Molecular and Genetic Analysis of *Cryptosporidium* spp. Oocysts: Sources and Genotypes in the Environment

by

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B. S. Civil and Environmental Engineering
Cornell University, 1997

SUBMITTED TO THE DEPARTMENT OF CIVIL AND ENVIRONMENTAL ENGINEERING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN CIVIL AND ENVIRONMENTAL ENGINEERING AT THE MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 2003

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ABSTRACT

Cryptosporidium parvum is responsible for an acute gastrointestinal disease that is self-limiting in immunocompetent people but potentially life-threatening for the immunocompromised. Until recently, *C. parvum* was the only species of *Cryptosporidium* known to cause disease in people, however, reports of *C. muris*, *C. felis*, and *C. meleagridis* in immunocompetent adults have raised questions about the extent to which *Cryptosporidium* spp. are infectious for humans. Until more is known, presence of any *Cryptosporidium* oocysts in the environment should be considered a potential public health risk. *Cryptosporidium* spp. can infect a wide range of animal hosts, and environmental sources may include wildlife, agricultural animals, or human sewage. Transmission of *Cryptosporidium* spp. via fecally-contaminated food and water has been well-documented, and outbreaks of cryptosporidiosis have occurred around the world.

The exogenous stage of the organism, the oocyst, is difficult to remove from drinking water supplies because it is resistant to chlorine disinfection and inefficiently filtered. Therefore, a better understanding of the sources, fate, and transport of oocysts in the environment is critical to protect source waters from oocyst contamination. In this work, a sensitive and specific molecular detection assay for *Cryptosporidium* spp. in environmental samples was developed and applied to surface water and fecal samples from the Wachusett Reservoir watershed, the drinking water source for metropolitan Boston, to establish links between oocyst sources and surface water contamination. Multiple species of *Cryptosporidium* were detected, and previously uncharacterized genetic diversity at the 18S rRNA locus was observed. Each surface water site had a hypothesized oocyst source, but results showed that the sources detected were often very different from those hypothesized to be most important. *Cryptosporidium* spp. from wildlife was detected in surface waters hypothesized to be contaminated by human sewage, and surface waters susceptible to agricultural runoff were observed to be more impacted by birds. In addition, *Cryptosporidium* spp. contamination occurred seasonally, with the seasonal pattern of detection distinct for surface waters with different oocyst sources. Results of this work contribute to a growing characterization of *Cryptosporidium* in the environment that will ultimately help minimize public exposure to this waterborne parasite.

Thesis Supervisors:
Harold F. Hemond, William E. Leonhard Professor of Civil and Environmental Engineering
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Acknowledgements

I am extremely grateful for the support of my thesis advisors, Harry Hemond and David Schauer, who helped me pursue a thesis project outside the scope of their immediate interests and supported my work with enthusiasm and encouragement. They have inspired me to persevere in research and to always keep sight of the big picture. I am thankful for the six years I have had to learn from them, and I aspire to be the kind of mentor for my future students that they have been for me.

I thank my thesis committee, Martin Polz and Giovanni Widmer, who taught me much of what I needed to know to undertake this thesis. The countless hours they spent teaching me, attending meetings, and reviewing manuscripts are greatly appreciated.

I appreciate the help of the Metropolitan District Commission of the Commonwealth of Massachusetts for their help in many aspects of my work: Vincent Vignaly, for identification of field sites; Dave Worden, for coordination of water sample collections; Dave Getman, for help with agricultural fecal sampling; and Dan Clark, for assistance with wildlife fecal sampling.

Members of the Hemond and Schauer labs, past and present, were great teachers, supporters, and friends, making the work a little easier and life a lot more fun! I'd like to extend special thanks to the following people: Jessica Hart, for laughter in the lab and help in the field (especially for pulling me out of the Quinapoxet River and not taking a picture!); Jane Sohn, for helpful input and research advice over the years; Nicole Keon-Blute, a fellow van sheriff and Izzy's fanatic, who set a great example and became a valued friend along the way; and Jenny Jay, who has taught me more than she knows (about research and teaching, being a mentor, and finding the best plantains in town!) and who has inspired me from my very first day at MIT.

I sincerely thank Sheila Frankel, for hours of advice and wisdom, and for making me call her the first night of my General Exam so she could be sure I hadn't folded under the pressure!

Finally, my family – my parents, who have always encouraged me to pursue my goals, regardless of where they took me, and who have been my anchor in life; my grandparents, for their love and support in helping to make my education a reality; and my in-laws, whose support and pride has always made me feel like someone special. Thank you! Most of all, I thank my husband Darin for his never-ending support, encouragement, flexibility, sense of humor, and love. He has been beside me the whole way, through the highest of the highs and the lowest of the lows, and words can't express the depth of my love and gratitude.

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Chapter 1: Introduction

Background: *Cryptosporidium parvum* and Cryptosporidiosis.

Cryptosporidium parvum is an intracellular protozoan parasite responsible for an acute gastroenteritis that is self-limiting for otherwise healthy people but prolonged and life-threatening for the immunocompromised population. The life cycle of *C. parvum* is shown in Figure 1. The exogenous stage is an oocyst, a hardy organism capable of survival for months in the environment. Oocysts are transmitted via the fecal-oral route, and exposure via contaminated recreational water or ingestion of contaminated food or water has been well documented [1-3, 8, 9, 13]. Once the oocyst is ingested, contact with digestive enzymes and bile salts causes excystation and the release of four infective sporozoites. Sporozoites penetrate host epithelial cells and develop into trophozoites within parasitopherous vacuoles that are intracellular but extracytoplasmic. Trophozoites undergo asexual division to form merozoites, and merozoites either penetrate adjacent epithelial cells (creating an asexual cycle) or develop into type II meronts. Type II meronts enter host cells to form the sexual stages, microgamonts and macrogamonts. Microgametes, released from the microgamont, fertilize macrogamonts to create a zygote. About 80% of zygotes develop into thick-walled oocysts that are excreted back to the environment; the other 20% develop into thin-walled oocysts that excyst within the host to create an autoinfectious cycle. [5, 7] The existence of both asexual and autoinfectious cycles explains how ingestion of small numbers of oocysts can cause severe disease, particularly among immunocompromised patients. While the mean infectious dose for healthy human adults varies with the strain of *Cryptosporidium*, studies have shown it can range from 9 to 1042 oocysts [6, 11, 12].

Symptoms of cryptosporidiosis are nonspecific and may include diarrhea (often watery and profuse), abdominal cramps, nausea, vomiting, weight loss, and low-grade fever. Manifestation of symptoms may begin two to fourteen days after ingestion of oocysts, and for immunocompetent people can last for up to two weeks before clearing. However, infected individuals may also be asymptomatic. Due to the similarity of symptoms with those of other common illnesses, and the potential for infected individuals to be asymptomatic, the disease is likely underdiagnosed and underreported. No curative drug therapy currently exists for

cryptosporidiosis. At best, oral and parenteral rehydration in combination with anti-diarrheal medication can be administered to treat the symptoms of the disease.

Outbreaks of cryptosporidiosis have been attributed to contaminated food [1, 9] and contaminated recreational and drinking water [3, 8, 13]. Outbreaks have occurred worldwide, and within the United States they have spanned the country from coast to coast. Contaminated drinking water has been associated with a variety of water sources (both surface water and groundwater supplies) and water treatment methods (from disinfection only to inclusive coagulation, flocculation, sedimentation, filtration, and disinfection). Cryptosporidium is a challenge for water treatment plants because its small size (4-8 μm diameter) makes it inefficiently filtered and the exogenous oocyst stage is resistant to chlorine, the conventional disinfectant used in water treatment. The largest waterborne outbreak occurred in 1993 in Milwaukee, Wisconsin. While the exact source of oocyst contamination was never identified, likely sources included cattle wastes, slaughterhouse wastes, and human sewage that were flushed into Lake Michigan during a period of high flow resulting from spring rains and snowmelt runoff. Water treatment for Milwaukee included alum coagulation, flocculation, sedimentation, rapid sand filtration, and chlorination. Approximately 403,000 people (of a total 840,000 served by Milwaukee Water Works) became ill, 4,000 people were hospitalized, and at least 69 people (most of whom were HIV-positive) died. The outbreak in Milwaukee shows that waterborne cryptosporidiosis can occur even when rigorous water treatment strategies are in place and illustrates the impact of such an outbreak on a community.

Although many species of *Cryptosporidium* have been identified, until recently, *C. parvum* was considered the only species of concern for human health. In the past few years, human infections with *C. meleagridis*, *C. muris*, and *C. felis* have been reported (a detailed taxonomic review, including a discussion of the *Cryptosporidium* species associated with human health risks, follows in Chapter 2). Until we are sure about the extent to which *Cryptosporidium* species other than *C. parvum* are infectious for people, the presence of any species of *Cryptosporidium* in the environment should be considered a potential public health risk.

Thesis Goals

Given the potential devastation of waterborne cryptosporidiosis and the difficulty in removing or inactivating *Cryptosporidium* spp. oocysts once they enter drinking water supplies, the goal of this work was to characterize the behavior of *Cryptosporidium* spp. oocysts in the watershed. A better understanding of the sources, transport processes, and fate of oocysts in watersheds will ultimately aid in the development of watershed management strategies to minimize surface water contamination and public exposure to this parasite.

The Wachusett Reservoir watershed was chosen as the primary study location because it is the drinking water source for metropolitan Boston and contains a number of potential *Cryptosporidium* spp. sources, including wildlife, dairy farms, and sewage inputs from old septic systems. Within the scope of this thesis, the specific aims were to:

1. identify the species and/or genotypes of *Cryptosporidium* oocysts in surface waters susceptible to wildlife, agriculture, and sewage impacts,

2. determine the sources of oocysts in surface waters by examining fecal samples from suspected animal hosts in the watershed, and

3. investigate the potential of water quality parameters to serve as indicators of *Cryptosporidium* contamination to reduce the need for costly and time-intensive parasite detection and potentially elucidate transport processes or oocyst dynamics in the watershed.

Field Sites: Wachusett Reservoir and Boston Water Supply

Field studies in this thesis were conducted in the Wachusett Reservoir watershed, an integral part of the water supply system for eastern Massachusetts. A year-long watershed study was conducted at the Stillwater and Quinapoxet Rivers (susceptible to wildlife shedding) from February 2000 to January 2001. A second year-long watershed study was conducted at Gates Brook (susceptible to failed septic systems) and Brooks JF and SF (impacted by agricultural

runoff) from June 2001 to May 2002. Surface waters were sampled monthly, and fecal samples were collected intermittently.

The Wachusett Reservoir was constructed at the turn of the 20th century to supply drinking water to the growing Boston metropolitan area. In 1897, the Nashua River above the town of Clinton was impounded by the Wachusett Dam, and 6.5 square miles were flooded in the towns of Boylston, West Boylston, Clinton, and Sterling (Figure 2). Water from the reservoir, which is fed by the Stillwater and Quinapoxet Rivers, was conveyed by the Wachusett/Weston Aqueduct to Weston Reservoir and then by pipeline to the Chestnut Hill and Spot Pond Reservoirs. Work was completed in 1905 and the reservoir first filled in May 1908. The 65 billion gallon Wachusett Reservoir was the largest public water supply reservoir in the world at the time, and the system was built to service 29 municipalities within a 10 mile radius of the State House in Boston. [10]

As the demand for water grew in eastern Massachusetts, the Quabbin Reservoir in western Massachusetts was created and brought on-line. The reservoir was constructed by impounding the Swift River and flooding 39 square miles in the towns of Dana, Enfield, Greenwich, and Prescott. Construction began in 1936, filling commenced in August 1939, and the reservoir was completed in 1946. At the time, the 412 billion gallon Quabbin Reservoir was the largest man-made reservoir in the world devoted solely to water supply. [10]

Both the Quabbin and Wachusett Reservoirs contribute to the current Massachusetts Water Resource Authority's (MWRA) water supply system (Figure 3). The Quabbin Reservoir is fed by the Swift River and by flood flows diverted from the Ware River during the high-water months spanning October through June. Water entering the Quabbin Reservoir can take up to four years to circulate and enter the main intake to the 25-mile-long Quabbin Aqueduct, which flows underground to the Wachusett Reservoir. Quabbin water enters the Wachusett Reservoir and circulates for approximately eight months before exiting the reservoir and passing through underground pipes to Southborough. At Southborough, additions of fluoride (to prevent tooth decay) and sodium carbonate and carbon dioxide (to buffer the water and lessen corrosion of lead from pipes and plumbing fixtures) are made to the water before it continues through the

Hultman Aqueduct (85%) or the Weston Aqueduct (15%). Water empties into the Norumbega and Weston Reservoirs, is chlorinated as it is drawn into distribution mains, and feeds nine small distribution reservoirs and storage tanks and smaller pipes serving each community. The Quabbin and Wachusett Reservoirs can safely provide about 300 million gallons per day (mgd) of water, and the MWRA projects that the system demand will remain in the 240-260 mgd range. Thus, the current water supply system will be sufficient to meet the needs of the metropolitan Boston area for the foreseeable future. [14]

The MWRA water supply is not filtered. The Massachusetts Department of Environmental Protection found that filtration was not needed for the Quabbin and Wachusett Reservoirs in 1991 and 1998, respectively. The United States Environmental Protection Agency (EPA) recently sued the MWRA to build a costly filtration system, but in May 2000, Federal District Court Judge Richard Stearns ruled against the EPA, stating that the MWRA was already implementing a comprehensive program to protect public health and ensure high quality drinking water. This program includes watershed protection measures, pipeline replacement and rehabilitation projects, the phasing out of open storage reservoirs and the construction of new covered storage facilities, and the construction of two new water treatment plants. One recently completed new water treatment plant, the Quabbin, utilizes chlorine for primary disinfection and serves communities receiving water directly from the pristine Quabbin Reservoir. The second new treatment plant, Walnut Hill, is under construction and will use ozone, a much more effective disinfectant for organisms like *Giardia* and *Cryptosporidium*, to treat water delivered to the majority of MWRA customers in metropolitan Boston. [14]

Thesis Format

Chapters 2-6 are individual manuscripts with their own abstracts, introductions, conclusions, and reference lists. Each of these chapters is formatted for the journal to which the manuscript has been or will be submitted. Chapter 7 provides a comprehensive phylogenetic analysis of all *Cryptosporidium* isolates recovered in these studies, summarizes the conclusions from each of the individual studies, and offers a projection of future work to be done. The appendix details

how the molecular analyses were performed and includes sequence data and proposed 18S rRNA secondary structures for each analyzed *Cryptosporidium* isolate.

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Figure Captions.

Figure 1. Life cycle of *Cryptosporidium parvum* (adapted from [4]).

Figure 2. Maps of West Boylston before and after the construction of the Wachusett Reservoir. Panel A: West Boylston in 1892, before construction. Panel B: West Boylston in 1917, after construction (maps adapted from [15]).

Figure 3. Map of the Massachusetts Water Resource Authority water supply system (adapted from [4]).

Figure 1

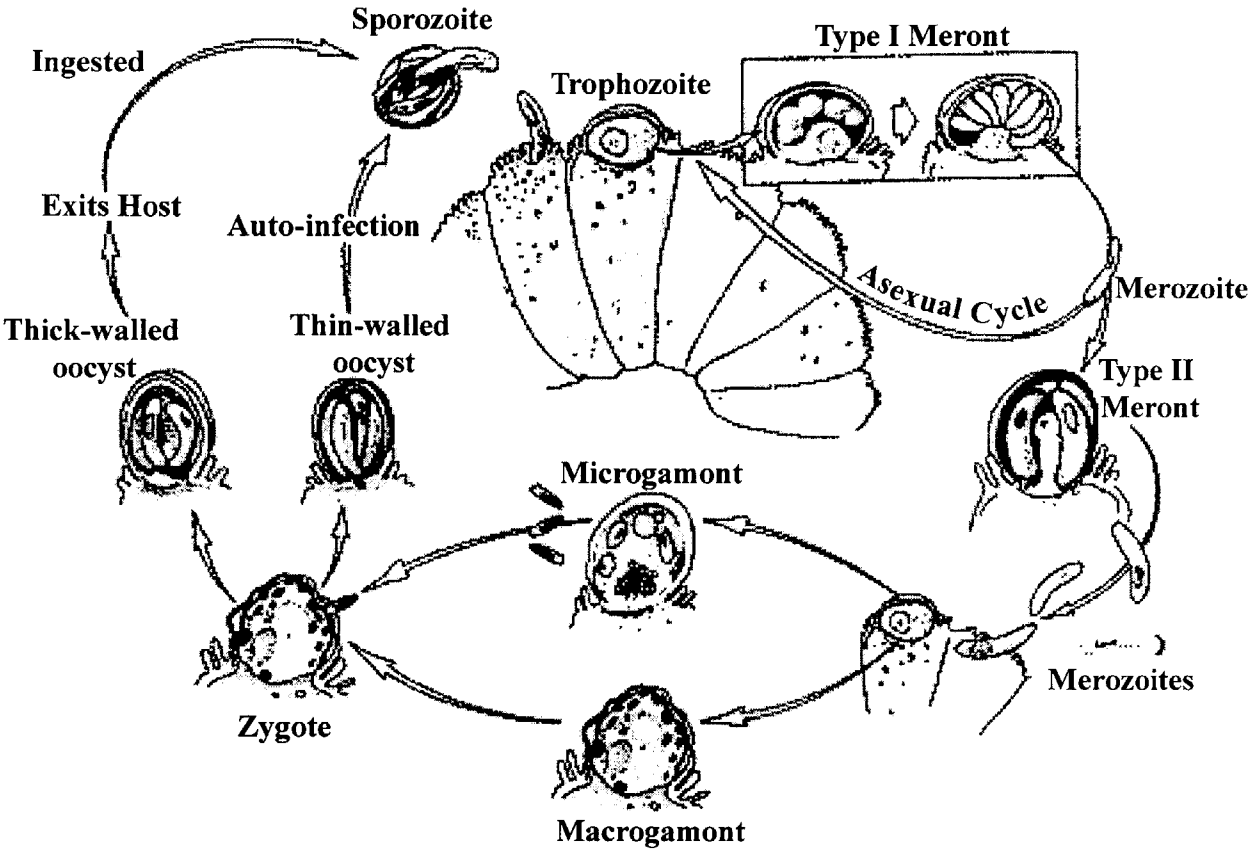

Sporozoite
Ingested
Type I Meront
Trophozoite
Exits Host
Auto-infection
Asexual Cycle
Merozoite
Thick-walled
oocyst
Thin-walled
oocyst
Type II
Meront
Microgamont
Merozoites
Zygote
Macrogamont

Figure 2

A

B

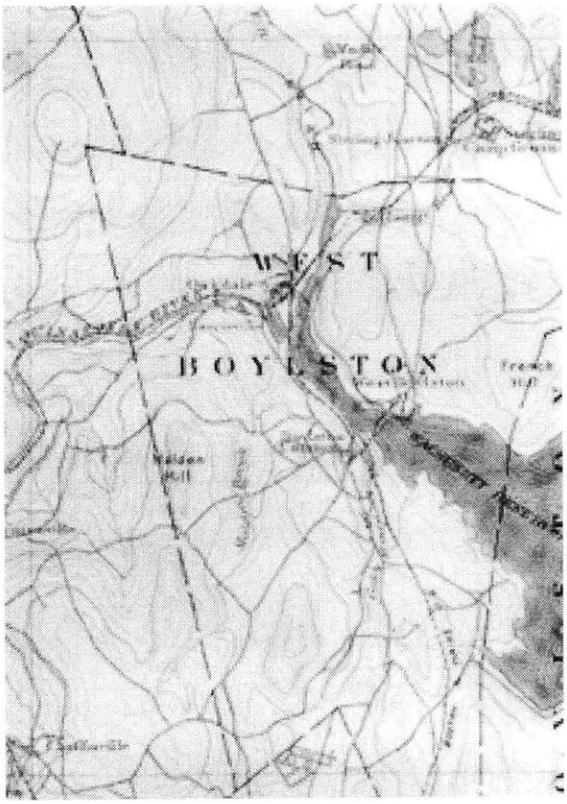
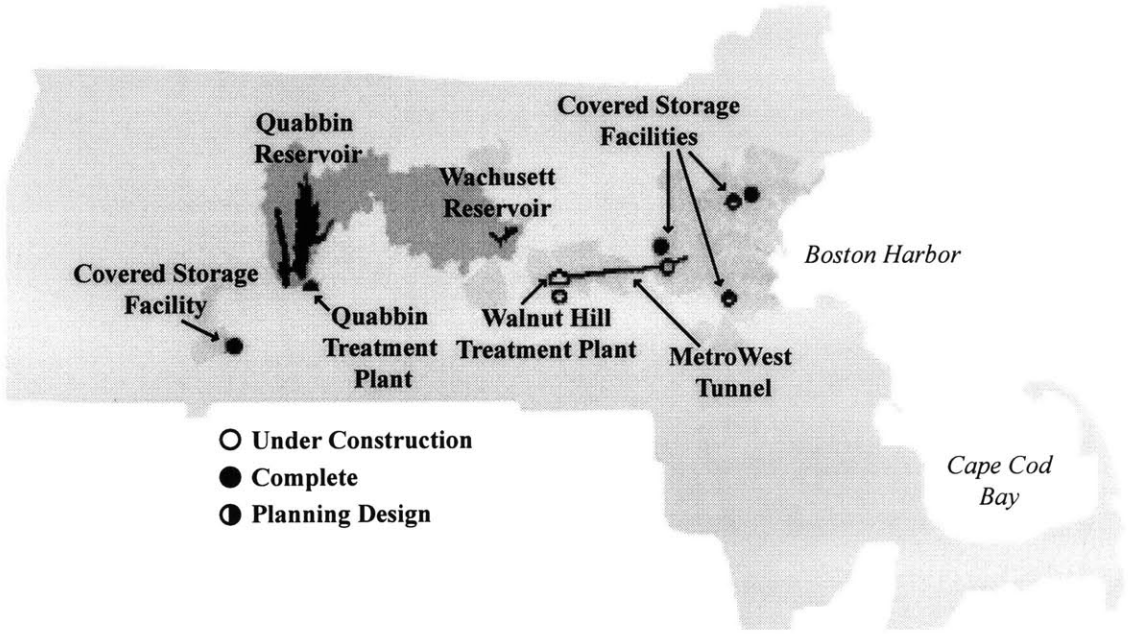


Figure 3


Quabbin Reservoir
Wachusett Reservoir
Covered Storage Facilities
Boston Harbor
Covered Storage Facility
Quabbin Treatment Plant
Walnut Hill Treatment Plant
MetroWest Tunnel
Cape Cod Bay
Under Construction
Complete
Planning Design

Chapter 2: Taxonomic Classifications of *Cryptosporidium* spp. Oocysts: Basis, Limitations, and Implications for Epidemiology

Manuscript to be submitted to ***Microbes and Infection***

Abstract. The current taxonomy of the genus *Cryptosporidium* lacks a set of standardized, uniform criteria by which species status can be assigned to various isolates. To date, taxonomic classifications have been made using varying combinations of oocyst morphology, host specificity, organ location, and genetic characterizations. This review addresses the difficulties associated with polyphasic morphological, biological, and genetic characterizations of *Cryptosporidium*, the existing state of *Cryptosporidium* taxonomy, and the implications of the current taxonomic system for environmental and epidemiological studies. A standardized, polyphasic approach to *Cryptosporidium* taxonomy, using well-defined criteria for oocyst morphology, host specificity, organ location, and genetic characterization, is recommended to eradicate the confusion surrounding the existing system.

Introduction

In 1907, E. E. Tyzzer first described oocysts of *Cryptosporidium muris* in the gastric glands of laboratory mice [1]. Five years later, Tyzzer described a new species, *C. parvum*, distinguishable from *C. muris* by smaller oocysts and colonization of the small intestine of laboratory mice [2]. Despite these early reports, there was very little interest in *Cryptosporidium* until the first human cases of cryptosporidiosis were reported in 1976 [3, 4]. Since the association of *Cryptosporidium* spp. with human infection, in particular humans with compromised immune systems, the level of biological and molecular characterization of the genus has increased dramatically.

Early taxonomic classifications of *Cryptosporidium* species were based on oocyst morphology and biology, including organism host range and localization of infection. However, *Cryptosporidium* is a challenging organism to classify based solely on morphometric and biological data. Oocysts do not possess many distinguishable morphometric characteristics; nearly spherical in shape, they sort into one of two size groups: larger oocysts (6 to 8 μm diameter) characteristic of *C. baileyi*, *C. muris*, *C. andersoni*, and *C. serpentis*, and smaller oocysts (4 to 6 μm diameter) characteristic of all other species. In addition, the parasite has not been cultured and requires passage through a host for reproduction. Characterization of oocyst host range and localization of infection necessitates the appropriate facilities and resources for animal infections in a range of potential hosts. The number of oocysts available for infection

studies (each animal infection requires typically $1x10^5$ to $1x10^7$ oocysts [5]) may also limit the scope of biological characterizations. With the advent of molecular tools like polymerase chain reaction (PCR) and DNA sequencing, increasing molecular characterization of the genus has occurred. These molecular tools permit characterization of small numbers of oocysts, provide greater specificity than morphometric analysis, and are less resource-intensive than animal infection studies. Genetic loci that have been used for taxonomical classifications of *Cryptosporidium* spp. include the 18S ribosomal RNA [6-9] and adjacent internal transcribed spacer 1 [9-11], heat shock protein 70 [9, 12], actin [13], dihydrofolate reductase [6], thrombospondin-related adhesive protein 1 [14], and *Cryptosporidium* oocyst wall protein [15, 16].

Increasing dependence on molecular data for species identification has contributed to confusion regarding the taxonomy of *Cryptosporidium*. Molecular characterization of *Cryptosporidium* oocysts has revealed extensive genetic diversity within the genus and raised questions about the validity of current taxonomic classifications. The possibility of genetic recombination during sexual reproduction, however, confounds interpretation of the observed genetic variability and makes it difficult to define an acceptable level of intraspecies genetic variability. Increasing reports of *Cryptosporidium* spp. oocysts recovered from hosts outside of the expected host range also challenge the legitimacy of the current classification system. This review describes the current status of *Cryptosporidium* taxonomy, including accepted species classifications, novel genotypes and host ranges of current species, the most recent reports of new species identifications, and the lack of standardization in species characterization. Implications of *Cryptosporidium* taxonomy for environmental studies and human cryptosporidiosis risk assessments are also addressed.

Current Status of Taxonomy

Cryptosporidium is a protozoan in the phylum Apicomplexa, class Coccidea, order Eucoccidiorida, family Cryptosporidiidae. Although there is no consensus on the number of legitimate *Cryptosporidium* species, Fayer et al. [17] recently listed ten species as valid. These species (and their primary hosts) include *C. parvum* (mammals), *C. meleagridis* (birds), *C. wrairi*

(guinea pigs), *C. felis* (cats), and *C. saurophilum* (skink), all of which colonize the small intestine; *C. baileyi* (birds), which colonizes the respiratory tract; *C. muris* (rodents), *C. andersoni* (cattle), and *C. serpentis* (reptiles), responsible for gastric infections; and *C. nasorum* (fish), which can infect either the stomach or the small intestine. The differentiation of one species from another has become less clear as broader host ranges and increasing genetic heterogeneity are revealed within many taxonomic groups. A summary of the biological data for currently accepted and proposed *Cryptosporidium* species and genotypes is given in Table 1; a more detailed description of the taxonomic groups, including genetic and phylogenetic characterizations, is provided below.

C. parvum. *C. parvum* is the species that has been traditionally associated with cryptosporidiosis among otherwise healthy adults. Given its impact on public health, *C. parvum* is the most extensively characterized species of *Cryptosporidium* to date. The species has been grouped into two distinct genotypes based on both biological and molecular data: "human" genotype-1, infectious for humans only, and "animal" genotype-2, infectious for both humans and animals [10, 18-21]. *C. parvum* human and bovine isolates were first differentiated in the early 1990s. Ortega et al. [22] reported different restriction fragment length polymorphism patterns between human and bovine *C. parvum* isolates in 1991, and the following year a phenotypic distinction between human and bovine *C. parvum* was made when Pozio et al. [18] observed that bovine isolates of *Cryptosporidium* caused severe diarrhea and a high production of oocysts in neonatal calves, while human isolates in the same host caused mild diarrhea and low oocyst production. The advent of molecular genetic characterization has continued to support the distinction between human and bovine *C. parvum* genotypes. These genotype classifications are continuously evolving, however; C. parvum genotype 1 was successfully propagated in a gnotobiotic pig [23], and the first reports of a *C. parvum* human genotype in nonprimate hosts [24, 25] and a *C. parvum* bovine genotype in a wildebeest [26] were made recently, possibly extending the range of potential reservoirs for these genotypes. Additional *C. parvum* animal-adapted genotypes, including pig, marsupial, mouse, ferret, and dog [6, 8, 27], have been reported.

Given the differences between the human and animal genotypes of *C. parvum*, Morgan-Ryan et al. [28] recently proposed they be considered distinct species and designated the human genotype *C. hominis*. Morphologically, oocysts of *C. hominis* and *C. parvum* bovine genotype are indistinguishable. Differences between *C. hominis* and *C. parvum* include the limited host range of *C. hominis* (it is not transmissible to mice, rats, cats, or dogs) [20, 21, 28] and parasite-associated lesion distribution and intensity of infection in a gnotobiotic pig model (intensity of infection was greater in pigs infected with *C. parvum*, with lesions of *C. parvum* observed throughout the small and large intestine compared to lesions of *C. hominis* observed only in the ileum and colon) [29]. Genetic analysis at multiple loci also supports the distinction between *C. hominis* and *C. parvum* [6-8, 12, 13, 27, 30, 31].

Cryptosporidium oocysts undergo both asexual and sexual reproduction in a host, and the observation of genetic recombination between two distinct *C. parvum* animal genotype-2 oocysts was recently reported [32]. Mixed infections of interferon-gamma knockout mice with two distinct *C. parvum* genotype-2 isolates resulted in recombinant progeny with multilocus genotypes containing alleles inherited from each parental line. In contrast, no recombinants between *C. parvum* genotypes 1 and 2 were identified in a multilocus analysis of *C. parvum* isolates from different hosts and geographic origins [33]. This observation suggests reproductive incompatibility between the two genotypes and supports the view that *C. parvum* genotypes 1 and 2 are distinct species.

Pig Genotype. Pigs have been shown harbor both the bovine and pig genotypes of *C. parvum* [34], and the pig genotype has been isolated from pigs with both symptomatic and asympomatic cryptosporidial infections [27]. While the pig-derived bovine genotype of *C. parvum* produced a strong infection in nude mice, the pig genotype failed to produce infection. Small subunit ribosomal RNA gene sequences of *Cryptosporidium* pig isolates from Switzerland, Western Australia, and the United States were found to be identical [27, 34], indicating genetic conservation of the pig isolate across wide geographical areas. In addition, phylogenetic analyses of the 18S rRNA and dihydrofolate reductase loci showed the pig genotype to be genetically distant from the majority of *C. parvum* isolates, leading some to suggest the pig genotype may represent a distinct species of *Cryptosporidium* [6, 8, 27].

Marsupial Genotype. The marsupial genotype has not been well characterized; only three marsupial isolates of *C. parvum* have been analyzed to date, but sequence analysis of the 18S rRNA, internal transcribed spacer region 1, and dihydrofolate reductase loci have confirmed its genetic distinctness from other *Cryptosporidium* species and genotypes [6, 8, 11, 35]. The genetic difference at the 18S rRNA locus between *C. parvum* and the marsupial genotype was reportedly larger than the difference between *C. parvum* and *C. wrairi* [6], suggesting that the marsupial genotype may be a distinct species. However, further biological and genetic characterization is necessary to confirm the taxonomic status of the marsupial genotype.

Mouse Genotype. Oocysts of the *C. parvum* mouse genotype are slightly smaller than other *C. parvum* oocysts (4.5 x 4.0 μm vs. 5.0 x 4.5 μm) and are genetically different from *C. parvum* human and bovine genotypes [26, 27]. Morgan et al. [26, 27] found that the mouse genotype, recovered from mice (*Mus musculus* syn. *domesticus*) and analyzed at both the rDNA and acetyl-CoA synthetase loci, was conserved across widely separated geographic areas. Sequence analysis of the internal transcribed spacer region 1 and dihydrofolate reductase loci have also confirmed the genetic distinctness of the mouse genotype [6, 11]. However, mice are also susceptible to other *C. parvum* genotypes [26]; five of 19 mice analyzed exhibited the bovine genotype, which is known to infect humans, yet the mouse genotype has not been identified in cattle. In addition, the mouse genotype was identified in a large-footed mouse-eared bat, extending the host range of the genotype [26].

Ferret Genotype. *C. parvum*-like oocysts from a ferret have been shown to exhibit distinct genotypes at both the 18S rRNA and heat shock protein 70 loci [8, 36]. Although the ferret genotype was most closely related to *C. wrairi* upon phylogenetic analysis of the 18S rRNA gene, the distance of the ferret genotype to *C. wrairi* was similar to the distance between *C. wrairi* and the *C. parvum* bovine genotype. Extensive biological characterization of the ferret genotype is necessary before a species distinction can be made.

Dog Genotype (*C. canis*). The *Cryptosporidium* dog genotype, while morphologically indistinguishable from the *C. parvum* human and bovine genotypes, is distinct from established species and genotypes of *Cryptosporidium* in both host specificity and genetics [8, 13, 37, 38]

and has been recently designated *C. canis* [37]. *C. canis* is genetically distinct at the 18S rRNA [8, 37, 38], heat shock protein 70 (HSP70) [37, 38], and actin [13] loci. Sequence analysis of the 18S rDNA and a short region of the HSP70 gene shows that *C. canis* is conserved among isolates from the United States and Australia [38]. In addition, the GC content of the HSP70 gene supports the uniqueness of *C. canis* as a valid species. Most *Cryptosporidium* are AT-rich in the HSP 70 gene (58-66% A or T), but *C. canis* has 48.2% A or T content at this locus [37]. *C. canis* differs from the *C. parvum* bovine genotype in that it is not infectious for mice, even when they have been immunosuppressed. *C. canis* is infectious for cattle, however, which distinguishes it from the *C. parvum* human genotype [37]. Mixed infections of *C. canis* and the *C. parvum* bovine genotype in both dogs and calves indicates that the oocysts remain genetically distinct with no recombination occurring [37]. *C. canis* has recently been recovered from both immunocompromised [39] and immunocompetent [40, 41] humans, thus extending its host range and significance for human health.

C. wrairi. *C. wrairi* was first described as a new species in guinea pigs in 1971 [42, 43], however, no morphological details distinguished it from other *Cryptosporidium* species. Two decades later, biological differences between *C. wrairi* and *C. parvum* were reported [44]. While all suckling mice inoculated with *C. parvum* became infected, not all mice fed *C. wrairi* became infected. Mice inoculated with *C. wrairi* produced on average 100-fold fewer oocysts by day 7 post-inoculation than mice fed *C. parvum*, and infections with *C. wrairi* were patchy with sparse endogenous stages compared to infections with *C. parvum*. In addition, striking differences were identified in oocyst wall proteins of *C. parvum* and *C. wrairi*. Other distinctive traits of *C. wrairi* included the ability to infect immunocompetent adult guinea pigs and localization of infection to the small intestine (*C. parvum* infections in infant guinea pigs were restricted to the large intestine) [45]. While *C. wrairi* is closely related to *C. parvum* phylogenetically [6, 8, 12, 13, 16], molecular genetic characterizations have revealed differences between *C. wrairi* and other *Cryptosporidium* spp. [8, 12, 13, 15, 46, 47].

C. meleagridis. *C. meleagridis* was first described in turkeys in 1955 [48], and along with *C. baileyi*, is one of the two established *Cryptosporidium* species associated with infection in birds. *C. meleagridis* is distinct from *C. baileyi* both morphologically [49] and biologically [48, 50].

Oocysts of *C. meleagridis* are smaller than those of *C. baileyi* (5.2 vs. 6.2 μm diameter), and *C. meleagridis* infects the small intestine of birds as opposed to the respiratory tract. However, oocysts of *C. meleagridis* are similar to those of *C. felis*, *C. wrairi*, and *C. parvum* in terms of size and morphology [51]. Bovine *C. parvum* has been successfully transmitted to birds [51, 52], and oocysts of *C. meleagridis* have been shown infectious for mammals as well, including mice, rats, rabbits, and cattle [51, 53]. In addition, *C. meleagridis* has recently been identified in both immunocompromised and immunocompetent [41, 54-60] humans. Both *C. meleagridis* and *C. parvum* infect the small intestine, and the duration of the prepatent and patent periods, as well as the number of oocysts excreted, were almost identical for mice infected with *C. meleagridis* or *C. parvum* [51]. Molecular genetic analyses have shown the *C. meleagridis* and *C. parvum* are closely related [61] but distinct [8, 12, 13, 16]. Two *C. meleagridis* isolates from Hungary and the United States, respectively, showed identical DNA sequences in a portion of the 18S rRNA gene [51], supporting conservation of the gene across wide geographic areas. Further genotypic analysis of eleven *C. meleagridis* isolates showed two and six distinct genotypes at the 18S rRNA and 60-kDa glycoprotein loci, respectively; six genotypes at the HSP70 gene were also identified from analysis of eight *C. meleagridis* isolates [62].

