine	5.2 (5.0 - 5.6)	4.6 (4.5 – 4.8)	1.13 (1.07 – 1.18)	(Lindsay et al., 1989a), (Tacconi et al., 2001)
C. baileyi	Cloaca, Bursa, Trachea	6.6	5.0	1.33	(Lindsay et al., 1989a)
C. galli	Proventriculus	8.25 (8.0 - 8.5)	6.3 (6.2 -6.4)	1.3	(Ryan et al., 2003b)
avian genotype I	NA	NA	NA	NA	NA
avian genotype II	NA	6.6 (6.0-6.5)	6.5 (4.8 -6.6)	1-1.25	(Meireles et al., 2006; Ng et al., 2006)
avian genotype III	NA	7.5	6.0	1.25	(Ng et al., 2006)
avian genotype IV	NA	8.25	6.3	1.30	(Ng et al., 2006)
avian genotype V	NA	5.8 (5.0 -6.6)	4.5 (4.1-5.2)	1.37 (1.16 – 1.53)	(Qi et al., 2011)
Eurasian woodcock genotype	Proventriculus	8.5	6.4	1.32	(Ryan et al., 2003a)

NA = Not available

Problem statement

There are approximately 5,400 extant mammalian species in 29 orders (Wilson and Reeder, 2005) and about 10,000 species of birds in 26 orders. Of the 26 *Cryptosporidium* species described, 18 have mammalian species as their type hosts and only 3 have avian species as their type host (Ryan et al., 2014). Eight species and 27 genotypes of *Cryptosporidium* have been described in members of the class Rodentia, which comprises more than 2,200 species that correspond to 40% of mammalian diversity (Kváč et al., 2014b). However, only 3 species and 3 genotypes have been described in the order Passeriformes, the most diverse avian order with more than 5,700 extant species,

accounting for 60% of avian diversity. There is a gap in our understanding of the diversity of *Cryptosporidium* in birds in general, and in the passerines in particular. There is no molecular data of *Cryptosporidium* infections available from passerines in North America. Also, the public health implications of the vectorial capacity of birds has not been fully elucidated. Thus, a greater knowledge of *Cryptosporidium* diversity in birds will enhance our understanding of how this parasite has co-evolved with its hosts, and will enhance our knowledge of the potential sources of human cryptosporidiosis.

THESIS OBJECTIVES

1. Determine the prevalence of *Cryptosporidium* in red-winged blackbirds (*Agelaius phoeniceus*), a member of the order Passeriformes that is native to North America, and characterize isolates at multiple genetic loci.
2. Undertake the morphological and biological characterization of *Cryptosporidium* from red-winged blackbirds.

CRYPTOSPORIDIUM IN NORTH AMERICAN RED-WINGED BLACKBIRDS *(AGELAIUS PHOENICEUS)*

Introduction

Cryptosporidium is an apicomplexan parasite that infects all major vertebrate groups (Ng et al., 2006; Nime et al., 1976; Wang et al., 2014; Widerström et al., 2014) and causes the disease cryptosporidiosis. Between 26 and 30 species are recognized; in addition, more than 70 genotypes have been described from sequences of rRNA and protein coding genes (Ryan et al., 2014; Šlapeta, 2013). Two major clades are evident in *Cryptosporidium* phylogenies: a basal clade comprising species that infect the gastric epithelium and a clade comprising species that infect the intestinal epithelium (Xiao et al., 2002). Gastric species tend to have larger oocysts than the intestinal species (Upton and Current, 1985).

Cryptosporidium species exhibit varying specificities for hosts, and the factors contributing to host specificity are poorly understood. Although the intestinal species *C. hominis* and *C. parvum*, which collectively cause more than 90% of human cryptosporidiosis cases, have the same genome organization and share approximately 98% nucleotide sequence similarity, they have very different host ranges. Natural *C. hominis* infections are rarely detected in non-human hosts; in contrast, *C. parvum* is a broadly specific zoonotic pathogen, infecting neonatal ruminants and a number of other mammal species in addition to humans (Feng et al., 2007; Hajdušek et al., 2004; Quah et al., 2011). Other *Cryptosporidium* species, such as *C. suis* in pigs and *C. cuniculus* in rabbits, appear to have a narrow host range yet are capable of causing human disease (Hadfield and Chalmers, 2012; Zhang et al., 2013).

Cryptosporidium has been identified in 17 of the 26 extant avian orders (Kváč et al., 2014b). Passeriformes, the largest avian order, comprises almost 60% of avian diversity, in 96 families, with more than 1200 genera, and 5700 species (Ericson et al., 2014). Passerines have been identified as hosts of the three avian-adapted species *C. galli* (Ryan,

2010; Ryan et al., 2003b; Sevá et al., 2011), *C. meleagridis* (Slavin, 1955), and *C. baileyi* (Current et al., 1986; Qi et al., 2011; Sevá et al., 2011). They also host *Cryptosporidium* avian genotype I (Nakamura et al., 2009), avian genotype III (Gomes et al., 2012; Nakamura et al., 2014; Qi et al., 2011), and avian genotype IV (Ng et al., 2006). Less frequently, they have been identified as hosts of *C. parvum* (Gomes et al., 2012).

There have been no reports to date identifying the *Cryptosporidium* species or genotypes infecting North American passerines. However, cryptosporidiosis has been diagnosed in North American passerines based on histopathology or detection of oocysts. Endogenous developmental stages were detected in the proventriculus of an Australian diamond fire tail finch (*Stagonopleura guttata*) following necropsy. The bird, which was from a pet store aviary, had died from acute onset of severe diarrhea (Blagburn et al., 1990). Cryptosporidiosis also has been diagnosed in white lored euphonias (*Euphonia chrysopata*), bronze mannikin finches (*Lonchura cucullata*), black-throated finch (*Poephila cincta*), and cliff swallows (*Petrochelidon pyrrhonota*) (Gardiner and Imes, 1984; Ley et al., 2012; Lindsay et al., 1991).

Red-winged blackbirds (*Agelaius pheonicius*) are members of the New World passerine family Icteridae, which has 95 species in 23 genera, including oropendolas (*Psarocolius* sps.), caciques (*Cacicus* sps.), orioles (*Icterus* sps.), meadowlarks (*Sturnella* sps.), grackles (*Quiscalus* sps., *Hypopyrrhus* sps., *Lampropsar* sps., *Macroagelius* sps.), and cowbirds (*Molothrus* sps.) (Lowther, 1975). Red-winged blackbirds are abundant in North America with a range that extends as far north as Alaska and as far south as Cuba. Higher latitude populations migrate to the lower latitudes in the winter (Ball et al., 1988). They nest in marshes, wetland areas, and hayfields, and populations are influenced by ecological factors such as regional hay production and climatic phenomena such as the North American Oscillation (Weatherhead, 2005). Although there have been no reports of *Cryptosporidium* in North American Icteridae, *C. galli* was identified in chopi blackbirds (*Gnorimopsar chopi*) in Brazil (Nakamura et al., 2014) and *C. baileyi* was identified in a red rumped cacique and

a crested oropendola (*Psarocolius decumanus*) in the Czech Republic (*Cacicus haemorrhous*) (Ryan et al., 2003a).

In the present study, proventricular and intestinal contents from North American red-winged blackbirds were examined for *Cryptosporidium* species by amplification and sequencing of fragments of the 18S rRNA, actin, and heat shock protein 70 (HSP70) genes. Phylogenetic analyses reveal that North American red-winged black birds host a novel *Cryptosporidium* genotype that is closely related to *C. galli* and they may be vectors of the zoonotic pathogen *C. parvum*.

Materials and methods

Sample collection and DNA isolation

Carcasses of 70 red-winged blackbirds originating from areas of Kansas, North Dakota, and Minnesota, were dissected and samples were obtained from the proventriculus and intestine. DNA was isolated from 200 µg of sampled contents by alkaline digestion and phenol-chloroform extraction and purified using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA) as described previously (Feltus et al., 2008; Peng et al., 2003).

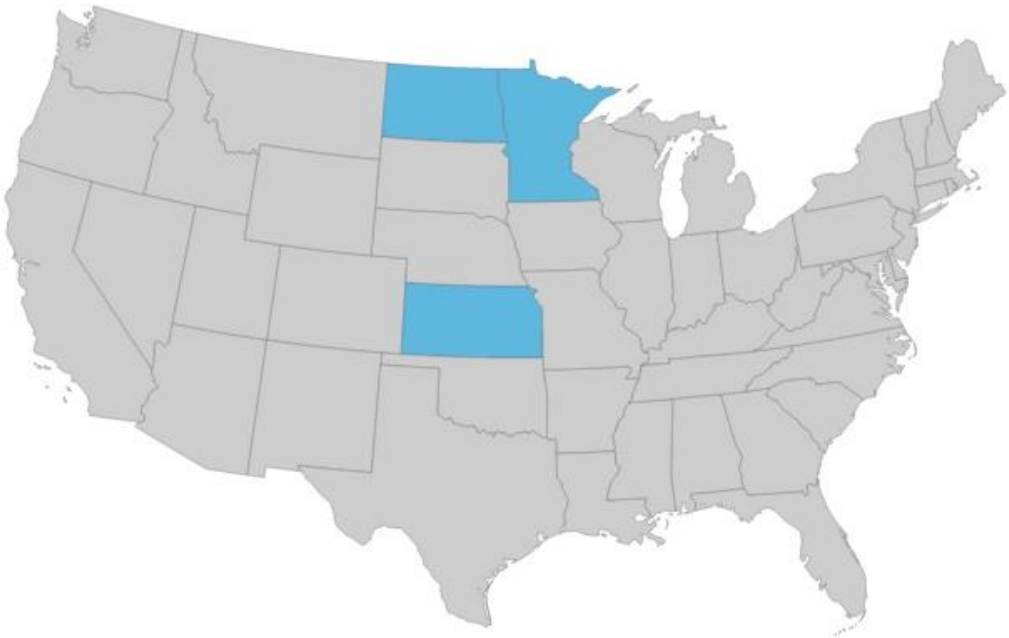

Figure 2. Sampling locations

PCR amplification and sequencing

The 18S rRNA, actin, HSP70, *Cryptosporidium* oocyst wall protein (COWP1), 60 kilodalton glycoprotein (gp60), and thrombospondin related adhesive protein of *Cryptosporidium* 1 (TRAPC1) genes were amplified using nested PCR protocols and the primers listed in Table 3.

An approximately 830 bp fragment of the 18S rRNA gene was amplified as described previously (Sulaiman et al., 2002; Xiao et al., 1999).

An approximately 1066 bp fragment of the actin gene was amplified using the protocol previously described (Sulaiman et al., 2002).

A 515bp fragment of the HSP70 gene was amplified using a nested PCR protocol developed to target gastric *Cryptosporidium* species. Nested PCR primers were designed to be specific for HSP70 sequences that are conserved in *C. galli* (accession no. AY168849), *Cryptosporidium* sp. CzechB1 Eurasian woodcock (accession no. AY273773), *C. muris* (accession no. AF221542), *C. andersoni* (accession no. AY954894), *C. serpentis* (accession no. AF221541), and *C. scrofarum* (accession no. JX424842) (Table 3). The primary reaction was carried out in 1x PCR buffer, 0.2mM of dNTP, 1.5mM of $MgCl_2$, 2.5U of Taq DNA polymerase, 10 pmol of primers HSPAvAF1 and HSPAvAR1, and 0.5µL, 1µL or 2µL of template DNA in a 100µL reaction. The PCR conditions for the primary reaction were initial denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 45 seconds, 52°C for 45 seconds, and 72°C for 1 min; and a final extension at 72°C for 10 min. The secondary reaction was carried out with 1x PCR buffer, 0.2 mM of dNTP, 1.5mM $MgCl_2$, 2.5U Taq DNA polymerase, 10pmol of primers HSPAvAF2 and HSPAvAR2, and 2µL of primary PCR product as DNA template. PCR cycling conditions were the same as the primary reaction with the exception that the annealing temperature was 50°C.

The COWP1 gene was targeted using the protocol described by (Spano et al., 1997).

The gp60 gene was targeted using the protocol described by (Strong et al., 2000).

The TRAPC1 gene was targeted using a protocol previously described by Spano et al. (1998).

DNA from *C. hominis* was used as a positive control for all nested PCR assays. Water was included instead of DNA template as a negative control. Secondary PCR products were visualized using EZ Vision One DNA Dye (Amresco, Solon, OH), following agarose gel electrophoresis. Products of expected size were purified (Wizard SV, Promega, Madison, WI) and stored at 4°C until they were sequenced.