C. baileyi. *Cryptosporidium* was described in the ceca of chickens in 1929 [63] but was not identified as *C. baileyi* until 1986 [64]. Oocysts of *C. baileyi* are morphologically distinct from other *Cryptosporidium* species, and host specificity is unique and limited to certain birds. *C. baileyi* does not cause infection in mice, rats, pigs, goats, or quail, but has been reported to cause mild infection in turkeys and heavy infection in ducks and geese [64, 65]. Similarly, Egyed et al. [66] found that *C. baileyi* was not infectious for mice, carp, frogs, and turtles but infectious for chicken, ducklings, and turkeys. *C. baileyi* causes a respiratory infection in birds, with parasite location in the bursa of Fabricius, cloaca, trachea, bronchi, and air sacs [66, 67]. Sequence and phylogenetic analyses at various loci have shown that *C. baileyi* is distinct from other *Cryptosporidium* species [6-8, 12, 13, 16, 66]. Given the distinct oocyst morphology, host specificity, organ location, and genetic characterization, *C. baileyi* is considered a valid species.

C. felis. *Cryptosporidium* oocysts from cat feces are slightly smaller (average: 4.6 x 4.0 μm) than those from humans (average: 5.0 x 4.5 μm) [68]. In addition, multiple feline

Cryptosporidium isolates from different continents are virtually genetically identical within a portion of the 18S rRNA locus [68, 69] and phylogenetic analyses at the 18S rRNA, dihydrofolate reductase, and actin loci provide strong support for *C. felis* as a distinct and valid species [6, 8, 13]. Additional support for unique species status is the GC content of the heat shock protein 70 (HSP70) gene; most *Cryptosporidium* species are AT-rich (58-66% A or T) in the HSP70 gene, but *C. felis* has 51.0% A or T content at this locus [37]. Feline *Cryptosporidium* oocysts are not infectious for mice, rats, guinea pigs, or dogs [5], but the host specificity of the species has come into question with the recent identification of *C. felis* in the feces of a cow [70] and both immunocompetent and immunosuppressed humans [39-41, 55-58, 71].

C. serpentis. *Cryptosporidium* was first described in snakes in 1977 [72] and designated a new species, *C. serpentis*, in 1980 [73]. Morphologically, oocyst size (6.2 x 5.3 μm) and electrophoretic protein profiles differentiate *C. serpentis* from *C. parvum* [74]. Biologically, oocysts of *C. serpentis* are not infective for mice [74], causing a gastric infection in reptiles that is usually asymptomatic in lizards but symptomatic in snakes [75]. Genetic analysis of the *Cryptosporidium* oocyst wall protein shows that *C. serpentis* is closely related to the other gastric species of *Cryptosporidium*, *C. muris* and *C. andersoni*, but has significant polymorphisms from the intestinal and respiratory *Cryptosporidium* species [16]. Further genetic analyses of the HSP70, 18S rRNA, and actin loci confirm the distinctness of *C. serpentis* [7, 8, 12, 13]. Some intraspecies genetic variation has been reported in the 18S rRNA gene, with two snake isolates differing from two lizard isolates [7, 76]. In addition, morphometric studies of oocysts recovered from snakes and lizards have shown the occurrence of at least 5 morphological types [77]. It has been suggested that these morphologically-distinct isolates may represent oocysts of *C. parvum* and *C. muris* from ingestion of infected prey [78], identifying a limitation of using host information as a primary indication of oocyst species.

C. muris. *C. muris* differs from *C. parvum* and the other intestinal *Cryptosporidium* parasites morphologically, biologically, and genetically. Oocysts of *C. muris* (8.0 x 6.2 μm) are larger than those of *C. parvum* (5.0 x 4.5 μm) and cause a gastric rather than intestinal infection [1]. Molecular genetic analyses at numerous loci have confirmed the validity of *C. muris* as a distinct

species, revealing it to be the most divergent species of *Cryptosporidium* and most closely related to *C. serpentis* [6-8, 12, 13, 16].

C. muris has been identified in both rodents and ruminants, and results of several biological and phylogenetic analyses have suggested the existence of two distinct *C. muris* genotypes, a bovine genotype (associated with cattle and potentially camel hosts) and a murine genotype (associated with mouse, hamster, rock hyrax, and camel hosts). *C. muris* isolates from rodents, a camel, and a rock hyrax were infectious for mice, but *C. muris* bovine isolates did not readily infect mice [79-81]. In addition, genetic differences between bovine and murine *C. muris* were identified at the 18S rRNA, internal transcribed spacer 1 region, and HSP70 loci [7, 9, 12]. More recently, the bovine genotype of *C. muris* has been renamed *C. andersoni* [82].

A number of non-rodent hosts have been infected with the *C. muris* murine genotype, including dogs, guinea pigs, rabbits, lambs, and cats [83-85]. Recently, *C. muris* has been identified in both immunocompetent and immunocompromised humans [56, 58, 86, 87], further extending the host range of this species and increasing its importance for human health.

C. andersoni. Lindsay et al. [82] recently proposed the bovine genotype of *C. muris* to be a distinct species, *C. andersoni*, based on oocyst morphology, host specificity, and genetic analysis. Oocysts of *C. andersoni* (7.4 x 5.5 μm) were found to be significantly different from oocysts of *C. muris* (8.4 x 6.3 μm) and *C. parvum* (5.0 x 4.5 μm) in terms of lengths, widths, and length/width ratios. A slight flattening on one side of *C. muris* oocysts was also noted as a distinguishing feature between *C. andersoni* and *C. muris*. In addition, Lindsay et al. [82] reported that *C. andersoni* oocysts were not infectious for mice, chickens, or goats. Based on these data, in addition to molecular analyses distinguishing the murine and bovine genotypes of *C. muris*, Lindsay et al. [82] proposed that *C. muris*-like oocysts in cattle are actually a distinct species, *C. andersoni*. Sreter et al. [88] confirmed the authenticity of the *C. andersoni* described by Lindsay et al. [82] by morphologic, host specificity, and genetic characterization of a European *C. muris*-like isolate from cattle. The genetic distinction of *C. andersoni* has been further shown in sequence analysis of the *Cryptosporidium* oocyst wall protein [16] and the actin gene [13]. A recent report [89] of a *C. andersoni* isolate that was infectious for

immunocompromised mice, however, may be indicative of heterogeneity among *C. andersoni* isolates and suggests that the host range of *C. andersoni* might be more extensive than originally reported.

C. saurophilum. *Cryptosporidium saurophilum* was described as a new species of *Cryptosporidium* from lizards, skinks (*Eumeces Schneideri*), and desert monitors in 1998 [90]. Although associated with reptiles, *C. saurophilum* is distinct from *C. serpentis* in that it has smaller oocysts (5.0 x 4.7 μm), develops in the small intestine, and is not infectious for snakes [90]. Sequence and phylogenetic analysis of the actin locus show that *C. saurophilum* is genetically distinct from *C. serpentis* and the intestinal *Cryptosporidium* parasites [13], but further investigations of host specificity and molecular genetics will be necessary to confirm its status as a valid species.

C. nasorum. *Cryptosporidium* sp. was first identified in a tropical marine fish, *Naso lituratus*, in 1981 [91] and has since been reported in both the stomachs and intestines of multiple species of freshwater and marine fish [75, 92, 93]. Oocysts are slightly smaller than those of *C. parvum* [94]. The name *C. nasorum* was given to the species of *Cryptosporidium* in fish in 1984 [95], but little is known about the morphological details, host range, and molecular genetics of the species.

C. molnari. A new *Cryptosporidium* species in fish, based on detailed morphological studies of oocysts and endogenous studies, has been described as *C. molnari* [96]. Oocysts of *C. molnari* are within the size range of *C. parvum* oocysts (and larger than oocysts of *C. nasorum*) but likely possess a distinct protein profile as monoclonal antibodies against *C. parvum* oocysts did not react with *C. molnari*. In contrast to other *Cryptosporidium* spp., which develop intracellularly but extracytoplasmically, endogenous stages of *C. molnari* were found deep within the epithelial cell. *C. molnari* was found preferentially in the stomach and seldom in the intestine. No *Cryptosporidium* sp. from fish has been genotyped yet, and molecular genetic characterization will be necessary to validate the taxonomical classification of *C. molnari*.

C. blagburni. Morgan et al. [97] described a new avian species of *Cryptosporidium*, *C. blagburni*, based on sequence and phylogenetic analysis of the 18S rRNA and HSP70 loci. In addition to the molecular analysis, the finch-derived *C. blagburni* isolates were found only in the proventriculus, a glandular portion of the avian compound stomach. This organ location is distinct from the locations of the other avian *Cryptosporidium* species, *C. baileyi* (respiratory tract) and *C. meleagridis* (intestine). Additional genetic, biological, and morphological data are necessary to confirm the species status of *C. blagburni*.

Implications of Taxonomy for Epidemiological Studies

The *C. parvum* dog and pig genotypes, *C. meleagridis*, *C. muris*, *C. felis*, and unrecognized *Cryptosporidium* species have been identified in both immunocompromised and immunocompetent humans by a combination of morphological and genetic methods [39-41, 54-54-60, 86, 87, 98-101]. These reports contradict conventional wisdom that only *C. parvum* human and bovine genotypes infect people and have raised questions about which species of *Cryptosporidium* are important for public health.

Studies involving the sources, fate, and transport of *Cryptosporidium* spp. in the environment are critical to the understanding of oocyst dynamics and the prevention of human exposure. Field studies to date have revealed the presence of many *Cryptosporidium* species and genotypes in surface waters, animal reservoirs, and fecal samples [33, 102-110], and increasing parasite diversity has been identified [102, 103, 107, 109]. Yet, until the taxonomy of the genus is standardized and complete, environmental detection of novel genotypes and non-*parvum* species will provide little information with respect to the potential health risks posed by those parasites.

Standardization of *Cryptosporidium* spp. Taxonomy

A critical problem with the current taxonomy of the genus *Cryptosporidium* is the lack of standardization. Taxonomic classifications have been made on the basis of host range, morphological data, biological characterization, and/or genetic analysis, but few taxa have been named on the basis of all four criteria. Egyed et al. [66] have suggested a polyphasic model for

characterization of cryptosporidia based on oocyst morphology, host specificity, organ location, virulence, and genetic characterization at multiple loci. Addressing each of these criteria is often outside the scope of any one laboratory, and thus, such rigorous taxonomic classifications may require collaborations among researchers.

Because the oocyst morphologies of many *Cryptosporidium* isolates are indistinguishable, and because the potential for genetic recombination between two isolates is not currently observable if those isolates do not infect the same host, a polyphasic approach to taxonomy, such as the one suggested by Egyed et al. [66], is warranted. However, a balance must be found between including enough criteria to make sound judgments of taxonomic status and including so many criteria that finding two isolates with common traits becomes rare. For example, the virulence of isolates may not an ideal factor to include in a polyphasic typing system because of its variability from host to host and its dependence on the host immune status. A classification system based on oocyst morphology, host specificity, organ location, and genetic characterization at multiple loci seems reasonable and is suggested. Host specificity is not easily addressed, given an unlimited number of potential hosts and the facilities required to undertake experimental infections, and thus, it may be appropriate to identify a condensed list of hosts to include in the analysis. Regardless of the criteria ultimately selected for a new taxonomic system, we must adopt uniform guidelines. The standardization of criteria to assign species status to *Cryptosporidium* isolates will allow complete comparisons of different isolates and will greatly improve the impact of future clinical, environmental, and epidemiological studies.

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Table 1. Summary of biological data for *Cryptosporidium* species and genotypes.

Species /Genotype	*Main Host*	*Other Hosts*	*Infection*	*Oocyst Size*[a]
C. parvum genotype 1/ *C. hominis*	Humans	Gnotobiotic pig [Ref. 23] Lamb [Ref. 25] Dugong [Ref. 24]	Intestinal	4.4-5.9x4.4-5.4 µm (5.2x4.86 µm) SI=1.0-1.09 (1.07) N=100 [Ref. 28]
C. parvum genotype 2	Mammals	-	Intestinal	4.8-5.6x4.2-4.8 µm (5.2x4.6 µm) SI=1.04-1.33 (1.16) N=30 [Ref. 44]
C. parvum pig genotype	Pigs	Humans [Ref. 98] Squirrels [Ref. 104]	Intestinal	ND[b]
C. parvum marsupial genotype	Koalas Kangaroos	-	Intestinal	ND
C. parvum mouse genotype	Mice	Bat [Ref. 26]	Intestinal	4.5x4.0 µm [Ref. 26]
C. parvum ferret genotype	Ferret	-	Intestinal	ND
C. parvum dog genoype/ *C. canis*	Dog	Humans [Ref. 39-41] Cattle [Ref. 37]	Intestinal	3.68-5.88x3.68-5.88 µm (4.95x4.71 µm) SI=1.04-1.06 (1.05) N=200 [Ref. 37]
C. wrairi	Guinea pigs	-	Intestinal	4.8-5.6x4.0-5.0 µm (5.4x4.6 µm) SI=1.04-1.33 (1.17) N=30 [Ref. 44]
C. meleagridis	Birds	Humans [Ref. 41, 54-60]	Intestinal	4.5-6.0x4.2-5.3 µm (5.2x4.6 µm) SI=1.00-1.33 (1.13) N=40 [Ref. 49]
C. felis	Cats	Cow [Ref. 70] Humans [Ref. 39-41, 55-58, 71]	Intestinal	3.2-5.1x3.0-4.0 µm (4.6x4.0 µm) N=40 [Ref. 68]
C. saurophilum	Reptiles	-	Intestinal	4.4-5.6x4.2-5.2 µm (5.0x4.7 µm) SI=1.04-1.12 (1.09) N=30 [Ref. 90]
C. baileyi	Birds	-	Respiratory (cloaca, bursa, respiratory tract)	6.0-7.5x4.8-5.7 µm (6.6x5.0 µm) SI=1.05-1.79 (1.33) N=40 [Ref. 49]

Table 1 (continued).

Species /Genotype	*Main Host*	*Other Hosts*	*Infection*	*Oocyst Size*[a]
C. muris	Rodents	Dogs, Guinea pigs, Rabbits, Lambs, Cats [Ref. 83-85] Humans [Ref. 56, 58, 86, 87]	Gastric	7.4-8.8x5.8-6.6 µm (8.0x6.2 µm) SI=1.19-1.40 (1.28) N=20 [Ref. 82]
C. andersoni	Cattle	Mice [Ref. 82]	Gastric (abomasum)	6.0-8.1x5.0-6.5 µm (7.4x5.5 µm) SI=1.07-1.50 (1.35) N=50 [Ref. 82]
C. serpentis	Reptiles	-	Gastric	5.6-6.6x4.8-5.6 µm (6.2x5.3 µm) SI=1.04-1.33 (1.16) N=30 [Ref. 74]
C. nasorum	Fish	-	Gastric and Intestinal	3.5-4.7x2.5-4.0 µm (4.3x3.3 µm) N=6 [Ref. 94]
C. molnari	Fish	-	Mainly gastric; seldom intestinal	3.23-5.45x3.02-5.04 µm (4.72x4.47 µm) SI=1-1.17 (1.05) N=22 [Ref. 96]
C. blagburni	Birds	-	Gastric (proventriculus)	ND

[a]Data range of oocyst length x width is given, followed by mean values in parentheses. SI = data range of shape index (length-to-width ratio), followed by mean value in parentheses. N = number of oocysts examined. References denoted in brackets.
[b]ND = not determined.

Chapter 3: Sources and Species of *Cryptosporidium* Oocysts in the Wachusett Reservoir Watershed

Jellison, K. L., H. F. Hemond, and D. B. Schauer. 2002.
Applied and Environmental Microbiology 68:569-575.

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Abstract

Understanding the behavior of *Cryptosporidium* oocysts in the environment is critical to developing improved watershed management practices for protection of the public from waterborne cryptosporidiosis. Analytical methods of improved specificity and sensitivity are essential to this task. We developed a nested polymerase chain reaction/restriction fragment length polymorphism assay that allows detection of a single oocyst in environmental samples and differentiates the human pathogen *C. parvum* from other *Cryptosporidium* species. We tested our method on surface water and animal fecal samples from the Wachusett Reservoir watershed in central Massachusetts. We also directly compared results from our method with those from the immunofluorescence microscopy assay recommended in the Information Collection Rule. Results suggest that immunofluorescence microscopy may not be a reliable indicator of public health risk for waterborne cryptosporidiosis. Molecular and environmental data identify both wildlife and dairy farms as sources of oocysts in the watershed, implicate times of cold water temperatures as high-risk periods for oocyst contamination of surface waters, and suggest that not all oocysts in the environment pose a threat to public health.

Introduction

Cryptosporidium parvum is an intracellular protozoan parasite responsible for an acute gastrointestinal, and less frequently, respiratory infection in humans that is self-limiting in immunocompetent people but prolonged and potentially life-threatening for the immunocompromised population (32). Gastrointestinal cryptosporidiosis is characterized by watery diarrhea, abdominal pain, low-grade fever (<39°C), general malaise, weakness, fatigue, loss of appetite, nausea, vomiting, and weight loss (10, 40). Symptomatic infection may last from a few days to a few weeks in immunocompetent individuals, although extreme cases of up to 12 weeks of severe diarrhea have been reported (40). Cryptosporidiosis is particularly serious for immunosuppressed people because no curative treatment presently exists.

The existence of multiple species of *Cryptosporidium*, including *C. parvum*, *C. muris*, *C. felis*, *C. wrairi*, and *C. andersoni* (mammals), *C. baileyi* and *C. meleagridis* (birds), *C. serpentis* (reptiles), and *C. nasorum* (fish), has been suggested on the basis of oocyst morphology, host specificity, infectivity, and 18S rRNA sequence comparisons (34, 35, 40). There is some uncertainty with respect to the validity of these taxa. For example, *C. wrairi* appears to be a strain of *C. parvum* that is isolated from guinea pigs, while *C. andersoni* is a recently proposed species characterized by *C. muris*-like oocysts that infect cattle (21). Classifications based on host species may not be appropriate given that *C. felis*, associated with cryptosporidial infection in cats, was recently isolated from a cow (4). There are now multiple reports of species other than *C. parvum* infecting humans, particularly immunocompromised people (11, 17, 26, 28, 29, 38). Due to the confusion surrounding the taxonomy of *Cryptosporidium*, it is difficult to conclusively assess the human public health threat attributable to *Cryptosporidium* species other than *C. parvum*

Numerous outbreaks of waterborne cryptosporidiosis in the United States have occurred over the past 20 years (6, 32) in both rural and urban areas, spanning the nation from Pennsylvania to Oregon. *Cryptosporidium* species are a threat to water supplies because they are resistant to chlorine disinfection, small (~5μm diameter) and thus difficult to filter, and harbored in many animal species (10). The largest waterborne outbreak in U.S. history occurred in Milwaukee in

the spring of 1993 and affected an estimated 403,000 people served by the Milwaukee Water Works. The Wisconsin Division of Health found that the outbreak was responsible for the premature deaths of at least 69 individuals, most of whom were HIV-positive. The sources of oocyst contamination, though not identified conclusively, were suspected to include cattle waste, slaughterhouse waste, and human sewage. The combination of severe spring rains and snowmelt runoff that occurred just prior to the outbreak could have carried oocysts from these suspected sources into Lake Michigan and subsequently into the intakes of the Milwaukee Water Works treatment plants. Treatment processes at the South Milwaukee Water Works plant included: chlorine and permanganate addition at the raw water intake, polyaluminum chloride coagulation, rapid mixing, flocculation, sedimentation, rapid sand filtration, chlorination, and fluoride addition. Despite such thorough water treatment, turbidity of the South Milwaukee Water Works plant effluent exceeded the 1993 EPA limit of 1.0 NTU, peaking at 1.7 NTU in late March 1993. (9, 23, 32)

This episode of *Cryptosporidium* oocysts passing through a water treatment plant bolsters the argument that successful public health measures must include appropriate watershed management. Improved watershed management requires a better understanding of the behavior of *Cryptosporidium* oocysts in the environment, and this in turn requires improved analytical detection methods. We now report a sensitive and specific nested polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) assay for detection of *Cryptosporidium* oocysts in environmental samples. This nested PCR targets a 434-bp hypervariable region of the 18S rRNA gene, a multi-copy gene (20 copies per oocyst) ideal for species identification. Application to surface water and animal fecal samples from the Wachusett Reservoir watershed in central Massachusetts confirms the method's high degree of sensitivity and specificity and provides new hypotheses regarding controls of *Cryptosporidium* oocyst contamination in surface waters.

Molecular methods for detection of *Cryptosporidium* oocysts in wastewater and surface water have been reported (22, 37, 39), and we have extended these studies with the development of a novel assay and its application to the investigation of sources and species of oocysts in a geographic area that has not been previously described. The Wachusett Reservoir, a drinking

water source for Boston and surrounding cities, has recently been the subject of litigation concerning appropriate measures to protect against waterborne parasites such as *C. parvum* and *Giardia lamblia*. Our goal of understanding the sources, species, and seasonal trends of oocyst contamination in watersheds will contribute to the development of better watershed management practices to prevent waterborne outbreaks of cryptosporidiosis in drinking water watersheds.

Materials and Methods

Oocysts. GCH1 *Cryptosporidium parvum* oocysts were a kind gift of Giovanni Widmer at Tufts University School of Veterinary Medicine in North Grafton, Massachusetts.

Surface Water Sample Selection. Sampling sites in the Wachusett Reservoir watershed in central Massachusetts (Figure 1) were chosen to encompass a variety of potential sources of *Cryptosporidium* contamination. Surface water sites (and their suspected source of contamination) included Stillwater River (wildlife); Quinapoxet River (wildlife); Gates Brook (sewage); and two small, unnamed brooks, designated Brook JF and Brook SF, downgradient from dairy farms (agricultural runoff). Stillwater River and Quinapoxet River were sampled monthly from February 2000 to January 2001, often side-by-side with the Metropolitan District Commission (MDC) of the Commonwealth of Massachusetts. The MDC followed the Information Collection Rule (13), using conventional yarn-wound filters and immunofluorescence microscopy (IFA) for oocyst detection. Gates Brook, Brook JF, and Brook SF were sampled periodically, but not as frequently, from March 1999 to January 2001.

Sample Collection. Surface waters were filtered through Gelman Envirochek Sampling Capsules (Pall Gelman Sciences Inc., Ann Arbor, Michigan) at 1-2 L min^{-1} according to manufacturer's recommendations. During filtration, water temperature was recorded. Filtration continued for one hour or until the backpressure exceeded the filter rating (30 psid), whichever came first. Typically, 40 to 80 L of water were filtered. Filters were transported to the laboratory on ice and samples were eluted according to manufacturer's recommendations within 36 h of sample collection. Eluted solids were resuspended in 10 mL laboratory-grade water (Milli-Q System, Millipore Corporation, Bedford, MA) for each 0.5 mL solids, stored at 4°C, and processed within 24 h.

Fecal samples were collected in sterile 50 mL polypropylene tubes and transported to the laboratory on ice. Fecal samples were suspended in 10 mL laboratory-grade water for each 0.5 mL solids, stored at 4°C, and processed within 24 h of collection.

Immunomagnetic Separation of Oocysts. Oocysts were purified from water and fecal samples using immunomagnetic separation (IMS) with the Crypto-Scan IMS kit (ImmuCell, Portland, ME) according to the recommendations of the manufacturer. After being dissociated from magnetic beads, oocysts were transferred to a new microcentrifuge tube and treated with 5 µL of 1N NaOH to neutralize pH. The oocysts were pelleted for 2 to 3 min at 16000xG, resuspended in 50 µL laboratory-grade water, and stored at 4°C.

Positive and negative IMS controls were processed with each set of field samples. Positive IMS controls consisted of 9.9 mL laboratory-grade water and 100 µL of a 10^4 mL^{-1} oocyst suspension; negative IMS controls consisted of 10 mL laboratory-grade water. IMS controls were processed as described above.

Genomic DNA Extraction. Oocysts were lysed by adding 25 µL IMS product to 475 µL Tris-EDTA (TE) buffer containing 0.2 g L^{-1} proteinase K and 0.4% SDS and incubating overnight at 45°C. (Positive and negative DNA extraction controls were included for each set of field samples. Positive DNA extraction controls consisted of 25 µL of a 10^4 mL^{-1} oocyst suspension in 475 µL TE buffer; negative DNA extraction controls consisted of 25 µL laboratory-grade water in 475 µL TE buffer.) DNA was extracted several times with phenol-chloroform, precipitated with 0.2M NaCl and 2 volumes of absolute ethanol, and resuspended in 30 µL TE buffer.

Nested PCR Assay. PCR amplification was performed in a 50 µL volume containing 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.015 mM each dNTP (Perkin Elmer, Wellesley, MA), 0.2 µM each primer, and 2 U of Taq DNA polymerase (Promega Corporation, Madison, WI). The initial amplification reaction was performed with 15 µL of DNA template, and 3 µL of the initial amplification product was used as template for the secondary PCR. Positive and negative PCR controls were included with each set of samples. For the initial amplification reaction, positive PCR controls contained 12 µL laboratory-grade water and 3 µL of genomic *C. parvum* DNA (at a concentration equivalent to 10^4 oocysts μL^{-1}); negative PCR controls contained 15 µL laboratory-grade water. For the secondary amplification reaction,

positive PCR controls contained 3 μL of genomic *C. parvum* DNA (at a concentration equivalent to 10^4 oocysts μL^{-1}); negative PCR controls contained 3 μL laboratory-grade water.

Both amplification reactions used forward and reverse oligonucleotide primers that are complementary to *Cryptosporidium* 18S rRNA gene sequences (Figure 2). The initial 1056-bp product was obtained with a forward primer (5'-CCACATCTAAGGAAGGCAGC-3'; KLJ1) corresponding to nucleotides 389 to 408 and a reverse primer (5'-ATGGATGCATCAGTGTAGCG-3'; KLJ2) corresponding to nucleotides 1422 to 1441 of *C. parvum* L16996 in GenBank (3). The final 434-bp product was obtained using forward and reverse primers CPB-DIAGF and CPB-DIAGR, respectively (16). Cycling conditions consisted of an initial denaturation (5 min at 80°C followed by 30 sec at 98°C), 40 cycles of amplification (denaturation for 30 sec at 94°C, annealing for 30 sec at 53°C, extension for 1 min at 72°C), and a final extension (10 min at 72°C). Secondary PCR products were visualized following electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Restriction Fragment Length Polymorphism Analysis. Digestion of amplified 18S rRNA gene products with *Nde*I can be used to differentiate most *C. parvum* isolates from non-*parvum* species of *Cryptosporidium*. The 434-bp final amplicon of most *C. parvum* isolates (with the exception of GenBank accession numbers AF112570 and AF108860, isolates from a kangaroo and a koala in Australia, respectively, and AF112576, the dog genotype) contains a single *Nde*I site (Figure 2), while the amplicons from other *Cryptosporidium* species (*C. muris*, *C. baileyi*, *C. serpentis*, and *C. felis*) do not. Restriction digestion was carried out in a 20 μL volume containing 10 μL of secondary PCR product, 20 U *Nde*I (New England Biolabs, Beverly, MA), 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, and 100 $\mu g\ mL^{-1}$ BSA and incubated at 37°C for 1 h. Digestion products were visualized after electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Cloning. Secondary PCR products from water or fecal samples positive for *Cryptosporidium* were cloned into the pGEM-T Easy Vector System (Promega Corporation, Madison, WI) and used to transform XL-1 Blue *E. coli* cells (Stratagene, La Jolla, California). Clones were selected on LB agar supplemented with 100 $\mu g\ mL^{-1}$ ampicillin and cultured overnight in LB

broth supplemented with 100 μg mL^{-1} ampicillin. Plasmid DNA was isolated from clones using the QIAPrep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA) and digested with *Not*I (New England Biolabs) to verify the presence of the secondary PCR amplicon insert. Plasmids with the insert were further digested with *Nde*I. All digestion products were visualized after electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Sequencing. Representative clones of the secondary PCR products were sequenced on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PE Applied Biosystems). If multiple *Nde*I digestion patterns existed among clones from a given sample, at least one clone of each digestion pattern was sequenced. At least two clones for each positive sample were sequenced in any case and confirmed by sequencing both strands. The basic local alignment search tool (BLAST) algorithm was used to compare cloned DNA sequences with GenBank sequences and to determine the species of *Cryptosporidium* present in the sample (1, 3). Multiple sequence alignments and phylogenetic trees were generated with MacVector 7.0 (Genetics Computer Group, Madison, WI) with manual adjustment.

Results

By seeding PCR reactions with known quantities of oocyst DNA, initial PCR amplification of the 18S rRNA gene was found to detect as few as 500 oocysts; the lower limit of detection of nested PCR was a single oocyst (Figure 3). This detection limit assay, however, was performed under ideal conditions and did not account for the possible presence of PCR inhibitors in environmental samples. The potential for PCR inhibition was tested by processing two filters side-by-side for a single surface water source: one filter contained the surface water only, and the second filter contained the surface water seeded with 500 *C. parvum* oocysts. Using one-half of the eluted water pellets for IMS, one-half of the IMS products for DNA extraction, and one-thirtieth of the DNA extract for PCR, the initial PCR of the seeded sample received the DNA equivalent of 4.2 oocysts. Following the secondary amplification reactions, no oocysts were detected in the surface water sample alone; oocysts were clearly detected in the spiked surface water sample (Figure 4).

For the year spanning February 2000 to January 2001, 34 surface water samples were collected for *Cryptosporidium* detection and 5 (14.7%) were positive by nested PCR. In addition, 44 water samples were collected by the MDC and 5 (11.4%) were positive by IFA. Table 1 shows all surface water samples positive for *Cryptosporidium* by either nested PCR or IFA and two additional samples analyzed in March and July of 1999. Of the 7 samples positive by nested PCR, *C. parvum* was identified in 3 samples (2/1/00, 4/4/00, and 11/7/00). The sample collected on 2/1/00 was a mixed population of *C. parvum* and *C. muris*, and *C. muris* appeared to be more prevalent since only one of the 12 clones could be digested with *Nde*I (the single clone containing an *Nde*I site was sequenced and identified as *C. parvum*, and 2 of the remaining clones were identified as *C. muris*). *C. muris* and *C. baileyi* were identified in 3 and 1 of the 7 positive samples, respectively. One positive sample could not be cloned and sequenced due to insufficient sample quantity.

Agricultural and wildlife fecal samples were collected in June and August of 2000. Results are summarized in Table 2. Among wildlife samples, *C. parvum* was found only in fresh deer feces and *C. baileyi* was identified in the feces from cormorants alone. No *Cryptosporidium* were

isolated from adult cattle on farm SF or from calves on farm JF. *C. baileyi* and *C. muris* were identified in adult cattle and in the manure pit, respectively, on farm JF.

Discussion

Nested PCR targeting the variable region of the 18S rRNA gene enabled detection of a single *Cryptosporidium* oocyst (Figure 3); this compares favorably to other sensitive PCR-RFLP methods for detection of *Cryptosporidium* (16, 22). Given an ID_{50} of 132 oocysts (7), our nested PCR should allow detection of oocysts in environmental samples at and below infectious levels. For all water and fecal samples that tested positive for *Cryptosporidium* oocysts, nested PCR was necessary for detection (i.e., no signal was detected in any sample after initial PCR amplification). Our findings suggest that single PCR, which has been used for both laboratory and environmental samples (2, 16, 19, 20, 22, 27, 33), may not be sensitive enough for detection of commonly-occurring levels of oocyst contamination in the environment.

This *Cryptosporidium* detection assay offers a high degree of sensitivity and species-level oocyst identification. Although the assay does not provide information about oocyst viability, detection of any *C. parvum* oocysts in environmental samples from source water watersheds is a warning that precautionary measures should be considered to protect public health. Oocyst viability is influenced by many environmental factors, including temperature, hydration, starvation, predation, and UV exposure (8, 14, 24, 30). The presence of oocysts in the environment, even if non-viable at one time, is an indication that potentially viable oocysts may be present under different environmental conditions in the future.

We were able to detect multiple species of *Cryptosporidium* oocysts in water and fecal samples, including *C. parvum*, *C. muris*, and *C. baileyi* (Tables 1 and 2). The 434-bp secondary PCR product is ideal for species identification because it spans the most hypervariable region of the 18S rRNA gene but also includes recognizable, conserved anchors (Mitchell L. Sogin, personal communication).

U.S. EPA Method 1622 for *Cryptosporidium* analysis in water (25) uses IFA for detection of oocysts in environmental samples. Comparison of our results to those obtained by IFA illustrates that IFA may not be a reliable indicator of public health risk (Table 1). First, IFA results are based on visual identification of oocysts and do not classify the *Cryptosporidium*

species. Thus, oocysts identified by IFA must be assumed to be infectious in order to protect public health. With our molecular method, we identified *C. muris* in a sample presumed positive for *C. parvum* by IFA on 7/12/99, illustrating the importance of species-level oocyst detection. A second limitation of IFA is the possibility that sample debris cross-reacting with the fluorescent antibodies may lead to false-positive reports. We believe this is the most likely explanation for samples that were positive for *Cryptosporidium* by IFA on 2/22/00 and 12/5/00 but negative by our molecular method. We believe our results on those dates are true negatives because we have shown that a single oocyst can be detected under ideal circumstances (Figure 3) and have run controls that discount the likelihood of PCR inhibitors (Figure 4). Though we do not routinely run controls for PCR inhibitors, they should be sufficiently removed during filtration and IMS (12, 16, 31). Third, low numbers of oocysts in the environment may go undetected by IFA due to sample dilution and competition of sample debris with fluorescent antibodies. We also identified *Cryptosporidium* oocysts (*C. parvum* and *C. baileyi* on 11/7/00 and 12/5/00, respectively) in water samples that were negative by IFA.

Although some of the differences between IFA and our molecular method may be explained by the random distribution of oocysts in the water (i.e., if the concentration of oocysts in surface water is low, one filter may trap an oocyst while another filter running simultaneously does not), our data suggest that it is possible to incorrectly estimate the public health threat for cryptosporidiosis using conventional IFA. Not all *Cryptosporidium* species in the environment are *C. parvum*. In fact, *C. baileyi* and *C. muris* have been identified more often than *C. parvum* in our water samples (Table 1). Of the wildlife fecal samples analyzed (Table 2), *C. parvum* oocysts were found in fresh deer stool only. In contrast, *C. baileyi* was found in fecal samples from cormorant and adult dairy cattle, and *C. muris* was identified in a dairy farm manure pit. To our knowledge, infection by *C. baileyi* has never been described in cattle. We speculate that the feed may have been contaminated with *C. baileyi* by birds on the farm and that the oocysts passed transiently through the cattle (the cattle were passing normal feces). The fact that no *C. parvum* oocysts were isolated from the dairy farm cattle or manure pit is especially pertinent since dairy cattle are considered a major source of infectious oocysts. Also relevant is the fact that *C. muris* (and not *C. parvum*) was identified in the manure pit on farm JF and in Brook SF (where the suspected source of oocysts is agricultural runoff) on 3/1/99. A recent study (21)

proposed that the large form of *Cryptosporidium* (previously thought to be *C. muris*) infecting the abomasum of cattle is a new species, *C. andersoni*; however, the lack of 18S rRNA sequence data in GenBank precludes the identification of *Cryptosporidium* oocysts in our samples as *C. andersoni* instead of *C. muris*.

Phylogenetic analysis of the sequence data derived from our water and fecal samples indicate that the oocysts isolated from both wildlife and dairy farm fecal samples are closely related to the oocysts found in surface waters in the Wachusett Reservoir watershed (Figure 5). The fact that we found a mixed population of oocysts on 2/1/00 at Stillwater River (*C. parvum* and *C. muris*) suggests that either one source may harbor multiple oocyst species or that multiple sources exist for this site. Because wildlife are abundant in the area, the existence of multiple sources is plausible. *C. muris* appeared to be more abundant than *C. parvum* at this site (as indicated by the fact that only 1 of the 12 nested PCR clones had the *C. parvum*-like *Nde*I restriction pattern). Additional studies to determine if wildlife are a significant source of oocysts pathogenic for people are therefore needed.

Our data also indicate a seasonal pattern in oocyst contamination of surface waters. Water samples positive for oocysts were limited to late fall, winter, and early spring (Table 1). No oocysts were found in water samples between mid-April and mid-October with one exception on 7/12/99. High-risk periods for oocyst contamination are often thought to be linked to calving season in late winter and early spring, but the detection of oocysts in late fall and early winter suggests that additional factors are operating. The observed seasonal pattern correlates well with temperature; the maximum water temperature at which positive samples were found during 2000 was 9°C. Given that wildlife and dairy farm fecal samples collected in the summer (when water temperatures were above 9°C) were positive for *Cryptosporidium* oocysts, it appears that oocysts are present in the watershed year-round. Although hydrologic factors are often and probably correctly thought to influence oocyst transport to streams, it is also possible that grazers or predators may limit surface water populations of *Cryptosporidium* in the summer. Possibly other chemical or biotic factors limit oocyst survival in surface waters in warmer temperatures.

The nested PCR protocol described here can be helpful in the identification of sources and species of oocysts in watersheds, as well as the times of year when surface waters are most susceptible to oocyst contamination. Such information will aid in the development and implementation of the most appropriate watershed management policies and water treatment technologies to protect the public from exposure to *C. parvum*.