Table 3: Details of primers used in this study

Gene	**Primer name**	**Sequence**	**Reference**
18S rRNA	18-1S For	5' TTC TAG AGC TAA TAC ATG CG	(Xiao et al., 1999)
	18-1S Rev	5' CCC ATT TCC TTC GAA ACA GGA '	
	18-2S For	5' GGA AGG GTT GTA TTT ATT AGA TAA AG	
	18-2S Rev	5' AAG GAG TAA GGA ACA ACC TCC A	
Actin	Actin1 For	5'-ATG RGW GAA GAA GWA RYW CAA GC	(Sulaiman et al., 2002)
	Actin1 Rev	5'-AGA ARC AYT TTC TGT GKA CAA T	
	Actin2 For	5'-CAA GCW TTR GTT GTT GAY AA	
	Actin2 Rev	5'-TTT CTG TGK ACA ATW SWT GG	
HSP70	HSPAvAF1	5'-GCT CGT GGT CCT AAA GAT AA	This study
	HSPAvAR1	5'-ACG GGT TGA ACC ACC TAC TAA T	
	HSPAvAF2	5'-ACA GTT CCT GCC TAT TTC	
	HSPAvAR2	5'-GCT AAT GTA CCA CGG AAA TAA TC	
COWP1	BCOWPR	5'-CGC ACC TGT TCC CAC TCA ATG TAA ACC C	(Spano et al., 1997)
	BCOWPF	5'-ACC GCT TCT CAA CAA CCA TCT TGT CCT G	
	Cry9	5'-GGA CTG AAA TAC AGG CAT TAT CTT G	
	Cry15	5'-GTA GAT AAT GGA AGA GAT TGT G	
gp60	AL3531	5'-ATG GTC TCC GCT GTA ATT C	(Strong et al., 2000)
	AL3535	5'-GGA AGG AAC GAT GTA TCT	
	AL3532	5'-TCC GCT GTA TTC TCA GCC	
	AL3534	5'-GCA GAG GAA CCA GCA TC	
TRAPC1	CpE	5'-GGA TGG GTA TCA GGT AAT AAG AA	(Spano et al., 1998)
	CpZ	5'-CAA CTA GCC CAG TTC TGA CTC TCT GG	
	TrapSecF	5'-AGA AAG TTG TAA TAA AGA TGT GGA ATG	
	TrapSecR	5'-CCA ATT TCT TGA ACA TAT TCT GAG TG	

Sequencing and phylogenetic analyses

Purified amplicons were sequenced directly in both directions using a BigDye Terminator v3.1 cycle sequencing kit with secondary PCR primers in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Carlsbad, CA).

Sequences were assembled using SeqMan (DNAStar, Madison, WI), aligned with previously published sequences using the MAFFT version 7 online server with automatic selection of alignment mode (**http://mafft.cbrc.jp/alignment/server/**) (Katoh and Standley, 2013). The evolutionary history of aligned sequences was inferred using neighbor joining (NJ) (Saitou and Nei, 1987) based on the Kimura 2-parameter (K2P) distances model (Kimura, 1980) with pairwise deletions. The bootstrap consensus tree was inferred from 1000 pseudoreplicates. Phylogenetic trees were constructed using MEGA5 (Tamura et al., 2011) and edited for style using Adobe Illustrator CS5.1 (San Jose, CA).

Results

Prevalence of *Cryptosporidium* in red winged blackbirds

Forty out of 140 (28.6%) samples from 25/70 (35.7%) birds were positive for the *Cryptosporidium* 18S rRNA gene. Six out of 70 (8.6%) birds were positive at the proventriculus site only, 4/70 (5.7%) birds were positive at the intestine site only, and 15/70 (21.4%) birds were positive at both sites.

Two *Cryptosporidium* 18S rRNA clades were present in red-winged blackbirds

A NJ tree constructed from sequences of 20 18S rRNA amplicons and representative *Cryptosporidium* 18S rRNA sequences in GenBank showed the presence of two major *Cryptosporidium* clades in red-winged blackbirds (Figure 3A). Sequences comprising one of the clades, which were obtained from 3/70 (4.2%) and 6/70 (8.5%) proventriculus samples and intestine samples, respectively, shared 100% identity with *C. parvum* (accession no. AF161857). The second clade represented a novel genotype, which we have named *Cryptosporidium* red-winged blackbird genotype. This genotype clustered with gastric

Cryptosporidium species and was most similar to *C. galli* and avian genotype IV, sharing 97.8 ± 0.6% and 96.3 ± 1.0% sequence similarity, respectively (Figure 3B). The red-winged blackbird genotype was identified in 7/70 (10%) and 4/70 (5.7%) of proventriculus and intestine samples, respectively. Two birds (2.8%) had the red-winged blackbird genotype at both the proventriculus and intestine sites, and two birds (2.8%) had the red winged black bird genotype at the proventriculus site and *C. parvum* at the intestine site.

Phylogenies of actin and HSP70 sequences were consistent with the 18S rRNA phylogeny.

Nested PCR reactions failed to amplify COWP1, TRAPC1, or gp60 genes from samples that were positive for *Cryptosporidium* 18S rRNA.

Nested PCR amplification for the actin gene was successful in 13/40 samples that were positive for the *Cryptosporidium* 18S rRNA gene. Of these, only three were successfully sequenced.

Similar to the 18S rRNA phylogeny, actin sequences from this study formed an intestinal clade with *C. parvum* and a gastric clade that was most similar to *C. galli*. The actin sequence that clustered with *C. parvum* was from proventriculus sample PV-3551, which had been identified as the red-winged blackbird genotype at the 18S rRNA locus. IN-3552, an intestine sample from the same bird as PV-3551, was positive for *C. parvum* at the 18S rRNA locus, suggesting that the bird had a mixed infection of the red-winged blackbird genotype and *C. parvum*. The actin sequence of the red-winged blackbird genotype shared 99.4 ± 0.3%, 96.0 ± 0.7%, and 93.0 ± 1.0% similarity with *C. galli, C. serpentis*, and *C. muris*, respectively (Figure 4).

The HSP70 gene was amplified from 7/40 samples that were positive for the *Cryptosporidium* 18S rRNA gene, and sequences were obtained from five amplicons. The HSP70 gene was not amplified from *C. parvum*, which was expected because the primers were designed to be specific for gastric *Cryptosporidium* spp. Similar to the 18S rRNA and actin phylogenies, HSP70 sequences clustered with the gastric *Cryptosporidium* species,

sharing 98.0 ± 0.3%, 94.6 ± 1.1%, and 92.0 ± 1.3% similarity, respectively, with *C. galli, C. serpentis*, and *C. muris* (Figure 5).

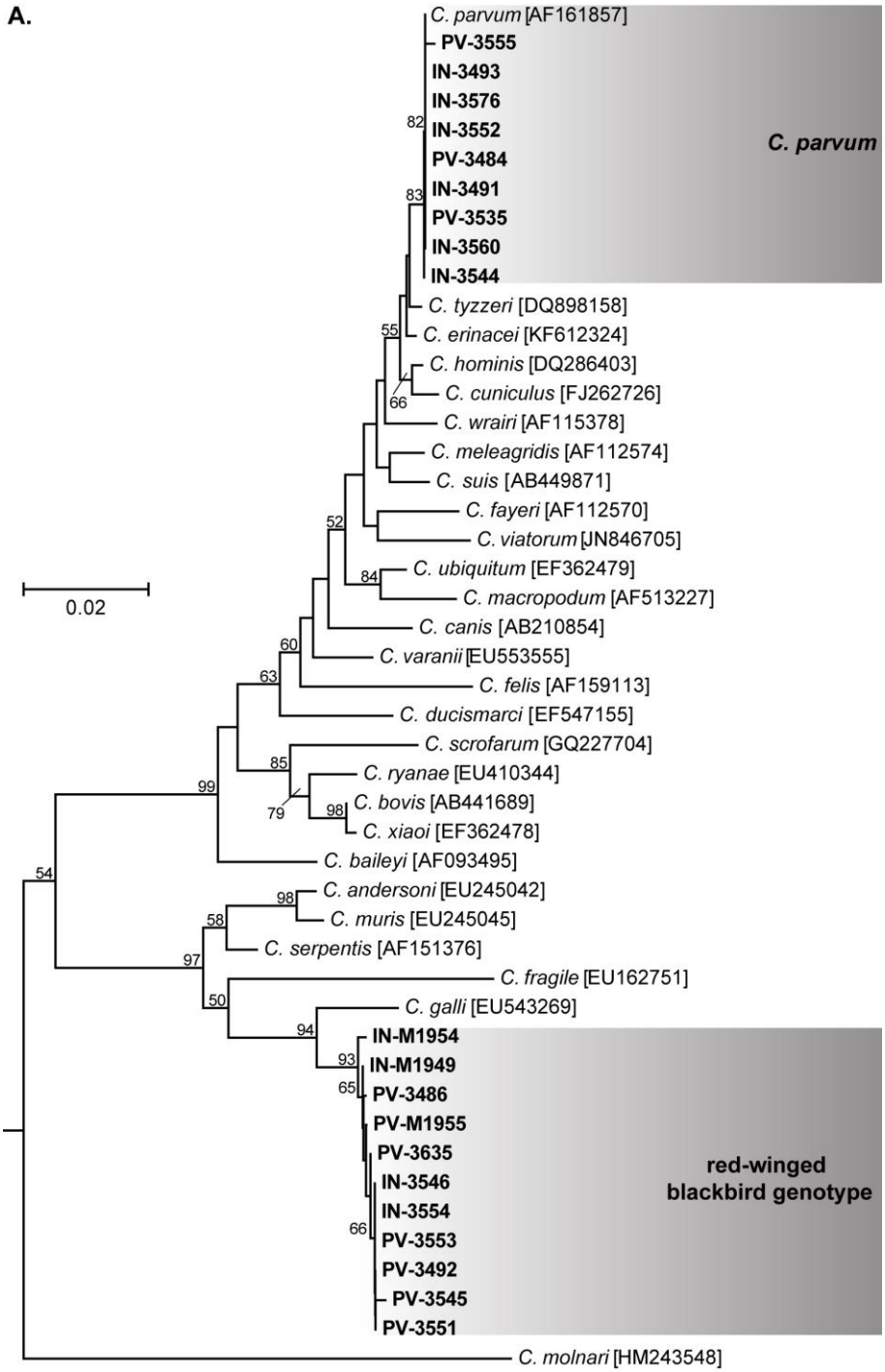

Figure 3. Neighbor-joining tree of 18S rRNA sequences from this study and [A] described *Cryptosporidium* species or [B] *Cryptosporidium* species and genotypes identified previously in birds

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. Only values >50% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The tree was rooted with 18S rRNA from *Monocystis agilis* (accession no. AF457127). Sequences from this study are bolded. The prefix 'PV' indicates a sequence obtained from a proventriculus sample. The prefix 'IN' indicates a sequence obtained from an intestinal sample.

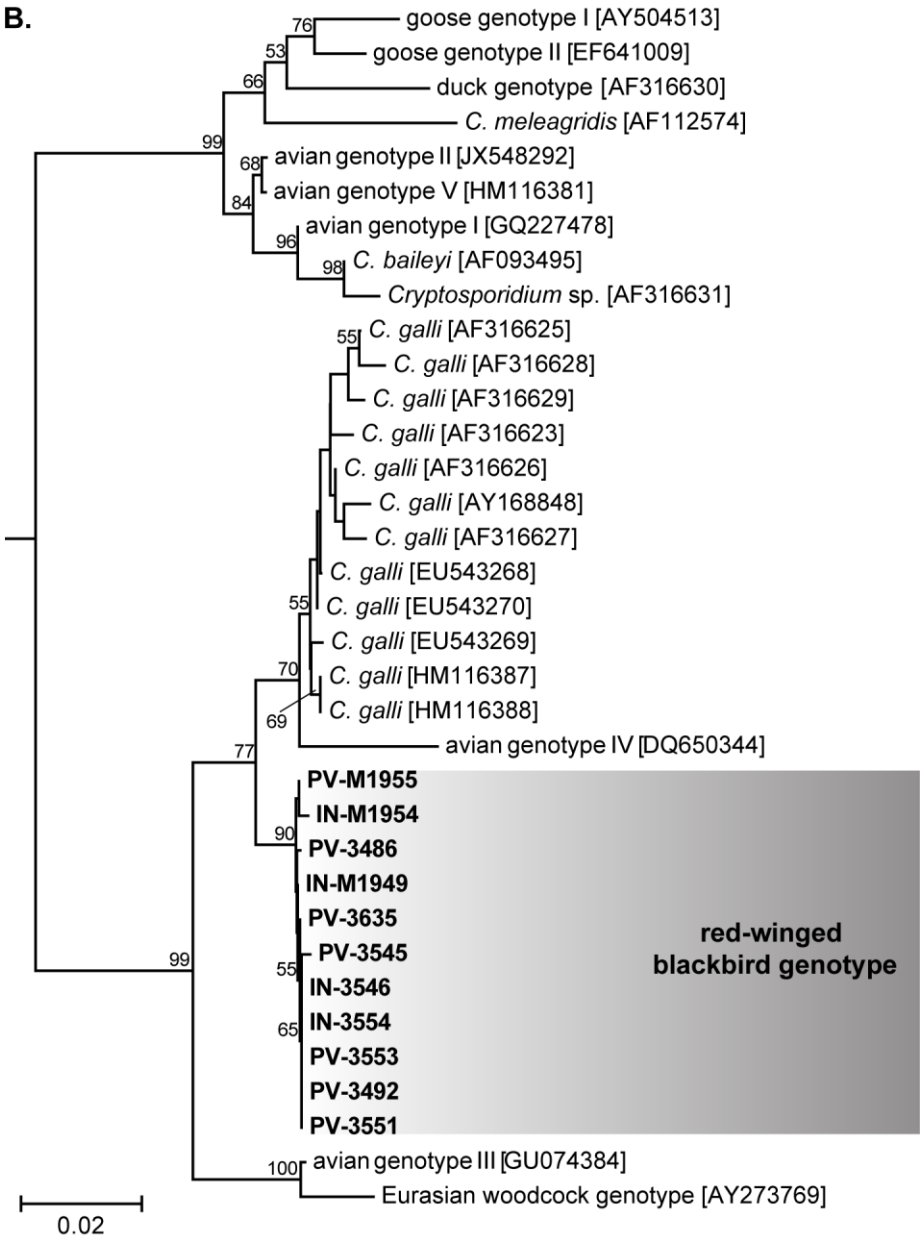

Figure 3. Neighbor-joining tree of 18S rRNA sequences from this study and [A] described *Cryptosporidium* species or [B] *Cryptosporidium* species and genotypes identified previously in birds (continued)

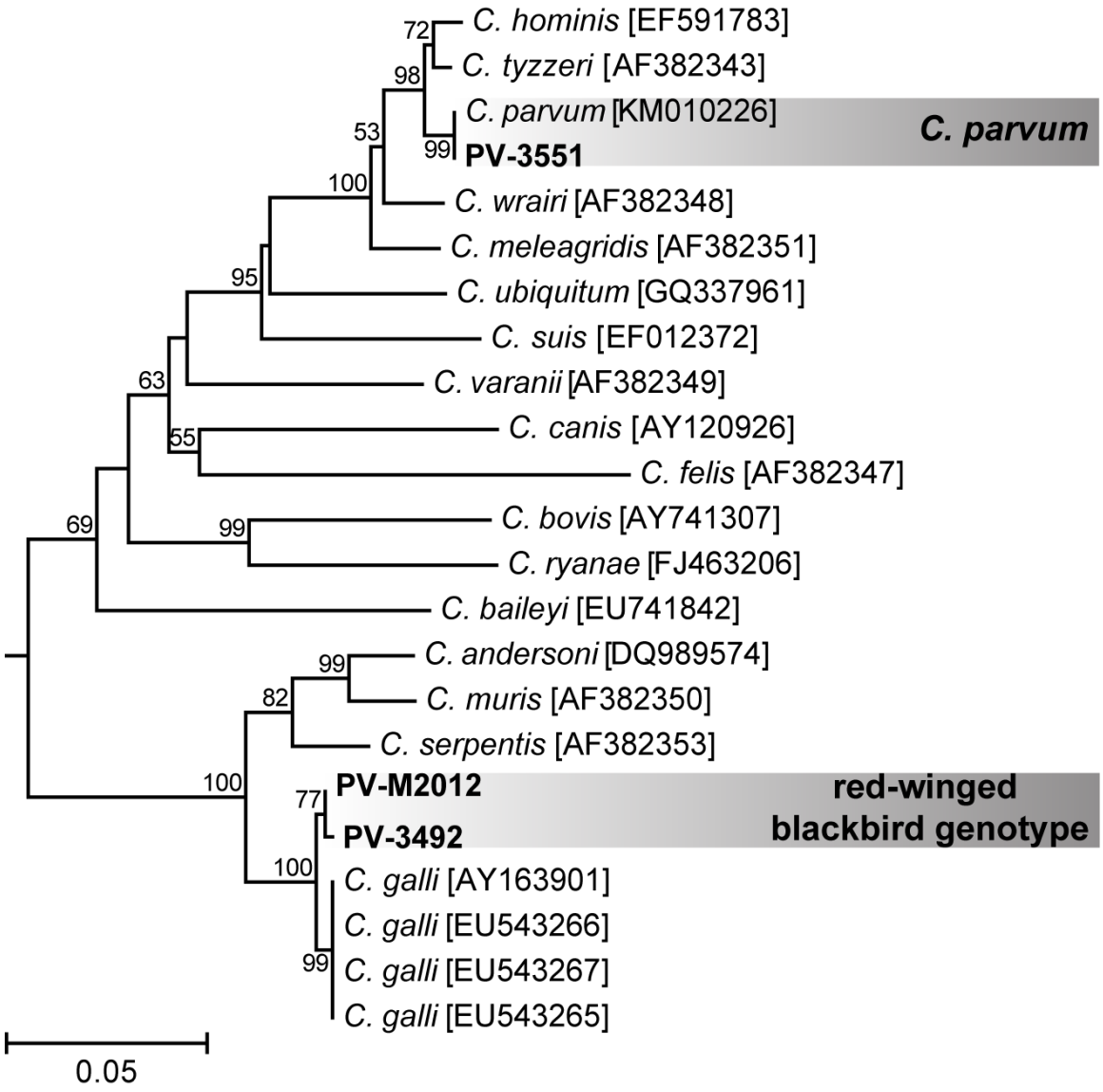

Figure 4. Neighbor-joining tree of actin gene sequences
The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. Only values >50% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The tree was rooted with 18S rRNA from Monocystis agilis (accession no. AY391264). Sequences from this study are bolded. The prefix 'PV' indicates a sequence obtained from a proventriculus sample. The prefix 'IN' indicates a sequence obtained from an intestinal sample.

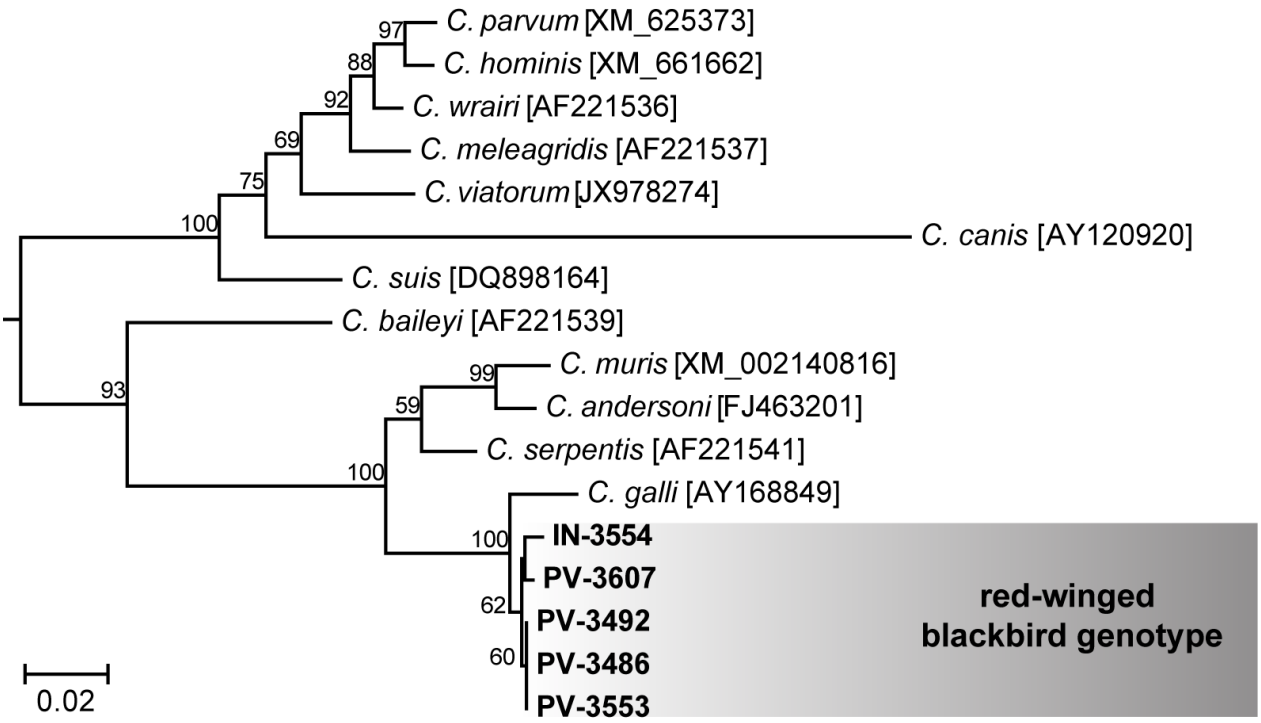

Figure 5. Neighbor-joining tree of heat shock protein 70 gene sequences
The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. Only values >50% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The tree was rooted with 18S rRNA from *Plasmodium falciparum* [accession no. M19753]. Sequences from this study are bolded. The prefix 'PV' indicates a sequence obtained from a proventriculus sample. The prefix 'IN' indicates a sequence obtained from an intestinal sample.

Discussion

This is the first study to isolate and characterize *Cryptosporidium* spp. from a wild North American passerine species. The findings contribute to the understanding of *Cryptosporidium* diversification in birds and suggest that red-winged blackbirds may play a role in the dissemination of human- and livestock-pathogenic *C. parvum*.

More than one in three free-living red-winged blackbirds (35.7%) were positive for *Cryptosporidium* spp. Most previous studies on *Cryptosporidium* in passerines were carried out on captive birds, limiting the value of comparisons with the present study. The only

other study to determine the prevalence of *Cryptosporidium* solely in passerines, reported a rate of 56.5% of all avian gastric *Cryptosporidium* species, but a mixed group of captive and wild passerines were used (Nakamura et al., 2014). The *Cryptosporidium* prevalence in wild red-winged blackbirds was higher than the 6.8% prevalence reported in wild Canada Geese from various US States (Jellison et al., 2004), but lower than the 49% reported in wild ducks in New Mexico, suggesting that the *Cryptosporidium* prevalence in North American migratory birds is variable (Kuhn et al., 2002). But, these comparisons may not yield valuable information, because in the first study, actual sites of infection in the bird are not accounted for, because fecal samples were collected from the environment, and although novel isolates formed a clade distinct from the basal gastric clade or grouped with *C. baileyi*, which can cause extraintestinal infections, differences in actual infection site cannot be determined (Jellison et al., 2004). In the second study, no sequencing was performed (Kuhn et al., 2002).

The extent of genetic diversity among the *Cryptosporidium* species infecting birds is limited compared to that in mammals, and proportionately more gastric *Cryptosporidium* spp. have been identified in birds. Sixteen of the species that primarily infect mammals (*C. cuniculus, C. hominis, C. parvum, C. wrairi, C. fayeri, C. viatorum, C. canis, C. macropodum, C. suis, C. ubiquitum, C. felis, C. scrofarum, C. ryanae, C. xaioi, C. bovis, C. erinacei)* belong to the intestinal clade, while *C. muris* and *C. andersoni* are the only two gastric species found in mammals. Three species and about twelve genotypes infect birds. Of the three species, *C. meleagridis* is intestinal, *C. galli* is proventicular and *C. baileyi* is capable of causing extraintestinal infections . In previous studies, *C. galli*, *C. meleagridis, C. baileyi,* avian genotype I, avian genotype III and avian genotype IV have been found to be naturally present in the Passeriformes (Antunes et al., 2008; Nakamura et al., 2014; Nakamura et al., 2009; Ng et al., 2006; Qi et al., 2011; Sevá et al., 2011). On the 18S rRNA phylogenetic trees, *C. meleagridis, C. baileyi* and avian genotype I cluster within the intestinal clade, whereas *C. galli*, red-winged black bird genotype (this study), avian

genotypes III and IV cluster within the monophyletic gastric species. This data on diversity is obtained from studies conducted in Asia, Europe, Australia, and South America, as no molecular data of *Cryptosporidium* has been available from passerines that live in North America thus far.