Acknowledgements

We are grateful for materials and technical assistance provided by Giovanni Widmer, Tufts University School of Veterinary Medicine, North Grafton, MA.

We thank the Metropolitan District Commission of the Commonwealth of Massachusetts for their generous help in identifying sampling locations and sharing the results of their IFA studies.

This work was supported by a National Science Foundation Graduate Research Fellowship, a United States Environmental Protection Agency "Science to Achieve Results" (STAR) Fellowship, and a feasibility project funded by the National Institute for Environmental Health Sciences.

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Table 1. Surface water samples that tested positive for *Cryptosporidium* spp.

Date	Location	Sample ID	MDC[a]	PCR-RFLP Results		
				Nested PCR	*Nde*I Digest[b]	Sequence Results[c]
3/1/99	Brook SF	SF	ND[d]	+	-	3/*C. muris*
7/12/99	Quinapoxet River	QR	+	+	-	3/*C. muris*
2/1/00	Stillwater River	SR	ND	+	1/+	1/*C. parvum*
					11/-	2/*C. muris*
2/22/00	Quinapoxet River		+	-		
3/7/00	Quinapoxet River		+	ND		
4/3/00	Quinapoxet River		+	ND		
4/4/00	Gates Brook	GB	ND	+	11/-	5/*C. parvum*
4/4/00	Brook JF		ND	+	ND[e]	ND[e]
10/23/00	Stillwater River		+	ND		
11/7/00	Quinapoxet River	QR1.5, QR2	-	+	6/-	2/*C. parvum*
12/5/00	Stillwater River	SR1.5, SR2	-	+	10/-	6/*C. baileyi*
12/5/00	Quinapoxet River		+	-		

[a]Results of MDC samples processed by IFA. + denotes presumptive positive for *C. parvum*.
[b]1/+: 1 nested PCR clone cut with *Nde*I; 11/-: 11 nested PCR clones did not cut with *Nde*I. For samples collected on 3/1/99 and 7/12/99, the complete nested PCR products did not cut with *Nde*I (the nested PCR clones were not digested individually).
[c]3/*C. muris*: the nucleotide sequences of 3 nested PCR clones were most closely related to *C. muris*.
[d]ND=Not done.
[e]Not done due to insufficient sample quantity.

Table 2. Results of fecal sampling.

Date	Location	Sample ID	Source	Nested PCR	*Nde*I Digest[a]	Sequence Results[b]
6/26/00	Farm SF		Adult Cattle	-		
6/26/00	Farm JF	Cow	Adult Cattle	+	5/-	2/*C. baileyi*
			Calves	-		
		Manure	Manure Pit	+	11/-	3/*C. muris*
8/21/00	Wachusett Reservoir		Geese	-		
			Deer (old)[c]	-		
		Deer	Deer (fresh)	+	3/-	3/*C. parvum*
			Geese/Corm[d]	-		
		Cormorant	Cormorant	+	9/-	3/*C. baileyi*

[a]5/-: 5 nested PCR clones did not digest with *Nde*I.
[b]2/*C. baileyi*: the nucleotide sequences of 2 nested PCR clones were most closely related to *C. baileyi*.
[c]Dessicated deer feces.
[d]Mixture of geese and cormorant feces.

Figure Captions

Figure 1. Schematic of the Wachusett Reservoir watershed sampling sites in central Massachusetts. SR = Stillwater River; QR = Quinapoxet River, GB = Gates Brook; SF = Brook SF; JF = Brook JF. Suspected sources of oocyst contamination include wildlife (SR and QR), sewage (GB), and agricultural runoff from dairy farms (SF and JF).

Figure 2. Schematic of the 1746-bp *Cryptosporidium* 18S rRNA gene (based on Genbank accession number L16996). Black rectangles depict regions of sequence variability within the gene. Primer binding locations are indicated above the gene (1 = KLJ1, 2 = CPB-DIAGF, 3 = CPB-DIAGR, 4 = KLJ2). Asterisk (*) identifies *Nde*I digest site.

Figure 3. Detection limit of nested PCR assay. (A) Initial PCR products (primers KLJ1/2). (B) Secondary PCR products (primers CPB-DIAGF/R). PCR reactions were spiked with known quantities of DNA representative of 1 to 10,000 oocysts (indicated at the top of each lane). Corresponding lanes on gels A and B represent the same spiked sample. The first lanes of gels A and B are molecular weight standards.

Figure 4. The potential for PCR inhibition was tested by seeding a surface water sample with 500 oocysts. From left to right, lanes are as follows: molecular weight standard; negative and positive control for secondary (2°) PCR, respectively; negative and positive control for initial (1°) PCR, respectively; negative and positive control for DNA extraction, respectively; negative and positive control for IMS, respectively; surface water sample (W); seeded surface water sample (W+).

Figure 5. Phylogenetic relationships among field samples and GenBank *Cryptosporidium* sequences (2, 10, 15, 25, 32-35). Phylogeny based upon multiple sequence alignments performed with MacVector 7.0 using the Tamura-Nei algorithm. A distance of 0.10 indicates a 10% difference between sequences. Field samples

labeled as: Sample ID-Clone# Date Sampled (e.g., "Manure-4 6/26/00" denotes clone #4 of Manure sampled on 6/26/00).

Figure 1

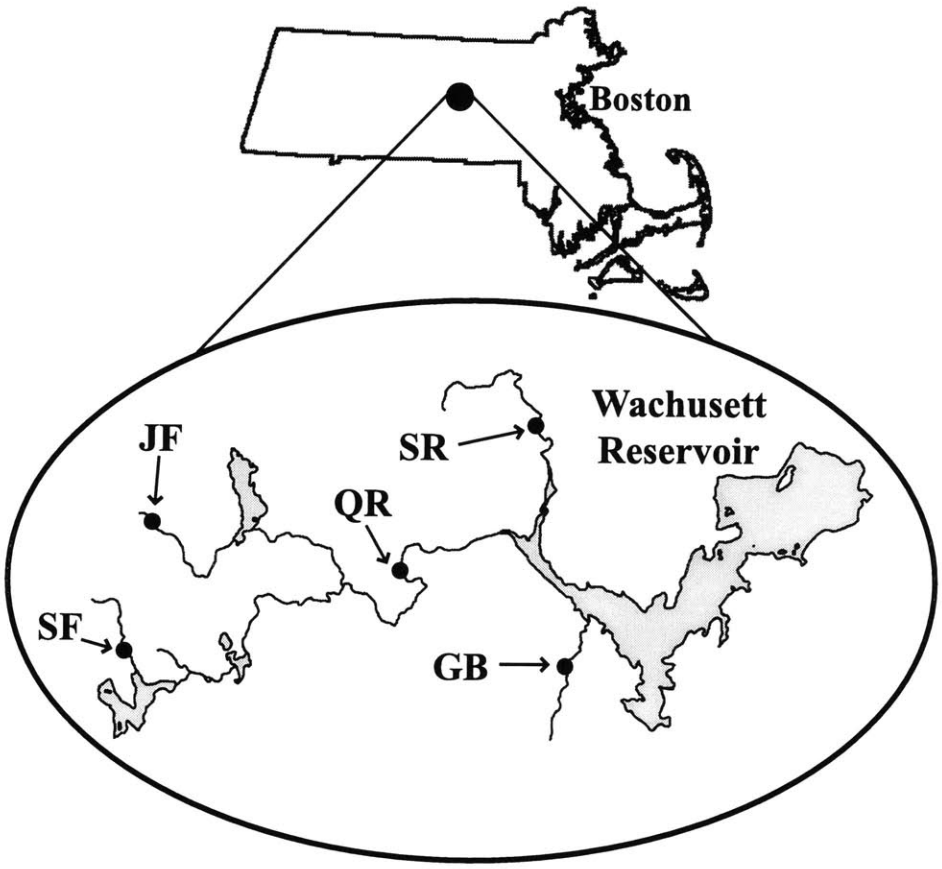


Figure 2

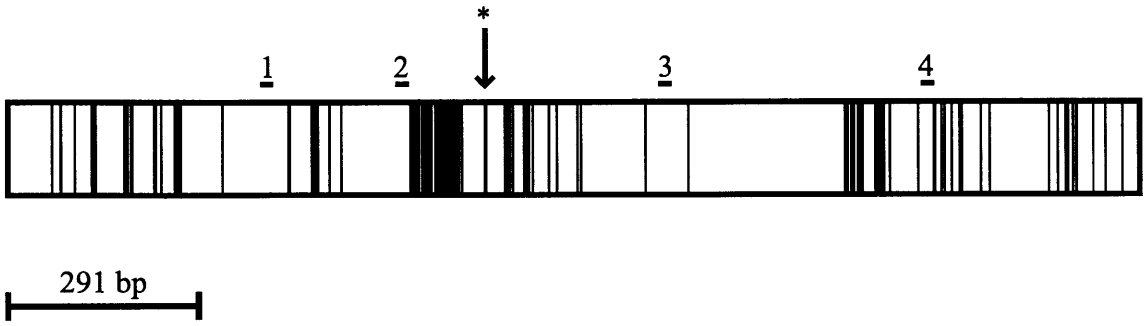


Figure 3

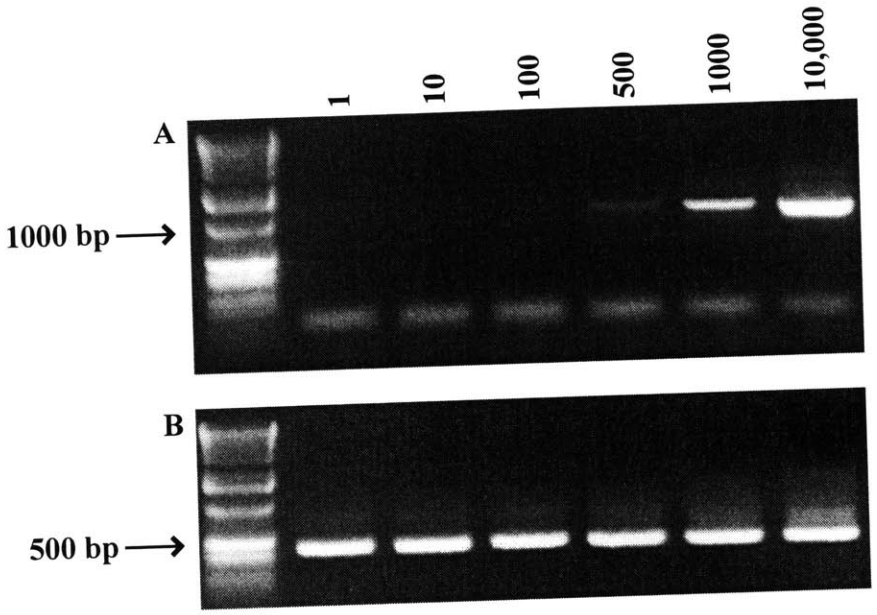


Figure 4

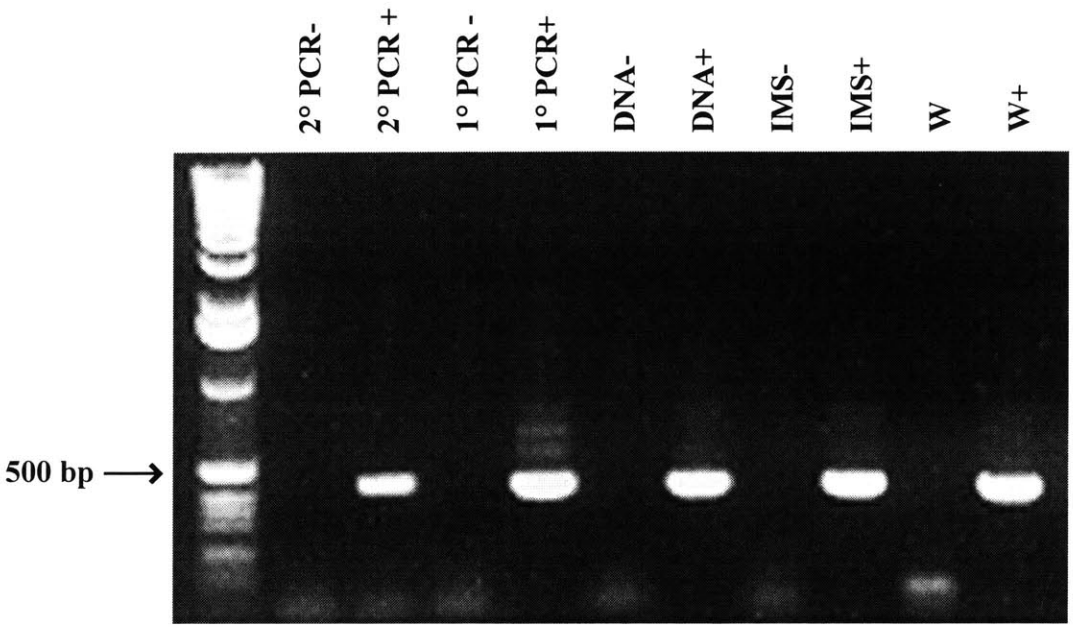
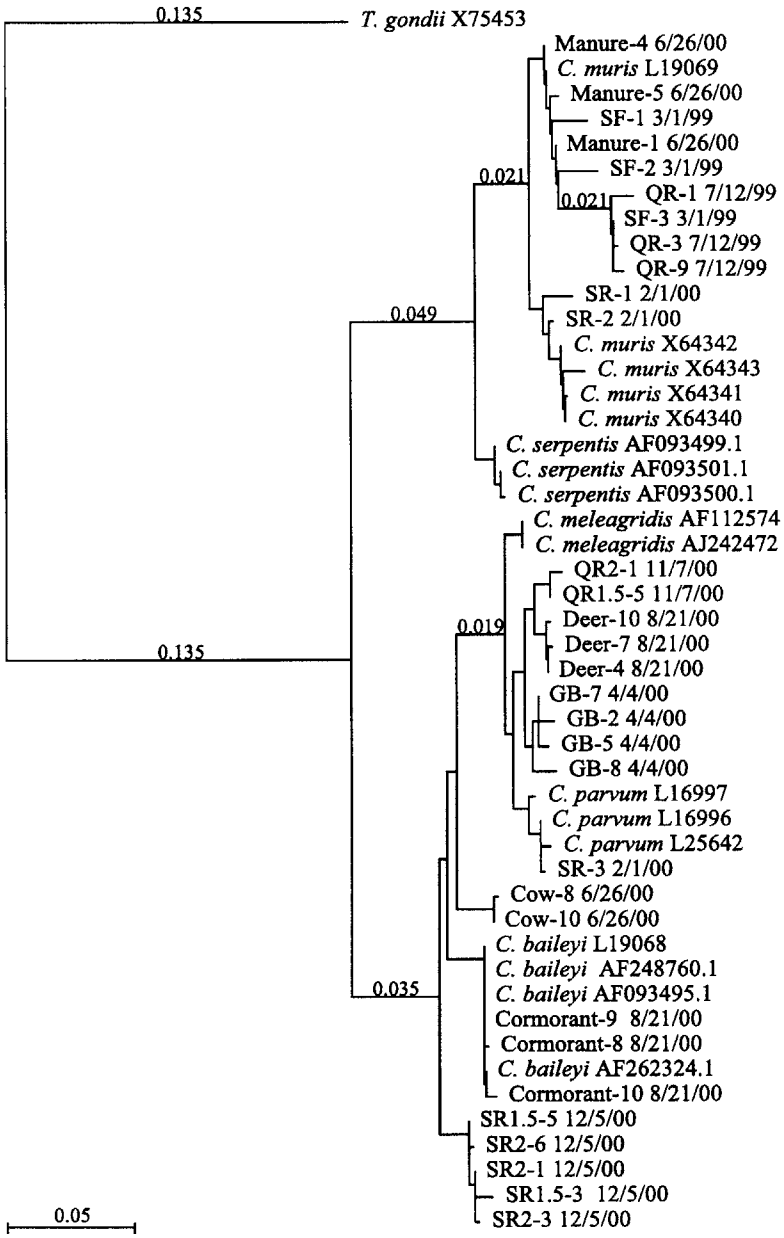


Figure 5


0.135
T. gondii X75453
Manure-4 6/26/00
C. muris L19069
Manure-5 6/26/00
SF-1 3/1/99
Manure-1 6/26/00
SF-2 3/1/99
0.021
0.021
QR-1 7/12/99
SF-3 3/1/99
QR-3 7/12/99
QR-9 7/12/99
SR-1 2/1/00
0.049
SR-2 2/1/00
C. muris X64342
C. muris X64343
C. muris X64341
C. muris X64340
C. serpentis AF093499.1
C. serpentis AF093501.1
C. serpentis AF093500.1
C. meleagridis AF112574
C. meleagridis AJ242472
QR2-1 11/7/00
QR1.5-5 11/7/00
0.019
Deer-10 8/21/00
Deer-7 8/21/00
0.135
Deer-4 8/21/00
GB-7 4/4/00
GB-2 4/4/00
GB-5 4/4/00
GB-8 4/4/00
C. parvum L16997
C. parvum L16996
C. parvum L25642
SR-3 2/1/00
Cow-8 6/26/00
Cow-10 6/26/00
C. baileyi L19068
C. baileyi AF248760.1
0.035
C. baileyi AF093495.1
Cormorant-9 8/21/00
Cormorant-8 8/21/00
C. baileyi AF262324.1
Cormorant-10 8/21/00
SR1.5-5 12/5/00
SR2-6 12/5/00
SR2-1 12/5/00
SR1.5-3 12/5/00
SR2-3 12/5/00
0.05

Chapter 4: Identification of *Cryptosporidium* spp. Oocysts in an Agricultural Watershed by 18S rRNA Phylogeny Implicates Birds as an Important Source

Manuscript to be submitted to ***Applied and Environmental Microbiology***

Abstract. The goal of the present study was to examine sources and genotypes of *Cryptosporidium* oocysts in samples collected from two dairy farms, SF and JF, and nearby surface waters (Brook SF and Brook JF, respectively) in the Wachusett Reservoir watershed. For one year, Brooks SF and JF were sampled monthly; *Cryptosporidium* spp. oocysts were detected in 5 (41.7%) out of 12 samples from Brook JF, and no oocysts were detected in any of the 12 Brook SF samples. Cryptosporidium was detected in an adult cow and a manure pit on Farm JF, but no oocysts were detected in adult cattle on Farm SF or calves on Farm JF. Oocysts from surface waters were compared to those from Farm JF by phylogenetic analysis of the hypervariable region of the 18S rRNA gene by both neighbor-joining and parsimony methods. Phylogenetic trees show extensive heterogeneity among cryptosporidium 18S rRNA sequences recovered from these environmental samples and suggest that birds are an important oocyst source in this agricultural watershed. The impact of birds on oocyst shedding is further supported by the seasonal detection of *Cryptosporidium* spp. oocysts in Brook JF: oocysts were detected in the summer through late fall, coincident with the presence of migratory birds in this northern watershed. Data from this study provide greater insight into the level of 18S rRNA heterogeneity among *Cryptosporidium* spp. oocysts and suggest that protection of surface waters from fecal droppings of birds may help prevent human exposure to waterborne *Cryptosporidium* spp. oocysts.

Introduction. *Cryptosporidium parvum* is a protozoan parasite responsible for cryptosporidiosis, an acute gastrointestinal illness that can be life-threatening for the immunocompromised population. Although *C. parvum* is the species most often associated with human cryptosporidiosis, multiple species of *Cryptosporidium* are recognized [10], and some of these other *Cryptosporidium* species have been associated with illness among immunocompromised people [3, 12, 25]. Oocysts are spread from host to host via fecal-oral routes of transmission, and outbreaks have been associated with ingestion of contaminated food and water and exposure to contaminated recreational water [5-7, 13, 19, 26, 29, 32].

Agriculture is widely recognized as a source of *Cryptosporidium* spp. oocysts in the environment. A recent study by Heitman et al. [17] looked at *Cryptosporidium* spp. in wildlife, sewage, and agricultural sources and found the highest *Cryptosporidium* spp. concentrations from agricultural sources. Agricultural runoff has been identified as the source of oocysts in a number of waterborne outbreaks of cryptosporidiosis [32], and a foodborne cryptosporidiosis outbreak from fresh-pressed apple cider [26] was attributed to contamination of apples with fecal material from an infected calf on the farm. Environmental studies of *Cryptosporidium* spp. oocysts in agricultural watersheds can provide critical insight into the dynamics of oocyst sources, transport, and fate and ultimately aid in improved watershed management to safeguard water supplies from oocyst contamination.

The goal of the present study was to assess the sources and species of *Cryptosporidium* oocysts in two agricultural areas within the Wachusett Reservoir watershed in central Massachusetts. Two brooks, down-gradient from two respective dairy farms, were chosen as sample sites. *Cryptosporidium* phylogeny based on the 18S rRNA gene has been described [36], and molecular characterization of the 18S rRNA gene has been applied to environmental studies [17, 21, 28, 38, 39]. In the present study, we sequenced the hypervariable region of the 18S rRNA gene of oocysts recovered from both farm and surface water samples and used phylogenetic analysis to propose likely sources and genotypes of oocysts in this watershed.

Materials and Methods.

Site selection and sample collection. Two dairy farms in the Wachusett Reservoir watershed in central Massachusetts, Farms SF and JF, were chosen as sample sites (Figure 1). Farms SF and JF are located upgradient from small brooks, designated Brook SF and Brook JF, respectively. Surface water samples were collected monthly from Brooks SF and JF for one year beginning June 2001 and ending May 2002 (Table 1). One additional water sample from Brook SF in March 1999 was included in the study as well [21]. Fecal samples were collected from adult cattle on Farm SF and adult cattle, calves, and a manure pit on Farm JF in June 2000 [21].

Surface waters were filtered through Gelman Envirochek Sampling Capsules (Pall Gelman Sciences, Inc., Ann Arbor, Mich.) at 1-2 liters min^{-1} according to manufacturer's recommendations. Filtration continued for one hour or until the backpressure exceeded the filter rating (30 lb/in^2 [psi]), whichever came first. On average, 75.5 liters of water were filtered (s.d. = 32.1 liters). Filters were transported to the laboratory on ice, and samples were eluted according to manufacturer's recommendations within 24 h of sample collection. Eluted solids were resuspended in 10 mL laboratory-grade water (Milli-Q System; Millipore Corp., Bedford, Mass.) for each 0.5 mL solids, stored at 4°C, and processed within 24 h.

Samples of animal feces and the Farm JF manure pit were collected in sterile 50 mL polypropylene tubes and transported to the laboratory on ice. Fecal samples were suspended in 10 mL laboratory-grade water for each 0.5 mL solids, stored at 4°C, and processed within 24 h of collection.

Immunomagnetic separation of oocysts. Oocysts were purified from water and fecal samples by using immunomagnetic separation (IMS) with the Crypto-Scan IMS kit (ImmuCell, Portland, Maine) according to the recommendations of the manufacturer. After being dissociated from magnetic beads, oocysts were transferred to a new microcentrifuge tube and treated with 5 µL of 1N NaOH to neutralize pH. The oocysts were pelleted for 2 to 3 min at 16000 x *g*, resuspended in 50 µl of laboratory-grade water, and stored at 4°C.

Positive and negative IMS controls were processed with each set of field samples. Positive IMS controls consisted of 9.5 mL laboratory-grade water and 500 µL of a 10^4 oocyst ml^{-1} suspension; negative IMS controls consisted of 10 ml of laboratory-grade water. IMS controls were processed as described above.

Genomic DNA extraction. Oocysts were lysed by adding 25 µl of IMS product to 475 µl of Tris-EDTA (TE) buffer containing 0.2 g proteinase K $liter^{-1}$ and 0.4% sodium dodecyl sulfate and incubating the mixture overnight at 45°C. Positive and negative DNA extraction controls were included for each set of field samples. Positive DNA extraction controls consisted of 25 µL of a suspension of 10^4 oocysts ml^{-1} in 475 µl of TE buffer; negative DNA extraction controls consisted of 25 µl of laboratory-grade water in 475 µl of TE buffer. DNA was extracted several times with phenol-chloroform, precipitated with 0.2M NaCl and 2 volumes of absolute ethanol, and resuspended in 30 µl of TE buffer.

Nested PCR assay. Nested PCR amplification of the hypervariable region of the 18S rRNA gene was performed as previously described [21] with the following modifications. The concentration of each deoxynucleoside triphosphate (Perkin-Elmer, Wellesley, Mass.) was 0.15 mM. The initial amplification reaction was performed with 15 µl of DNA template, and 1 µl of the initial amplification product was used as template in the secondary PCR. Positive and negative PCR controls were included with each set of water or fecal samples. For the initial amplification reaction, positive PCR controls contained 14 µl of laboratory-grade water and 1 µl of genomic *C. parvum* DNA (at a concentration equivalent to 10^4 oocysts μl^{-1}); negative PCR controls contained 15 µl of laboratory-grade water. For the secondary amplification reaction, positive PCR controls contained 1 µl of genomic *C. parvum* DNA (at a concentration equivalent to 10^4 oocysts μl^{-1}); negative PCR controls contained 1 µl of laboratory-grade water.

Both amplification reactions used forward and reverse oligonucleotide primers that are complementary to all *Cryptosporidium* spp. 18S rRNA gene sequences. For the primary PCR, an approximately 1,056-bp product (dependent on *Cryptosporidium* species) was obtained using forward and reverse primers KLJ1 and KLJ2, respectively [21]; for the secondary PCR, an

approximately 434-bp product was obtained using forward and reverse primers CPB-DIAGF and CPB-DIAGR, respectively [24]. Cycling conditions for both the primary and secondary PCRs consisted of an initial denaturation (5 min at 80°C, followed by 30 s at 98°C), 25 cycles of amplification (denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C), and a final extension (10 min at 72°C). Secondary PCR products were visualized after electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Cloning. Secondary PCR products positive for *Cryptosporidium* spp. were cloned into the pGEM-T Easy Vector System (Promega Corporation, Madison, Wis.) and used to transform XL-1 Blue *E. coli* cells (Stratagene, La Jolla, Calif.). Clones were selected on Luria-Bertani (LB) agar supplemented with 100 μg of ampicillin ml^{-1} and cultured overnight in LB broth supplemented with 100 μg of ampicillin ml^{-1}. Plasmid DNA was isolated from clones by using the QIAPrep Spin Miniprep Kit (Qiagen, Inc., Valencia, Calif.) and digested with *Not*I (New England Biolabs, Beverly, Mass.) to verify the presence of the secondary PCR amplicon insert and *Nde*I (New England Biolabs) to identify any heterogeneity among the clones [21]. Restriction digestion was carried out in a 20-μl volume containing 4 μl of plasmid DNA, 20 U of *Not*I, 10 U of *Nde*I, 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg of bovine serum albumin ml^{-1} and then incubated at 37°C for 1 h. Digestion products were visualized after electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Sequencing. Representative clones of the secondary PCR products were sequenced on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, Calif.) using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PE Applied Biosystems). If multiple *Nde*I digestion patterns existed among clones from a given sample, at least one clone of each digestion pattern was sequenced. The number of clones sequenced for each Brook JF sample is shown in Table 1. For the sample collected from Brook SF in March 1999 and the adult cow and manure pit fecal samples collected from Farm JF in June 2000, three, two, and three clones were sequenced, respectively. Data were confirmed by sequencing both strands of each clone. When multiple clones were sequenced with less than 1% difference, the consensus sequence was used in the phylogenetic analysis.

Phylogenetic Analysis. Sequences were aligned manually, based on the secondary structure of the 18S rRNA, using the GCG sequence editor (Genetics Computer Group, Madison, WI). Variable length loop regions were masked and excluded from the phylogenetic analysis. Phylogenetic Analysis Using Parsimony (PAUP), beta version 4.0 [34], was used to create both neighbor-joining and parsimony trees from the GCG alignments. *C. felis* was designated an outgroup, and construction of neighbor-joining trees was based on the evolutionary distances between different isolates calculated by the Kimura two-parameter analysis. Statistical support for the resulting trees was tested using 1000 pseudoreplicates of the bootstrap test; only values above 50% were reported, and bootstrap values greater than 70% were considered significant [18]. GenBank accession numbers used in the phylogenetic analysis are noted in the caption of Figure 2.

Results.

Prevalence of Oocyst Contamination. Brook JF was sampled 12 times during the year (Table 1), and 5 samples (41.7%) were positive for *Cryptosporidium* spp. oocysts by nested PCR. Three of the 5 positive samples were successfully cloned and sequenced. Brook SF was sampled 12 times throughout the year and was never positive for *Cryptosporidium* spp. oocysts. No *Cryptosporidium* spp. oocysts were detected in the feces of adult cattle from Farm SF or the calves from Farm JF. *Cryptosporidium* spp. oocysts were detected in one adult cow and the manure pit from Farm JF.

Phylogenetic Analysis. Phylogenetic trees constructed by both neighbor-joining and parsimony methods (Figure 2) show several taxonomic groups of *Cryptosporidium* spp.: *C. andersoni*, *C. muris*, and *C. serpentis* form one statistically significant clade, *C. parvum*, *C. meleagridis*, and *C. wrairi* form another group (although not statistically significant), and *C. baileyi* is on a distinct branch (*C. felis* is the outgroup). Sequences from the Farm JF manure pit and Brook SF cluster within the *C. andersoni/C. muris/C. serpentis* clade with a bootstrap value of 100%. The sequence from the manure pit was indistinguishable from the bovine-type *C. muris* (evolutionary distance = 0.00), and the sequence from Brook SF (SF Mar. 1999) was closest to the bovine-type *C. muris*, with an evolutionary distance (0.005) identical to that between the *C. parvum* human and ferret genotypes (Tables 2 and 3).

Although the sequence from the adult cow on Farm JF clustered with *C. baileyi* in the neighbor-joining tree, the bootstrap value was not significant. The cow-derived sequence was most closely related to *C. baileyi* with an evolutionary distance of 0.035, identical to the distance between *C. baileyi* and *C. meleagridis* (Table 3).

The sequences recovered from Brook JF in June 2001 (JF #1 and 2) did not cluster with any existing taxonomic group, yet they grouped significantly with a sequence recovered from a Canada goose in New York (Jellison et al., 2003a, in preparation) by both neighbor-joining and parsimony analyses (bootstrap value = 100%). JF #1 was indistinguishable from the goose-derived sequence (evolutionary distance = 0.000), but had an evolutionary distance to JF #2 (0.025) identical to that between the *C. parvum* human and dog genotypes (Tables 2 and 3). The

evolutionary distances between JF #1 and *C. baileyi* (0.074) and *C. meleagridis* (0.069), respectively, were larger than the evolutionary distance between *C. baileyi* and human-type *C. parvum* (0.042).

Similarly, the sequences from Brook JF in November 2001 did not significantly cluster with any existing taxonomic group. The evolutionary distance between JF #5 and JF #6 (0.029) was slightly greater than the distance between the *C. parvum* human and dog genotypes (0.025) and greater than the distance between *C. serpentis* and *C. andersoni* (0.017) (Tables 2 and 3). Similarly, the evolutionary distances between JF #5 and JF #7 (0.022), and JF # 6 and JF #7 (0.017) were comparable to the distances between closely-related genotypes and species. JF #5 was most closely related to *C. wrairi* and *C. meleagridis* with equal evolutionary distances of 0.032.

The sequence recovered from Brook JF in August 2001 formed an independent phylogenetic branch that clustered significantly with a *Cryptosporidium* spp. sequence recovered from a goose in Illinois [22] with bootstrap values of 100% by neighbor-joining and parsimony analyses. The evolutionary distance between the goose-derived sequence and JF #4 was 0.007, identical to the distance between *C. parvum* human genotype and *C. meleagridis*. The evolutionary distance between JF #4 and all other sequences in the analysis ranged from 0.045 to 0.114, on par with the distances between biologically-distinct species of *Cryptosporidium* (Table 3).

Discussion.

Environmental sampling has shown extensive heterogeneity among 18S rRNA gene sequences for *Cryptosporidium* spp. [28, 37, 38]. The majority of the sequences recovered from the present study did not cluster significantly with any well-defined taxonomic group, and although additional biological and phenotypic data are needed to make conclusive identifications, the phylogenetic analysis suggests that these sequences may represent novel genotypes or perhaps even uncharacterized species of *Cryptosporidium*. Continued environmental sampling will be critical to characterize the full extent of this heterogeneity and aid in the interpretation of oocyst sources and species from limited molecular data.

The data from this study provide insight into the dynamics of cryptosporidium in agricultural watersheds. Cattle are susceptible to infection with *C. parvum* and *C. andersoni* [1, 4, 9, 11, 33], and identification of *C. andersoni*-like 18S rDNA in the manure pit confirms the presence of *C. andersoni* or bovine-type *C. muris* oocysts on Farm JF. More surprising, however, was the identification of a novel 18S rRNA gene sequence from an adult cow on the farm. The evolutionary distance of this 18S rRNA gene sequence from known *Cryptosporidium* species supports the idea that this may represent a previously uncharacterized species, although additional morphological and biological data are needed to confirm a taxonomic designation. This finding suggests that cows may act as mechanical vectors of *Cryptosporidium* species other than *C. parvum*, *C. muris*, and *C. andersoni* in agricultural watersheds. The importance of these non-*parvum* species for human health requires further investigation.

Birds may be an important source of *Cryptosporidium* spp. oocysts in this agricultural watershed given the phylogenetic analysis of oocysts recovered from Brook JF and the fact that the cow-derived 18S rRNA gene sequence was most similar to that of *C. baileyi*. The extent to which birds impact *Cryptosporidium* spp. oocyst transmission is becoming increasingly recognized. Traditionally, *C. baileyi* and *C. meleagridis* were the only two *Cryptosporidium* species known to infect birds, but recent studies have proposed two novel species of *Cryptosporidium*, *C. galli* and *C. blagburni*, in finches [27, 30] and have shown that Canada geese shed oocysts with a much broader range of 18S rRNA genotypes than previously characterized [22]. *Cryptosporidium* spp. oocysts have also been recovered from the feces of gulls [31], and a report

by Graczyk et al. [16] of zoonotic *C. parvum* in Canada geese showed that birds can be carriers of infectious oocysts. Since the infectivity of *C. parvum* oocysts for neonatal BALB/c mice is retained upon intestinal passage through ducks [14] and geese [15, 16], birds have been identified as potential vectors of infectious oocysts in the environment.

The link between birds and agricultural watersheds is explicable. Canada geese are primarily grazers and as such reside in large grassy areas typical of farms [8]. Geese and other birds have been observed in the agricultural watersheds of the current study, and in the agricultural region near the Chesapeake Bay, geese were actually observed to wander behind cattle and pick up undigested corn from their feces [16]. The observed phylogenetic grouping of 18S rRNA gene sequences from geese and Brook JF further supports the significance of birds in the fate and transport of oocysts in agricultural watersheds.

The seasonal occurrence of *Cryptosporidium* spp. oocysts in Brook JF also supports the theory that birds are an important source of oocysts in this watershed. Wade et al. [35] found no seasonal pattern of *C. parvum* or *C. andersoni* infection of dairy cattle, and Bodley-Tickell et al. [2] found oocysts in surface waters draining a livestock farm throughout the year, suggesting that cattle can shed *Cryptosporidium* spp. oocysts year-round. Oocyst shedding by dairy herds was found to be higher in the winter than in the summer [20], however, and maximum concentrations is surface waters draining a livestock farm were found during the autumn and winter [2]. By contrast, *Cryptosporidium* spp. were detected in Brook JF from summer through late fall, and no oocysts were detected in the winter or early spring. This seasonal pattern was also in contrast to the detection of oocysts in wildlife-influenced surface waters in the late fall through early spring [23]. The varying seasonal pattern in surface waters susceptible to oocyst contamination via different sources suggests that source dynamics have a role in the presence of cryptosporidium in surface waters. Migratory Canada geese are present in this northern agricultural watershed during the warmer summer months and fly south for the colder winter months, coinciding well with the seasonal detection of *Cryptosporidium* spp. oocysts in Brook JF.

The data from this study strongly implicate birds as an important source of *Cryptosporidium* spp. oocysts in this agricultural watershed. The heterogeneity of *Cryptosporidium* 18S rRNA

genotypes recovered from birds has been extensive, and the public health significance of these oocysts is not immediately clear. While oocysts recovered from farms or agriculture-influenced surface waters can not be presumed infectious without further molecular and biological characterization, watershed management aimed at controlling the numbers of birds in source watersheds will likely contribute to reduced *Cryptosporidium* spp. concentrations and protect against waterborne cryptosporidiosis.

Acknowledgements. We thank the Metropolitan District Commission of the Commonwealth of Massachusetts for help in locating sample sites and assistance in obtaining samples from the dairy farms. We thank the farmers for their permission to collect samples and their cooperation in the study. We also gratefully acknowledge Giovanni Widmer of Tufts University School of Veterinary Medicine, North Grafton, Mass., for materials and technical assistance.

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Table 1. Summary of Brook JF samples from June 2001 to May 2002.