The finding that red-winged blackbirds are carriers of *C. parvum* suggests that these birds could play a role in the dissemination of this important human pathogen. Red-winged blackbirds cross the major cattle-producing states of Nebraska and Kansas during migration and are frequently observed on cattle feedlots at certain times of the year, where they are exposed to infected cattle and their feces. Point prevalence studies estimate that 0.99 to 1.08 % of feedlot pens from the central and western United States had *C. parvum* in fecal matter, with about 1.3 to 3.6 *Cryptosporidium* oocysts per gram of feces on average, and an estimated oocyst output of 2.8×10^4 to 1.4×10^5 oocysts per animal per day (Atwill et al., 2006). It has been shown that when *Cryptosporidium* oocysts remain infective following passage through refractory avian hosts such as ducks and geese (Graczyk et al., 1996; Graczyk et al., 1997). Water-associated birds such as seagulls and Canada geese (*Branta canadensis)* can act as carriers of *C. parvum* and potentially be a source of viable oocysts in water catchment and wetland areas (Graczyk et al., 1998; Smith et al., 1993). In multispecies agricultural complexes, mechanical vectors can carry infectious oocysts of *Cryptosporidium* to other surfaces (Conn et al., 2007). Red-winged blackbirds could be mechanical vectors that transmit *Cryptosporidium* oocysts from cattle feedlots to wetland areas, given their habitat and migratory habits.

In summary, a novel *Cryptosporidium* genotype that clustered with gastric *Cryptosporidium* spp. at the 18S rRNA, actin, and HSP70 loci, was identified in North American red-winged blackbirds. Consistent with its phylogenetic position, the prevalence of the genotype in proventriculus samples was almost twice that in intestinal samples. Human pathogenic *C. parvum* also was detected in red-winged blackbirds, and the frequency in intestinal samples was twice that in proventricular samples. These findings

broaden our understanding of the *Cryptosporidium* species and genotypes in North American passerines.

ATTEMPTED MORPHOLOGICAL AND BIOLOGICAL CHARACTERIZATION OF THE *CRYPTOSPORIDIUM* RED-WINGED BLACKBIRD GENOTYPE

Introduction

The genetic diversity in *Cryptosporidium* has been increasingly well described over the past decade because of the widespread use of molecular tools to characterize isolates (Kváč et al., 2014b). More than 70 genotypes have been identified, primarily from sequences of the 18S rRNA locus. However, a genotype is a temporary descriptor and is not a valid taxonomic classification (Plutzer and Karanis, 2009); therefore, a critical next step to clarify taxonomy and better understand evolution is to undertake the morphological and biological characterization that is necessary to describe genotypes as new species or assign them to already described species. Phylogenies have shown that many genotypes are genetically more different from recognized species, than recognized species are from each other, and it is therefore anticipated that they will be described as separated species.

The standard of evidence to support descriptions of new *Cryptosporidium* species has been agreed by research workers in the field (Egyed et al., 2003; Jirků et al., 2008). The evidence includes (a) a description of oocyst morphology including length, width and oocyst index, (b) sequence information from multiple genetic loci, (c) a description of host range, predilection site, and patent and prepatent periods from natural and experimental infections, and (d) adherence to International Commission on Zoological Nomenclature (ICZN) rules. Using this standard, a number of new species have been described in recent years, including *C. ryanae* in cattle (previously deer-like genotype; Fayer et al., 2008), *C. scrofarum* in pigs (previously pig genotype II; Kváč et al., 2013), *C. erinacei* in hedgehogs (previously hedgehog genotype; Kváč et al., 2014a), and *C. galli* in birds (previously various finch genotypes (Ryan et al., 2003b).

The experimental studies necessary to describe a new species require an adequate supply of purified oocysts. In many cases, oocysts numbers from natural infections are

insufficient to meet the needs of experimental studies; therefore, a method is required to propagate the isolate. Although *in vitro* cultivation can be used to study the endogenous development of some *Cryptosporidium* spp., this approach cannot be used for propagation because of inadequate oocysts production: the number of oocysts produced during *in vitro* infection does not exceed the oocysts used to establish the infection (Arrowood, 2002). Therefore, propagation of *Cryptosporidium* oocysts must be carried out *in vivo*.

Few *Cryptosporidium* isolates are propagated routinely in animals. Of these, *C. parvum* is most frequently propagated because of its clinical importance and the ease of propagation in various animals including neonatal calves and mice. Limited propagation of *C. hominis* is carried out at a single lab using a gnotobiotic pig model; consequently, oocysts of this species are not easily obtained for research. Other *Cryptosporidium* species are either propagated commercially (e.g. *C. muris* in mice) or for limited periods to support experimental studies (Kváč et al., 2009; Kváč et al., 2008).

Our objective in this study was to determine the oocyst morphology and host specificity of *Cryptosporidium* red-winged black bird genotype in order to address two of the four criteria (parts A and C above) necessary for its description as a new species. Genetic characterization at the 18S rRNA, actin, and HSP70 loci (Chapter 2) fulfilled the requirement for sequence information from multiple genetic loci (part B above). Achieving our objective required propagation of a red-winged blackbird isolate in a susceptible host to obtain sufficient numbers of oocysts for experimental studies. Infectivity studies and studies of oocyst morphology were carried out at the laboratory of Dr. Martin Kváč, Biology Center, Academy of Sciences of the Czech Republic. Although the propagation of red-winged blackbird genotype oocysts was unsuccessful, a number of techniques relating to animal infectivity and oocyst characterization were mastered at the laboratory of Dr. Kváč, and these will be described in this chapter.

Materials and methods

Sample preparation

Eleven samples of the proventriculus from red-winged blackbirds that were positive for *Cryptosporidium* red-winged blackbird genotype DNA (Chapter 2) were sent to the laboratory of Dr. Kváč for biological characterization. Samples were placed in separate 2mL eppendorf tubes, washed twice with sterile PBS (pH 7.0), and pelleted by centrifugation at 3000G for 3 mins. Each tube was individually wrapped with Parafilm M (Bemis NA, Neenah, WI) and placed between absorbent sheets of paper in plastic sealed bags. Samples were further packaged in accordance with IATA shipping protocols and shipped by FedEX to the Laboratory of Veterinary and Medical Protistology (head Dr. M. Kváč) at the Institute of Parasitology BC ASCR, České Budějovice, Czech Republic. All sample tubes were stored at 4°C upon arrival at the Institute of Parasitology BC ASCR. Smears were made from all the positive samples, and examined at high power with oil immersion objectives, after aniline carbol methyl violet staining (Milácek and Vítovec, 1985).

Propagation studies

The red-winged blackbird is not present in the Czech Republic, so we had to consider alternative hosts for oocyst propagation. Because propagation is most likely to be successful between homologous species (Karanis and Aldeyarbi, 2011), we examined experimental hosts with demonstrated susceptibility to *C. galli*, a genetically closely related species. Natural *C. galli* infections have been reported in Passeriformes (O'Donoghue et al., 1987), Galliformes (Antunes et al., 2008; da Silva et al., 2010; Morgan et al., 2001; Nakamura et al., 2014; Nakamura et al., 2009; Ng et al., 2006; Qi et al., 2011; Ryan et al., 2003a; Ryan et al., 2003b; Ryan et al., 2003b; Sevá et al., 2011), and Psittaciformes (Ng et al., 2006; Ryan et al., 2003b). Therefore, representative avian species from these three orders were examined for susceptibility to *Cryptosporidium* red-winged blackbird genotype. These were the domestic chicken (*Gallus domesticus*; Family: Phasianidae; Order: Galliformes), zebra

finch (*Taeniopygia guttata*; Family: Estrildidae; Order: Passeriformes), and cockatiel (*Nymphicus hollandicus*; Family: Cacatuidae; Order: Psittaciformes).

Five-day-old chickens were purchased from a commercial hatchery. The parents were negative for *Cryptosporidium*. The birds were housed in individual cages on deep litter. Litter, water, and feed were changed every day during the study period. Chickens were fed a commercial chicken feed, and purified water *ad libitum*.

Three zebra finches and one cockatiel of unknown age purchased from a commercial pet shop were housed in individual commercial avian pet cages with perches. The cage floor was lined with clean paper that was changed every day, which facilitated the hygienic collection of fecal pellets for analysis. Zebra finches and cockatiels were fed a commercial pet bird feed, and purified water *ad libitum*.

Four out of the five 9-day-old chickens were orally fed red-winged blackbird genotype positive samples mixed with 1 mL of sterile PBS. One chick was used as a negative control, and was left untreated. The negative control chick was housed in a separate cage in a different part of the experimental animal room. Fresh droppings were collected from the birds from days 3 to 12 post infection (p.i.) and stored at 4°C.

Two Zebra finches and one cockatiel were orally fed red-winged Black bird genotype sample diluted with sterile PBS. Fecal samples were collected from each bird from days 3 to 12 p.i. and stored at 4°C. One Zebra finch was used as a negative control, and was left untreated. It was housed in a separate cage in a different part of the experimental animal room.

An isolate of *Cryptosporidium* avian genotype V originally obtained from an infected cockatiel in the Czech Republic was used as a positive control for *Cryptosporidium* infection in the cockatiel. The bird was inoculated orally and fecal samples collected from each bird from days 3 to 12 p.i. and stored at 4°C

Two hundred micrograms of feces from each sample was used to make a fecal smear for staining with aniline carbol methyl violet (Antunes et al., 2008; Milácek and Vítovec,

1985; Nakamura et al., 2009; Ng et al., 2006). Slides were examined at 1,000x total magnification under oil immersion. DNA was extracted from 200 µg fecal samples using the Stratec PSP Spin Stool DNA Kit (Buch, Germany) following bead disruption (Sak et al., 2008). DNA was eluted in 200µL of deionized nuclease free water and stored at -20°C. Nested PCR amplification of the 18S rRNA gene was carried out as described previously (Xiao et al., 1999), and amplified products were visualized following agarose gel electrophoresis on 1% agarose gels.

Results and discussion

Aniline carbol methyl violet stained fecal smears from red-winged blackbird genotype infected birds were negative for oocysts by microscopic examination. In contrast, *Cryptosporidium* oocysts were detected in fecal smears from the cockatiel infected with *Cryptosporidium* avian genotype V (positive control).

None of the fecal samples from birds infected with the red-winged blackbird genotype were positive for amplicons of the 18S rRNA gene. In contrast, amplicons were obtained from fecal samples of the cockatiel infected with avian genotype V. Sequencing of the secondary PCR product from the positive control showed that it was 100% identical to avian genotype V.

The failure to infect chickens, a zebra finch, and a cockatiel with *Cryptosporidium* red-winged blackbird genotype may be due to a lack of susceptibility in the hosts and/or an inadequate infectious dose in the inoculum.

Only 0.2 to 1.2 grams of proventricular and intestinal samples were obtained from red-winged blackbirds because of their small size. Oocyst numbers in the PCR positive samples used to infect birds were below the detection limit of the microscopy-based aniline carbol methyl violet staining method. Methods based on microscopy of non-concentrated fecal smears have detection limits of 50,000 oocysts per gram of feces (Weber et al., 1991), far higher than the detection limit of 10 oocysts per gram by nested PCR (Sturbaum et al.,

2001). The intensity of oocyst shedding in red-winged blackbirds is unknown. Also, no macroscopic changes had been detectable at necropsy.

The infectious dose in inocula could have been further reduced by loss of oocyst viability during transport and storage, as the oocysts were stored in water and subjected to environmental temperature variations (Fayer et al., 1998). Standard microscopy-based methods for viability assessment, including *in vitro* excystation and dye permeability assays, were not possible because oocyst numbers were below levels that were detectable by microscopy (Weber et al., 1991). In addition, purified oocysts are necessary for *in vitro* excystation assays, which would result in further oocyst losses.

The closely related species, *C. galli* was able to infect juvenile birds but not adult chicken (Ryan et al., 2003b) The age at which cockatiels become susceptible to the gastric species - *C. galli* and avian genotype III, is unknown (Antunes et al., 2008; Ng et al., 2006). Similarly, the age at which finches become susceptible to *C. galli* is also unknown (Antunes et al., 2008) The exact age of the zebra finches and cockatiel acquired for this study from the commercial aviaries were unknown, but they were considered adults based on presence of full adult plumage and size.

This study highlights a major impediment to biological studies of novel *Cryptosporidium* genotypes: obtaining sufficient numbers of infectious oocysts for infectivity assays. This can be viewed as a catch-22 paradox because the most effective way to obtain infectious oocysts is from an experimental infection. However, an alternative approach would be to use pooled samples collected on multiple occasions from a naturally infected animal held in captivity. Greater numbers of viable oocysts would be expected following concentration of pooled fecal samples. This approach has the added benefit of purifying oocysts, which should result in greater control of the inoculum and permit microscopy-based estimates of viability.