Date	*Nested PCR Result*[a]	*No. Clones Sequenced*	*Sequence Identifier*
6/25/01	+	2	JF #1
		1	JF #2
		2	JF #3
7/24/01	+	ND[b]	
8/20/01	+	6	JF #4
9/24/01	-		
10/29/01	+	ND	
11/28/01	+	1	JF #5
		1	JF #6
		1	JF #7
12/17/01	-		
1/3/02	-		
2/21/02	-		
3/18/02	-		
4/22/02	-		
5/20/02	-		

[a]+, the cryptosporidium-specific 434-bp nested PCR amplicon was present; -, the 434-bp nested PCR amplicon was absent

[b]ND = Not done

Table 2. Kimura two-parameter distance matrix (substitutions/site) for *C. parvum* genotypes. GenBank accession numbers for *C. parvum* genotypes are AF093489 (human), AF093493 (bovine), AF112570 (kangaroo), AF112571 (mouse), AF112572 (ferret), AF112576 (dog), and AF115377 (pig).

	1 *C. parvum* (human)	**2 *C. parvum* (bovine)**	**3 *C. parvum* (kangaroo)**	**4 *C. parvum* (mouse)**	**5 *C. parvum* (ferret)**	**6 *C. parvum* (dog)**	**7 *C. parvum* (pig)**
1	-						
2	0.002	-					
3	0.017	0.015	-				
4	0.002	0.000	0.015	-			
5	0.005	0.002	0.017	0.002	-		
6	0.025	0.022	0.025	0.022	0.025	-	
7	0.010	0.012	0.022	0.012	0.015	0.025	-

Table 3. Kimura two-parameter distance matrix (substitutions/site) for GenBank and sample sequences. GenBank accession numbers are AF093489 and AF093493 (*C. parvum* human and bovine genotypes, respectively), U11440 (*C. wrairi*), AF112574 (*C. meleagridis*), AF112575 (*C. felis*), L19068 (*C. baileyi*), AF093499 (*C. serpentis*), AB089284 and L19069 (*C. muris* mouse and bovine genotypes, respectively), and AB089285 (*C. andersoni*).

	1 *C. parvum* (himan)	2 *C. parvum* (bovine)	3 *C. wrairi*	4 *C. meleagridis*	5 *C. felis*	6 *C. baileyi*	7 *C. serpentis*	8 *C. muris* (mouse)	9 *C. muris* (bovine)	10 *C. andersoni*	11 Manure	12 SF (Mar. 1999)	13 JF #1 (June 2001)	14 JF #2 (June 2001)	15 JF #4 (Aug. 2001)	16 JF #5 (Nov. 2001)	17 JF #6 (Nov. 2001)	18 JF #7 (Nov. 2001)	19 Cow	20 Goose (Illinois)	21 Goose (New York)
1	-																				
2	0.002	-																			
3	0.007	0.005	-																		
4	0.007	0.005	0.005	-																	
5	0.042	0.045	0.045	0.040	-																
6	0.042	0.040	0.037	0.035	0.050	-															
7	0.100	0.097	0.095	0.092	0.112	0.087	-														
8	0.103	0.100	0.098	0.095	0.115	0.087	0.017	-													
9	0.097	0.095	0.092	0.089	0.109	0.087	0.017	0.010	-												
10	0.095	0.092	0.089	0.087	0.106	0.087	0.017	0.010	0.000	-											
11	0.097	0.095	0.092	0.089	0.109	0.087	0.017	0.010	0.000	0.000	-										
12	0.103	0.100	0.097	0.095	0.114	0.092	0.022	0.014	0.005	0.005	0.005	-									
13	0.077	0.074	0.069	0.069	0.085	0.074	0.127	0.129	0.126	0.124	0.126	0.126	-								
14	0.105	0.102	0.097	0.097	0.111	0.094	0.133	0.129	0.127	0.124	0.127	0.127	0.025	-							
15	0.053	0.050	0.050	0.045	0.056	0.060	0.095	0.100	0.098	0.095	0.098	0.103	0.096	0.114	-						
16	0.040	0.037	0.032	0.032	0.055	0.042	0.100	0.109	0.103	0.101	0.103	0.109	0.072	0.094	0.055	-					
17	0.050	0.047	0.042	0.042	0.058	0.055	0.111	0.120	0.120	0.118	0.120	0.126	0.091	0.111	0.047	0.029	-				
18	0.042	0.040	0.035	0.035	0.055	0.050	0.103	0.114	0.112	0.109	0.112	0.117	0.082	0.102	0.047	0.022	0.017	-			
19	0.047	0.045	0.042	0.040	0.063	0.035	0.090	0.098	0.098	0.098	0.098	0.103	0.085	0.094	0.058	0.047	0.060	0.052	-		
20	0.060	0.058	0.058	0.052	0.063	0.063	0.098	0.103	0.100	0.098	0.100	0.106	0.099	0.117	0.007	0.058	0.055	0.055	0.061	-	
21	0.077	0.074	0.069	0.069	0.085	0.074	0.127	0.129	0.126	0.124	0.126	0.126	0.000	0.025	0.096	0.072	0.091	0.082	0.085	0.099	-

Figure Captions.

Figure 1. Locations of farms and surface water sampling sites for (A) Farm JF and (B) Farm SF. Farm areas are designated by boxes; surface waters are highlighted by bold black lines. Panels A and B are adapted from United States Geographical Survey topographic maps. Scale: 1 cm = 250 m. Contour intervals = 3 m.

Figure 2. (A) Neighbor-joining and (B) parsimony trees based on the hypervariable region of the 18S rRNA gene. GenBank accession numbers of sequences included in the trees are AB089285 (*C. andersoni*), L19068 (*C. baileyi*), AF112575 (*C. felis*), AF112574 (*C. meleagridis*), L19069 (*C. muris* bovine genotype), AB089284 (*C. muris* murine genotype), AF093489 (*C. parvum* human genotype), AF093493 (*C. parvum* bovine genotype), AF112571 (*C. parvum* mouse genotype), AF112572 (*C. parvum* ferret genotype), AF115377 (*C. parvum* pig genotype), AF112576 (*C. parvum* dog genotype), AF112570 (*C. parvum* kangaroo genotype), AF093499 (*C. serpentis*), and U11440 (*C. wrairi*). Geese (New York and Illinois) sequences refer to Goose #7 and Goose #3 (sequence b), respectively, reported elsewhere [22]. Bootstrap values greater than 50% are indicated in bold at their respective nodes.

Figure 1

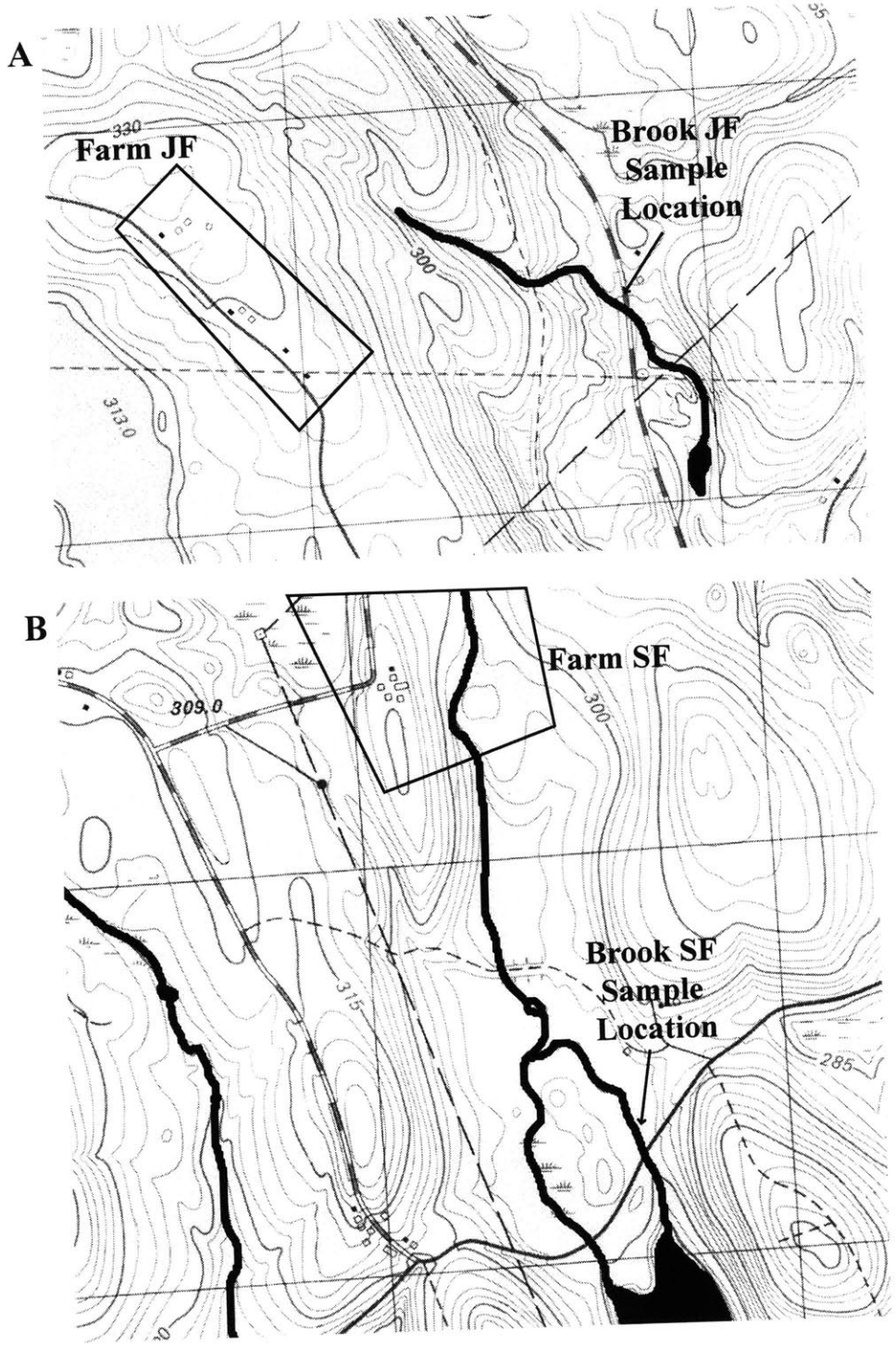
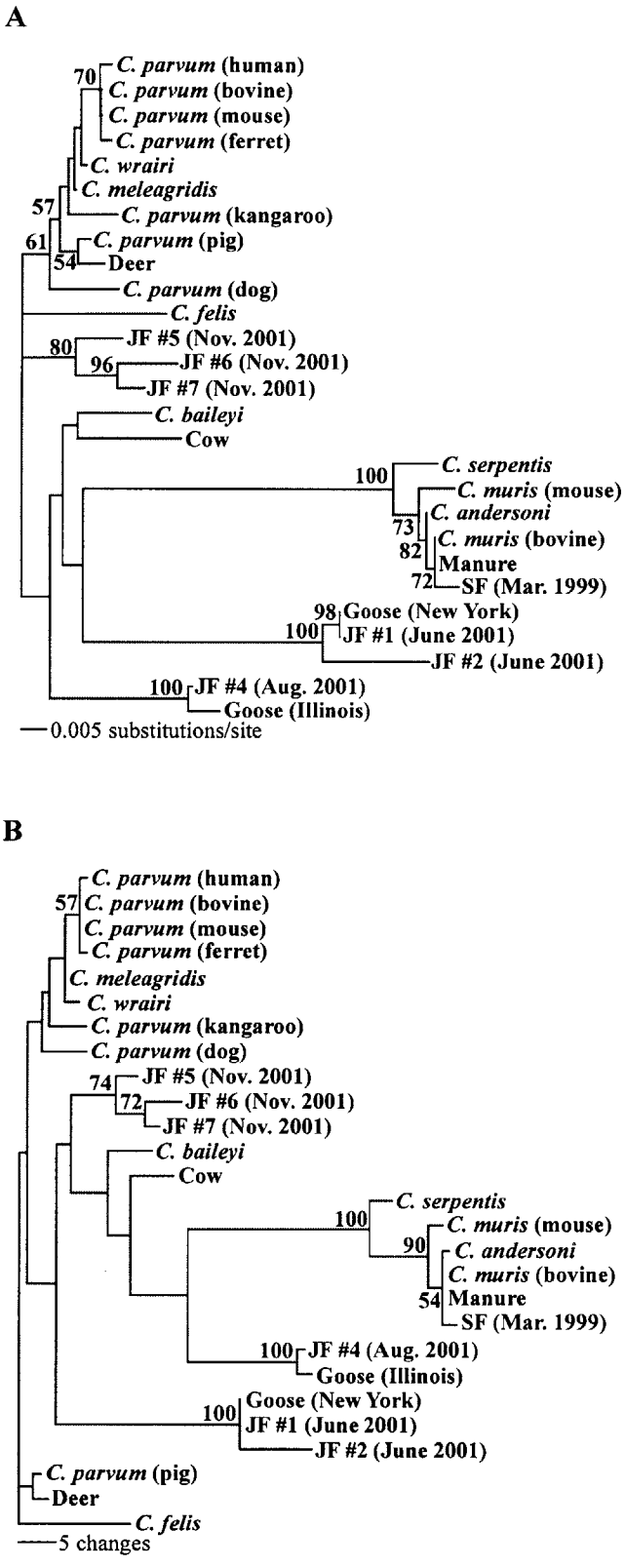

A
330
Farm JF
Brook JF
Sample
Location
300
313.0
B
Farm SF
309.0
300
315
Brook SF
Sample
Location
285

Figure 2


A
C. parvum (human)
C. parvum (bovine)
C. parvum (mouse)
C. parvum (ferret)
C. wrairi
C. meleagridis
C. parvum (kangaroo)
C. parvum (pig)
Deer
C. parvum (dog)
C. felis
JF #5 (Nov. 2001)
JF #6 (Nov. 2001)
JF #7 (Nov. 2001)
C. baileyi
Cow
C. serpentis
C. muris (mouse)
C. andersoni
C. muris (bovine)
Manure
SF (Mar. 1999)
Goose (New York)
JF #1 (June 2001)
JF #2 (June 2001)
JF #4 (Aug. 2001)
Goose (Illinois)
0.005 substitutions/site
B
C. parvum (human)
C. parvum (bovine)
C. parvum (mouse)
C. parvum (ferret)
C. meleagridis
C. wrairi
C. parvum (kangaroo)
C. parvum (dog)
JF #5 (Nov. 2001)
JF #6 (Nov. 2001)
JF #7 (Nov. 2001)
C. baileyi
Cow
C. serpentis
C. muris (mouse)
C. andersoni
C. muris (bovine)
Manure
SF (Mar. 1999)
JF #4 (Aug. 2001)
Goose (Illinois)
Goose (New York)
JF #1 (June 2001)
JF #2 (June 2001)
C. parvum (pig)
Deer
C. felis
5 changes

Chapter 5: Correlation of *Cryptosporidium* spp. Contamination of Surface Waters with Physical Water Quality Parameters

ABSTRACT. Physical water quality parameters that relate to the presence of *Cryptosporidium* spp. oocysts in surface waters may provide insight into the factors affecting oocyst transport and survival in a watershed and can serve as indicators of potential water contamination, thereby reducing the need for regular *Cryptosporidium* monitoring. The relationships between *Cryptosporidium* contamination and temperature, pH, specific conductivity, turbidity, flow rate, and dissolved oxygen were analyzed at five surface water locations in the Wachusett Reservoir watershed. The selected surface water sites were susceptible to oocyst contamination by wildlife shedding, agricultural runoff, or human sewage. At the wildlife-influenced surface water sites, the mean temperature of positive samples (3.6°C) was significantly lower ($p=0.0125$) than the mean temperature of negative samples (10.8°C), although the presence of oocysts did not correlate significantly with water temperature; no significant relationships were observed between cryptosporidium and pH, specific conductivity, turbidity, dissolved oxygen, and flow rate. At the agriculture-influenced surface water sites, cryptosporidium presence correlated positively with dissolved oxygen ($p=0.0507$) and pH ($p=0.0095$) and negatively with specific conductivity ($p=0.0082$); although not reaching statistical significance ($p=0.1062$), the mean temperature of positive samples (12.1°C) was higher than that of negative samples (6.9°C). When the data from all surface water sites were combined, no significant relationships between cryptosporidium and any of the recorded water quality parameters emerged. It appears that cryptosporidium contamination of surface waters is impacted by seasonal stimuli (e.g., oocyst sources and sinks or land-use impacts) that may vary among surface waters within a watershed.

INTRODUCTION. *Cryptosporidium parvum* is a protozoan parasite responsible for a gastrointestinal disease that is self-limiting for otherwise healthy individuals but severe and life-threatening for the immunocompromised. The parasite has many environmental sources, including wildlife, farm animals, and humans, and can be transmitted anthroponotically or zoonotically by ingestion of contaminated food and water. Oocysts, the environmental life stage of cryptosporidium, are difficult to remove from drinking water with conventional technology because they are resistant to chemical disinfectants and, given their small size, not effectively removed by filtration. Thus, efforts must be made to control oocyst contamination of surface waters to minimize the risk of public exposure to waterborne cryptosporidium.

Water quality parameters that correlate strongly with oocyst presence can serve as indicators of potential water contamination and may reduce the need for regular cryptosporidium monitoring, which can be costly and time-consuming. A number of previous studies have investigated possible correlations between cryptosporidium contamination of surface waters and various physical and biological water quality indicators, with variable results. Rose et al. [1] studied a river susceptible to oocyst contamination from animal wastes and sewage treatment plant discharges and found a significant correlation between surface water concentrations of *Cryptosporidium* spp. and *Giardia* spp. but no correlations between *Cryptosporidium* spp. levels and total coliforms, fecal coliforms, or turbidity. By contrast, LeChevallier et al. [2] reported significant correlations between *Cryptosporidium* spp. densities in surface waters and total coliforms, fecal coliforms, and turbidity. Chauret et al. [3] analyzed water samples from three rivers susceptible to oocyst contamination from agriculture, sewage, and wildlife and found significant watershed-dependent correlations between *Cryptosporidium* spp. and *Giardia* spp. and *Cryptosporidium* spp. and fecal streptococci; no significant correlations were detected between *Cryptosporidium* spp. and fecal and total coliforms, *Aeromonas* sp., *Pseudomonas aeruginosa*, *Clostridium perfringens*, algae, or coliphages. LeChevallier et al. [4] monitored the Delaware River for a full year and observed a significant correlation between *Cryptosporidium* spp. and turbidity. Small, but significant, correlations were also identified between *Cryptosporidium* spp. and *Giardia* spp., total and fecal coliforms, *E. coli*, *C. perfringens*, coliphage, alkalinity, hardness, pH, dissolved oxygen, and river flow.

The physical water quality parameters analyzed in this study were temperature, flow rate, pH, specific conductivity, turbidity, and dissolved oxygen. Fayer et al. [9] recently reported that oocysts were recovered from a greater percentage of oysters in the Chesapeake Bay at low water temperatures. These data, in addition to the fact that oocyst viability has been shown to decrease as water temperature increases from 4°C [5-8], led us to hypothesize that oocysts would be present more often in cold waters. Rainfall events have been associated with increased *Cryptosporidium* spp. detection in a number of studies [4, 9-11], and thus we expected to see an association between high flow rate and oocyst contamination. We expected to find oocysts in surface waters of near-neutral pH since Robertson et al. [12] found that extreme pH levels (i.e., pH 1.5 and 10.5) adversely impact oocyst survival. Furthermore, we expected fewer surface water samples to be positive for *Cryptosporidium* spp. at acidic pH given an oocyst isoelectric point near 3.0 and an increase in oocyst hydrophobicity (and thus greater potential for oocyst sedimentation from the water column) as pH declines from neutral [13, 14]. Oocyst hydrophobicity also modestly increased with higher specific conductivity [13, 14]; thus, we hypothesized that *Cryptosporidium* spp. oocysts would be detected more often in surface waters of lower specific conductivity. We expected to see a strong positive correlation between cryptosporidium contamination and turbidity. Surface water turbidity is a strong indicator of precipitation and runoff events, and, as mentioned above, cryptosporidium detection in surface waters has been found to correlate strongly with turbidity in previous studies [2, 4]. Little information is available regarding the impact of dissolved oxygen on oocyst survival, but we hypothesized that oocysts would survive longer in waters of high dissolved oxygen and thus anticipated a positive correlation between dissolved oxygen concentrations and cryptosporidium presence.

The purpose of the current study was to test the validity of these hypotheses by identifying significant relationships between cryptosporidium contamination and physical water quality parameters in the Wachusett Reservoir watershed, the drinking water source for Boston, Massachusetts and surrounding cities. Surface water sites within the watershed were selected to encompass a variety of oocyst sources, including wildlife shedding, agricultural runoff, and human sewage. The identification of significant relationships between cryptosporidium detection and water quality parameters will elucidate environmental conditions under which oocyst

contamination of surface waters is likely to occur and provide insight into the fate and transport mechanisms of oocysts.

MATERIALS AND METHODS.

Sample Times and Locations. Five sampling sites in the Wachusett Reservoir watershed in central Massachusetts were chosen to encompass a variety of potential sources of *Cryptosporidium* spp. oocyst contamination. Surface water sites (and their suspected source of contamination) included Stillwater River (wildlife); Quinapoxet River (wildlife); Gates Brook (sewage); and two small, unnamed brooks, designated Brook JF and Brook SF, downgradient from dairy farms (agricultural runoff). The wildlife-influenced sites, Stillwater River (SR) and Quinapoxet River (QR), were sampled monthly from February 2000 to January 2001, often side-by-side with the Metropolitan District Commission (MDC) of the Commonwealth of Massachusetts. The agriculture-influenced sites, Brook JF (JF) and Brook SF (SF), and the human-influenced site, Gates Brook (GB), were sampled monthly from June 2001 to May 2002.

***Cryptosporidium* Detection.** Samples collected by the MDC from QR and SR were analyzed according to the Information Collection Rule [15], using conventional yarn-wound filters and an immunofluorescence microscopy assay for oocyst detection. All other samples were collected and analyzed as described previously [16]. Briefly, surface water was filtered through Gelman Envirochek Sampling Capsules (Pall Gelman Sciences, Inc., Ann Arbor, Mich.) and filters were eluted according to manufacturer's recommendations. Surface waters were filtered until the Envirochek filter clogged or for a maximum of one hour. Depending on surface water turbidity, 10.6-177.6 L of water were filtered (mean = 84.8, s.d. = 32.3). Eluted water pellets were resuspended in laboratory-grade water (Milli-Q System; Millipore Corp., Bedford, Mass.) and oocysts were purified from sample debris using the Crypto-Scan Immunomagnetic Separation (IMS) kit (ImmuCell, Portland, Maine). DNA was extracted from IMS products with phenol-chloroform, precipitated with ethanol, and detected with a nested PCR assay targeting a 434-bp region of the 18S rRNA gene [16]. Nested PCR products were cloned and sequenced to identify the species of *Cryptosporidium* in the original surface water sample. Although the detection limit of the assay was estimated to be about 1 oocyst per liter of filtered surface water, the detection of cryptosporidium was not quantitative; samples were identified as either positive or negative for *Cryptosporidium* spp. oocysts. Samples that were identified as positive for *Cryptosporidium* spp. oocysts by either one or both detection methods were treated as positive in the statistical analyses.

An aliquot of the original filtration volume was carried through each step of the cryptosporidium detection assay, with typically one-half of the filtration volume used in IMS, one-half of the IMS product used for DNA extraction, and one-half of the extracted DNA used as PCR target. Thus, sample volume (mean = 11.8 L, s.d. = 5.8), as used in the following statistical analyses, is a corrected value corresponding to the fraction of the original filtration volume that was used as template in the primary PCR.

Water Quality Data Collection. During surface water collection, temperature (°C), dissolved oxygen (mg/L and % saturation), pH, specific conductivity (µS/cm), and turbidity (ntu) were recorded. Temperature, dissolved oxygen, pH, and specific conductivity were measured with Minisonde probes and a Surveyor 4 (Hydrolab Corporation, Austin, TX). Turbidity was measured with a DRT-15CE Portable Turbidimeter (HF Scientific, Inc., Fort Myers, FL). The MDC also recorded temperature, pH, specific conductivity, and turbidity for the QR and SR samples they collected; temperature and specific conductivity were measured with a YSI Model 30 field instrument (YSI Incorporated, Yellow Springs, OH), pH was measured with an Orion Model 520A bench instrument (MG Scientific, Pleasant Prairie, WI), and turbidity was measured with a Hach Model 2100A bench instrument (Hach Company, Loveland, CO). Flow rate data were recorded from United States Geological Survey (USGS) gauging stations for QR (USGS station no. 01095375 in Holden, Mass.) and SR (USGS station no. 01095220 in Sterling, Mass.) only. When data from both our study and the MDC study were available, values were averaged and included in the statistical analysis.

Statistical Analyses. All statistical analyses were performed with StatView software (SAS Institute, Cary, NC) using a significance level of 0.05. Significant differences between physical parameters of surface waters that tested positive and negative for cryptosporidium were identified with the unpaired t-test. Pearson correlation coefficients were derived to describe the relationships among cryptosporidium contamination, water quality parameters, and sample volumes. Principle components analysis was used for factor extraction to describe interrelationships among cryptosporidium contamination and the recorded parameters. Samples were assigned a value of 1 (positive for *Cryptosporidium* spp. oocysts) or 0 (negative for

Cryptosporidium spp. oocysts) for both the derivation of correlation coefficients and the principle components analysis.

RESULTS. From February 2000 to January 2001, the wildlife-influenced sites, QR and SR, were each sampled 23 times. *Cryptosporidium* spp. oocysts were detected in 5 (22%) and 3 (13%) samples from QR and SR, respectively. From June 2001 to May 2002, the agriculture-influenced sites, SF and JF, and the human-influenced site, GB, were each sampled 12 times. *Cryptosporidium* spp. oocysts were detected in 6 (50%) and 2 (17%) samples from JF and GB, respectively. No *Cryptosporidium* spp. oocysts were detected in any of the samples collected from SF.

Water quality data for surface water sites susceptible to *Cryptosporidium* spp. oocyst contamination by wildlife shedding, agricultural runoff, and human activities are shown in Table 1. The mean data values for all positive and all negative samples in each source category were compared using the unpaired t-test. Consideration of the wildlife-influenced sites alone showed a significant difference between the mean water temperatures of samples that tested positive (3.6°C) and negative (10.8°C) for *Cryptosporidium* spp. oocysts ($p=0.0125$). The mean values of flow rate, pH, turbidity, specific conductivity, and dissolved oxygen (mg/L) were not significantly different for wildlife-influenced surface waters that tested positive and negative for *Cryptosporidium* spp. oocysts. No statistical analysis was performed on the dissolved oxygen (% saturation) data collected from wildlife-influenced surface waters because of the limited number of data points. Consideration of the agriculture-influenced sites alone revealed a significant difference between the mean dissolved oxygen content (% saturation) of surface waters that tested positive (87.0%) and negative (63.1%) for *Cryptosporidium* spp. oocysts ($p=0.0513$). The mean pH (6.96 and 6.45) and specific conductivity (86.7μS/cm and 289.1μS/cm) were also statistically different ($p=0.0105$ and $p=0.0091$) between agriculture-influenced surface waters that were positive and negative, respectively, for oocysts. Mean values of surface water temperature, turbidity, and dissolved oxygen (mg/L) were not significantly different between agriculture-influenced surface waters identified as positive and negative for *Cryptosporidium* spp. oocysts. Data from the human-influenced surface waters were not considered alone because of the small number of positive samples collected. However, consideration of data from wildlife, agriculture-, and human-influenced surface waters combined showed no significant difference in the mean values of temperature, pH, turbidity, specific conductivity, and dissolved oxygen (mg/L and % saturation) for positive and negative samples.

Because surface waters were filtered until the filter clogged or for a maximum of one hour, sample volumes were not uniform throughout the study. When wildlife-influenced sites were considered alone, the mean volume of positive samples (7.5L) was not statistically different from that of negative samples (10.3L, p=0.2590). However, when agriculture-influenced sites were considered alone, the mean volume of positive samples (17.6L) was significantly higher than that of negative samples (10.4L, p=0.0189). Similarly, the mean volume of positive samples (15.6L) was significantly higher than that of negative samples (10.9L, p=0.0118) when the wildlife-, agriculture-, and human-influenced sites were considered together.

Parameters that were significantly different among cryptosporidium-positive and -negative surface waters were plotted against the date of sample collection to identify seasonal trends (Figure 1). Temperatures of both wildlife- (Figure 1A) and agriculture-influenced (Figure 1B) surface waters showed a seasonal pattern, with colder temperatures from October through April and warmer temperatures from May through September. Wildlife-influenced surface waters were positive for *Cryptosporidium* spp. oocysts during colder temperatures, while agriculture-influenced surface waters were positive during warmer temperatures. The pH values of the agriculture-influenced surface water sites (Figure 1C), while close to neutral throughout the year, also showed a seasonal pattern of higher pH in the warmer months and lower pH during the colder months. The seasonal pH patterns of both agriculture-influenced sites were similar, but JF had consistently higher pH values than SF. Dissolved oxygen content (% saturation) varied dramatically between JF and SF, with JF showing high values throughout the year and SF showing a range of values with no obvious seasonal pattern (Figure 1D). Similarly, the specific conductivities of JF and SF were quite different (Figure 1E), with JF values low and constant throughout the year and SF values considerably higher and peaking during the winter months.

Correlation coefficients for the presence or absence of cryptosporidium and the measured water quality parameters were derived. When the data from all surface waters were analyzed together, a slight, but significant, positive correlation between oocyst presence and sample volume was revealed (0.386, p=0.0030); no significant correlations were identified between oocyst presence and any of the water quality parameters. When data from the wildlife-influenced sites were analyzed alone, oocyst presence did not correlate significantly with sample volume or any of the

water quality parameters. Pearson correlation coefficients for data from agriculture-influenced surface water sites are shown in Table 2. Slight, but significant, positive correlations were identified between oocyst presence and sample volume, dissolved oxygen (% saturation), and pH. A slight, but significant, inverse correlation was seen between oocyst presence and specific conductivity. Increasing dissolved oxygen (% saturation), decreasing specific conductivity, and increasing sample volume correlated significantly. Increasing dissolved oxygen also correlated significantly with decreasing turbidity, and decreasing turbidity correlated significantly with increasing sample volume. Increasing pH correlated significantly with decreasing specific conductivity but did not show any relationship with dissolved oxygen or sample volume. A significant positive correlation (0.618) between pH and temperature was also seen ($p=0.0010$, data not shown in Table 2); similar correlations between pH and temperature were also seen among the wildlife-influenced sample data (0.585, $p<0.0001$) and the combined wildlife-, agriculture-, and human-influenced sample data (0.494, $p<0.0001$).

Results of the principle components analysis for the combined data set (wildlife-, agriculture-, and human-influence sites), as well as the wildlife-influenced and agriculture-influenced data sets alone, are shown in Table 3. For the combined data set, three factors (F1, F2, and F3) were extracted that describe the cleanliness (F1), season (F2), and ionic strength (F3) of the samples. Oocyst presence did not contribute significantly (i.e., factor loading > 0.50) to any of the three factors. For the wildlife-influenced surface water data, three factors describing season (F1), cleanliness (F2), and ionic strength (F3) were also extracted. Oocyst presence contributed significantly to F1, the seasonal factor, only. For the agriculture-influenced surface water data, two factors that describe cleanliness (F1) and a combination of season and ionic strength (F2) were extracted. Oocyst presence contributed significantly to F2, the seasonal and ionic strength factor, only.

DISCUSSION. Different associations between cryptosporidium contamination and water quality parameters were observed when the surface water data were grouped by oocyst source or considered as a whole. Water quality associations may be specific to individual surface water sites, given differences in the range of data values for each parameter at sites impacted by different land uses. For example, the range of data values for a particular parameter may be sufficiently narrow at one site such that an association between extreme values of that parameter and cryptosporidium detection would be imperceptible. Water quality associations may also be unique for each oocyst source, given the broad range of potential cryptosporidium sources in the environment (wildlife, birds, farm animals, and humans) and the specific behavioral patterns of each. Finally, water quality associations may be specific to the species of *Cryptosporidium*, since each species of the parasite has a particular range of animal hosts and may be best adapted for survival under a unique set of physical conditions.

The relationship between surface water temperature and oocyst contamination was disparate for wildlife- and agriculture-influenced surface waters. Wildlife-influenced sites showed a significant association between cold water temperatures and oocyst contamination, while agriculture-influenced sites showed a trend toward warm water temperatures and oocyst contamination (although it did not reach statistical significance). Although we hypothesized that oocysts would be found more often in colder waters, the data from this study show that oocysts were detected in surface waters of ambient temperature throughout the year (Figure 1, panels A and B). Thus, the different temperature trends seen for wildlife- and agriculture-influenced surface waters are likely due to factors other than the effect of temperature on oocyst survival.

Water temperature and season are related (Figure 1, panels A and B), and thus the recorded temperature trends may be an indicator of the seasonal presence of oocyst sources in the watershed or the seasonal shedding of oocysts from those sources. For example, birds have been identified as a significant source of *Cryptosporidium* spp. oocysts on dairy farm JF [unpublished data]. The largest bird populations in this northern watershed are found in the warm summer months, coincident with the observed trend of oocyst contamination of agriculture-influenced surface waters during higher water temperatures. Temperature trends may also be an indicator of the seasonal presence of an oocyst predator or reservoir that removes the parasite from the water

column. Recent reports have shown that free-living ciliated protozoa are predators of *C. parvum* oocysts in wastewater [17] and that freshwater benthic clams ingest *C. parvum* and release oocysts in their feces [18]. The seasonal presence and feeding patterns of these and other aquatic organisms may play a role in the temporal detection of cryptosporidium in various surface waters. Similar to the combined analysis of the wildlife-, agriculture-, and human-influenced surface waters, a 1996 study of the Delaware River [4] found no correlation between water temperature and cryptosporidium contamination. We have previously shown that multiple sources and species of *Cryptosporidium* spp. oocysts exist in the Wachusett Reservoir watershed [16]; the confounding influence of multiple sources, species, and predators may explain the absence of a distinct temperature association when data from a large watershed are analyzed as a whole.

The correlation of increasing pH with cryptosporidium contamination among agriculture-influenced surface waters (pH range: 5.93 to 7.43) is in contrast to the findings of the 1996 Delaware River study [4] in which the authors report a significant negative Spearman Rank correlation (-0.184) for pH and cryptosporidium (pH range: 6.7 to 8.5). However, taking into account the different pH ranges for the two studies, the data from both seem to suggest an association of cryptosporidium with surface waters near neutral pH; this observation supports the original hypothesis that oocysts would be found in waters of neutral pH. Although no significant association between neutral pH and oocyst detection was identified when analyzing data from the wildlife-influenced sites alone or the wildlife-, agriculture-, and human-influenced sites combined, the pH ranges of all data sets (for both positive and negative samples) were very close to neutral and may not have been large enough to discern a significant relationship. Because the recorded pH never fell below 5.80 at any of the surface water sites, the original hypothesis that fewer oocysts would be detected at acidic pH, due to an increase in oocyst hydrophobicity and sedimentation, was not addressed.

The significant positive correlation between pH and temperature at all sample sites suggests that pH is a seasonal indicator as well. Seasonal fluctuations of surface water pH are common: an influx of nitrate and organic acids via snowmelt runoff and heavy rains often causes springtime acidification, and increased nitrate and sulfate reduction in the warm summer months

leads to increases in pH. Thus, the relationship of pH to oocyst contamination may have less to do with the effect of pH on survival and more to do with seasonal influences. The theory of seasonal influences on oocyst contamination is further supported by the results of the factor analysis (Table 3), which shows oocyst presence correlating strongly with seasonal factors among the wildlife-influenced and agriculture-influenced data sets. For F1 of the wildlife-influenced sites, oocyst presence (-0.77) shows a strong negative correlation with temperature (0.81) and pH (0.74); for F2 of the agriculture-influenced sites, oocyst presence (0.74) shows a strong positive correlation with temperature (1.04) and pH (1.05). The equal and opposite correlations observed from these two data sets most likely explains why, when the data sets are combined, no strong correlation of oocyst presence with the seasonal factor (F2) is seen.

The original hypotheses that cryptosporidium contamination would be associated with high turbidity and high flow rate were not supported by the data from this study. Data from the Delaware River study [4] show a significant positive Spearman Rank correlation (0.571) for turbidity and *Cryptosporidium* spp. oocysts. The fact that the turbidity range in the Delaware River (0 to 97 ntu) was much broader than that in the present work (0.30 to 12.60 ntu) most likely explains the different findings of the two studies. Similarly, cryptosporidium and flow rate showed a significant positive correlation in the Delaware River with a Spearman Rank coefficient of 0.194. Although no association between flow rate and cryptosporidium was found in the present study, flow rate data were limited to the wildlife-influenced sites and the range of data was about 100-fold lower than that of the Delaware River work (4300 to 100,000 cfs). The narrow ranges of flow rate and turbidity data recorded in the present study may explain why no significant correlations with cryptosporidium contamination were observed. Further environmental sampling needs to be done in surface waters with broader ranges of turbidity and flow rate to establish the uniformity of the correlations reported in the Delaware River study.