CONCLUSION

The broad diversity of *Cryptosporidium* spp. infecting vertebrates is testimony to a long history of parasitism. Characterizing diversification, and determining how it differs along the major phylogenetic branches, will increase our understanding of *Cryptosporidium* evolution and coevolution with vertebrate hosts. Because *Cryptosporidium* causes human and livestock disease, much more is known about parasite diversity in mammals than in other major vertebrate groups. A number of *Cryptosporidium* spp. that infect avians, reptiles, and amphibians belong to the significantly understudied gastric clade, which diverged from the intestinal group quite early during *Cryptosporidium* evolution. In addition to oocyst size and infection site, gastric and intestinal species have major differences in their biochemistry. For example, the gastric species, *C. muris* has a working a mitochondrion and a functional TCA cycle and oxidative phosphorylation, all of which have been lost in the intestinal species *C. parvum*. Given these biological differences, it is probable that the mechanisms of, and perhaps even capacity for, diversification in gastric *Cryptosporidium* spp. are entirely different to those in intestinal species. Our study of *Cryptosporidium* spp. in red-winged blackbirds, the first to characterize *Cryptosporidium* in North American passerines, begins to address the gap in knowledge between intestinal and gastric species and between mammalian and avian species.

Coevolution of *Cryptosporidium* with avian hosts

To understand host-parasite coevolution between passerine birds and *Cryptosporidium*, it is necessary to consider the evolution of birds. Most evidence supports the hypothesis that passerines originated in Gondwana and radiated following the breakup of Gondwanaland (Ericson et al., 2003). The passerines are divided into three major monophyletic clades; the basal New Zealand wrens (Acanthisittidae), the suboscines (Tyranni) and the oscines (Passeri and the songbirds). The suboscines are morphologically distinct because of the presence of a bulbular base of the columella bone in the inner ear.

They are considered more primitive than the oscines and include the broadbills and pittas of the Old World, and the manakins and woodcreepers of the New World (Ericson et al., 2003). The ancestor of the passerines split from their sister group the swifts about 76 million years ago (Mya) (Haddrath and Baker, 2012). The New World suboscines diverged from their ancestors when the split between the Old World and New World suboscines occurred 63.6 Mya, and they reached South America about 40 Mya, aided by continental drift and migration (Ericson et al., 2003). They migrated from there to North America 3 Mya (Ericson et al., 2014). Differences in divergence rates have been observed leading to morphologically hypervariable passerine lineages (Barker et al., 2013).

The oscines, comprising the Corvoidea and the Eupasseri, radiated in Australia, spreading northwards aided by the tectonic collision of the Australian and South East Asian plate. According to models, the oscines reached North America during the passerine radiation via the Beringian track, or via Greenland from Europe, and later spread southward into the Americas (Ericson et al., 2003). The core Corvoidea clade is estimated to have radiated around 37.4 Mya (Ericson et al., 2014). The Eupasseri, which comprises the Sylvioidea (larks, swallows, bulbuls, babblers, white-eyes), Muscicapoidea (thrushes, old world flycatchers, starlings), and the Passeroidea (sparrows, finches, nine- primaried oscines, weavers), is estimated to have radiated 43.5 Mya (Ericson et al., 2014).

The New World nine-primaried oscine clade, to which the family Icteridae belongs, is an assemblage of related but highly diverse families comprising 8% of extant avian species, 15% of passerine species, and 17% of New World bird species (Barker et al., 2013). The clade comprises the Fringillidae (finches), Emberizidae (sparrows), Parulidae (warblers), Thraupidae (tangers), Cardinalidae (cardinals) and Icteridae (blackbirds) (Yuri and Mindell, 2002).

To test the hypothesis that *C. galli* and related gastric *Cryptosporidium* spp. coevolved with their passerine hosts, a phylogenetic analysis was constructed from *Cryptosporidium* 18S rRNA gene sequences obtained from birds in different geographic

regions (Figure 6). Since most hosts from which sequences are available were captive species, it is likely that they were host to *Cryptosporidium* spp. from their location of origin (Antunes et al., 2008; da Silva et al., 2010; Nakamura et al., 2014; Nakamura et al., 2009; Ng et al., 2006; Qi et al., 2011; Ryan et al., 2003a).

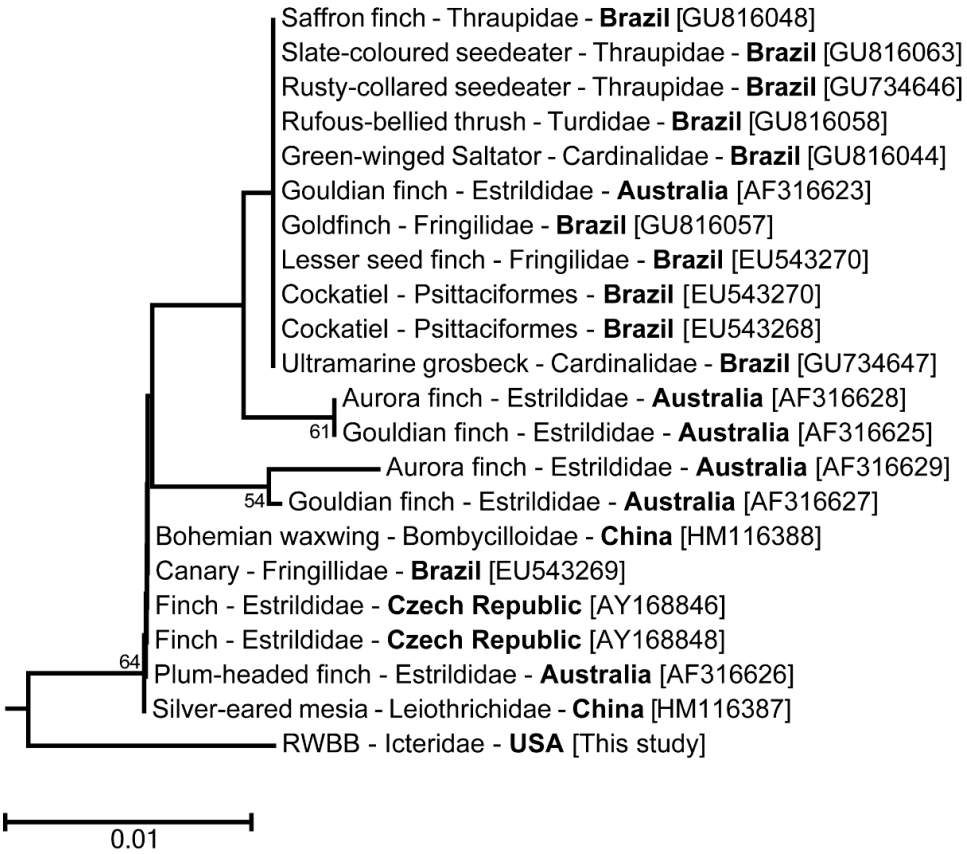

Figure 6. Neighbor-joining tree of 18S rRNA sequences of *Cryptosporidium galli* derived from passerine birds
The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. Only values >50% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The tree was rooted with 18S rRNA from *Cryptosporidium andersoni* [accession no. AB362934]. The family that each bird belongs to is provided, along with the location of sampling.

Cryptosporidium galli sequences that originate from Brazil cluster separately from the sequences that originate from Australia and Czech Republic. The sequence of the red-winged black bird genotype forms a sister group to all the *C. galli* sequences, indicating that

it is evolutionarily distinct. There is a marked diversity among *C. galli* and similar isolates from different continents, which reflects the divergence of hosts, suggesting that the hosts and parasites have coevolved due to geographical separation from other similar populations.

Cryptosporidium red-winged black bird genotype which was isolated in this study, is evolutionarily distinct, and is from a nine-primaried oscines endemic to North America. Since the host is distinct and evolved in isolation from other oscines, the genetic distinctiveness of the novel genotype could be attributed to coevolution with its host.

Taxonomic nomenclature

The criteria for naming a new species in the genus *Cryptosporidium* have been widely accepted and reviewed (Ryan et al., 2003b). These include morphometrics of oocysts, deposition of gene sequences, demonstration of infections in natural and experimental hosts and compliance with ICZN rules. Many genotypes have been redescribed as valid species including *C. xiaoi, C. ubiquitum, C. ryanae, C. erinacei, C. scrofarum, C. hominis, C. macropodum, C. suis, and C. galli* (Egyed et al., 2003; Jirků et al., 2008). However, not all newly described species satisfy the four criteria for a valid species. *Cryptosporidium viatorum* was described in humans with only genetic and morphometric information (Elwin et al., 2012). No biological data are available for this species. Some species that were previously described have been invalidated (Ryan et al., 2014). Most recently, *C. pestis* (Slapeta, 2006), which does not obey the ICZN rule of priority, and *C. ducismarci* (Traversa, 2010), which lacks biological data and a formal description, have been invalidated.

In summary, the work in this thesis has contributed to knowledge of *Cryptosporidium* diversity in, and coevolution with, passerines. The difficulties in obtaining the necessary morphological and biological data to redescribe a genotype as a new species greatly hinder progress towards a more complete understanding of *Cryptosporidium* diversity. The vectorial capacity of birds as carriers of *C. parvum* has been explored, and further work is warranted to address the public health implications of these findings.

REFERENCES

Abe, N., Iseki, M., 2004. Identification of *Cryptosporidium* isolates from cockatiels by direct sequencing of the PCR-amplified small subunit ribosomal RNA gene. Parasitol. Res. 92, 523-526.

Abe, N., Makino, I., 2010. Multilocus genotypic analysis of *Cryptosporidium* isolates from cockatiels, Japan. Parasitol. Res. 106, 1491-1497.

Adams, R.B., Guerrant, R.L., Zu, S., Fang, G., Roche, J.K., 1994. *Cryptosporidium parvum* infection of intestinal epithelium: morphologic and functional studies in an in vitro model. J. Infect. Dis. 169, 170-177.

Aguirre, S.A., Mason, P.H., Perryman, L.E., 1994. Susceptibility of major histocompatibility complex (MHC) class I- and MHC class II-deficient mice to *Cryptosporidium parvum* infection. Infect Immun 62, 697-699.

Akiyoshi, D.E., Dilo, J., Pearson, C., Chapman, S., Tumwine, J., Tzipori, S., 2003. Characterization of *Cryptosporidium meleagridis* of human origin passaged through different host species. Infect Immun 71, 1828-1832.

Alvarez-Pellitero, P., Quiroga, M.I., Sitjà-Bobadilla, A., Redondo, M.J., Palenzuela, O., Padrós, F., Vázquez, S., Nieto, J.M., 2004. *Cryptosporidium scophthalmi* n. sp. (Apicomplexa: Cryptosporidiidae) from cultured turbot *Scophthalmus maximus*. Light and electron microscope description and histopathological study. Dis. Aquat. Organ. 62, 133-145.

Alvarez-Pellitero, P., Sitjà-Bobadilla, A., 2002. *Cryptosporidium molnari* n. sp. (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata L*. and *Dicentrarchus labrax L*. Int. J. Parasitol. 32, 1007-1021.

Antunes, R.G., Simões, D.C., Nakamura, A.A., Meireles, M.V., 2008. Natural infection with *Cryptosporidium galli* in canaries (*Serinus canaria*), in a cockatiel (*Nymphicus*

hollandicus), and in lesser seed-finches (*Oryzoborus angolensis*) from Brazil. Avian Diseases Digest 3, e40-e42.

Appelbee, A.J., Thompson, R.C., Olson, M.E., 2005. *Giardia* and *Cryptosporidium* in mammalian wildlife - current status and future needs. Trends Parasitol. 21, 370-376.

Arrowood, M.J., 2002. In vitro cultivation of *Cryptosporidium* species. Clin. Microbiol. Rev. 15, 390-400.

Atwill, E.R., Pereira, M.D., Alonso, L.H., Elmi, C., Epperson, W.B., Smith, R., Riggs, W., Carpenter, L.V., Dargatz, D.A., Hoar, B., 2006. Environmental load of *Cryptosporidium parvum* oocysts from cattle manure in feedlots from the central and western United States. Journal of environmental quality 35, 200-206.

Auray, G., Lacroix-Lamandé, S., Mancassola, R., Dimier-Poisson, I., Laurent, F., 2007. Involvement of intestinal epithelial cells in dendritic cell recruitment during *C. parvum* infection. Microbes Infect 9, 574-582.

Ball, R.M., Freeman, S., James, F.C., Bermingham, E., Avise, J.C., 1988. Phylogeographic population structure of Red-winged Blackbirds assessed by mitochondrial DNA. Proc Natl Acad Sci U S A 85, 1558-1562.

Barker, F.K., Burns, K.J., Klicka, J., Lanyon, S.M., Lovette, I.J., 2013. Going to extremes: contrasting rates of diversification in a recent radiation of new world passerine birds. Syst Biol 62, 298-320.

Barker, I.K., Carbonell, P.L., 1974. *Cryptosporidium agni* sp.n. from lambs, and *Cryptosporidium bovis* sp.n. from a calf, with observations on the oocyst. Z Parasitenkd 44, 289-298.

Blagburn, B.L., Lindsay, D.S., Hoerr, F.J., Atlas, A.L., Toivio-Kinnucan, M., 1990. *Cryptosporidium* sp. infection in the proventriculus of an Australian diamond firetail finch (*Staganoplura bella*: Passeriformes, Estrildidae). Avian Dis. 34, 1027-1030.

Bomfim, T., Gomes, R., Huber, F., Couto, M., 2013. The Importance of Poultry in Environmental Dissemination of *Cryptosporidium* spp. . The Open Veterinary Science Journa 7, 12 -17.

Bouzid, M., Hunter, P.R., Chalmers, R.M., Tyler, K.M., 2013. *Cryptosporidium* pathogenicity and virulence. Clin. Microbiol. Rev. 26, 115-134.

Carreno, R.A., Martin, D.S., Barta, J.R., 1999. *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. Parasitol. Res. 85, 899-904.

CDC 2013. CryptoNet: Molecular-based Tracking to Better Understand U.S. Cryptosporidiosis Transmission (Atlanta, GA, Centers for Disease Control and Prevention).

CDC/Alexander J. da Silva, P.M.M. 2002. Life cycle of *Cryptosporidium*, the causal agent of Cryptosporidiosis., 3386, I., ed. (Atlanta, GA, USA, CDC), pp. This illustration depicts the life cycle of different species of *Cryptosporidium*, the causal agents of Cryptosporidiosis.

Chalmers, R.M., Elwin, K., Thomas, A.L., Guy, E.C., Mason, B., 2009. Long-term *Cryptosporidium* typing reveals the aetiology and species-specific epidemiology of human cryptosporidiosis in England and Wales, 2000 to 2003. Euro Surveill 14.

Chappell, C.L., Okhuysen, P.C., Langer-Curry, R.C., Akiyoshi, D.E., Widmer, G., Tzipori, S., 2011. *Cryptosporidium meleagridis*: infectivity in healthy adult volunteers. Am J Trop Med Hyg 85, 238-242.

Chen, W., Harp, J.A., Harmsen, A.G., 1993. Requirements for CD4+ cells and gamma interferon in resolution of established *Cryptosporidium parvum* infection in mice. Infect Immun 61, 3928-3932.

Chvala, S., Fragner, K., Hackl, R., Hess, M., Weissenbock, H., 2006. *Cryptosporidium* infection in domestic geese (*Anser anser f. domestica*) detected by in-situ hybridization. J. Comp. Pathol. 134, 211-218.

Conn, D.B., Weaver, J., Tamang, L., Graczyk, T.K., 2007. Synanthropic flies as vectors of *Cryptosporidium* and *Giardia* among livestock and wildlife in a multispecies agricultural complex. Vector Borne Zoonotic Dis 7, 643-651.

Current, W.L., Garcia, L.S., 1991. Cryptosporidiosis. Clin Microbiol Rev 4, 325-358.

Current, W.L., Reese, N.C., 1986. A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. J Protozool 33, 98-108.

Current, W.L., Upton, S.J., Haynes, T.B., 1986. The life cycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. J. Protozool. 33, 289–296.

da Silva, D.C., Homem, C.G., Nakamura, A.A., Teixeira, W.F., Perri, S.H., Meireles, M.V., 2010. Physical, epidemiological, and molecular evaluation of infection by *Cryptosporidium galli* in Passeriformes. Parasitol. Res. 107, 271-277.

Darabus, G., Olariu, R., 2003. The homologous and interspecies transmission of *Cryptosporidium parvum* and *Cryptosporidium meleagridis*. Pol. J. Vet. Sci. 6, 225-228.

Egyed, Z., Sreter, T., Szell, Z., Beszteri, B., Dobos-Kovacs, M., Marialigeti, K., Cornelissen, A.W., Varga, I., 2002. Polyphasic typing of *Cryptosporidium baileyi*: a suggested model for characterization of cryptosporidia. J. Parasitol. 88, 237-243.

Egyed, Z., Sréter, T., Széll, Z., Varga, I., 2003. Characterization of *Cryptosporidium* spp.--recent developments and future needs. Vet Parasitol 111, 103-114.

Ehigiator, H.N., Romagnoli, P., Borgelt, K., Fernandez, M., McNair, N., Secor, W.E., Mead, J.R., 2005. Mucosal cytokine and antigen-specific responses to *Cryptosporidium parvum* in IL-12p40 KO mice. Parasite Immunol 27, 17-28.

Elwin, K., Hadfield, S.J., Robinson, G., Crouch, N.D., Chalmers, R.M., 2012. *Cryptosporidium viatorum* n. sp. (Apicomplexa: Cryptosporidiidae) among travellers

returning to Great Britain from the Indian subcontinent, 2007-2011. Int. J. Parasitol. 42, 675-682.

Ericson, P.G., Klopfstein, S., Irestedt, M., Nguyen, J.M., Nylander, J.A., 2014. Dating the diversification of the major lineages of Passeriformes (Aves). BMC Evol Biol 14, 8.

Ericson, P.G.P., Irestedt, M., Johansson, U.S., 2003. Evolution, biogeography, and patterns of diversification in passerine birds. Journal of Avian Biology 34, 3-15.

Fayer, R., Santín, M., 2009. *Cryptosporidium xiaoi* n. sp. (Apicomplexa: Cryptosporidiidae) in sheep (*Ovis aries*). Vet. Parasitol. 164, 192-200.

Fayer, R., Santín, M., Macarisin, D., 2010. *Cryptosporidium ubiquitum* n. sp. in animals and humans. Vet Parasitol 172, 23-32.

Fayer, R., Santín, M., Trout, J.M., 2008. *Cryptosporidium ryanae* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*). Vet. Parasitol. 156, 191-198.

Fayer, R., Santín, M., Xiao, L., 2005. *Cryptosporidium bovis* n. sp. (Apicomplexa: Cryptosporidiidae) in cattle (*Bos taurus*). J. Parasitol. 91, 624-629.

Fayer, R., Trout, J.M., Jenkins, M.C., 1998. Infectivity of *Cryptosporidium parvum* oocysts stored in water at environmental temperatures. J Parasitol 84, 1165-1169.

Fayer, R., Trout, J.M., Xiao, L., Morgan, U.M., Lai, A.A., Dubey, J.P., 2001. *Cryptosporidium canis* n. sp. from domestic dogs. J. Parasitol. 87, 1415-1422.

Feltus, D.C., Giddings, C.W., Khaitsa, M.L., McEvoy, J.M., 2008. High prevalence of *Cryptosporidium bovis* and the deer-like genotype in calves compared to mature cows in beef cow-calf operations. Vet Parasitol 151, 191-195.

Feng, Y., Alderisio, K.A., Yang, W., Blancero, L.A., Kuhne, W.G., Nadareski, C.A., Reid, M., Xiao, L., 2007. *Cryptosporidium* genotypes in wildlife from a new york watershed. Appl Environ Microbiol 73, 6475-6483.

Gardiner, C.H., Imes, G.D., 1984. *Cryptosporidium* sp in the kidneys of a black-throated finch. J Am Vet Med Assoc 185, 1401-1402.

Gargala, G., 2008. Drug treatment and novel drug target against *Cryptosporidium*. Parasite 15, 275-281.

Gomes, R.S., Huber, F., da Silva, S., do Bomfim, T.C., 2012. *Cryptosporidium* spp. parasitize exotic birds that are commercialized in markets, commercial aviaries, and pet shops. Parasitol. Res. 110, 1363-1370.

Goodwin, M.A., Krabill, V.A., 1989. Diarrhea associated with small-intestinal cryptosporidiosis in a budgerigar and in a cockatiel. Avian Dis. 33, 829-833.

Gookin, J.L., Duckett, L.L., Armstrong, M.U., Stauffer, S.H., Finnegan, C.P., Murtaugh, M.P., Argenzio, R.A., 2004. Nitric oxide synthase stimulates prostaglandin synthesis and barrier function in C. *parvum*-infected porcine ileum. Am J Physiol Gastrointest Liver Physiol 287, G571-581.

Graczyk, T.K., Cranfield, M.R., Fayer, R., Anderson, M.S., 1996. Viability and infectivity of *Cryptosporidium parvum* oocysts are retained upon intestinal passage through a refractory avian host. Appl. Environ. Microbiol. 62, 3234-3237.

Graczyk, T.K., Cranfield, M.R., Fayer, R., Trout, J., Goodale, H.J., 1997. Infectivity of *Cryptosporidium parvum* oocysts is retained upon intestinal passage through a migratory water-fowl species (Canada goose, *Branta canadensis*). Trop. Med. Int. Health 2, 341-347.

Graczyk, T.K., Fayer, R., Trout, J.M., Lewis, E.J., Farley, C.A., Sulaiman, I., Lal, A.A., 1998. Giardia sp. cysts and infectious *Cryptosporidium parvum* oocysts in the feces of migratory Canada geese (*Branta canadensis*). Appl. Environ. Microbiol. 64, 2736-2738.

Haddrath, O., Baker, A.J., 2012. Multiple nuclear genes and retroposons support vicariance and dispersal of the palaeognaths, and an Early Cretaceous origin of modern birds. Proc Biol Sci 279, 4617-4625.

Hadfield, S.J., Chalmers, R.M., 2012. Detection and characterization of *Cryptosporidium cuniculus* by real-time PCR. Parasitol. Res. 111, 1385-1390.

Hajdušek, O., Ditrich, O., Šlapeta, J., 2004. Molecular identification of *Cryptosporidium* spp. in animal and human hosts from the Czech Republic. Vet. Parasitol. 122, 183-192.

Harris, J.R., Petry, F., 1999. *Cryptosporidium parvum*: structural components of the oocyst wall. J Parasitol 85, 839-849.

Hoover, D.M., Hoerr, F.J., Carlton, W.W., Hinsman, E.J., Ferguson, H.W., 1981. Enteric cryptosporidiosis in a naso tang, Naso lituratus Bloch and Schneider. Journal of Fish Diseases 4, 425-428.

Hunter, P.R., Nichols, G., 2002. Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. Clin. Microbiol. Rev. 15, 145-154.

Inman, L.R., Takeuchi, A., 1979. Spontaneous cryptosporidiosis in an adult female rabbit. Vet Pathol 16, 89-95.

Iseki, M., 1979. *Cryptosporidium felis* sp. n. (Protozoa: Eimeriorina) from the domestic cat *Jpn. J. Parasitol* 28, 285-307.

Itakura, C., Nakamura, H., Umemura, T., Goryo, M., 1985. Ultrastructure of cryptosporidial life cycle in chicken host cells. Avian Pathol 14, 237-249.

Jellison, K.L., Distel, D.L., Hemond, H.F., Schauer, D.B., 2004. Phylogenetic analysis of the hypervariable region of the 18S rRNA gene of *Cryptosporidium* oocysts in feces of Canada geese (Branta canadensis): evidence for five novel genotypes. Appl Environ Microbiol 70, 452-458.

Jellison, K.L., Distel, D.L., Hemond, H.F., Schauer, D.B., 2007. Phylogenetic analysis implicates birds as a source of *Cryptosporidium* spp. oocysts in agricultural watersheds. Environ. Sci. Technol. 41, 3620-3625.

Jirků, M., Valigurová, A., Koudela, B., Křížek, J., Modrý, D., Šlapeta, J., 2008. New species of *Cryptosporidium* Tyzzer, 1907 (Apicomplexa) from amphibian host: morphology, biology and phylogeny. Folia Parasitol. (Praha) 55, 81-94.

Karanis, P., Aldeyarbi, H.M., 2011. Evolution of *Cryptosporidium in vitro* culture. Int. J. Parasitol. 41, 1231-1242.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772-780.

Kelly, P., Jack, D.L., Naeem, A., Mandanda, B., Pollok, R.C., Klein, N.J., Turner, M.W., Farthing, M.J., 2000. Mannose-binding lectin is a component of innate mucosal defense against *Cryptosporidium parvum* in AIDS. Gastroenterology 119, 1236-1242.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kotloff, K.L., Nataro, J.P., Blackwelder, W.C., Nasrin, D., Farag, T.H., Panchalingam, S., Wu, Y., Sow, S.O., Sur, D., Breiman, R.F., Faruque, A.S., Zaidi, A.K., Saha, D., Alonso, P.L., Tamboura, B., Sanogo, D., Onwuchekwa, U., Manna, B., Ramamurthy, T., Kanungo, S., Ochieng, J.B., Omore, R., Oundo, J.O., Hossain, A., Das, S.K., Ahmed, S., Qureshi, S., Quadri, F., Adegbola, R.A., Antonio, M., Hossain, M.J., Akinsola, A., Mandomando, I., Nhampossa, T., Acácio, S., Biswas, K., O'Reilly, C.E., Mintz, E.D., Berkeley, L.Y., Muhsen, K., Sommerfelt, H., Robins-Browne, R.M., Levine, M.M., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet 382, 209-222.

Kuhn, R.C., Rock, C.M., Oshima, K.H., 2002. Occurrence of *Cryptosporidium* and *Giardia* in wild ducks along the Rio Grande River valley in southern New Mexico. Appl. Environ. Microbiol. 68, 161-165.