The observed association between low specific conductivity and cryptosporidium contamination of agriculture-influenced surface waters supports the original hypothesis that oocysts are less hydrophobic and more likely to be detected in the water column at lower specific conductivity. The range of specific conductivity values among wildlife-influenced surface waters (50 to 270.6 µS/cm) was much narrower than the range of values among the agriculture-

influenced surface waters (63 to 642.0 μS/cm), which may easily explain why no significant difference was seen in the mean values of specific conductivity for wildlife-influenced surface waters that tested positive and negative for *Cryptosporidium* spp. oocysts. Similarly, no significant relationship between cryptosporidum and specific conductivity was found in the Delaware River study [4], but the data range of that study was also quite narrow (79 to 249 μS/cm). However, when the data from the present study were considered all together, no significant association between *Cryptosporidium* and specific conductivity was seen, suggesting that this relationship may be unique to agricultural sources of oocysts or watersheds impacted by agricultural land use.

The association of high dissolved oxygen (% saturation) with cryptosporidium contamination of agriculture-influenced surface waters is in agreement with the 1996 Delaware River study [4] in which a significant Spearman Rank coefficient of 0.424 was derived to describe the relationship between cryptosporidium and dissolved oxygen (mg/L). These data support the original hypothesis that oocysts would survive longer in waters of high dissolved oxygen content, although to date, no published data exist to characterize the oxygen needs of *Cryptosporidium* spp. oocysts for survival in surface waters. When the dissolved oxygen data from the wildlife-, agriculture-, and human-influenced surface waters were combined, no significant correlation with cryptosporidium contamination was observed. Although an explanation is not immediately evident, one possibility is that different species of oocysts may have different oxygen requirements.

It must be noted that low specific conductivity and high dissolved oxygen, both showing significant correlations with cryptosporidium contamination of agriculture-influenced surface waters, also correlated with high sample volume (Table 2). The correlation of specific conductivity and dissolved oxygen with sample volume is not entirely unexpected, since surface waters of low specific conductivity and high dissolved oxygen typically have fewer particulates, clog the filter less frequently, and yield larger filtration volumes. However, statistical analysis of agriculture-influenced sites alone, and wildlife-, agriculture-, and human-influenced sites combined, showed larger mean sample volumes of cryptosporidium-positive versus cryptosporidium-negative surface waters. Since a larger sample volume means a greater chance

of capturing and detecting oocysts, the authenticity of the observed correlations between oocyst presence, specific conductivity, and dissolved oxygen remains unclear. The principle component analysis (Table 3) extracted cleanliness factors for each data set (combined, wildlife, and agriculture); variables that contributed strongly to the cleanliness factors include turbidity (low), dissolved oxygen (high), and sample volume (high). Oocyst presence did not correlate strongly with the cleanliness factor from any of the data sets, suggesting that oocyst detection does not depend on sample volume. However, further environmental analysis, using surface water samples of equal volume, is needed to confirm the relationships between cryptosporidium, specific conductivity, and dissolved oxygen.

The data suggest that cryptosporidium contamination of surface waters is impacted strongly by seasonal stimuli, and that these seasonal stimuli can vary among surface waters within the same watershed. Although interesting relationships between cryptosporidium contamination and physical water quality parameters have emerged from the present study, the variability of these relationships among surface waters exposed to oocysts from different environmental sources precludes efforts to draw universal conclusions. An earlier study investigating correlations of cryptosporidium with microbial indicators [3] also found differences in statistical results when data from three rivers were analyzed separately or grouped as a whole, and these authors suggest that relationships may vary from one aquatic system to another or possibly from one site to another on the same river. The current study was limited in scope to one or two surface water sites for each cryptosporidium source group, and the differences seen among these wildlife-, agriculture-, and human-influenced surface waters may well be explained by phenomena specific to each site, such as land use impacts or source dynamics.

Further field studies are needed, in additional watersheds, to better elucidate universal relationships between cryptosporidium and water quality parameters and to understand which environmental phenomena (e.g., oocyst source, oocyst species, or watershed characteristics) are most responsible for oocyst transport and survival from source to surface waters. For example, studies targeting a number of surface water sites susceptible to oocyst contamination from the same source group, or parallel studies in a number of watersheds with similar land use patterns, will elucidate whether associations between cryptosporidium and water quality parameters are

conserved among similar source groups or land use impacts. In future studies, assessments of oocyst viability in surface waters will be important so that correlations between water quality parameters and cryptosporidium can be made even more specific to address public health risks. Results from this and future field studies will help identify surrogate water quality parameters that can be used to model and forecast periods of high risk for *Cryptosporidium* spp. oocyst contamination of surface waters. Ultimately, this knowledge will contribute to the development of standardized watershed management strategies to protect the public from waterborne cryptosporidiosis.

ACKNOWLEDGMENTS. We thank the people of the Metropolitan District Commission of the Commonwealth of Massachusetts for their help in identifying surface water sample locations and for sharing their water quality data for the Quinapoxet and Stillwater Rivers. We are also grateful to Melanie Ihrig of the Division of Comparative Medicine at Massachusetts Institute of Technology for her help with the statistical analyses.

This work was supported by a National Science Foundation Graduate Research Fellowship, a U.S. EPA "Science to Achieve Results" (STAR) Fellowship, and a feasibility project funded by the National Institute for Environmental Health Sciences.

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Table 1. Recorded water quality data for surface waters susceptible to *Cryptosporidium* spp. oocyst contamination via wildlife shedding, agricultural runoff, and human sewage. Positive sample values in **bold** are statistically different ($p<0.05$) from their negative counterparts by the unpaired t-test.

Sample Result[a]	Source[b]	Temperature (°C)	Flow Rate (cfs)	pH	Turbidity (ntu)	Specific Conductivity (µS/cm)	Dissolved Oxygen (mg/L)	Dissolved Oxygen (%sat)
Pos	W	**3.6** ± 3.7 (n=8)[c]	40.7 ± 24.4 (n=6)	6.77 ± 0.32 (n=8)	0.89 ± 0.18 (n=8)	152.6 ± 73.0 (n=5)	12.25 ± 1.18 (n=3)	88.4 (n=1)
Neg	W	10.8 ± 7.6 (n=38)	41.3 ± 46.0 (n=26)	6.77 ± 0.38 (n=38)	0.92 ± 0.32 (n=38)	144.4 ± 53.7 (n=31)	10.71 ± 1.37 (n=11)	89.9 ± 6.0 (n=7)
Pos	A	12.1 ± 5.1 (n=6)	NR[d]	**6.96** ± 0.33 (n=6)	1.15 ± 1.32 (n=6)	**86.7** ± 21.6 (n=6)	9.42 ± 1.13 (n=6)	**87.0** ± 7.8 (n=6)
Neg	A	6.9 ± 6.9 (n=18)	NR	6.45 ± 0.41 (n=18)	2.96 ± 3.00 (n=18)	289.1 ± 170.4 (n=18)	8.12 ± 4.03 (n=18)	63.1 ± 27.7 (n=18)
Pos	H	5.9 ± 5.6 (n=2)	NR	7.49 ± 0.01 (n=2)	1.40 ± 0.57 (n=2)	680.5 ± 98.3 (n=2)	11.97 ± 2.33 (n=2)	94.8 ± 5.2 (n=2)
Neg	H	10.7 ± 5.9 (n=10)	NR	7.32 ± 0.27 (n=10)	1.49 ± 1.30 (n=10)	1309.3 ± 1325.9 (n=10)	11.11 ± 1.51 (n=10)	98.9 ± 3.5 (n=10)

[a]Pos = positive for *Cryptosporidium* spp. oocysts; Neg = negative for *Cryptosporidium* spp. oocysts.
[b]Oocyst source. W = wildlife; A = agriculture; H = humans
[c]Mean data value ± standard deviation (n=number of data points)
[d]NR = not recorded

Table 2. Pearson correlation coefficients for water quality parameters recorded at sites susceptible to oocyst contamination via agricultural runoff.

	D.O.[a]	pH	Cond[b]	Turb[c]	Volume[d]
Oocysts[e]	**0.402**[f] **(0.0507)**[g]	**0.512** **(0.0095)**	**-0.521** **(0.0082)**	-0.289 (0.1721)	**0.475** **(0.0178)**
D.O.		0.272 (0.2006)[e]	**-0.611**[f] (0.0011)	**-0.782** (<0.0001)	**0.675** (0.0002)
pH			**-0.753** (<0.0001)	-0.115 (0.5958)	0.246 (0.2491)
Cond				0.393 (0.0573)	**-0.526** (0.0074)
Turb					**-0.720** (<0.0001)

[a]Dissolved oxygen (% saturation)
[b]Specific conductivity (µS/cm)
[c]Turbidity (ntu)
[d]Sample volume (L)
[e]Presence or absence of oocysts. For derivation of correlation coefficients, "Oocysts" variable was assigned a value of 1 (present) or 0 (absent).
[f]Values in bold text denote statistically significant correlations.
[g]Values in parentheses denote p values.

Table 3. Oblique factor extractions derived from principle component analysis.

	Combined Data			*Wildlife*			*Agriculture*	
	F1[a] (C[b])	F2 (S[c])	F3 (I[d])	F1 (S)	F2 (C)	F3 (I)	F1 (C)	F2 (S & I)[e]
Oocysts[f]	0.35	0.31	-0.50	***-0.77***	0.16	0.16	0.04	***0.74***
Volume[g]	***0.75***	0.14	-0.25	0.03	***-0.86***	0.18	***0.80***	0.08
Temp[h]	-0.30	***0.89***	-0.30	***0.81***	0.35	0.05	***-0.90***	***1.04***
pH	0.34	***0.79***	0.18	***0.74***	0.00	0.41	-0.31	***1.05***
Cond[i]	0.07	0.00	***0.89***	0.00	0.01	***0.97***	-0.21	***-0.76***
Turb[j]	***-0.84***	0.02	0.22	0.06	***0.85***	0.19	***-0.89***	0.08
D.O.[k]	***0.91***	-0.32	0.29	NI[m]	NI	NI	***1.12***	-0.38
D.O.[l] (%)	***0.90***	0.05	0.17	NI	NI	NI	***0.95***	0.00

[a]Factors (F1, F2, and F3) were derived for the combined data set (wildife-, agriculture-, and human-influenced sites), the wildlife-influenced sites, and the agriculture-influenced sites. Values in the table (i.e., factor loadings) show the correlation of each variable with each factor (values close to 0 indicate low correlation, values close to 1 indicate high correlation). High factor loadings (above 0.50) are shown in bold italics. Factors were assigned descriptive titles (C, S, or I) based upon the variables with highest factor loadings.

[b]C=Cleanliness factor. Samples of large volume, low turbidity, and high dissolved oxygen imply high quality, clean surface water.

[c]S=Seasonal factor. Samples of high temperature and high pH are indicative of late spring and summer seasons.

[d]I=Ionic strength factor. Samples of high specific conductivity suggest surface waters of high ionic strength.

[e]S&I=Seasonal and Ionic strength factor. Factor has high loadings from temperature, pH, and specific conductivity.

[f]Presence or absence of oocysts. For factor analysis, "Oocysts" variable was assigned a value of 1 (present) or 0 (absent).

[g]Sample volume (L)

[h]Temperature (°C)

[i]Specific conductivity (µS/cm)

[j]Turbidity (ntu)

[k]Dissolved oxygen (mg/L)

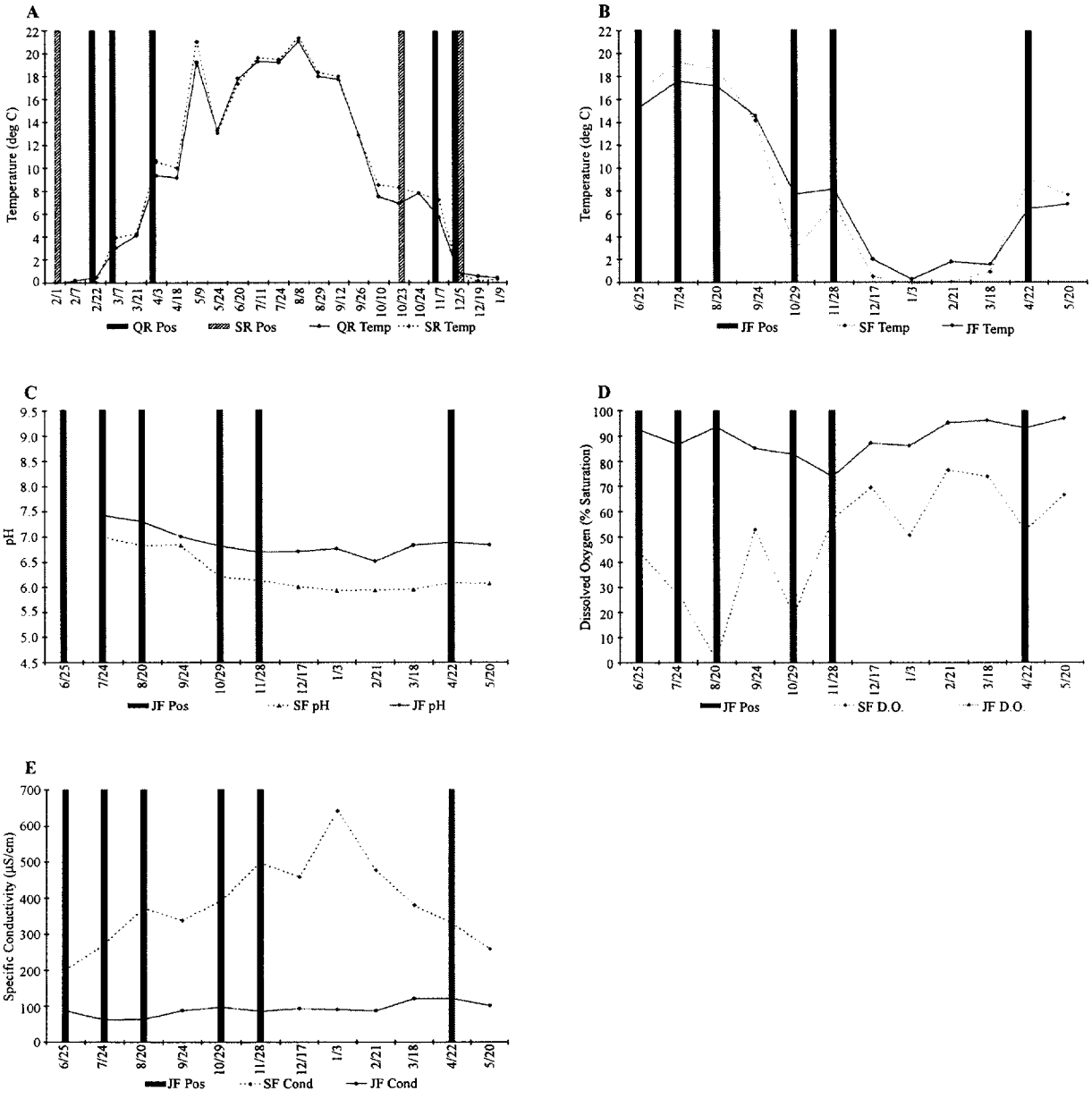
[l]Dissolved oxygen (% saturation)

[m]NI=Variable not included in factor analysis due to limited data points.

FIGURE CAPTION.

Figure 1. Selected water quality parameters of wildlife sites (panel A) and agricultural sites (panels B through E) plotted against sample date to illustrate any seasonal trends. (A) Results of Quinapoxet River (QR) and Stillwater River (SR) are plotted separately. Dark bars and shaded bars indicate dates when QR and SR, respectively, were positive for *Cryptosporidium*. Dark line is temperature data for QR; dotted line is temperature data for SR. (B through E). Results of Brook JF (JF) and Brook SF (SF) are plotted separately. Dark bars indicate dates when JF was positive for *Cryptosporidium*; SF was never positive for *Cryptosporidium*. Dark lines are water quality data for JF; shaded lines are water quality data for SF.

Figure 1


A
Temperature (deg C)
QR Pos
SR Pos
QR Temp
SR Temp
B
Temperature (deg C)
JF Pos
SF Temp
JF Temp
C
pH
JF Pos
SF pH
JF pH
D
Dissolved Oxygen (% Saturation)
JF Pos
SF D.O.
JF D.O.
E
Specific Conductivity (µS/cm)
JF Pos
SF Cond
JF Cond

Chapter 6: Phylogenetic Analysis of the Hypervariable Region of the 18S rRNA Gene of *Cryptosporidium* spp. Oocysts in the Feces of Canada Geese (*Branta Canadensis*): Evidence for Novel Genotypes

Manuscript to be submitted to ***Applied and Environmental Microbiology***

Abstract. In order to assess genetic diversity in the hypervariable region of the 18S rRNA gene of *Cryptosporidium* spp. oocysts in the feces of Canada geese, 161 fecal samples from Canada geese in the United States were analyzed. Eleven (6.8%) were positive for *Cryptosporidium* spp. following nested PCR amplification of the 18S rRNA hypervariable region. Nine PCR products from geese were cloned and sequenced, and all nine diverged from *Cryptosporidium* spp. 18S rRNA gene sequences in GenBank. Five sequences (Goose #1, 2, 3a, 6, and 8) were very similar or identical to each other but genetically distinct from *C. baileyi*, two (Goose #5 and 9) were most closely related to, but genetically distinct from, the first five, and two (Goose #3b and 7) were distinct from any other sequences analyzed. One additional sequence isolated from a cormorant was identical to *C. baileyi* in the hypervariable region of the 18S rRNA gene. Phylogenetic analysis suggested that *C. baileyi* may be conserved at the 18S rRNA locus and provided evidence for new genotypes of *Cryptosporidium* in Canada geese. Although *C. parvum* was not detected in any of the geese fecal samples, the potential for the oocysts recovered from geese in this study to infect humans is unknown. Results of this study revealed novel 18S rRNA genotypes among *Cryptosporidium* spp. and suggest that further work is necessary to fully characterize the extent of genetic diversity within the genus. A more complete understanding of genetic diversity among *Cryptosporidium* spp. is required before any information about oocyst source or species can be deduced from 18S rRNA sequence data.

Introduction. *Cryptosporidium parvum* is a protozoan parasite that causes self-limiting gastrointestinal disease in otherwise healthy adults but severe, prolonged, and potentially life-threatening illness in immunocompromised individuals. The parasite has a broad range of animal hosts and can be transmitted either anthroponotically or zoonotically following ingestion of contaminated food or water. Molecular characterization of *C. parvum* has revealed extensive genetic polymorphism, and the species has been classified into two distinct genotypes: human-adapted genotype 1 and animal-adapted genotype 2 [2, 4, 18, 21, 24]. Sufficient variation among the animal-adapted *C. parvum* group has led to further classifications of bovine, dog, pig, mice, deer, ferret, marsupial, and monkey genotypes [15, 19, 27].

Traditional taxonomic classification of *Cryptosporidium* spp. oocysts is based on oocyst morphology, host specificity, and the anatomical site of infection [7]. More recently, the polyphasic approach to taxonomy has included molecular genetic characterization as well as the traditional criteria [8]. The small number of *Cryptosporidium* spp. oocysts recovered from environmental samples often precludes traditional taxonomic analysis, resulting in species identification that is based solely on molecular characterization of one or more genes [13, 19, 28, 30]. Genes encoding actin [22], the 70-kilodalton heat shock protein [23], the *Cryptosporidium* oocyst wall protein [29], and the 18S small subunit rRNA [26] have all been used for molecular genetic characterization of *Cryptosporidium* spp..

Although the molecular genetic polymorphism of *C. parvum* has been extensively characterized, diversity in other *Cryptosporidium* species is not as well studied. Currently, there are over 140 18S rRNA sequence entries for *C. parvum* in the GenBank database [3]; by contrast, there are only 34, 12, 6, 5, 3, and 2 entries for *C. meleagridis*, *C. muris*, *C. serpentis*, *C. baileyi*, *C. wrairi*, and *C. andersoni*, respectively. Given the paucity of molecular genetic information for *Cryptosporidium* species other than *C. parvum*, identification of oocyst species from environmental samples using DNA sequence data alone can be difficult.
We hypothesize that the level of genetic polymorphism seen in *C. parvum* may also exist in other *Cryptosporidium* species. In order to test this hypothesis, the genetic variability of the 18S rRNA gene of *Cryptosporidium* spp. oocysts in the feces of Canada geese was determined. Geese were chosen as the target animal host because they are ubiquitous and impact surface

water quality. In addition, geese are known to be a host for non-*parvum* species; birds are susceptible to infection with only two *Cryptosporidium* species, *C. meleagridis* and *C. baileyi*, which are sufficiently different at the genetic level that DNA sequence data should elucidate the species to which the fecally-derived oocysts belong. Confining this study to geese eliminates the uncertainty of oocyst source and allows comparison of DNA sequences among particular species of *Cryptosporidium* from the environment.

Here we report the prevalence of *Cryptosporidium* spp. oocysts in geese from different parts of the United States and describe 18S rRNA polymorphism in oocysts recovered from goose feces. These findings will improve our understanding of the role of geese in the transmission of waterborne cryptosporidiosis and of the genetic variability of *Cryptosporidium* spp. in this host. A more complete understanding of the phylogeny of *Cryptosporidium* spp. will facilitate the identification of likely sources of oocysts detected by molecular genetic methods in the environment.

Materials and Methods.

Fecal Collection. Fresh fecal pellets from geese were collected from August 2001 to October 2002 at various geographic locations in the United States (Table 1). All fecal pellets were handled with disposable gloves to prevent cross-contamination of samples. Individual fecal pellets were stored on ice in sterile 50-ml polypropylene conical tubes and shipped to the MIT laboratory within 24 h of collection. One additional avian fecal sample, collected in August 2000 from a cormorant in Massachusetts, was included in the study as well.

Oocyst Isolation. Upon arrival at the MIT laboratory, a 1- to 3-gram sample of each fecal pellet was suspended in 20 ml of laboratory-grade water (Milli-Q System; Millipore Corp., Bedford, Mass.). A second 1- to 3-gram sample of one fecal pellet for each batch of samples was included as a positive control for the detection assay. This positive control pellet was resuspended in 19.5 ml laboratory-grade water and spiked with 500 µl of a 10^4 oocyst ml^{-1} suspension. Each fecal suspension was vortexed for 30 s to homogenize the fecal slurry and was then allowed to settle for 3 min to remove large fecal particles (mostly grass). After settling, 10 ml of supernatant were transferred to a glass Leighton tube (Bellco Glass, Inc., Vineland, NJ), and oocysts were purified from each fecal sample by immunomagnetic separation (IMS) using the Crypto-Scan IMS kit (ImmuCell, Portland, Maine) according to manufacturer's recommendations. After dissociation from the magnetic beads, oocysts were transferred to a microcentrifuge tube and treated with 5 µl of 1 N NaOH to neutralize the pH. The oocysts were pelleted for 2 to 3 min at 16,000 x g, resuspended in 50 µl of laboratory-grade water, and stored at 4°C.

Positive and negative IMS controls were processed with each set of fecal samples. Positive IMS controls consisted of 9.5 ml of laboratory-grade water spiked with 500 µl of a 10^4 oocyst ml^{-1} suspension; negative IMS controls consisted of 10 ml of laboratory-grade water. IMS controls were processed as described above.

Genomic DNA Extraction. Oocysts were lysed by adding 25 µL of IMS product to 475 µl Tris-EDTA (TE) buffer containing 0.2 g proteinase K $liter^{-1}$ and 0.4% sodium dodecyl sulfate and incubating the mixture overnight at 45°C. Positive and negative DNA extraction controls were included for each set of fecal samples. Positive DNA extraction controls consisted of 25 µL of a

suspension of 10^4 oocysts ml^{-1} in 475 µL of TE buffer; negative DNA extraction controls consisted of 25 µl of laboratory-grade water in 475 µL of TE buffer. DNA was extracted with phenol-chloroform, precipitated with 0.2M NaCl and 2 volumes of absolute ethanol, and resuspended in 30 µL of TE buffer.

Nested PCR Assay. Nested PCR amplification of the hypervariable region of the 18S rRNA gene was performed as previously described [13] with minor modifications. The concentration of each deoxynucleoside triphosphate (Perkin-Elmer, Wellesley, Mass.) was 0.15 mM. The initial amplification reaction was performed with 15 µl of DNA template, and 1 µl of the initial amplification product was used as template in the secondary PCR. Positive and negative PCR controls were included with each set of fecal samples. For the initial amplification reaction, positive PCR controls contained 14 µl of laboratory-grade water and 1 µl of genomic *C. parvum* DNA (at a concentration equivalent to 10^4 oocysts μl^{-1}); negative PCR controls contained 15 µl of laboratory-grade water. For the secondary amplification reaction, positive PCR controls contained 1 µl of genomic *C. parvum* DNA (at a concentration equivalent to 10^4 oocysts μl^{-1}); negative PCR controls contained 1 µl of laboratory-grade water.

Both amplification reactions used forward and reverse oligonucleotide primers that are complementary to all *Cryptosporidium* spp. 18S rRNA gene sequences. For the primary PCR, an approximately 1,056-bp product (dependent on *Cryptosporidium* species) was obtained using forward and reverse primers KLJ1 and KLJ2, respectively [13]; for the secondary PCR, an approximately 434-bp product was obtained using forward and reverse primers CPB-DIAGF and CPB-DIAGR, respectively [14]. Cycling conditions for both the primary and secondary PCRs consisted of an initial denaturation (5 min at 80°C, followed by 30 s at 98°C), 25 cycles of amplification (denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 1 min at 72°C), and a final extension (10 min at 72°C). Secondary PCR products were visualized after electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Cloning. Secondary PCR products were cloned into the pGEM-T Easy Vector System (Promega Corporation, Madison, Wis.) and used to transform XL-1 Blue *E. coli* cells (Stratagene, La Jolla, Calif.). Clones were selected on Luria-Bertani (LB) agar supplemented with 100 µg of

ampicillin ml^{-1} and cultured overnight in LB broth supplemented with 100 μg of ampicillin ml^{-1}. Plasmid DNA was isolated from clones by using the QIAPrep Spin Miniprep Kit (Qiagen, Inc., Valencia, Calif.) and digested with *Not*I (New England Biolabs, Beverly, Mass.) to verify the presence of the secondary PCR amplicon insert and *Nde*I (New England Biolabs) to identify any heterogeneity among the clones [13]. Restriction digestion was carried out in a 20-μl volume containing 4 μl plasmid DNA, 20 U of *Not*I, 10 U of *Nde*I, 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg of bovine serum albumin ml^{-1} and then incubated at 37°C for 1 h. Digestion products were visualized after electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Sequencing of Secondary PCR products. Representative clones of the secondary PCR products were sequenced on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, Calif.) using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PE Applied Biosystems). If multiple *Nde*I digestion patterns existed among clones from a given sample, at least one clone of each digestion pattern was sequenced. With the exception of Goose #7, at least three clones for each positive sample were sequenced and confirmed by sequencing both strands. For Goose #7, one clone was successfully sequenced and confirmed by sequencing both strands. The consensus sequences for the clones recovered from each bird were used in the phylogenetic analysis.

Phylogenetic Analysis. Sequences were aligned manually, based on the secondary structure of the 18S rRNA, using the GCG sequence editor (Genetics Computer Group, Madison, WI). Variable length regions were masked and excluded from the phylogenetic analysis. Phylogenetic Analysis Using Parsimony (PAUP), beta version 4.0 [25], was used to create both neighbor-joining and parsimony trees from the GCG alignments. Construction of neighbor-joining trees was based on the evolutionary distances between different isolates calculated by the Kimura two-parameter analysis and the designation of *C. felis* as an outgroup. Statistical support for the resulting trees was tested using 1000 pseudoreplicates of the bootstrap test; only values above 50% were reported, and bootstrap values greater than 70% were considered significant [12]. GenBank accession numbers used in the phylogenetic analyses are noted in the captions of Figures 2 and 3.

Oocyst Detection Limit. Pooled goose fecal samples negative for *Cryptosporidium* spp. oocysts were divided into 7 2-g aliquots and seeded with 1, 10, 50, 100, 500, 1000, or 5000 *C. parvum* oocysts. Laboratory-grade water was added to bring each spiked fecal sample to a final volume of 20 mL. Fecal suspensions were vortexed for 30 s to homogenize the fecal slurry and then allowed to settle for 3 min to remove large fecal particles. After settling, 10 ml of each sample were transferred to a glass Leighton tube and processed as described above (IMS, DNA extraction, and nested PCR). Secondary PCR products were visualized after electrophoresis on a 1.2% agarose gel stained with ethidium bromide to identify the sensitivity (oocysts g^{-1} feces) of the detection assay.

Results.

Oocyst Detection Limit. Using a nested PCR amplification method, the lower limit of detection for C. parvum was found to be 25 oocysts g^{-1} feces (i.e., 50 oocysts spiked into 2 g feces) (Figure 1). The detection limit assay was performed twice with identical results.

Prevalence of *Cryptosporidium* spp. oocysts in Geese. A total of 161 fecal samples from geese were collected and examined for the presence of *Cryptosporidium* spp. oocysts, and 11 (6.8 %) were positive for the parasite (Table 1). Of the 11 positive fecal specimens, 8 were successfully cloned and sequenced. For one sample, Goose #3 from Illinois, two different 18S rRNA sequences (designated a and b) were identified.

Phylogenetic Analysis. Both neighbor-joining and parsimony trees were created to determine the phylogenetic relationship of the parasites obtained from geese (Figure 2). Several distinct taxa of *Cryptosporidium* spp. are evident from the phylogenetic trees: *C. parvum*, *C. meleagridis*, and *C. wrairi* form one clade; *C. andersoni*, *C. muris*, and *C. serpentis* form another clade; and *C. baileyi* and *C. felis* are each on their own distinct branch. Evolutionary distances (Table 2) between clades are relatively large, ranging from 0.087 to 0.103 between the *C. andersoni* and *C. parvum* clades, 0.087 between *C. baileyi* and the *C. andersoni* clade, 0.035 to 0.042 between *C. baileyi* and the *C. parvum* clade, and 0.106 to 0.115 between *C. felis* and the *C. andersoni* clade. Within a clade, evolutionary distances are much smaller, with a range of 0.010 to 0.017 within the *C. andersoni* clade and 0.002 to 0.007 within the *C. parvum* clade.

The sequence recovered from the cormorant was 100% identical to *C. baileyi* L19068. Sequences from oocysts of Goose #1, 2, 6, and 8 were 100% identical to each other and had an evolutionary distance (Table 2) to *C. baileyi* L19068 (0.050) identical to the evolutionary distance between *C. baileyi* L19068 and *C. felis* AF112574. One of the sequences from Goose #3 (sequence a) was also very closely related to the sequences of Goose #1, 2, 6, and 8, and these five sequences formed a clade with a bootstrap values of 100% and 99%, respectively, by the neighbor-joining and parsimony methods (Figure 2). The sequence from Goose #9 clustered with the clade of Goose #1, 2, 3 (sequence a), 6, and 8 sequences with significant bootstrap values of 95% and 97% for neighbor-joining and parsimony analyses, respectively. The

sequence from Goose #5 also clustered with the sequence from Goose #9 and the clade of Goose #1, 2, 3 (sequence a), 6, and 8 sequences by both the neighbor-joining and parsimony analyses. The evolutionary distances of Goose #5 and 9 to the group of Goose #1, 2, 6, and 8 sequences were 0.032 and 0.024, respectively, greater than the distances between *C. parvum* to *C. wrairi* (0.007) or *C. serpentis* to *C. muris* (0.017). Similarly, the evolutionary distance between Goose #5 and Goose #9 (0.027) was greater than the distance between distinct *Cryptosporidium* species.

Two additional sequences, from Goose #7 and Goose #3 (sequence b), were very different from the sequences recovered from the other geese and from GenBank. The evolutionary distances between Goose #7 and Goose #1 (0.077), Goose #7 and *C. baileyi* (0.074), and Goose #7 and *C. meleagridis* (0.069) were greater than the evolutionary distance between *C. parvum* AF093489 and *C. baileyi* L19068 (0.042). In addition, the evolutionary distance between sequence b from Goose #3 and all other sequences in the phylogenetic analysis ranged from 0.052 to 0.103 (Table 2), similar to the range of evolutionary distances between *C. felis* and the other sequences in the trees (0.040 to 0.115).

Discussion.

A previous study by Graczyk at al. [11] reported the average concentration of *C. parvum* oocysts in goose feces to be 370 ± 197 oocysts g^{-1}. Since birds are refractory hosts of *C. parvum*, i.e., no life-cycle stages of *C. parvum* were found in the stomachs, jejunums, ilea, ceca, cloacae, larynges, tracheae, or lungs of ducks experimentally infected with *C. parvum* oocysts [9], even greater numbers of *C. baileyi* or *C. meleagridis* oocysts would be expected in goose feces. Thus, the detection limit of the current assay (25 g^{-1} feces) was acceptable for the concentrations of *Cryptosporidium* spp. oocysts expected in geese.

At the time of this study, only five *C. baileyi* 18S rRNA gene sequences had been deposited in GenBank; these five sequences are from U.S., Australian, and Hungarian strains and are identical, suggesting that the 18S rRNA gene of *C. baileyi* is conserved. Furthermore, the HSP70 and COWP gene sequences of these isolates are identical, suggesting that the genome of *C. baileyi* may be conserved. The genetic distinctness of *C. baileyi* was further supported by the recovery of an identical 18S rRNA sequence in this study from a cormorant in Massachusetts.

Unique 18S rRNA gene sequences, suggestive of new *Cryptosporidium* genotypes, were identified in geese feces as well. Identical sequences from Goose #1, 2, 6, and 8 (from Illinois, Illinois, Massachusetts, and Virginia, respectively) were recovered, showing conservation of another 18S rRNA gene sequence across broad geographic areas. The evolutionary distance of these sequences (and sequence "a" from Goose #3) to *C. baileyi* was similar to the evolutionary distance of *C. felis* to *C. baileyi*, suggesting that this clade of sequences (Figure 2) represents a new genotype or perhaps even a distinct species of *Cryptosporidium* in geese. The sequences from Goose #5 and 9 were most closely related to this clade, yet the evolutionary distances between Goose #5 and 9 and this clade were greater than the distance between *C. serpentis* and *C. muris*. Thus, the oocysts recovered from Goose #5 and 9 may represent two new genotypes, or two distinct but closely-related species, of the taxonomic group represented by the clade of Goose #1, 2, 3 (sequence a), 6, and 8 sequences. A definitive taxonomic classification of these oocysts requires morphological and biological characterizations that are not feasible given the limited oocyst quantities in environmental samples.

Further evidence for new *Cryptosporidium* genotypes in geese was found by the unique 18S rRNA gene sequences recovered from Goose #7 and 3 (sequence b). The integrity of the 18S rRNA secondary structure, given the nucleotide changes observed in the sequences from Goose #3 and 7, was verified, and the possibility of *Taq* polymerase error during PCR was eliminated as an explanation for the observed sequence differences. The sequences recovered from Goose #7 and 3 (sequence b) are valid and most likely represent two previously uncharacterized species of *Cryptosporidium*. The genetic heterogeneity observed among *Cryptosporidium* oocysts from geese in this study supports the increasing level of diversity continuously reported for this genus [5, 17, 19, 28].

Two new species of *Cryptosporidium* in birds have been recently proposed [16, 20]. Oocysts isolated from finches have been named *C. blagburni* based on the unique localization of the oocysts in the proventriculus of birds and phylogenetic analyses of both the 18S rRNA and heat shock protein 70 genes [16]. In a separate study, partial sequences for the 18S rRNA gene of oocysts isolated from finches have been submitted to GenBank under the name *C. galli*. Phylogenetic analysis of *C. blagburni*, *C. galli*, and the geese-derived sequences from the present study at the 18S rRNA locus (Figure 3) shows that the sequences from the present study are genetically distinct from those of *C. blagburni* and *C. galli* and also suggests that *C. blagburni* and *C. galli* may represent the same taxonomic group.

Although we set out to characterize the level of genetic heterogeneity in the 18S rRNA gene within the bird species of *Cryptosporidium*, *C. baileyi* and *C. meleagridis*, we ultimately showed the increasing level of genetic heterogeneity within the genus. Because all of the 18S rRNA gene sequences recovered in this study were distinct from existing *Cryptosporidium* sequences, little has been discovered about the level of genetic variation among *C. baileyi* and *C. meleagridis* from geese. The recovery of a sequence from a cormorant that was 100% identical to all of the *C. baileyi* 18S rRNA sequences in GenBank suggests that *C. baileyi* may be a conserved species. Given the observed low prevalence of *Cryptosporidium* spp. oocysts in geese, a more exhaustive sampling of birds will be required to ascertain the level of genetic heterogeneity of *C. baileyi* and *C. meleagridis* in the environment.

Although some conservation of *Cryptosporidium* spp. 18S rRNA sequence at different geographic locations was observed in the present study, the data suggest that geographic location is not indicative of 18S rRNA gene sequence. Different 18S rRNA sequences were recovered from closely related oocysts in geese from Illinois (Goose #1, #3(a), and #5) and Virginia (Goose #8 and #9), and one goose (#3) shed oocysts with two distinct 18S rRNA sequences. The data suggest that geese can be carriers of more than one species of oocyst simultaneously.