Kváč, M., Hofmannová, L., Hlásková, L., Květoňová, D., Vítovec, J., McEvoy, J., Sak, B., 2014a. *Cryptosporidium erinacei* n. sp. (Apicomplexa: Cryptosporidiidae) in hedgehogs. Vet. Parasitol. 201, 9-17.

Kváč, M., Kestřánová, M., Pinková, M., Květoňová, D., Kalinová, J., Wagnerová, P., Kotková, M., Vítovec, J., Ditrich, O., McEvoy, J., Stenger, B., Sak, B., 2013. *Cryptosporidium scrofarum* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic pigs (Sus scrofa). Vet Parasitol 191, 218-227.

Kváč, M., McEvoy, J., Stenger, B., Clark, M., 2014b. Cryptosporidiosis in other vertebrates, In: Cacciò, S.M., Widmer, G. (Eds.) *Cryptosporidium*: parasite and disease. Springer Vienna, Vienna, Austria, pp. 237-323.

Kváč, M., Sak, B., Kvetonová, D., Secor, W.E., 2009. Infectivity of gastric and intestinal *Cryptosporidium* species in immunocompetent Mongolian gerbils (Meriones unguiculatus). Vet. Parasitol. 163, 33-38.

Kváč, M., Sak, B., Květoňová, D., Ditrich, O., Hofmannová, L., Modrý, D., Vítovec, J., Xiao, L., 2008. Infectivity, pathogenicity, and genetic characteristics of mammalian gastric *Cryptosporidium* spp. in domestic ruminants. Vet. Parasitol. 153, 363-367.

Levine, N.D., 1980. Some corrections of coccidian (Apicomplexa: Protozoa) nomenclature. J. Parasitol. 66, 830-834.

Ley, D.H., Moresco, A., Frasca, S., Jr., 2012. Conjunctivitis, rhinitis, and sinusitis in cliff swallows (*Petrochelidon pyrrhonota*) found in association with *Mycoplasma sturni* infection and cryptosporidiosis. Avian Pathol. 41, 395-401.

Lindsay, D., Blagburn, B., Sundermann, C., 1989a. Morphometric Comparison of the Oocysts of *Cryptosporidium meleagridis* and *Cryptosporidium baileyi* from birds. Proc. Helminthol. Soc. Wash. 56, 91-92.

Lindsay, D.S., Blagburn, B.L., Hoerr, F.J., 1990. Small intestinal cryptosporidiosis in cockatiels associated with *Cryptosporidium baileyi*-like oocysts. Avian Dis. 34, 791-793.

Lindsay, D.S., Blagburn, B.L., Hoerr, F.J., Giambrone, J.J., 1987a. Experimental *Cryptosporidium baileyi* infections in chickens and turkeys produced by ocular inoculation of oocysts. Avian Dis. 31, 355-357.

Lindsay, D.S., Blagburn, B.L., Hoerr, F.J., Smith, P.C., 1991. Cryptosporidiosis in zoo and pet birds. J. Protozool. 38, 180S-181S.

Lindsay, D.S., Blagburn, B.L., Sundermann, C.A., Hoerr, F.J., 1989b. Experimental infections in domestic ducks with *Cryptosporidium baileyi* isolated from chickens. Avian Dis. 33, 69-73.

Lindsay, D.S., Blagburn, B.L., Sundermann, C.A., Hoerr, F.J., Ernest, J.A., 1986. Experimental *Cryptosporidium* infections in chickens: oocyst structure and tissue specificity. Am. J. Vet. Res. 47, 876-879.

Lindsay, D.S., Blagburn, B.L., Sundermann, C.A., Hoerr, F.J., Giambrone, J.J., 1987b. *Cryptosporidium baileyi*: effects of intra-abdominal and intravenous inoculation of oocysts on infectivity and site of development in broiler chickens. Avian Dis. 31, 841-843.

Lindsay, D.S., Upton, S.J., Owens, D.S., Morgan, U.M., Mead, J.R., Blagburn, B.L., 2000. *Cryptosporidium andersoni* n. sp. (Apicomplexa: Cryptosporiidae) from cattle, *Bos taurus*. J. Eukaryot. Microbiol. 47, 91-95.

Lowther, P.E. 1975. Geographic and ecological variation in the family Icteridae. In The Wilson Bulletin (Lawrence, KS, Wilson Ornithological Society), pp. 481 - 495.

López-Vélez, R., Tarazona, R., Garcia Camacho, A., Gomez-Mampaso, E., Guerrero, A., Moreira, V., Villanueva, R., 1995. Intestinal and extraintestinal cryptosporidiosis in AIDS patients. Eur J Clin Microbiol Infect Dis 14, 677-681.

Mac Kenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R., Rose, J.B., 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N Engl J Med 331, 161-167.

Majewska, A.C., Graczyk, T.K., Słodkowicz-Kowalska, A., Tamang, L., Jedrzejewski, S., Zduniak, P., Solarczyk, P., Nowosad, A., Nowosad, P., 2009. The role of free-ranging, captive, and domestic birds of Western Poland in environmental contamination with *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts. Parasitol Res 104, 1093-1099.

Makino, I., Abe, N., Reavill, D.R., 2010. *Cryptosporidium* avian genotype III as a possible causative agent of chronic vomiting in peach-faced lovebirds (*Agapornis roseicollis*). Avian Dis. 54, 1102-1107.

Manque, P.A., Tenjo, F., Woehlbier, U., Lara, A.M., Serrano, M.G., Xu, P., Alves, J.M., Smeltz, R.B., Conrad, D.H., Buck, G.A., 2011. Identification and immunological characterization of three potential vaccinogens against *Cryptosporidium* species. Clin. Vaccine Immunol. 18, 1796-1802.

Marcial, M.A., Madara, J.L., 1986. *Cryptosporidium*: cellular localization, structural analysis of absorptive cell-parasite membrane-membrane interactions in guinea pigs, and suggestion of protozoan transport by M cells. Gastroenterology 90, 583-594.

Matos, O., Alves, M., Xiao, L., Cama, V., Antunes, F., 2004. *Cryptosporidium felis* and C. *meleagridis* in persons with HIV, Portugal. Emerg Infect Dis 10, 2256-2257.

Meinhardt, P.L., Casemore, D.P., Miller, K.B., 1996. Epidemiologic aspects of human cryptosporidiosis and the role of waterborne transmission. Epidemiol Rev 18, 118-136.

Meireles, M.V., Soares, R.M., dos Santos, M.M., Gennari, S.M., 2006. Biological studies and molecular characterization of a *Cryptosporidium* isolate from ostriches (*Struthio camelus*). J. Parasitol. 92, 623-626.

Meisel, J.L., Perera, D.R., Meligro, C., Rubin, C.E., 1976. Overwhelming watery diarrhea associated with a *Cryptosporidium* in an immunosuppressed patient. Gastroenterology 70, 1156-1160.

Milácek, P., Vítovec, J., 1985. Differential staining of cryptosporidia by aniline-carbol-methyl violet and tartrazine in smears from feces and scrapings of intestinal mucosa. Folia Parasitol (Praha) 32, 50.

Molina-Lopez, R.A., Ramis, A., Martin-Vazquez, S., Gomez-Couso, H., Ares-Mazas, E., Caccio, S.M., Leiva, M., Darwich, L., 2010. *Cryptosporidium baileyi* infection

associated with an outbreak of ocular and respiratory disease in otus owls (*Otus scops*) in a rehabilitation centre. Avian Pathol. 39, 171-176.

Morgan, U.M., Monis, P.T., Xiao, L., Limor, J., Sulaiman, I., Raidal, S., O'Donoghue, P., Gasser, R., Murray, A., Fayer, R., Blagburn, B.L., Lal, A.A., Thompson, R.C., 2001. Molecular and phylogenetic characterisation of *Cryptosporidium* from birds. Int. J. Parasitol. 31, 289-296.

Morgan, U.M., Xiao, L., Limor, J., Gelis, S., Raidal, S.R., Fayer, R., Lal, A., Elliot, A., Thompson, R.C., 2000. *Cryptosporidium meleagridis* in an Indian ring-necked parrot (*Psittacula krameri*). Aust. Vet. J. 78, 182-183.

Morgan-Ryan, U.M., Fall, A., Ward, L.A., Hijjawi, N., Sulaiman, I., Fayer, R., Thompson, R.C., Olson, M., Lal, A., Xiao, L., 2002. *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from Homo sapiens. J Eukaryot Microbiol 49, 433-440.

Nakamura, A.A., Homem, C.G., da Silva, A.M., Meireles, M.V., 2014. Diagnosis of gastric cryptosporidiosis in birds using a duplex real-time PCR assay. Vet Parasitol 205, 7-13.

Nakamura, A.A., Simoes, D.C., Antunes, R.G., da Silva, D.C., Meireles, M.V., 2009. Molecular characterization of *Cryptosporidium* spp. from fecal samples of birds kept in captivity in Brazil. Vet. Parasitol. 166, 47-51.

Ng, J., Pavlásek, I., Ryan, U., 2006. Identification of novel *Cryptosporidium* genotypes from avian hosts. Appl. Environ. Microbiol. 72, 7548-7553.

Nguyen, S.T., Fukuda, Y., Tada, C., Huynh, V.V., Nguyen, D.T., Nakai, Y., 2013. Prevalence and molecular characterization of *Cryptosporidium* in ostriches (*Struthio camelus*) on a farm in central Vietnam. Exp. Parasitol. 133, 8-11.

Nime, F.A., Burek, J.D., Page, D.L., Holscher, M.A., Yardley, J.H., 1976. Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. Gastroenterology 70, 592-598.

Nime, F.A., J. D. Burek, D. L. Page, M. A. Holscher, and J. H. Yardley., 1976. Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. Gastroenterology 70, 592-598.

O'Donoghue, P.J., Tham, V.L., de Saram, W.G., Paull, K.L., McDermott, S., 1987. *Cryptosporidium* infections in birds and mammals and attempted cross-transmission studies. Vet. Parasitol. 26, 1-11.

Okhuysen, P.C., Chappell, C.L., 2002. *Cryptosporidium* virulence determinants--are we there yet? Int J Parasitol 32, 517-525.

Okhuysen, P.C., Chappell, C.L., Crabb, J.H., Sterling, C.R., DuPont, H.L., 1999. Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. J Infect Dis 180, 1275-1281.

Paperna, I., Vilenkin, M., 1996. Cryptosporidiosis in the gourami *Thrichogaster leeri* :description of a new species and a proposal for a new genus, *Piscicryptosporidium*, for species infecting fish. Dis. Aquat. Organ 27, 95-101.

Pavlasek, I., 1994. Localization of endogenous developmental stages of *Cryptosporidium meleagridis* Slavin, 1955 (Apicomplexa: Cryptosporidiidae) in birds. Vet. Med. (Praha) 39, 733-742.

Pavlásek, I., 1993. The black-headed gull (Larus ridibundus L.), a new host for *Cryptosporidium baileyi* (Apicomplexa: Cryptosporidiidae). Vet Med (Praha) 38, 629-638.

Pavlásek, I., Lavickova, M., Horak, P., Kral, J., Kral, B., 1995. *Cryptosporidium varanii*n. sp. (Apicomplexa: Cryptosporidiidae) in Emerald monitor (*Varanus prasinus* Schegel, 1893) in captivity at Prague zoo. Gazella 22, 99-108.

Pedraza-Diaz, S., Amar, C.F., McLauchlin, J., Nichols, G.L., Cotton, K.M., Godwin, P., Iversen, A.M., Milne, L., Mulla, J.R., Nye, K., Panigrahl, H., Venn, S.R., Wiggins, R., Williams, M., Youngs, E.R., 2001. *Cryptosporidium meleagridis* from humans: molecular analysis and description of affected patients. J. Infect. 42, 243-250.

Pedraza-Díaz, S., Amar, C., McLauchlin, J., 2000. The identification and characterisation of an unusual genotype of *Cryptosporidium* from human faeces as *Cryptosporidium meleagridis*. FEMS Microbiol Lett 189, 189-194.

Peng, M.M., Wilson, M.L., Holland, R.E., Meshnick, S.R., Lal, A.A., Xiao, L., 2003. Genetic diversity of *Cryptosporidium* spp. in cattle in Michigan: implications for understanding the transmission dynamics. Parasitol Res 90, 175-180.

Pitlik, S.D., Fainstein, V., Garza, D., Guarda, L., Bolivar, R., Rios, A., Hopfer, R.L., Mansell, P.A., 1983. Human cryptosporidiosis: spectrum of disease. Report of six cases and review of the literature. Arch. Intern. Med. 143, 2269-2275.