The heterogeneity observed among *Cryptosporidium* 18S rRNA gene sequences from geese highlights the need for more similar studies and offers insight into the use of 18S rRNA sequence data for species and source identification of oocysts in the environment. Most attention to date has been given to characterizing the biology and genetic composition of *C. parvum*, the species of concern for human health. The lack of information about *Cryptosporidium* species other than *C. parvum* represents a significant gap in the knowledge base that needs to be filled in order to make environmental studies more informative. As the present study shows, the potential is great for unique and undiscovered *Cryptosporidium* 18S rRNA gene sequences to exist in the environment. Yet identification of these new *Cryptosporidium* spp. DNA sequences in surface waters is confounding, because no information about the source, species, or potential health risk can be gained. Only with a broader knowledge of the genetic heterogeneity of each species, and the genus as a whole, can environmental studies be of greatest benefit to the identification of important watershed sources of *Cryptosporidium* and the development of appropriate watershed management strategies to protect surface waters from oocyst contamination.

Geese feces have been clearly identified as potential sources of microbiological contamination to surface waters [1, 6]. Graczyk et al. [9, 10] showed that *C. parvum* oocysts retained infectivity for neonatal BALB/c mice after intestinal passage through Peking ducks and Canada geese. A later field study near the Chesapeake Bay [11] identified infectious zoonotic *C. parvum* oocysts in geese feces, indicating that waterfowl can serve as mechanical vectors of *C. parvum* and disseminate infectious oocysts to the environment. Although we did not see evidence of *C. parvum* oocysts in goose feces in this study, we did isolate novel gene sequences of uncharacterized *Cryptosporidium* spp. oocysts with unknown potential to cause disease in humans. Further study is warranted to rigorously characterize the extent of *Cryptosporidium*

spp. diversity in goose feces, the ability of those species to cause infection in humans, and the role of geese in the epidemiology of waterborne cryptosporidiosis.

Acknowledgements.

We thank Giovanni Widmer (Tufts University School of Veterinary Medicine, North Grafton, Mass.) for materials and technical assistance. We gratefully acknowledge the efforts of the following individuals in the collection of fecal samples: Barbara Kostroff (Diagnostic Specialty Lab, New City, New York), Monica Tischler (Benedictine University, Lisle, Illinois), Thomas Lustig (National Wildlife Foundation, Boulder, Colorado), Jo Ann Jellison, Richard Ting, Ralph Capriola, Jacqueline Fletcher (Oklahoma State University, Stillwater, Oklahoma), and Thomas Roderick.

This work was supported by a U. S. EPA "Science to Achieve Results" (STAR) Fellowship.

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Table 1. Summary of fecal sample collections.

Date	*Location*	*No. Samples*	*No. Positive*[a]	*No. Cloned*	*Sequence Identifier(s)*
Aug. 2001	New York	12	4	1	Goose #7
Aug. 2001	Illinois	25	4	4	Goose #1, #2, #3(a&b), #5
Mar. 2002	Massachusetts	10	0		
Jun. 2002	Colorado	15	0		
Jun. 2002	Virginia	7	2	2	Goose #8, #9
Jun. 2002	Washington	5	0		
Jul. 2002	Massachusetts	25	0		
Jul. 2002	Colorado	1	0		
Aug. 2002	Pennsylvania	15	0		
Aug. 2002	Oklahoma	13	0		
Sep. 2002	Massachusetts	19	1	1	Goose #6
Oct. 2002	Colorado	14	0		
	Total:	161	11	8	

[a]Positive for *Cryptosporidium* spp. oocysts by nested PCR targeting the hypervariable region of the 18S rRNA gene.

Table 2. Kimura two-parameter distance matrix (substitutions/site). GenBank accession numbers of sequences included in the matrix are AF112575 (*C. felis*), AB089285 (*C. andersoni*), AB089284 (*C. muris* murine genotype), AF093499 (*C. serpentis*), L19068 (*C. baileyi*), AF112574 (*C. meleagridis*), AF093489 (*C. parvum* human genotype), AF093493 (*C. parvum* bovine genotype), and U11440 (*C. wrairi*).

	1 *C. felis*	**2 *C. andersoni***	**3 *C. muris* (murine)**	**4 *C. serpentis***	**5 *C. baileyi***	**6 Goose #1 (Illinois)**	**7 Goose #3a (Illinois)**	**8 Goose #3b (Illinois)**	**9 Goose #5 (Illinois)**	**10 Goose #7 (New York)**	**11 Goose #9 (Virginia)**	**12 *C. meleagridis***	**13 *C. parvum* (human)**	**14 *C. parvum* (bovine)**	**15 *C. wrairi***
1	-														
2	0.106	-													
3	0.115	0.010	-												
4	0.112	0.017	0.017	-											
5	0.050	0.087	0.087	0.087	-										
6	0.082	0.107	0.112	0.098	0.050	-									
7	0.083	0.107	0.113	0.099	0.050	0.000	-								
8	0.063	0.098	0.103	0.098	0.063	0.096	0.096	-							
9	0.069	0.079	0.084	0.071	0.027	0.032	0.032	0.068	-						
10	0.085	0.124	0.129	0.127	0.074	0.077	0.077	0.099	0.074	-					
11	0.077	0.104	0.104	0.095	0.042	0.024	0.025	0.088	0.027	0.074	-				
12	0.040	0.087	0.095	0.092	0.035	0.066	0.066	0.052	0.042	0.069	0.055	-			
13	0.042	0.095	0.103	0.100	0.042	0.069	0.069	0.060	0.050	0.077	0.058	0.007	-		
14	0.045	0.092	0.100	0.097	0.040	0.071	0.072	0.058	0.047	0.074	0.060	0.005	0.002	-	
15	0.045	0.089	0.098	0.095	0.037	0.069	0.069	0.058	0.045	0.069	0.058	0.005	0.007	0.005	-

Figure Captions.

Figure 1. The oocyst detection limit (oocysts g^{-1} feces) was determined by spiking geese feces with decreasing numbers of oocysts. Secondary PCR products are shown after electrophoresis on a 1.2% agarose gel stained with ethidium bromide. From left to right, the lanes are as follows: molecular weight standard; negative controls for secondary (2°) and initial (1°) PCRs, respectively; positive controls for 2° and 1° PCRs, respectively; negative and positive controls for DNA extraction, respectively; negative and positive controls for IMS, respectively; fecal samples spiked with 1, 10, 50, 100, 500, 1000, and 5000 oocysts, respectively.

Figure 2. (A) Neighbor-joining and (B) parsimony trees based on the hypervariable region of the 18S rRNA gene (created with PAUP 4.0 software). *C. felis* was designated an outgroup. Evolutionary distances were determined by the Kimura two-parameter method. GenBank accession numbers of sequences included in the trees are AB089285 (*C. andersoni*), L19068 (*C. baileyi*), AF112575 (*C. felis*), AF112574 (*C. meleagridis*), L19069 (*C. muris* bovine genotype), AB089284 (*C. muris* murine genotype), AF093489 (*C. parvum* human genotype), AF093493 (*C. parvum* bovine genotype), AF112571 (*C. parvum* mouse genotype), AF112572 (*C. parvum* ferret genotype), AF115377 (*C. parvum* pig genotype), AF112576 (*C. parvum* dog genotype), AF112570 (*C. parvum* kangaroo genotype), AF093499 (*C. serpentis*), and U11440 (*C. wrairi*). Bootstrap values greater than 50% are indicated in bold at each respective node.

Figure 3. Phylogenetic analysis of the partial hypervariable region of the 18S rRNA gene to assess the relationships between the geese-derived sequences in the current study and *C. galli* and *C. blagburni* in finches. (A) Neighbor-joining and (B) most parsimonious tree created with PAUP 4.0. *C. felis* was designated an outgroup. Evolutionary distances were determined by the Kimura two-parameter method. GenBank accession numbers of sequences included in the trees are AB089285 (*C. andersoni*), L19068 (*C. baileyi*), AF112575 (*C. felis*), AF112574 (*C. meleagridis*), L19069 (*C. muris* bovine genotype), AB089284 (*C. muris* murine genotype), AF093489 (*C. parvum* human genotype), AF093493 (*C. parvum* bovine genotype), AF112571 (*C. parvum* mouse genotype), AF112572 (*C. parvum* ferret genotype), AF115377 (*C. parvum* pig genotype), AF112576 (*C. parvum* dog genotype), AF112570 (*C. parvum* kangaroo genotype), AF093499 (*C. serpentis*), U11440 (*C. wrairi*), AY168846-8 (*C. galli*), and

AF316623-9 (*C. blagburni*). Bootstrap values greater than 50% are indicated in bold at each respective node.

Figure 1

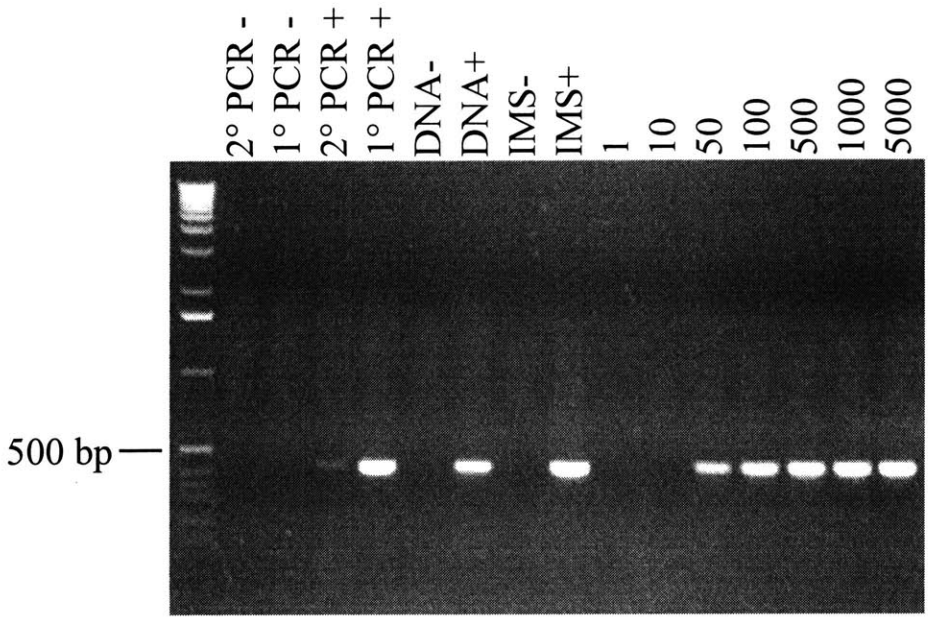

2° PCR -
1° PCR -
2° PCR +
1° PCR +
DNA-
DNA+
IMS-
IMS+
1
10
50
100
500
1000
5000
500 bp

Figure 2

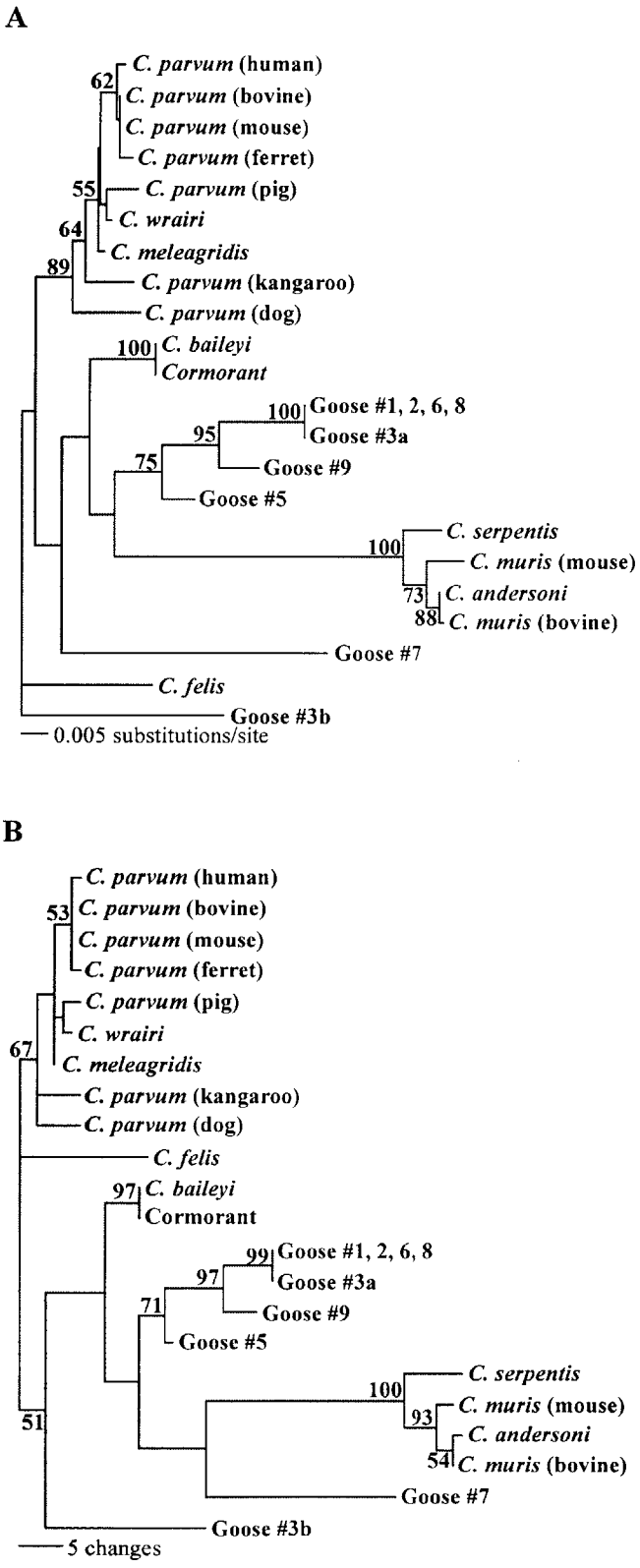
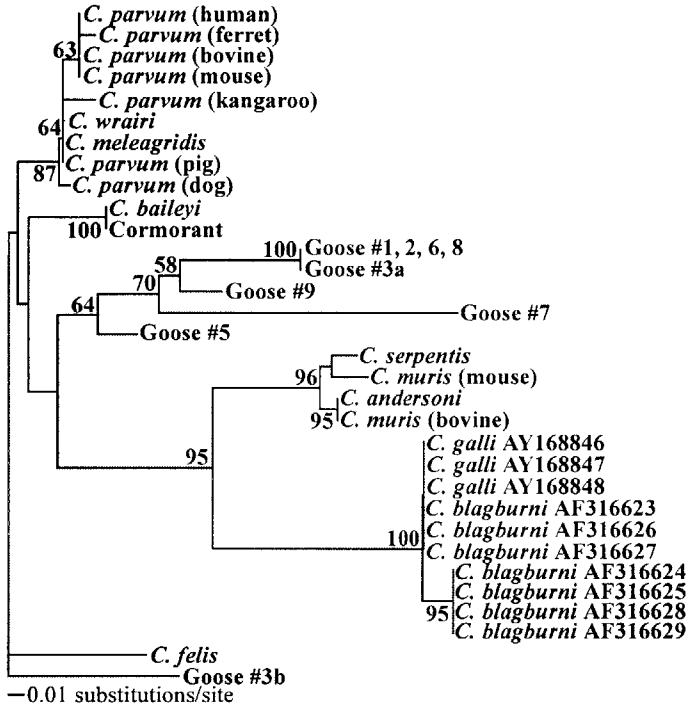
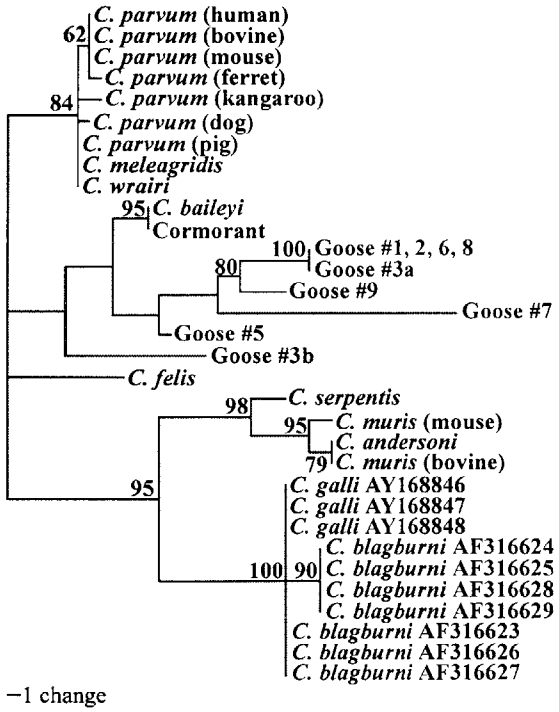

A
C. parvum (human)
C. parvum (bovine)
C. parvum (mouse)
C. parvum (ferret)
C. parvum (pig)
C. wrairi
C. meleagridis
C. parvum (kangaroo)
C. parvum (dog)
C. baileyi
Cormorant
Goose #1, 2, 6, 8
Goose #3a
Goose #9
Goose #5
C. serpentis
C. muris (mouse)
C. andersoni
C. muris (bovine)
Goose #7
C. felis
Goose #3b
0.005 substitutions/site
62
55
64
89
100
100
95
75
100
73
88
B
C. parvum (human)
C. parvum (bovine)
C. parvum (mouse)
C. parvum (ferret)
C. parvum (pig)
C. wrairi
C. meleagridis
C. parvum (kangaroo)
C. parvum (dog)
C. felis
C. baileyi
Cormorant
Goose #1, 2, 6, 8
Goose #3a
Goose #9
Goose #5
C. serpentis
C. muris (mouse)
C. andersoni
C. muris (bovine)
Goose #7
Goose #3b
5 changes
53
67
97
99
97
71
100
93
54
51

Figure 3

A


C. parvum (human)
C. parvum (ferret)
63
C. parvum (bovine)
C. parvum (mouse)
C. parvum (kangaroo)
64
C. wrairi
C. meleagridis
87
C. parvum (pig)
C. parvum (dog)
C. baileyi
100
Cormorant
100
Goose #1, 2, 6, 8
58
Goose #3a
70
Goose #9
Goose #7
64
Goose #5
C. serpentis
96
C. muris (mouse)
C. andersoni
95
C. muris (bovine)
C. galli AY168846
95
C. galli AY168847
C. galli AY168848
C. blagburni AF316623
100
C. blagburni AF316626
C. blagburni AF316627
C. blagburni AF316624
C. blagburni AF316625
95
C. blagburni AF316628
C. blagburni AF316629
C. felis
Goose #3b
0.01 substitutions/site

B


C. parvum (human)
62
C. parvum (bovine)
C. parvum (mouse)
C. parvum (ferret)
84
C. parvum (kangaroo)
C. parvum (dog)
C. parvum (pig)
C. meleagridis
C. wrairi
95
C. baileyi
Cormorant
100
Goose #1, 2, 6, 8
80
Goose #3a
Goose #9
Goose #7
Goose #5
Goose #3b
C. felis
98
C. serpentis
95
C. muris (mouse)
C. andersoni
79
C. muris (bovine)
95
C. galli AY168846
C. galli AY168847
C. galli AY168848
C. blagburni AF316624
100
90
C. blagburni AF316625
C. blagburni AF316628
C. blagburni AF316629
C. blagburni AF316623
C. blagburni AF316626
C. blagburni AF316627
1 change

Chapter 7: Conclusions

Summary

The molecular detection method used in these field studies is both sensitive and specific for *Cryptosporidium* detection in environmental samples. *Cryptosporidium* spp. were detected in a variety of surface waters and animal fecal samples, and detection of *Cryptosporidium* in environmental samples routinely required nested PCR (no DNA was detectable after a single PCR). The lack of DNA signal after a single PCR indicates the need for sensitive detection methods to characterize oocysts in baseline environmental samples, i.e., samples not subject to large quantities of oocysts, such as surface waters during non-storm conditions or animals that are not infected with *Cryptosporidium* but are shedding oocysts that have transiently passed through the body. The molecular method allowed detection of the genus *Cryptosporidium*, as a range of 18S rDNA genotypes similar to those previously characterized from the intestinal, respiratory, and gastric species were recovered.

A comprehensive phylogenetic analysis, based on data in the appendix and including all environmental *Cryptosporidium* 18S rRNA sequences recovered in this work, is shown in Figures 1 and 2. The neighbor-joining (Figure 1) and parsimony (Figure 2) trees show that both known and novel genotypes were detected. The sequence recovered from the cormorant in the Wachusett Reservoir in August 2000 was identical to *C. baileyi*, suggesting conservation of the hypervariable region of the 18S rRNA gene for C. baileyi in different geographic areas. SR #2, a surface water sample collected from Stillwater River in February 2000, grouped with the *C. parvum* clade by both analyses and most likely represents an animal genotype. An additional sequence recovered from Stillwater River in February 2000, SR #1, grouped with the *C. muris* murine genotype and was statistically supported by both neighbor-joining and parsimony analyses. These finding both support the hypothesis that Stillwater River is influenced by wildlife shedding. Sequences recovered from a deer in the Wachusett Reservoir watershed in August 2000 and Gates Brook (GB #2) in November 2001 grouped with the *C. parvum* genotypes, although the grouping was not statistically supported in the parsimony tree. However, the Deer and GB #2 sequences significantly grouped together by both analyses, suggesting that Gates Brook, originally hypothesized to be impacted by septic system failures, may be more heavily influenced by wildlife. Sequences recovered from Quinapoxet River (QR

#2) in July 1999, Brook SF (SF #1) in March 1999, and the Farm JF manure pit (Manure) in June 2000 all grouped with *C. andersoni* and the bovine genotype of *C. muris*. This finding supports the hypothesis that dairy cattle are influencing Brook SF and the dairy farm manure pit but suggests that agricultural influences can extend beyond the immediate vicinity of the farm, as the sampling site on the Quinapoxet River was significantly downstream from the dairy farms and hypothesized to be most significantly impacted by wildlife.

The sequence recovered from a cow on Farm JF in June 2000, in addition to all sequences recovered from Brook JF in 2001, represented novel genotypes. The three sequences recovered from Brook JF in November 2001, JF #5-7, grouped together significantly by both neighbor-joining and parsimony analyses but were genetically distinct from all other previously characterized genotypes. Similarly, sequence JF #4, collected in August 2001, was unique but closely related to a sequence recovered from a goose (Goose #3b) in Illinois in August 2001. JF #4 and Goose #3b grouped together significantly by both neighbor-joining and parsimony analyses but did not group significantly with any other *Cryptosporidium* spp. genotypes. Finally, sequences recovered from Brook JF in June 2001, JF #1 and 2, grouped significantly with a goose sequence (Goose #7) collected in New York in August 2001, but this clade was genetically distinct from all other *Cryptosporidium* spp. genotypes. These findings suggest that birds, in addition to dairy cattle and other farm animals, may play a significant role in *Cryptosporidium* dissemination in agricultural settings.

Additional novel genotypes were identified in geese. Identical, but novel, genotypes were collected from geese in Illinois (Geese # 1 and 2), Massachusetts (Goose #6), and Virginia (Goose #8), suggesting conservation of this variable region 18S rRNA genotype across different geographic locations. A very similar sequence was recovered from another goose in Illinois (Goose #3a), and these five sequences formed a clade with a bootstrap value of 100% by both neighbor-joining and parsimony analyses. The closely related, but distinct, sequence from a goose in Virginia (Goose #9) also grouped significantly with these five geese. An additional sequence from a goose in Illinois (Goose #5) did not cluster significantly with Geese #1, 2, 3a, 6, 8, and 9, but was most closely related to this group. These novel genotypes may represent oocysts characteristic of bird infections, or they may represent *Cryptosporidium* spp. that were

transiently carried and disseminated by these geese. These previously unidentified sequences illustrate extensive parasite diversity and highlight the need for further characterization of *Cryptosporidium* genotypes in the environment.

Analysis of water quality correlations with oocyst contamination of surface waters revealed that the strongest indicator of *Cryptosporidium* spp. presence was season, and that seasonal trends varied with the suspected source of oocyst contamination. The surface water sites susceptible to wildlife impacts, Stillwater and Quinapoxet Rivers, were positive for *Cryptosporidium* during colder months, from late fall through early spring, and never positive during the summer. While too few positive samples at Gates Brook precluded a statistical analysis, Gates Brook samples that were positive for *Cryptosporidium* occurred during the colder months as well. The fact that the seasonal trend of Cryptosporidium detection at Gates Brook agreed with the seasonal trend of wildlife-influenced surface waters, in addition to the recovery of 18S rRNA genotypes from Gates Brook that were similar to a sequence recovered from a deer, supports the conclusion that Gates Brook is more likely impacted by wildlife than humans. In contrast, Brook JF was positive for *Cryptosporidium* spp. during the summer and not during the cold winter months. The seasonal trend of oocysts in Brook JF is in agreement with the seasonal presence of birds in the watershed and supports the hypothesis that birds may be significantly impacting the dissemination of *Cryptosporidium* spp. in this agricultural area.

Conclusions

- The molecular detection method developed in this thesis is sensitive and specific for *Cryptosporidium* spp. in environmental samples.

- Multiple species of *Cryptosporidium* are present in the Wachusett Reservoir watershed, and 18S rRNA genotypes indicative of intestinal, gastric, and respiratory *Cryptosporidium* spp. were identified in water and fecal samples.

- Extensive and previously uncharacterized diversity exists among *Cryptosporidium* spp. oocysts at the 18S rRNA locus.

- *Cryptosporidium* spp. in Stillwater and Quinapoxet Rivers is likely due to wildlife shedding.

- *Cryptosporidium* spp. in Gates Brook is likely due to wildlife shedding, and no evidence of human impacts was seen.

- *Cryptosporidium* spp. in Brook JF is likely due to birds in the watershed, although more extensive characterization of the novel genotypes recovered from Brook JF and geese will be necessary to confirm.

- *Cryptosporidium* spp. contamination of surface waters varies seasonally, and the seasonal pattern of oocyst presence is different for sites impacted by distinct sources.

Future Work

The present environmental study has revealed extensive parasite diversity at the molecular level. Identification of novel genotypes from parasites in environmental samples can provide only speculation about the *Cryptosporidium* species to which that parasite is most-closely related and can not be used to identify public health risks associated with that parasite. Parasite diversity needs to be more fully characterized with respect to the host specificity of *Cryptosporidium* spp. genotypes so that health risks can be more accurately interpreted from environmental studies.

Additional environmental studies, in a variety of watersheds, will be useful to identify common features of *Cryptosporidium* spp. dynamics. Because the quantity of oocysts recovered from environmental samples often precludes a polyphasic taxonomic analysis, multi-locus genetic characterization will be necessary to make more conclusive statements about the species, genotype, sources, and potential health risks of oocysts in the environment.

Environmental studies, while necessary for understanding the natural dynamics of *Cryptosporidium* spp. oocysts, are subject to many unknown and uncontrolled variables that can confound interpretation of data. Thus, environmental studies should be coupled with controlled

microcosm experiments in which the effects of limited variables or conditions on oocyst fate and transport can be analyzed. For example, in this study, the water quality conditions that correlated significantly with *Cryptosporidium* spp. contamination of agriculture-influenced surface waters included high dissolved oxygen content, high pH (high relative to the pH of negative samples, but still within the neutral range), and low specific conductivity. However, when data from wildlife-influenced sites were considered alone, or when all sites (regardless of source) were considered together, these correlations were not seen. Further work needs to be done to assess the importance of dissolved oxygen, pH, and specific conductivity on oocyst fate in the environment. Are these factors only important under certain land-use conditions? Do oocysts have a dissolved oxygen requirement for survival? Is the dissolved oxygen requirement of oocysts the same for all species and genotypes? Do pH and specific conductivity impact oocyst settling in the water column by affecting oocyst hydrophobicity? These and other questions that arise from environmental findings can be most effectively addressed in a controlled environment.

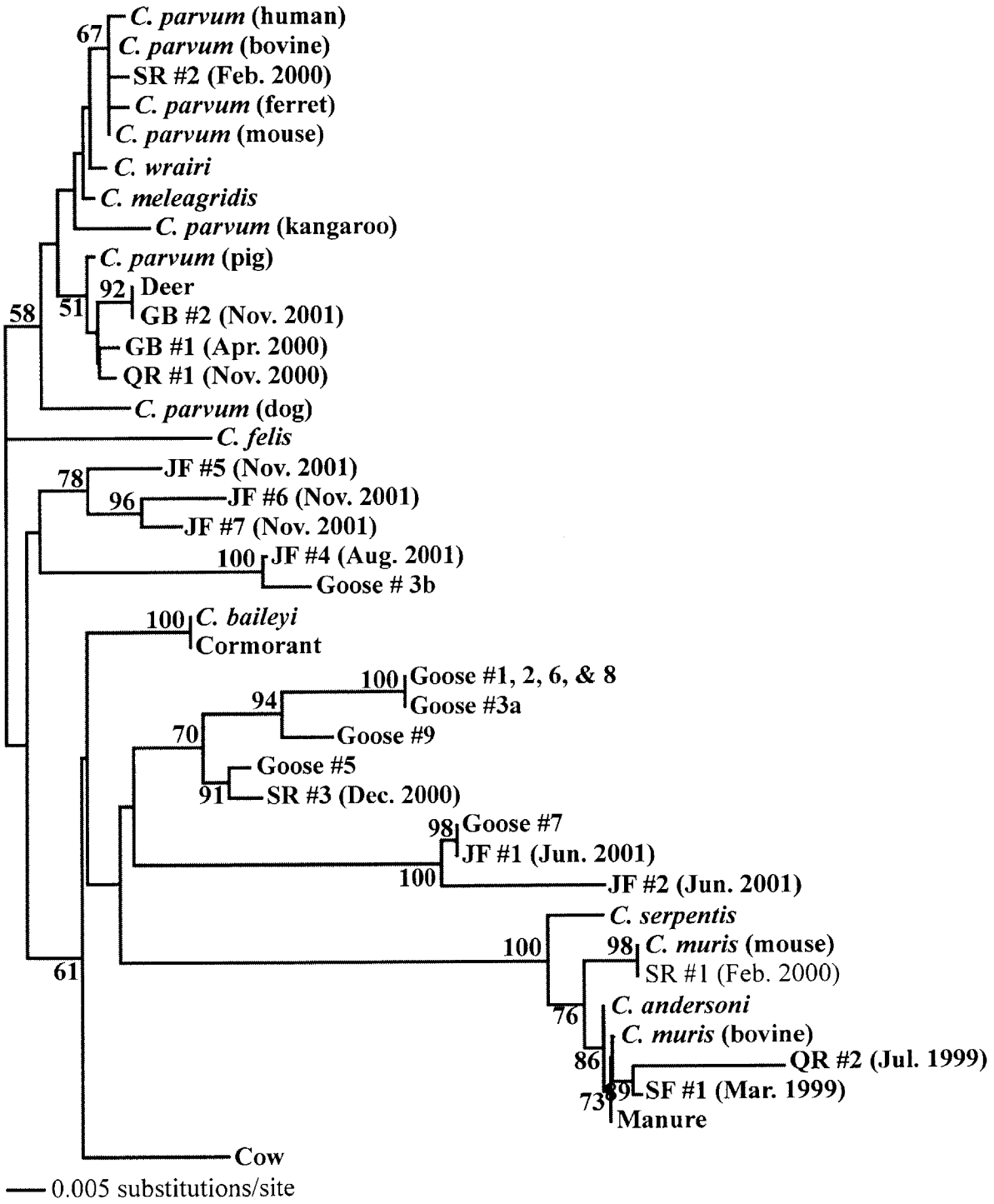
Ultimately, observations from environmental studies, in combination with quantitative data from controlled microcosm studies, will aid in the development of a transport model for oocysts in the environment. A more thorough understanding of oocyst transport will be a major step towards the development of watershed management strategies to minimize public exposure to waterborne *Cryptosporidium* spp. oocysts.

Figure Captions

Figure 1. Neighbor-joining tree based on the hypervariable region of the 18S rRNA gene (created with PAUP 4.0). *C. felis* was designated an outgroup. Evolutionary distances were determined by the Kimura two-parameter method. GenBank accession numbers of sequences included in the trees are AB089285 (*C. andersoni*), L19068 (*C. baileyi*), AF112575 (*C. felis*), AF112574 (*C. meleagridis*), L19069 (*C. muris* bovine genotype), AB089284 (*C. muris* murine genotype), AF093489 (*C. parvum* human genotype), AF093493 (*C. parvum* bovine genotype), AF112571 (*C. parvum* mouse genotype), AF112572 (*C. parvum* ferret genotype), AF115377 (*C. parvum* pig genotype), AF112576 (*C. parvum* dog genotype), AF112570 (*C. parvum* kangaroo genotype), AF093499 (*C. serpentis*), and U11440 (*C. wrairi*). Bootstrap values greater than 50% are indicated in bold at each respective node.

Figure 2. Parsimony tree based on the hypervariable region of the 18S rRNA gene (created with PAUP 4.0). *C. felis* was designated an outgroup. Evolutionary distances were determined by the Kimura two-parameter method. GenBank accession numbers of sequences included in the trees are AB089285 (*C. andersoni*), L19068 (*C. baileyi*), AF112575 (*C. felis*), AF112574 (*C. meleagridis*), L19069 (*C. muris* bovine genotype), AB089284 (*C. muris* murine genotype), AF093489 (*C. parvum* human genotype), AF093493 (*C. parvum* bovine genotype), AF112571 (*C. parvum* mouse genotype), AF112572 (*C. parvum* ferret genotype), AF115377 (*C. parvum* pig genotype), AF112576 (*C. parvum* dog genotype), AF112570 (*C. parvum* kangaroo genotype), AF093499 (*C. serpentis*), and U11440 (*C. wrairi*). Bootstrap values greater than 50% are indicated in bold at each respective node.

Figure 1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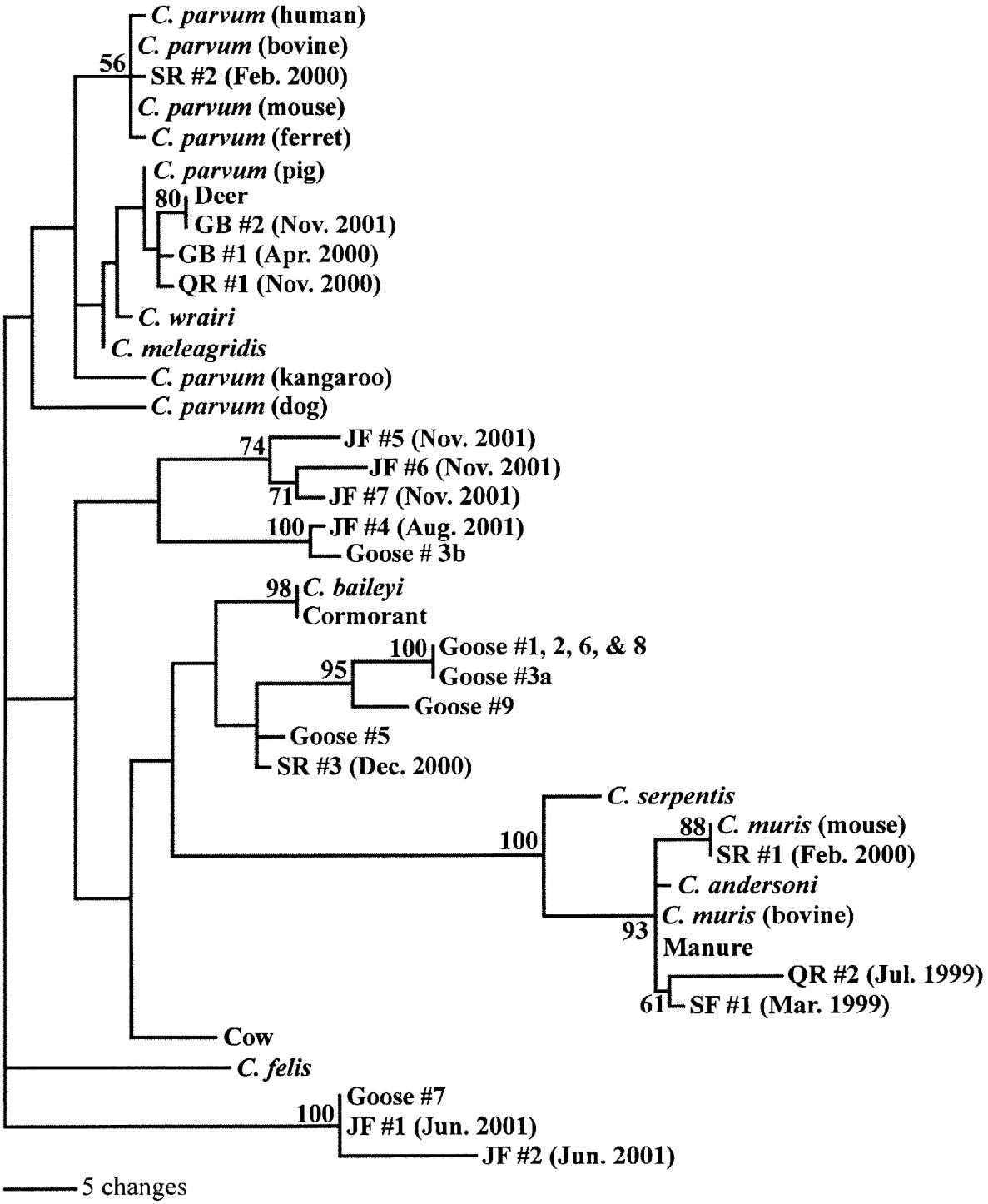
C. meleagridis
C. parvum (kangaroo)
C. parvum (pig)
Deer
GB #2 (Nov. 2001)
GB #1 (Apr. 2000)
QR #1 (Nov. 2000)
C. parvum (dog)
C. felis
JF #5 (Nov. 2001)
JF #6 (Nov. 2001)
JF #7 (Nov. 2001)
JF #4 (Aug. 2001)
Goose # 3b
C. baileyi
Cormorant
Goose #1, 2, 6, & 8
Goose #3a
Goose #9
Goose #5
SR #3 (Dec. 2000)
Goose #7
JF #1 (Jun. 2001)
JF #2 (Jun. 2001)
C. serpentis
C. muris (mouse)
SR #1 (Feb. 2000)
C. andersoni
C. muris (bovine)
QR #2 (Jul. 1999)
SF #1 (Mar. 1999)
Manure
Cow
0.005 substitutions/site

Figure 2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C. parvum (mouse)
C. parvum (ferret)
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Deer
GB #2 (Nov. 2001)
GB #1 (Apr. 2000)
QR #1 (Nov. 2000)
C. wrairi
C. meleagridis
C. parvum (kangaroo)
C. parvum (dog)
74
JF #5 (Nov. 2001)
JF #6 (Nov. 2001)
71
JF #7 (Nov. 2001)
100
JF #4 (Aug. 2001)
Goose # 3b
98
C. baileyi
Cormorant
100
Goose #1, 2, 6, & 8
95
Goose #3a
Goose #9
Goose #5
SR #3 (Dec. 2000)
C. serpentis
88
C. muris (mouse)
100
SR #1 (Feb. 2000)
C. andersoni
C. muris (bovine)
93
Manure
QR #2 (Jul. 1999)
61
SF #1 (Mar. 1999)
Cow
C. felis
Goose #7
100
JF #1 (Jun. 2001)
JF #2 (Jun. 2001)
5 changes

Appendix

DNA sequences recovered from the field studies were manually aligned with GenBank sequences by consideration of secondary structure of the 18S rRNA. The secondary structure of *Plasmodium vivax* U02079 was used as a model (Figure 1), since *Plasmodium* is closely related to *Cryptosporidium* and the secondary structure for *Cryptosporidium* has not yet been determined. Two regions, helices 1 and 2 near the 5' end of the nested PCR product, contained the vast majority of genetic diversity among isolates of *Cryptosporidium* and were the regions in which secondary structure was necessary to create strong alignments. The proposed secondary structures of helices 1 and 2 are shown in Figures 2 and 3, respectively. The nucleotide in a particular position of one structure was aligned with the nucleotides in that same position in other structures. Nucleotides that bulged out of the structure were inserted into the alignment, and if that bulging nucleotide was missing in another structure, a gap was inserted. The DNA sequence alignment, based on the secondary structures proposed in Figures 2 and 3, is shown in Figure 4. Phylogenetic trees in chapters 4, 6, and 7 are based on the sequence alignment shown in Figure 4.

Figure Captions

Figure 1. Secondary structure model of *Plasmodium vivax* (GenBank accession number U03079). Adapted from the European Ribosomal RNA Database (http://rrna.uia.ac.be). Nucleotide sequence of *P. vivax* is compared to that of *Cryptosporidium parvum* (Genbank accession number AF093489) and indicated with color. Red = identical nucleotides. Green = different nucleotides. Blue = nucleotides not present in *C. parvum*. Positions of the 5' and 3' ends of the nested PCR product analyzed in this study are indicated. Locations of the variable helices 1 and 2 are indicated as well.

Figure 2. Proposed secondary structures of helix 1 of the nested PCR products for all *Cryptosporidum* spp. isolated analyzed in this study. Structures are based on that of *Plasmodium vivax* U03079. Nucleotides in **bold** were included in the phylogenetic analyis; nucleotides in *italics* are part of the variable-length loop region and were masked out of the alignments.

Figure 3. Proposed secondary structures of helix 2 of the nested PCR products for all *Cryptosporidum* spp. isolated analyzed in this study. Structures are based on that of *Plasmodium vivax* U03079. Nucleotides in **bold** were included in the phylogenetic analyis; nucleotides in *italics* are part of the variable-length loop region and were masked out of the alignments.

Figure 4. DNA sequence alignment of the hypervariable region of *Cryptosporidium* spp. 18S rRNA (region shown in Figure 1), including both GenBank and field sequences. Sequences were manually aligned, based on the proposed secondary structures shown in Figures 2 and 3, using the GCG sequence editor. The starting, ending, and variable regions of helices 1 and 2 are indicated. Nucleotide positions shaded in gray were masked out of the phylogenetic analyses. GenBank accession numbers are AB089285 (*C. andersoni*), L19068 (*C. baileyi*), AF316623 (*C. blagburni* 1), AF316624 (*C. blagburni* 2), AF316625 (*C. blagburni* 3), AF316626 (*C. blagburni* 4), AF316627 (*C. blagburni* 5), AF316628 (*C. blagburni* 6), AF316629 (*C. blagburni* 7), AF112575 (*C. felis*), AY168846 (*C. galli* 1), AY168847 (*C. galli* 2), AY168848 (*C. galli* 3), AF112574 (*C. meleagridis*), L19069 (*C. muris* bovine genotype), AB089284 (*C. muris* mouse genotype), AF093493 (*C. parvum* bovine genotype), AF112576 (*C. parvum* dog genotype),

AF112572 (*C. parvum* ferret genotype), AF093489 (*C. parvum* human genotype), AF112570 (*C. parvum* kangaroo genotype), AF112571 (*C. parvum* mouse genotype), AF115377 (*C. parvum* pig genotype), AF093499 (*C. serpentis*), and U11440 (*C. wrairi*). GB = Gates Brook. JF = Brook JF. QR = Quinapoxet River. SF = Brook SF. SR = Stillwater River.

Figure 1

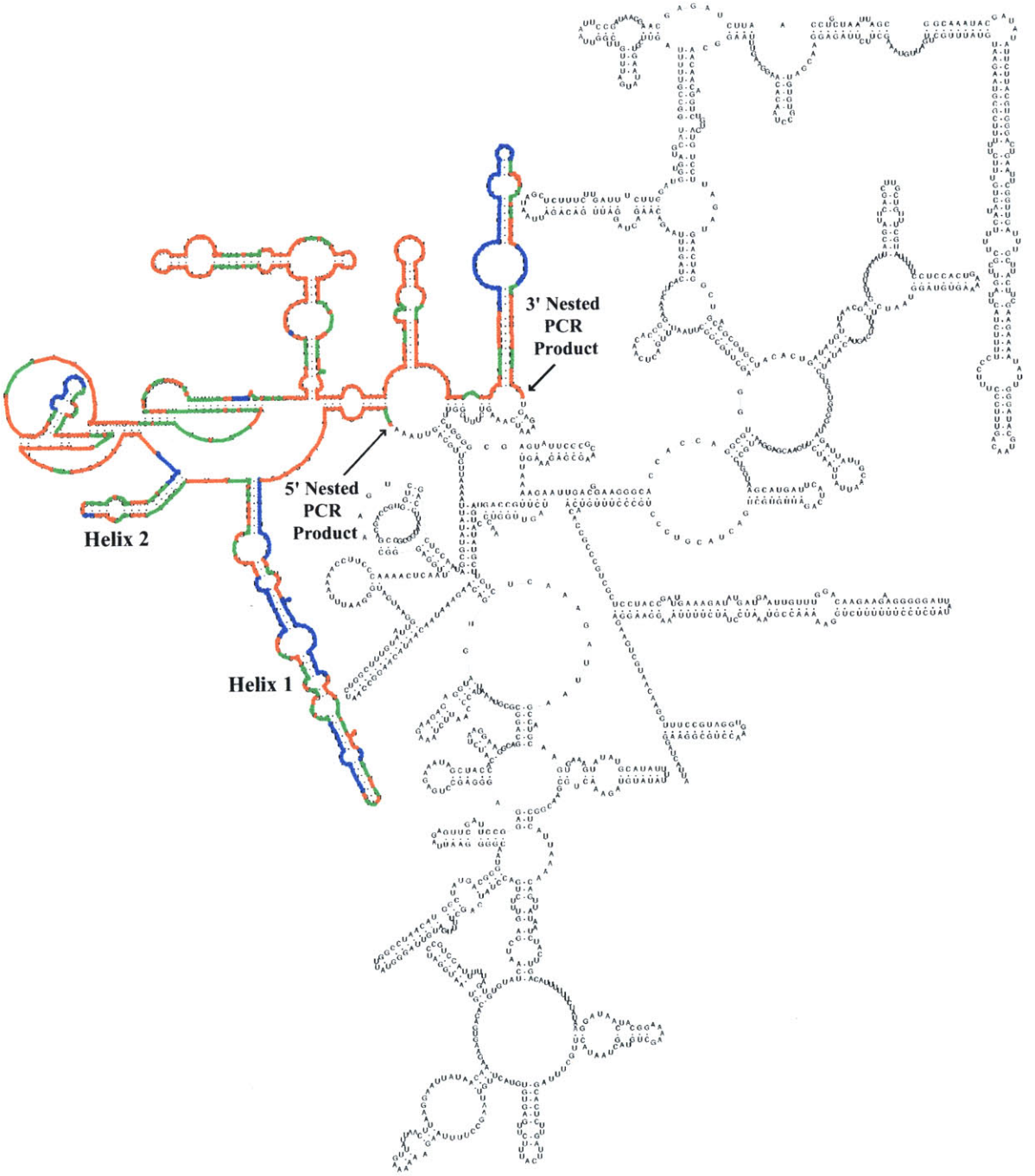
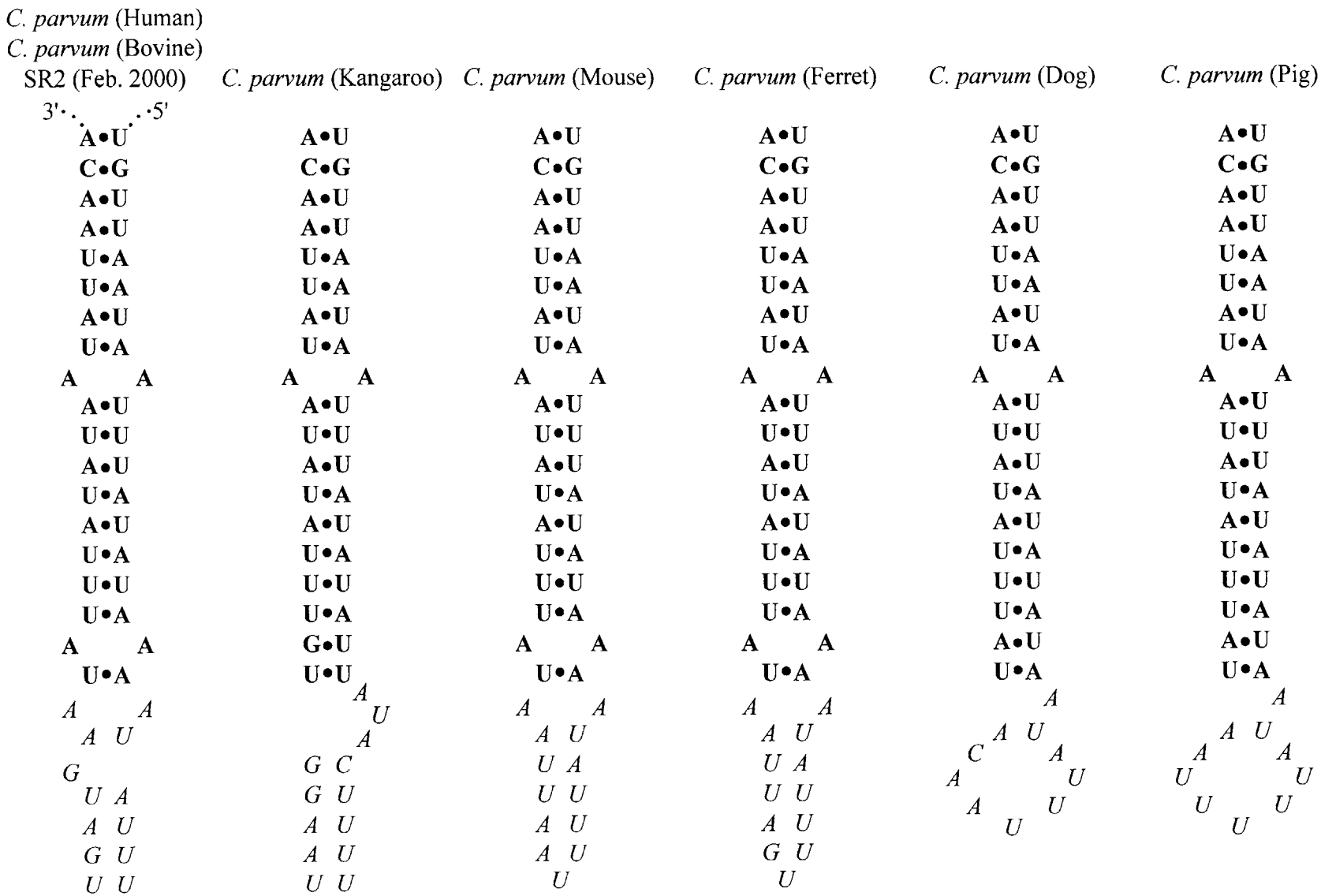


Figure 2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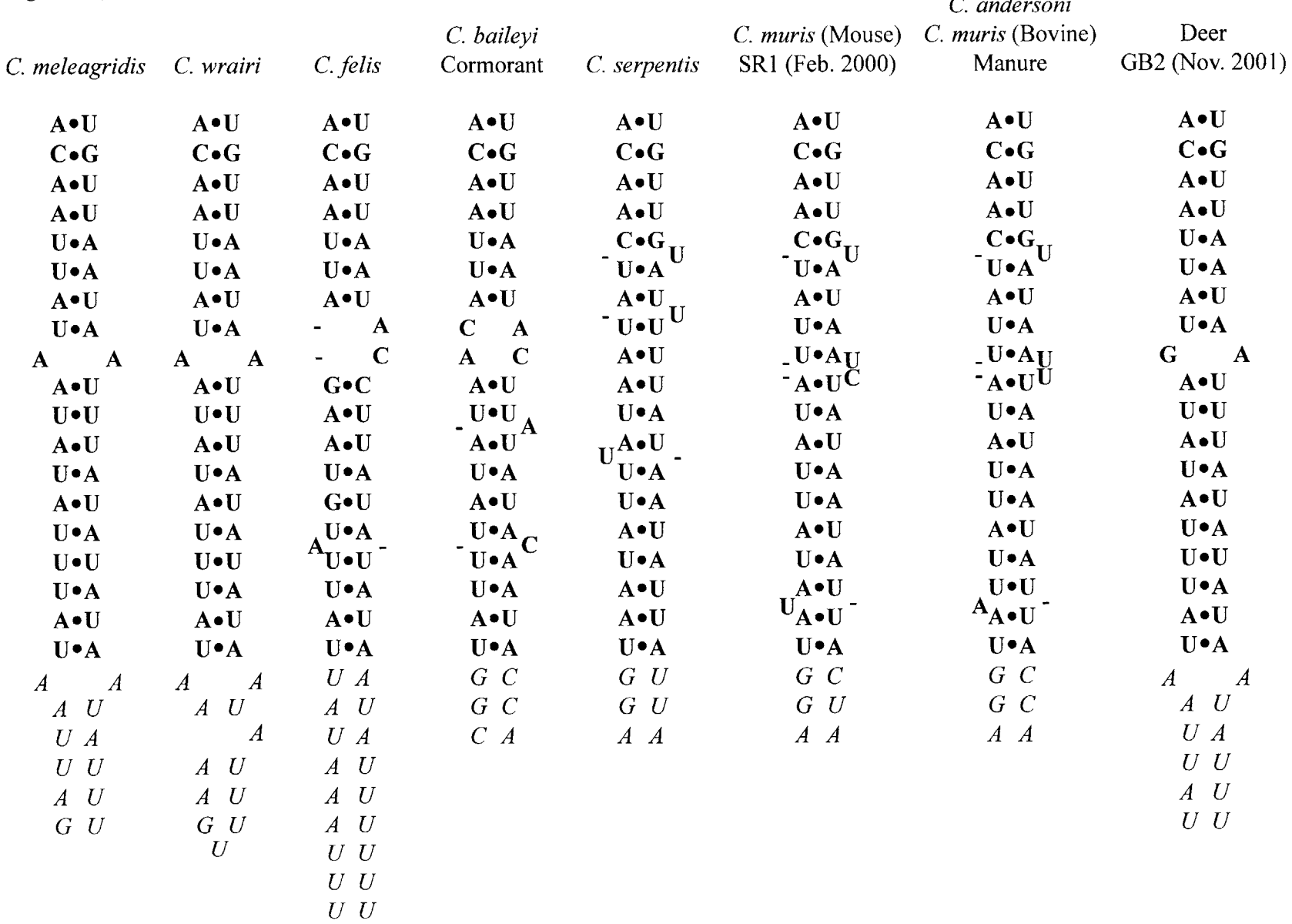
C. parvum (Bovine)
SR2 (Feb. 2000)
C. parvum (Kangaroo)
C. parvum (Mouse)
C. parvum (Ferret)
C. parvum (Dog)
C. parvum (Pig)
3'
5'

Figure 2 (continued)



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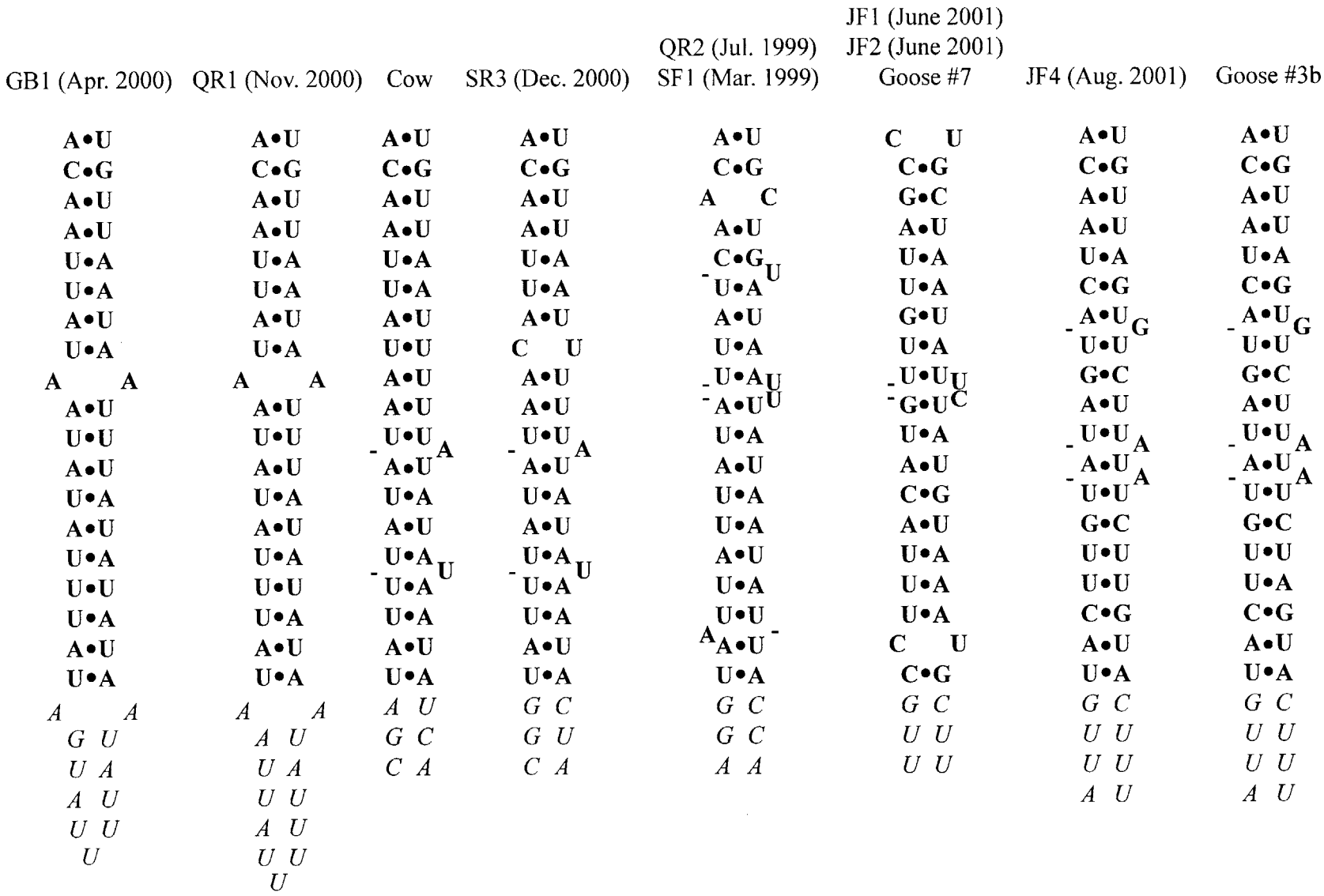

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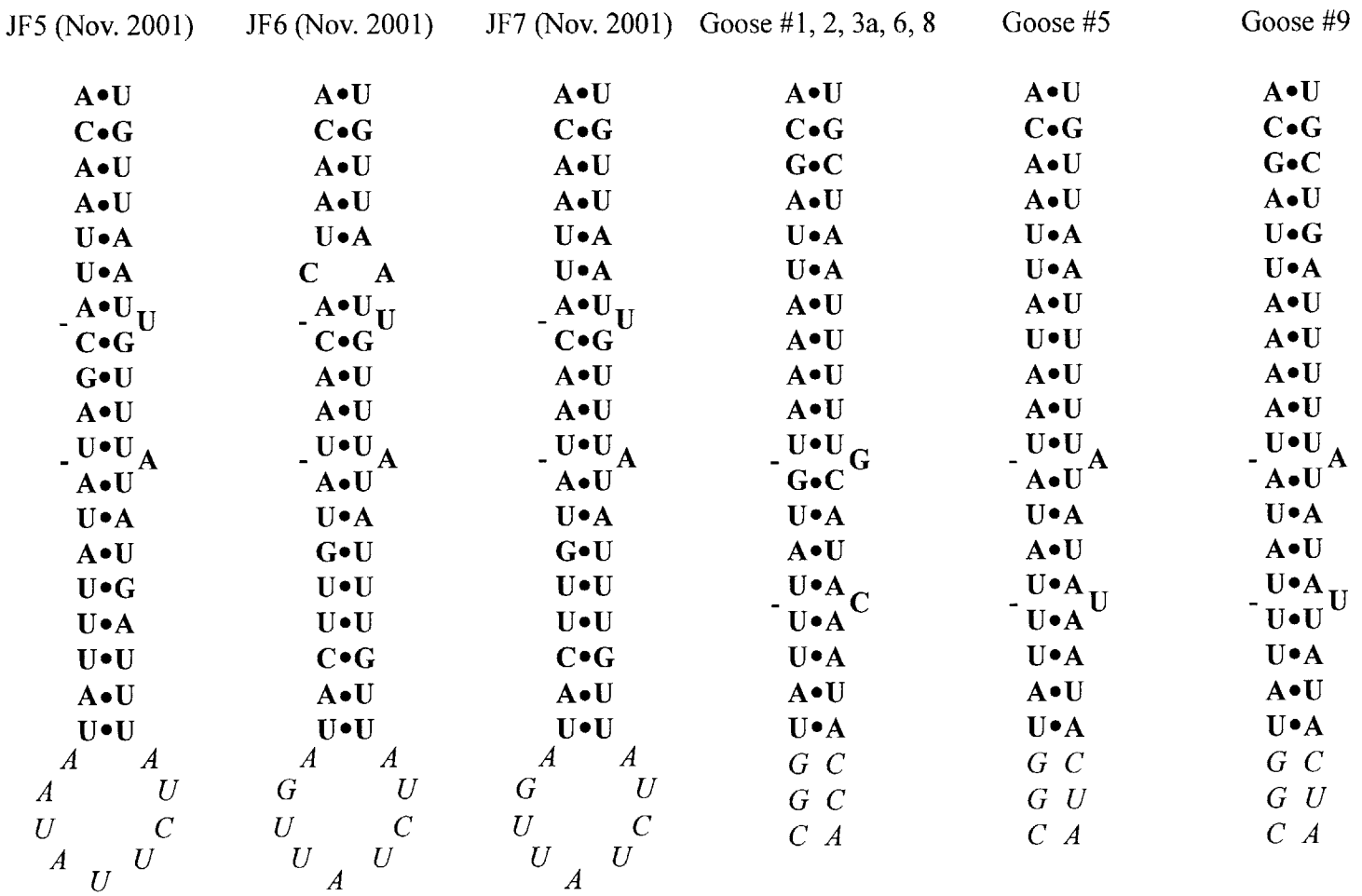
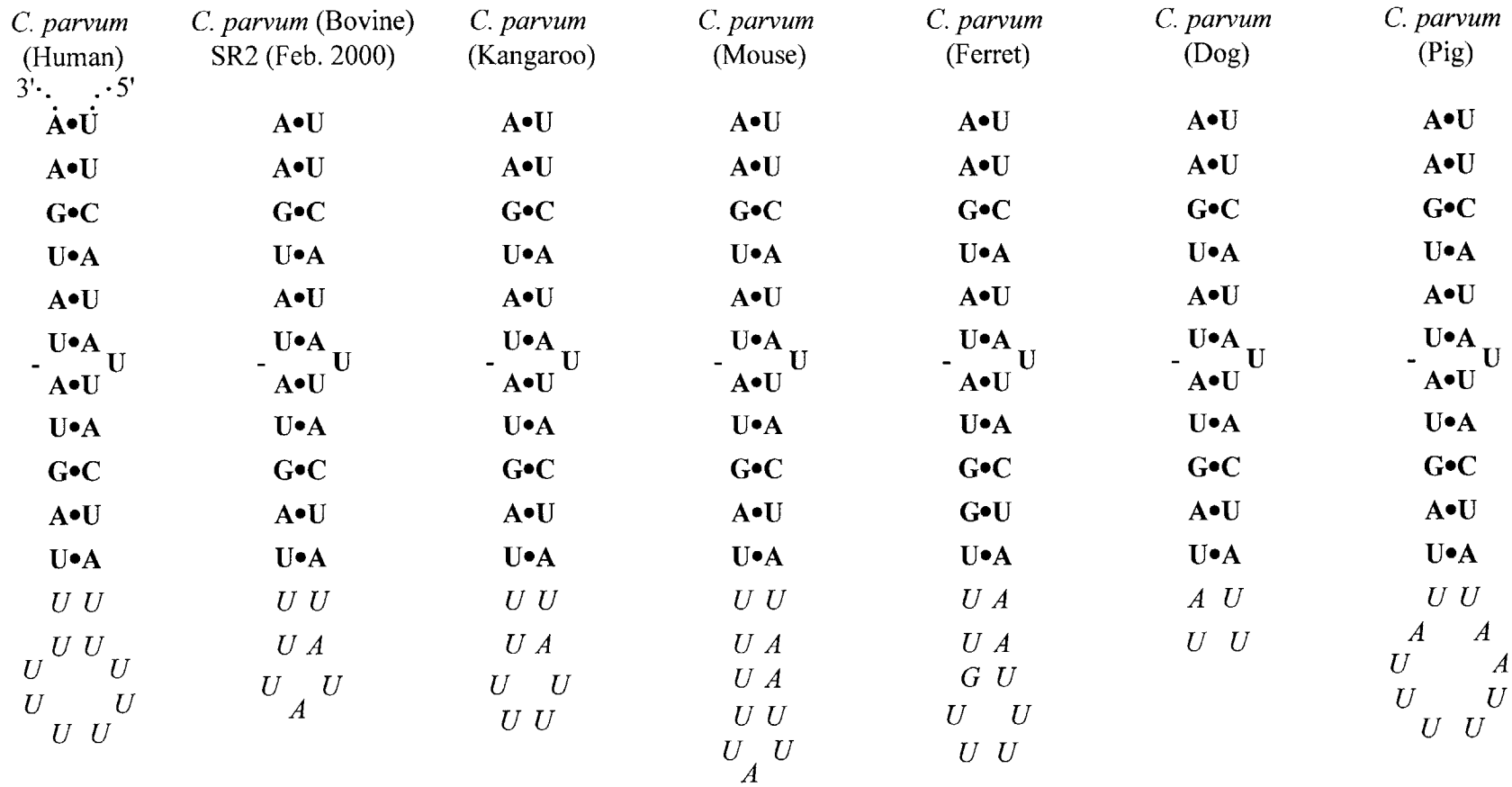


Figure 3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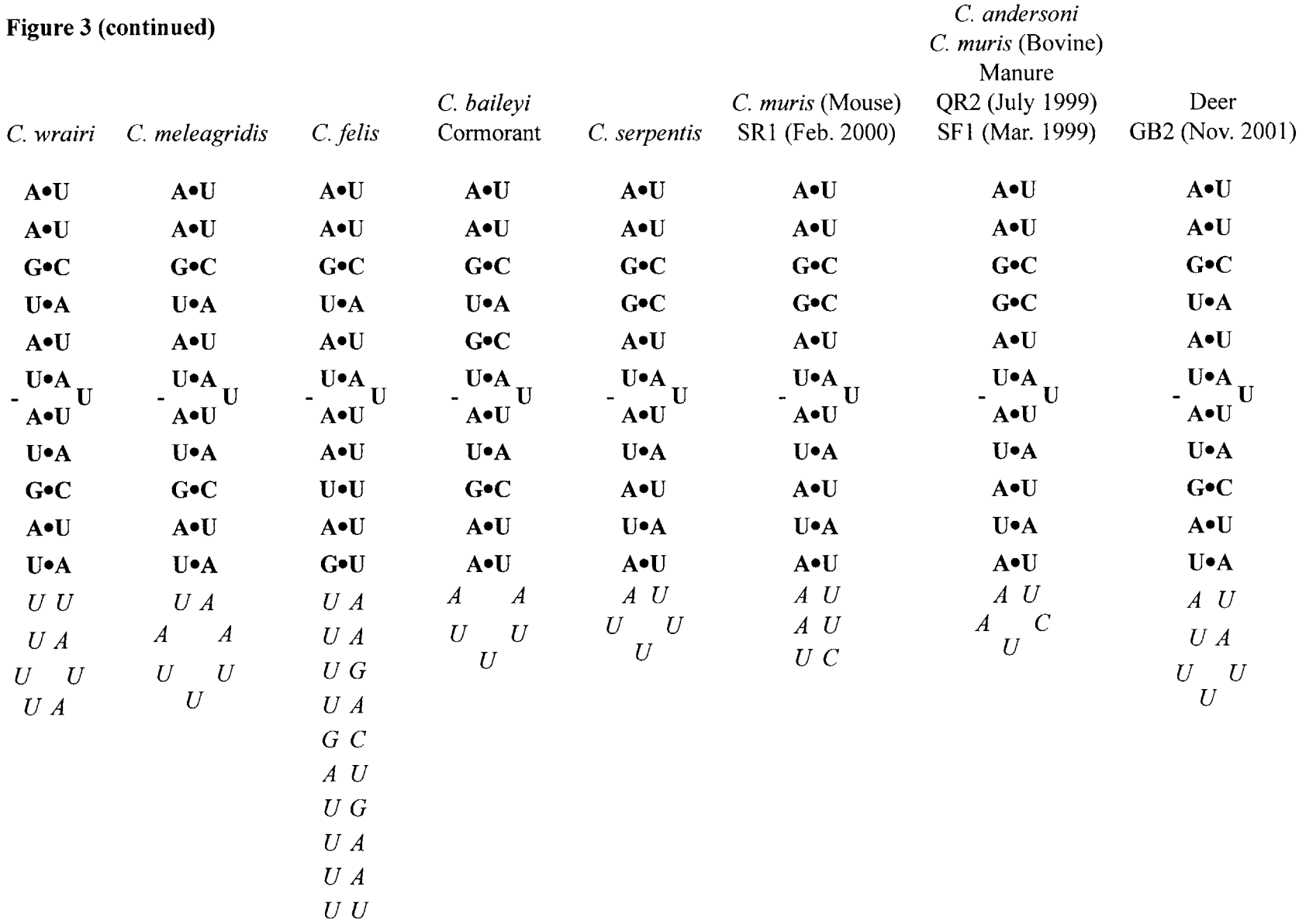
3'
5'
C. parvum (Bovine) SR2 (Feb. 2000)
C. parvum (Kangaroo)
C. parvum (Mouse)
C. parvum (Ferret)
C. parvum (Dog)
C. parvum (Pig)

Figure 3 (continued)



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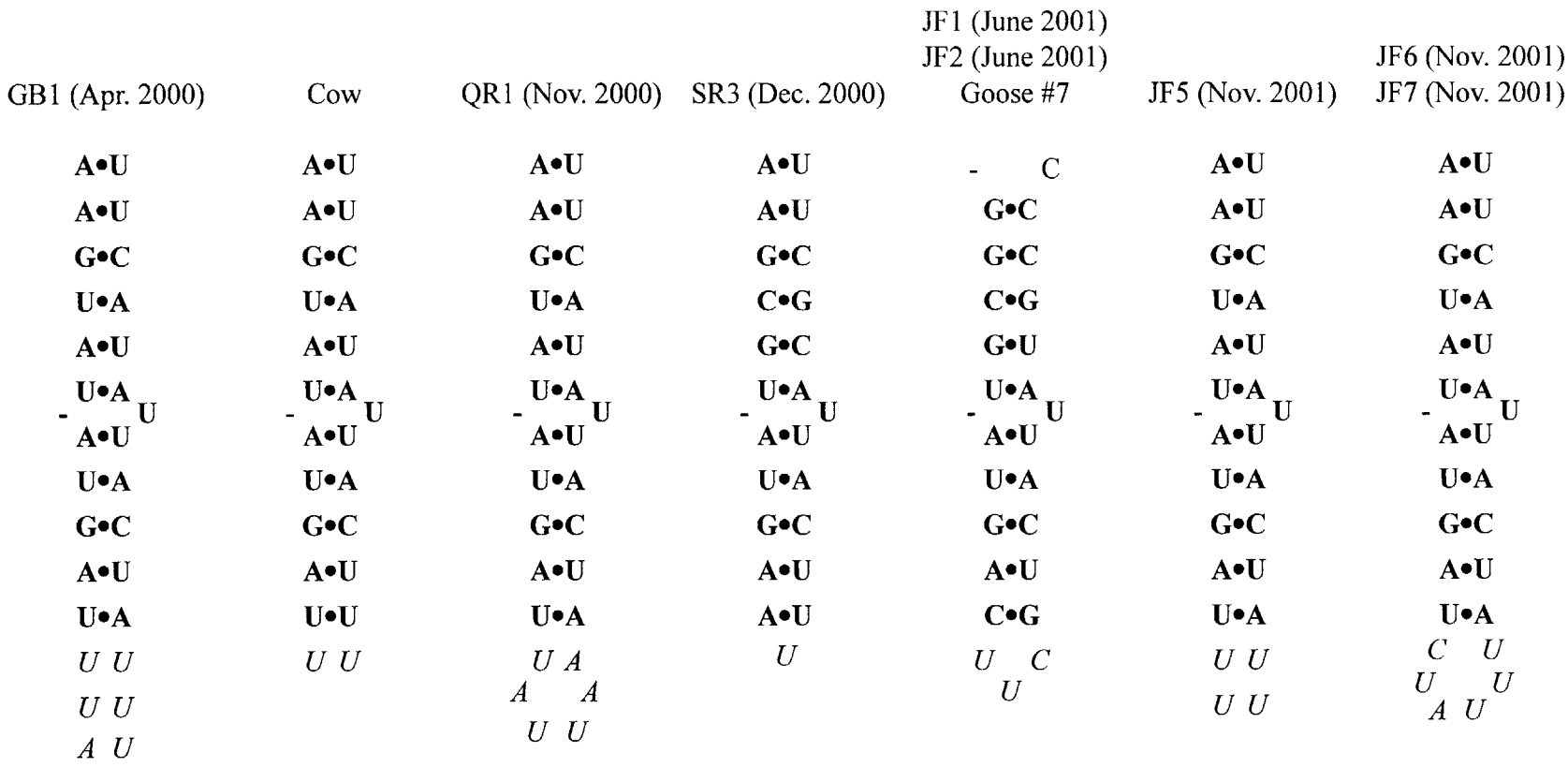