Plutzer, J., Karanis, P., 2009. Genetic polymorphism in *Cryptosporidium* species: an update. Vet. Parasitol. 165, 187-199.

Pohlenz, J., Bemrick, W.J., Moon, H.W., Cheville, N.F., 1978. Bovine cryptosporidiosis: a transmission and scanning electron microscopic study of some stages in the life cycle and of the host-parasite relationship. Vet. Pathol. 15, 417-427.

Power, M.L., Ryan, U.M., 2008. A new species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) from eastern grey kangaroos (*Macropus giganteus*). J. Parasitol. 94, 1114-1117.

Qi, M., Wang, R., Ning, C., Li, X., Zhang, L., Jian, F., Sun, Y., Xiao, L., 2011. *Cryptosporidium* spp. in pet birds: genetic diversity and potential public health significance. Exp. Parasitol. 128, 336-340.

Quah, J.X., Ambu, S., Lim, Y.A., Mahdy, M.A., Mak, J.W., 2011. Molecular identification of *Cryptosporidium parvum* from avian hosts. Parasitology 138, 573-577.

Ren, X., Zhao, J., Zhang, L., Ning, C., Jian, F., Wang, R., Lv, C., Wang, Q., Arrowood, M.J., Xiao, L., 2012. *Cryptosporidium tyzzeri* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic mice (*Mus musculus*). Exp. Parasitol. 130, 274-281.

Richardson, A.J., Frankenberg, R.A., Buck, A.C., Selkon, J.B., Colbourne, J.S., Parsons, J.W., Mayon-White, R.T., 1991. An outbreak of waterborne cryptosporidiosis in Swindon and Oxfordshire. Epidemiol. Infect. 107, 485-495.

Robinson, G., Wright, S., Elwin, K., Hadfield, S.J., Katzer, F., Bartley, P.M., Hunter, P.R., Nath, M., Innes, E.A., Chalmers, R.M., 2010. Re-description of *Cryptosporidium cuniculus* Inman and Takeuchi, 1979 (Apicomplexa: Cryptosporidiidae): morphology, biology and phylogeny. Int. J. Parasitol. 40, 1539-1548.

Ryan, U., 2010. *Cryptosporidium* in birds, fish and amphibians. Exp. Parasitol. 124, 113-120.

Ryan, U., Fayer, R., Xiao, L., 2014. *Cryptosporidium* species in humans and animals: current understanding and research needs. Parasitology 141, 1667-1685.

Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A.A., Pavlásek, I., 2003a. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl. Environ. Microbiol. 69, 4302-4307.

Ryan, U.M., Monis, P., Enemark, H.L., Sulaiman, I., Samarasinghe, B., Read, C., Buddle, R., Robertson, I., Zhou, L., Thompson, R.C., Xiao, L., 2004. *Cryptosporidium* suis n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa*). J Parasitol 90, 769-773.

Ryan, U.M., Power, M., Xiao, L., 2008. *Cryptosporidium fayeri* n. sp. (Apicomplexa: Cryptosporidiidae) from the Red Kangaroo (*Macropus rufus*). J. Eukaryot. Microbiol. 55, 22-26.

Ryan, U.M., Xiao, L., Read, C., Sulaiman, I.M., Monis, P., Lal, A.A., Fayer, R., Pavlasek, I., 2003b. A redescription of *Cryptosporidium galli* Pavlasek, 1999 (Apicomplexa: Cryptosporidiidae) from birds. J Parasitol 89, 809-813.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sak, B., Kvác, M., Hanzlíková, D., Cama, V., 2008. First report of *Enterocytozoon bieneusi* infection on a pig farm in the Czech Republic. Vet Parasitol 153, 220-224.

Sevá, A.a.P., Funada, M.R., Richtzenhain, L., Guimarães, M.B., Souza, S.e.O., Allegretti, L., Sinhorini, J.A., Duarte, V.V., Soares, R.M., 2011. Genotyping of *Cryptosporidium* spp. from free-living wild birds from Brazil. Vet Parasitol 175, 27-32.

Slapeta, J., 2006. *Cryptosporidium* species found in cattle: a proposal for a new species. Trends Parasitol 22, 469-474.

Slavin, D., 1955. *Cryptosporidium meleagridis* (sp. nov.). Journal of Comparitive Pathology 65, 262-266.

Smith, H.V., Brown, J., Coulson, J.C., Morris, G.P., Girdwood, R.W., 1993. Occurrence of oocysts of *Cryptosporidium* sp. in *Larus* spp. gulls. Epidemiol. Infect. 110, 135-143.

Spano, F., Putignani, L., McLauchlin, J., Casemore, D.P., Crisanti, A., 1997. PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between C. *parvum* isolates of human and animal origin. FEMS Microbiol Lett 150, 209-217.

Spano, F., Putignani, L., Naitza, S., Puri, C., Wright, S., Crisanti, A., 1998. Molecular cloning and expression analysis of a *Cryptosporidium parvum* gene encoding a new member of the thrombospondin family. Mol Biochem Parasitol 92, 147-162.

Sréter, T., Kovács, G., da Silva, A.J., Pieniazek, N.J., Széll, Z., Dobos-Kovács, M., Márialigeti, K., Varga, I., 2000. Morphologic, host specificity, and molecular characterization of a Hungarian *Cryptosporidium meleagridis* isolate. Appl. Environ. Microbiol. 66, 735-738.

Striepen, B., 2013. Parasitic infections: Time to tackle cryptosporidiosis. Nature 503, 189-191.

Strong, W.B., Gut, J., Nelson, R.G., 2000. Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. Infect Immun 68, 4117-4134.

Sturbaum, G.D., Reed, C., Hoover, P.J., Jost, B.H., Marshall, M.M., Sterling, C.R., 2001. Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts. Appl Environ Microbiol 67, 2665-2668.

Sulaiman, I.M., Lal, A.A., Xiao, L., 2002. Molecular phylogeny and evolutionary relationships of *Cryptosporidium* parasites at the actin locus. J Parasitol 88, 388-394.

Tacconi, G., Pedini, A.V., Gargiulo, A.M., Coletti, M., Piergili-Fioretti, D., 2001. Retrospective ultramicroscopic investigation on naturally cryptosporidial-infected commercial turkey poults. Avian Dis. 45, 688-695.

Tadeja-Simborio, L., Itakura, C., 1993. Ultrastructural changes of chick bursal epithelial cells experimentally infected with *Cryptosporidium* sp. Avian Pathol 22, 113-129.

Tadeja-Simborio, L., Ochiai, K., Itakura, C., 1993. Ultrastructural changes of tracheal epithelial cells of chicks experimentally infected with *Cryptosporidium* sp. Avian Pathol 22, 363-381.

Takeuchi, D., Jones, V.C., Kobayashi, M., Suzuki, F., 2008. Cooperative role of macrophages and neutrophils in host antiprotozoan resistance in mice acutely infected with *Cryptosporidium parvum*. Infect Immun 76, 3657-3663.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Traversa, D., 2010. Evidence for a new species of *Cryptosporidium* infecting tortoises: *Cryptosporidium ducismarci*. Parasites & vectors 3, 21.

Tyzzer, E. 1912. *Cryptosporidium parvum* (sp. nov.), a coccidium found in the small intestine of the common mouse In Archiv für Protistenkunde (Elsevier).

Tyzzer , E. 1912. *Cryptosporidium parvum* (sp. nov.), a coccidium found in the small intestine of the common mouse (Elsevier), pp. 394–412.

Tyzzer, E.E., 1907. A sporozoan found in the peptic glands of the common mouse. Experimental Biology and Medicine 5, 12-13.

Tyzzer, E.E., 1910. An extracellular Coccidium, *Cryptosporidium muris* (Gen. Et Sp. Nov.), of the gastric Glands of the Common Mouse. J Med Res 23, 487-510.483.

Tyzzer, E.E., 1929. Coccidiosis in *galli*naceous birds. American Journal of Epidemiology 10, 269-383.

Upton, S.J., Current, W.L., 1985. The species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) infecting mammals. J. Parasitol. 71, 625-629.

Urban, J.F., Fayer, R., Chen, S.J., Gause, W.C., Gately, M.K., Finkelman, F.D., 1996. IL-12 protects immunocompetent and immunodeficient neonatal mice against infection with *Cryptosporidium parvum*. J Immunol 156, 263-268.

van Zeeland, Y.R., Schoemaker, N.J., Kik, M.J., van der Giessend, J.W., 2008. Upper respiratory tract infection caused by *Cryptosporidium baileyi* in three mixed-bred falcons (*Falco rusticolus* x *Falco cherrug*). Avian Dis. 52, 357-363.

Vetterling, J.M., Jervis, H.R., Merrill, T.G., Sprinz, H., 1971a. *Cryptosporidium wrairi* sp. n. from the guinea pig *Cavia porcellus*, with an emendation of the genus. J. Protozool. 18, 243-247.

Vetterling, J.M., Takeuchi, A., Madden, P.A., 1971b. Ultrastructure of *Cryptosporidium* wrairi from the guinea pig. J Protozool 18, 248-260.

Wang, Y., Yang, W., Cama, V., Wang, L., Cabrera, L., Ortega, Y., Bern, C., Feng, Y., Gilman, R., Xiao, L., 2014. Population genetics of *Cryptosporidium meleagridis* in humans and birds: evidence for cross-species transmission. Int J Parasitol 44, 515-521.

Weatherhead, P.J., 2005. Long-term decline in a red-winged blackbird population: ecological causes and sexual selection consequences. Proc Biol Sci 272, 2313-2317.

Weber, R., Bryan, R.T., Bishop, H.S., Wahlquist, S.P., Sullivan, J.J., Juranek, D.D., 1991. Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: evidence for low sensitivity of current diagnostic methods. J Clin Microbiol 29, 1323-1327.

Widerström, M., Schönning, C., Lilja, M., Lebbad, M., Ljung, T., Allestam, G., Ferm, M., Björkholm, B., Hansen, A., Hiltula, J., Långmark, J., Löfdahl, M., Omberg, M., Reuterwall, C., Samuelsson, E., Widgren, K., Wallensten, A., Lindh, J., 2014. Large outbreak of *Cryptosporidium hominis* infection transmitted through the public water supply, Sweden. Emerg Infect Dis 20, 581-589.

Wilson, D.E., Reeder, D.M., 2005. Mammal species of the world : a taxonomic and geographic reference, 3rd Edition. Johns Hopkins University Press, Baltimore.

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R., Lal, A.A., 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. Appl. Environ. Microbiol. 65, 1578-1583.

Xiao, L., Fayer, R., Ryan, U., Upton, S.J., 2004a. *Cryptosporidium* taxonomy: recent advances and implications for public health. Clin Microbiol Rev 17, 72-97.

Xiao, L., Feng, Y., 2008. Zoonotic cryptosporidiosis. FEMS Immunol Med Microbiol 52, 309-323.

Xiao, L., Morgan, U.M., Fayer, R., Thompson, R.C., Lal, A.A., 2000. *Cryptosporidium* systematics and implications for public health. Parasitol Today 16, 287-292.

Xiao, L., Ryan, U.M., Graczyk, T.K., Limor, J., Li, L., Kombert, M., Junge, R., Sulaiman, I.M., Zhou, L., Arrowood, M.J., Koudela, B., Modry, D., Lal, A.A., 2004b. Genetic diversity of *Cryptosporidium* spp. in captive reptiles. Appl. Environ. Microbiol. 70, 891-899.

Xiao, L., Sulaiman, I.M., Ryan, U.M., Zhou, L., Atwill, E.R., Tischler, M.L., Zhang, X., Fayer, R., Lal, A.A., 2002. Host adaptation and host-parasite co-evolution in *Cryptosporidium*: implications for taxonomy and public health. Int J Parasitol 32, 1773-1785.

Yuan, L., Yan, W., Wang, T., Qian, W., Ding, K., Zhang, L., Han, L., Shao, X., 2014. Effects of different inoculation routes on the parasitic sites of *Cryptosporidium baileyi* infection in chickens. Exp Parasitol 145, 152-156.

Yuri, T., Mindell, D.P., 2002. Molecular phylogenetic analysis of Fringillidae, "New World nine-primaried oscines" (Aves: Passeriformes). Mol Phylogenet Evol 23, 229-243.

Zhang, W., Yang, F., Liu, A., Wang, R., Zhang, L., Shen, Y., Cao, J., Ling, H., 2013. Prevalence and genetic characterizations of *Cryptosporidium* spp. in pre-weaned and post-weaned piglets in Heilongjiang Province, China. PLoS One 8, e67564.

Zhou, L., Kassa, H., Tischler, M.L., Xiao, L., 2004. Host-adapted *Cryptosporidium* spp. in Canada geese (*Branta canadensis*). Appl. Environ. Microbiol. 70, 4211-4215.

Šlapeta, J., 2013. Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? Int J Parasitol 43, 957-970.