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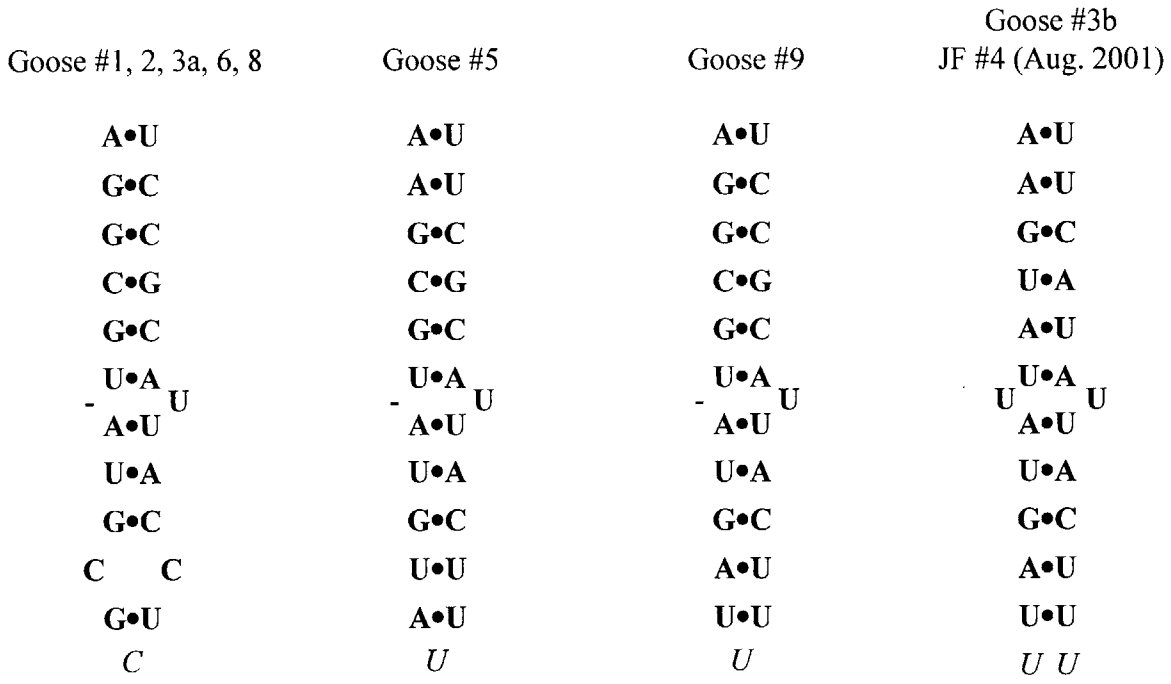
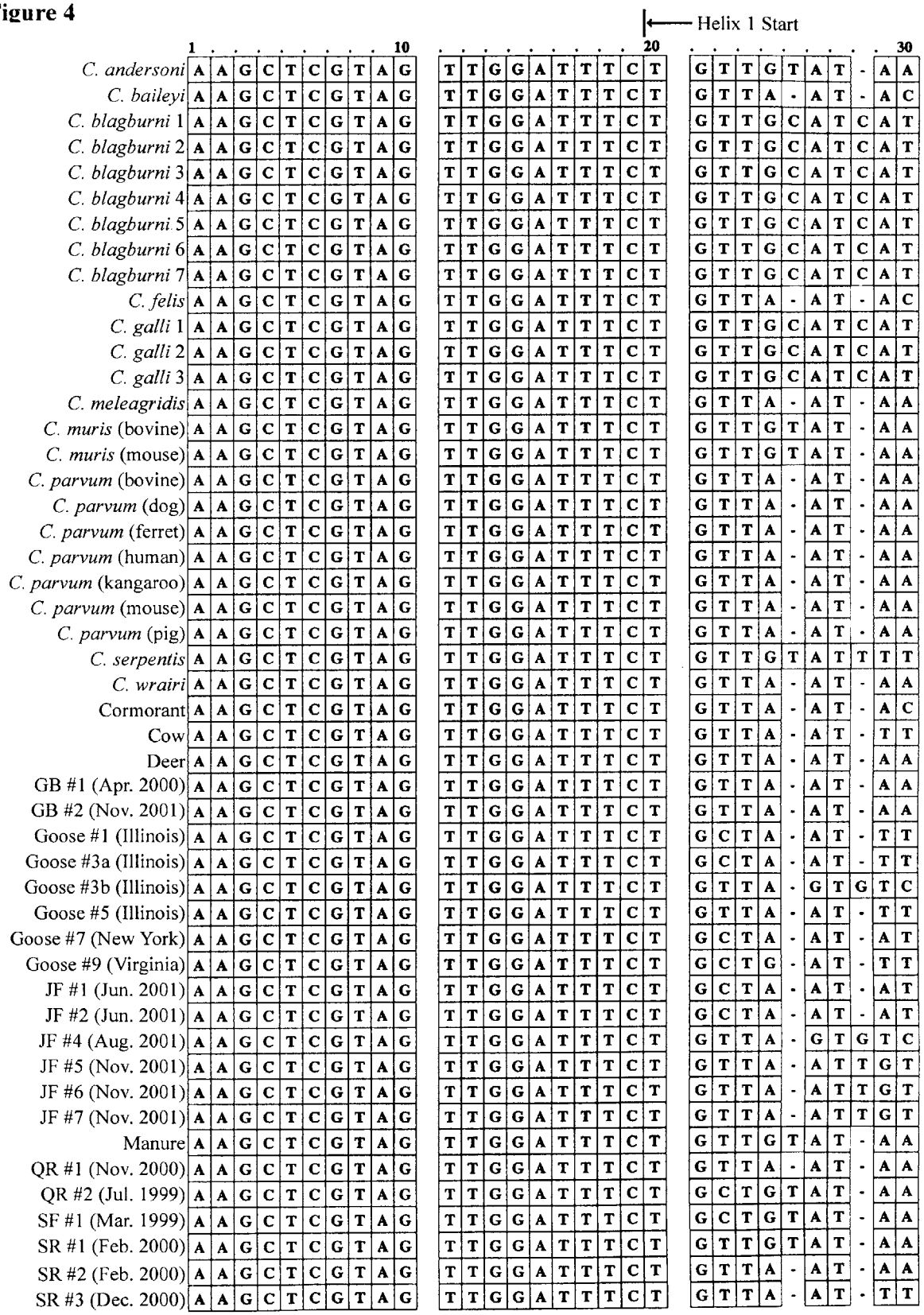


Figure 4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C. blagburni 1 A A G C T C G T A G T T G G A T T T C T G T T G C A T C A T
C. blagburni 2 A A G C T C G T A G T T G G A T T T C T G T T G C A T C A T
C. blagburni 3 A A G C T C G T A G T T G G A T T T C T G T T G C A T C A T
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C. blagburni 6 A A G C T C G T A G T T G G A T T T C T G T T G C A T C A T
C. blagburni 7 A A G C T C G T A G T T G G A T T T C T G T T G C A T C A T
C. felis A A G C T C G T A G T T G G A T T T C T G T T A - A T - A C
C. galli 1 A A G C T C G T A G T T G G A T T T C T G T T G C A T C A T
C. galli 2 A A G C T C G T A G T T G G A T T T C T G T T G C A T C A T
C. galli 3 A A G C T C G T A G T T G G A T T T C T G T T G C A T C A T
C. meleagridis A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
C. muris (bovine) A A G C T C G T A G T T G G A T T T C T G T T G T A T - A A
C. muris (mouse) A A G C T C G T A G T T G G A T T T C T G T T G T A T - A A
C. parvum (bovine) A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
C. parvum (dog) A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
C. parvum (ferret) A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
C. parvum (human) A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
C. parvum (kangaroo) A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
C. parvum (mouse) A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
C. parvum (pig) A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
C. serpentis A A G C T C G T A G T T G G A T T T C T G T T G T A T T T T
C. wrairi A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
Cormorant A A G C T C G T A G T T G G A T T T C T G T T A - A T - A C
Cow A A G C T C G T A G T T G G A T T T C T G T T A - A T - T T
Deer A A G C T C G T A G T T G G A T T T C T G T T A - A T - A A
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Manure A A G C T C G T A G T T G G A T T T C T G T T G T A T - A A
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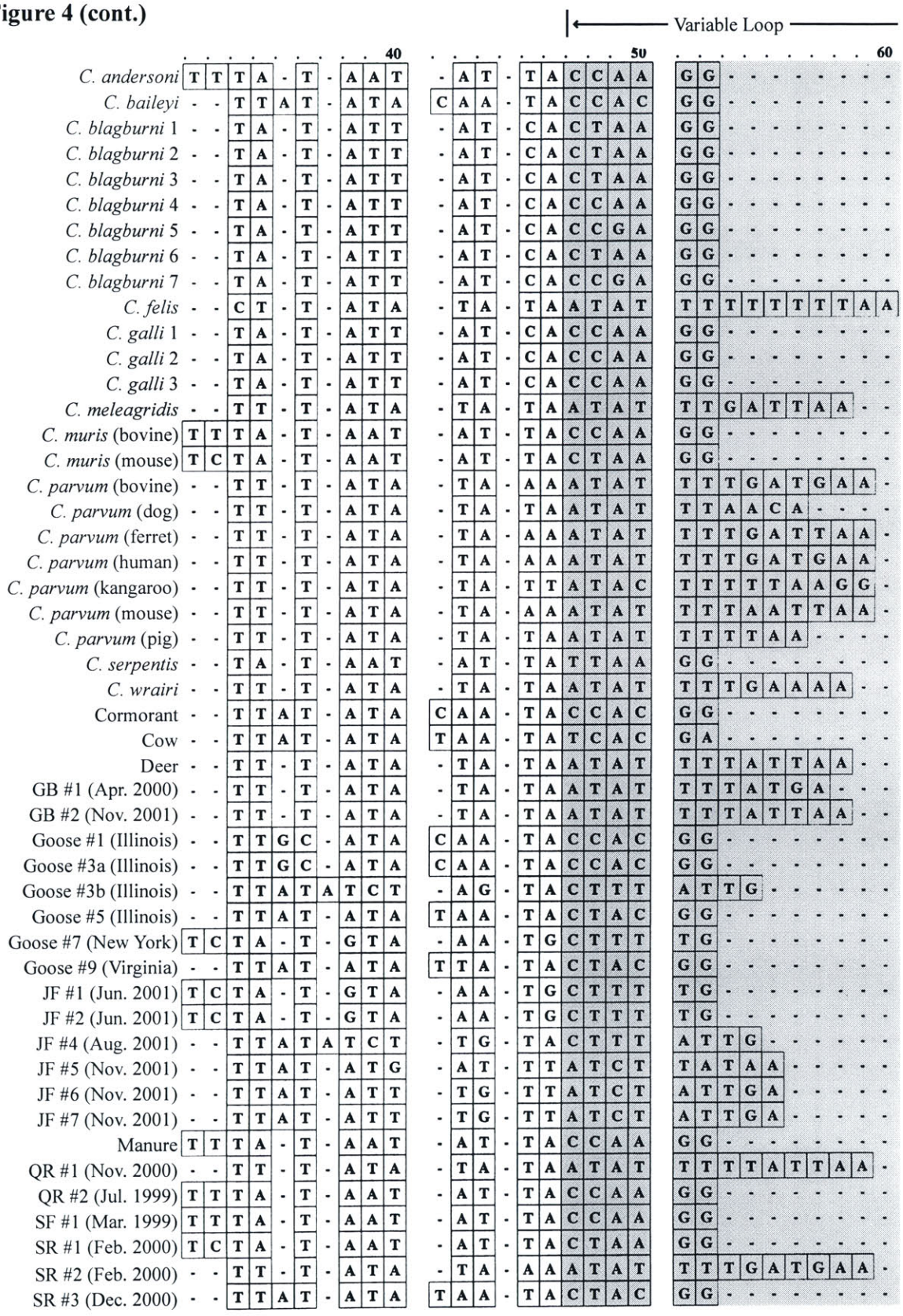

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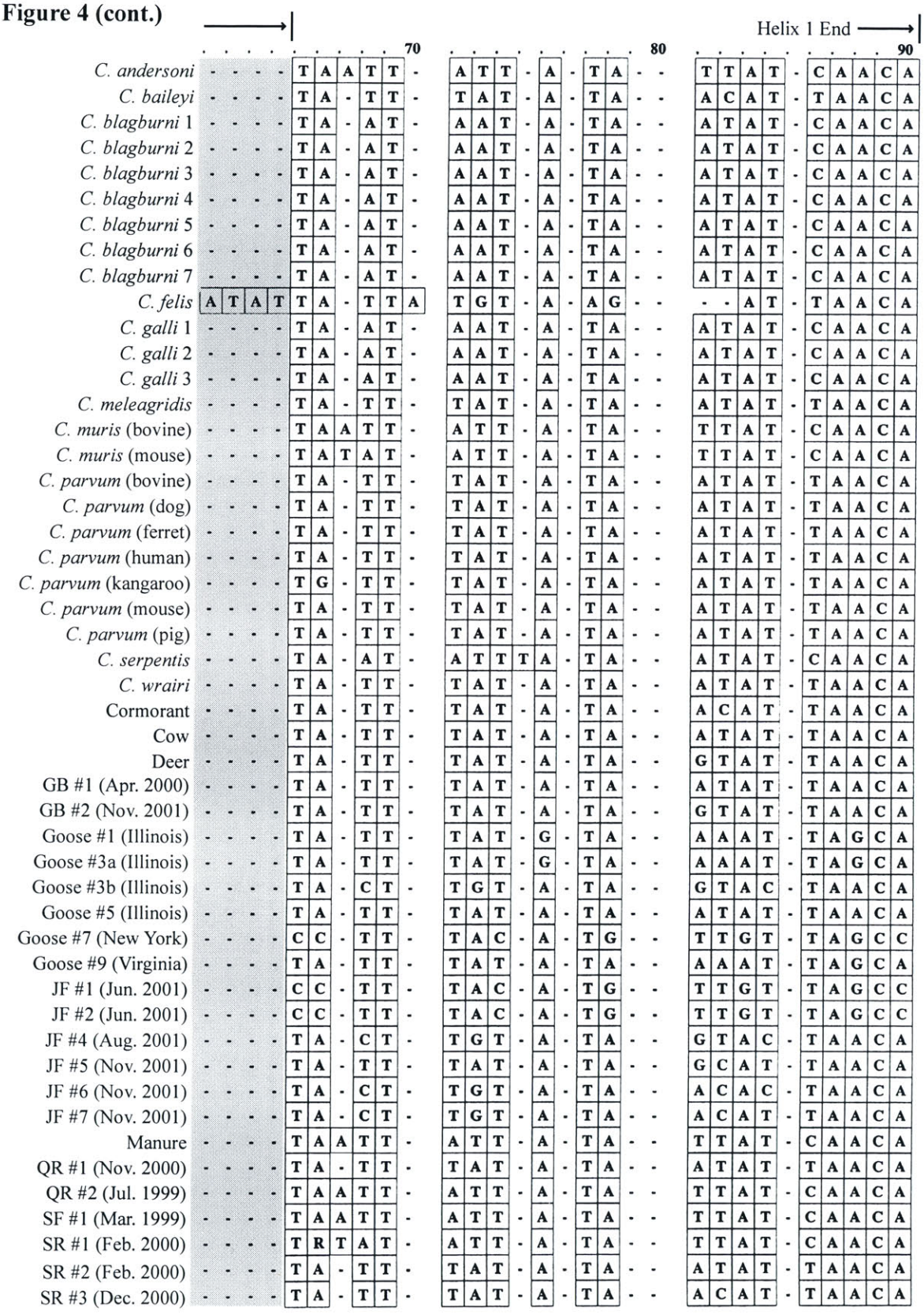

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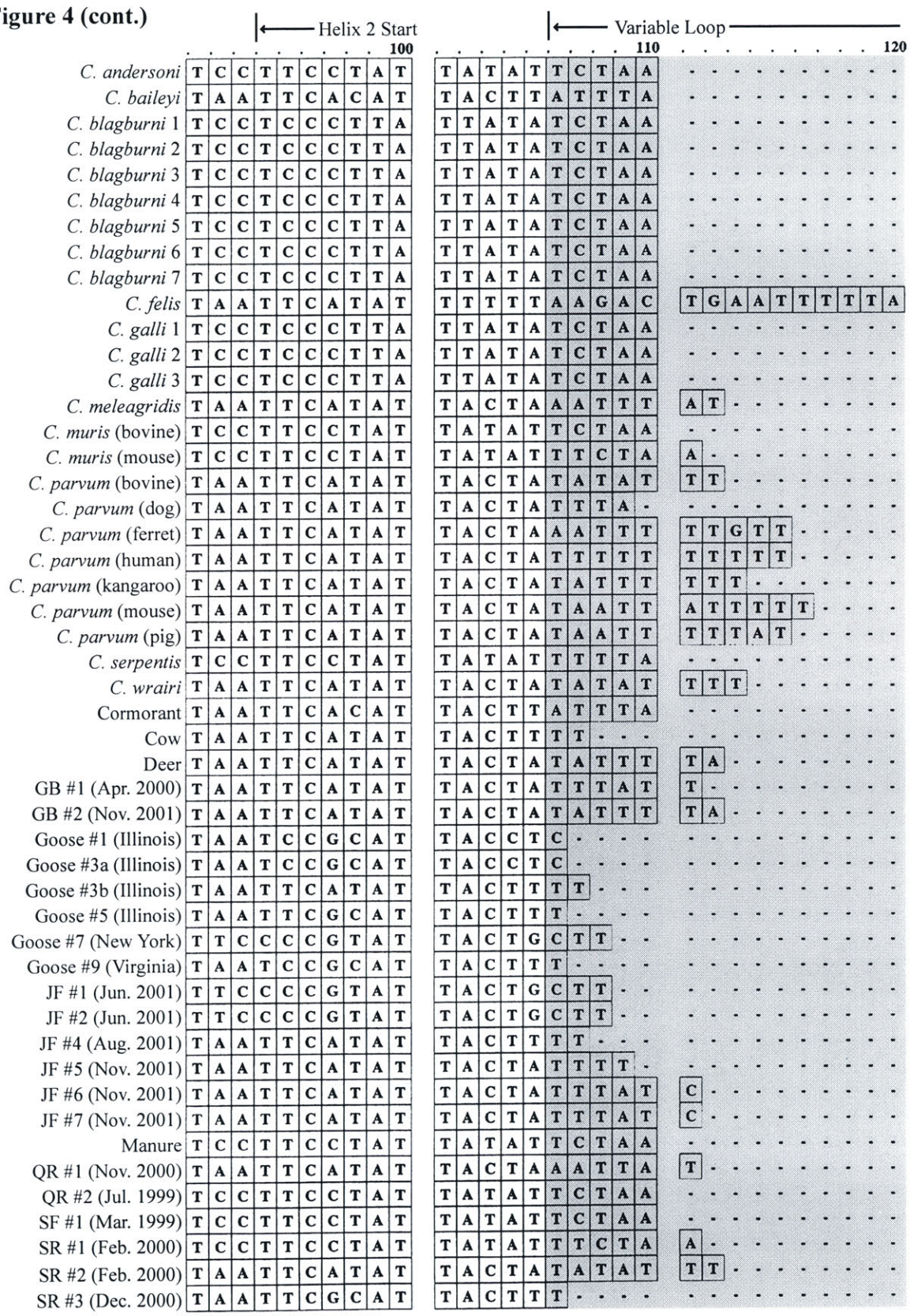

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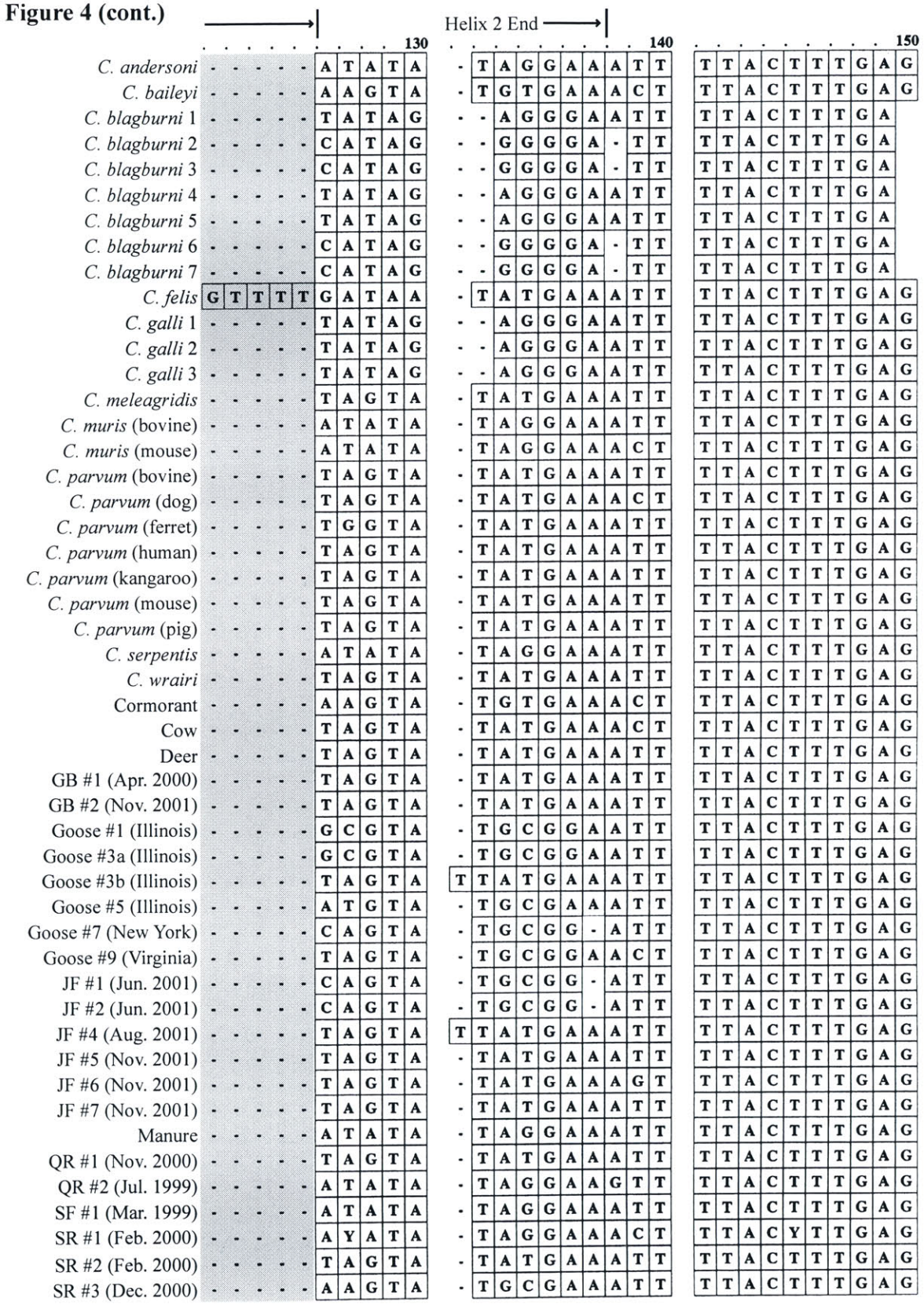

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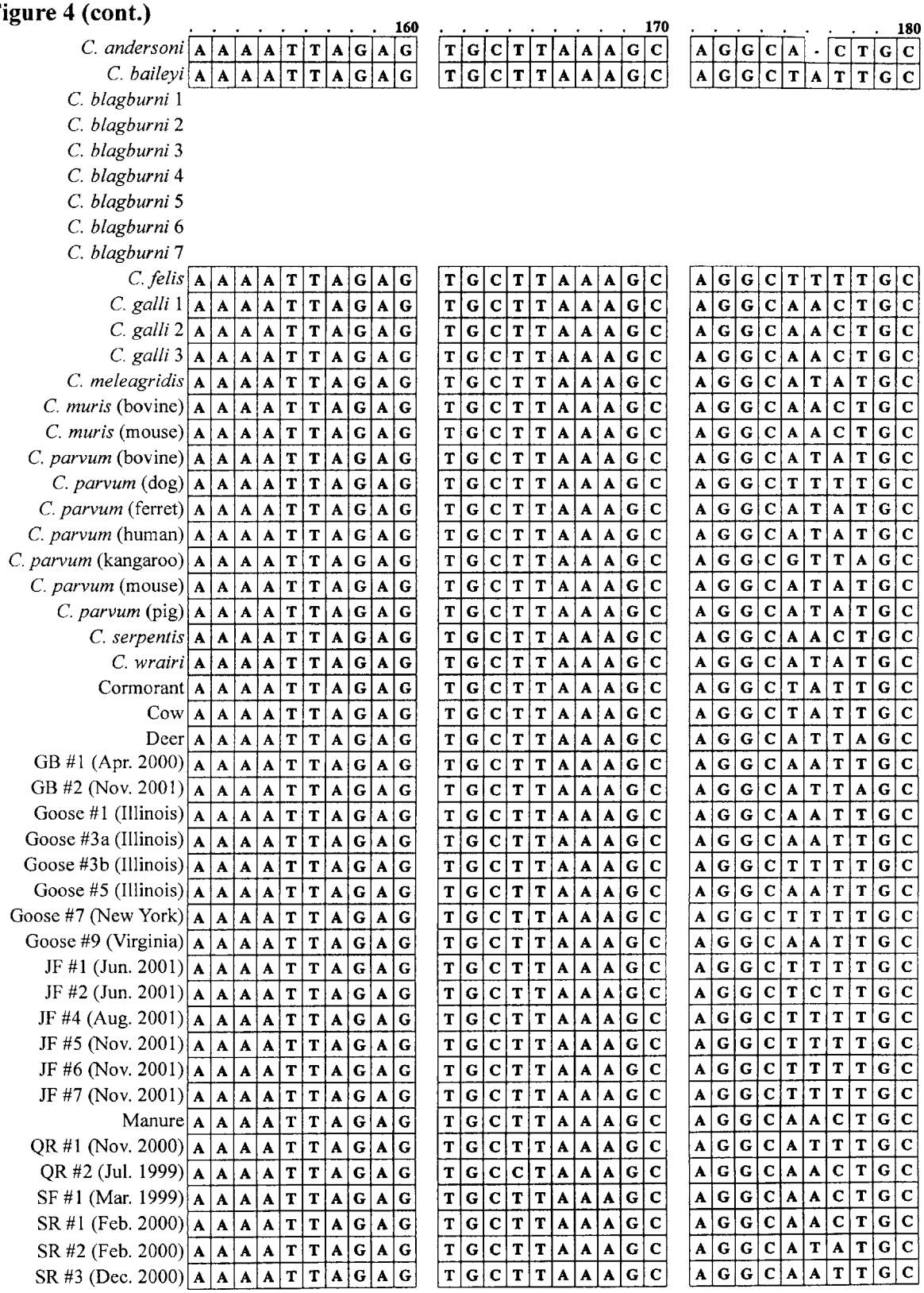
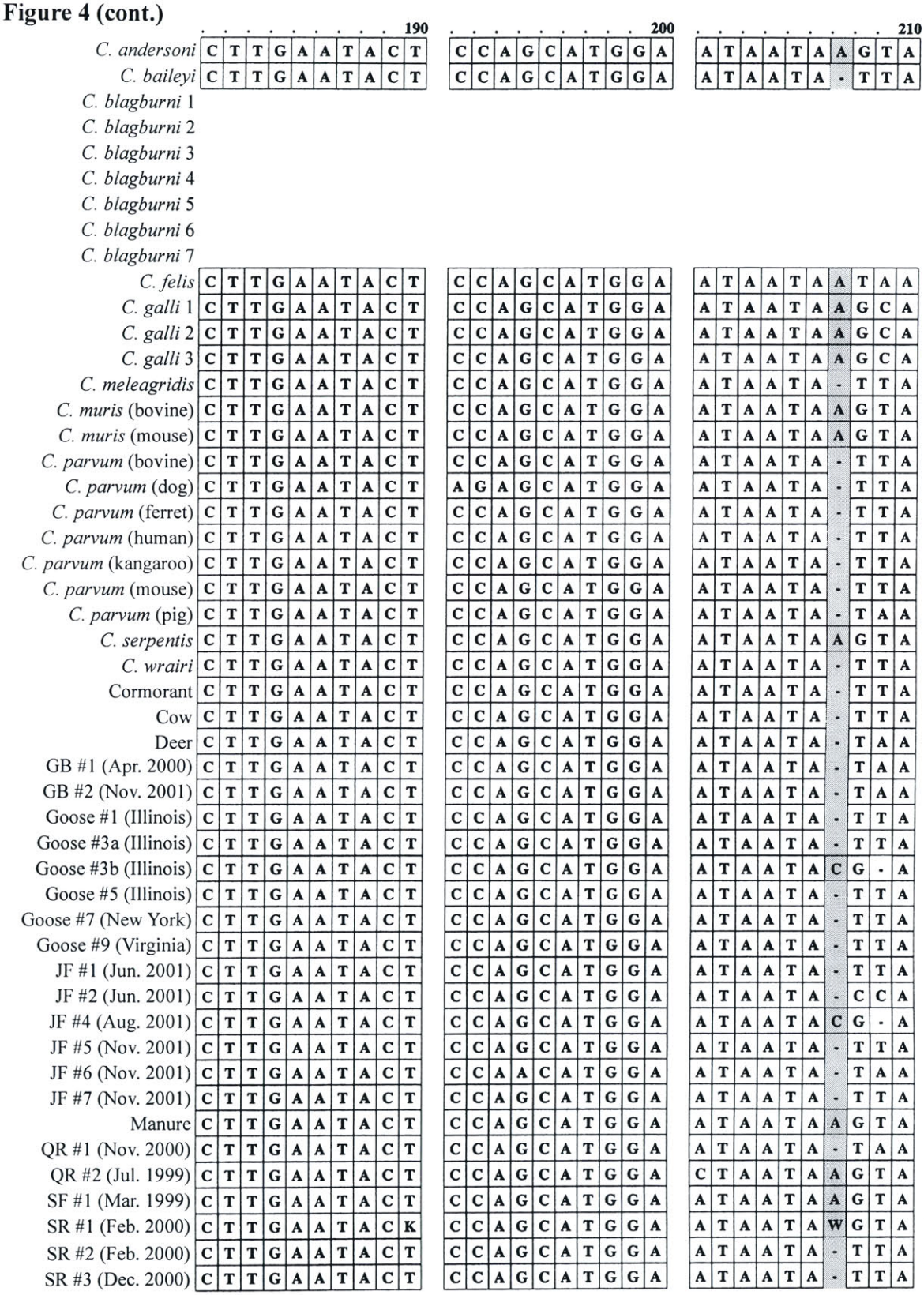


Figure 4 (cont.)



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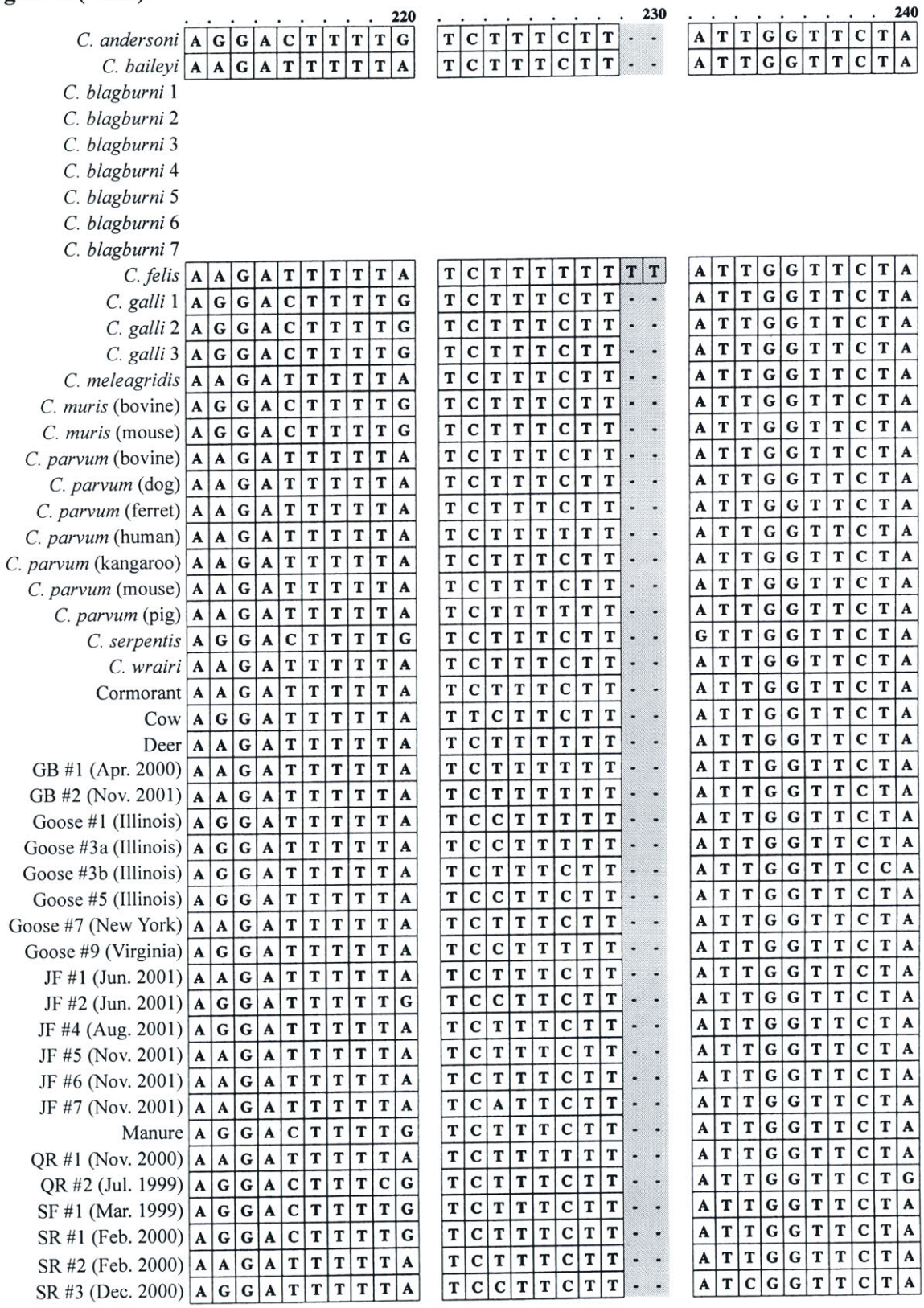

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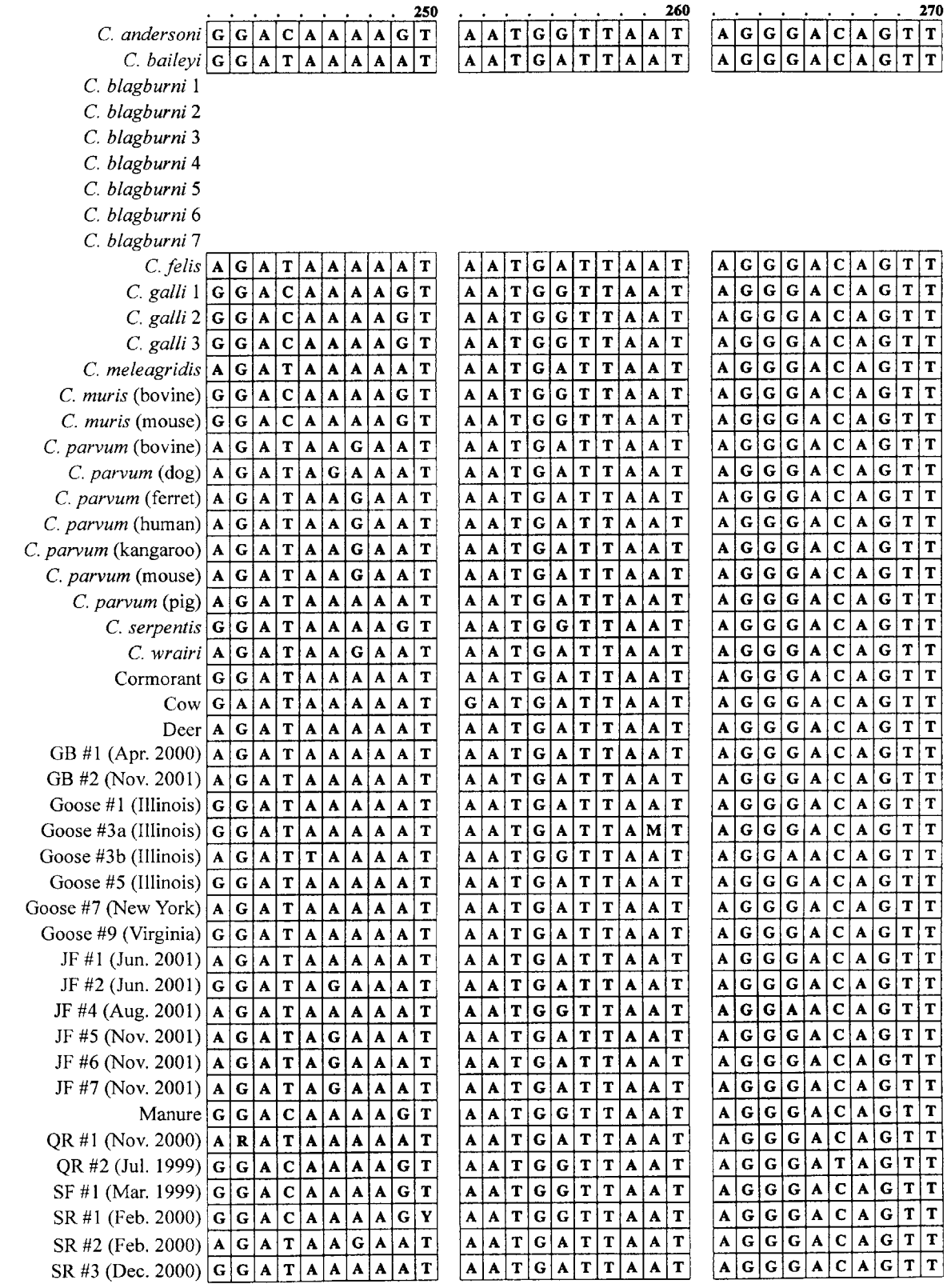

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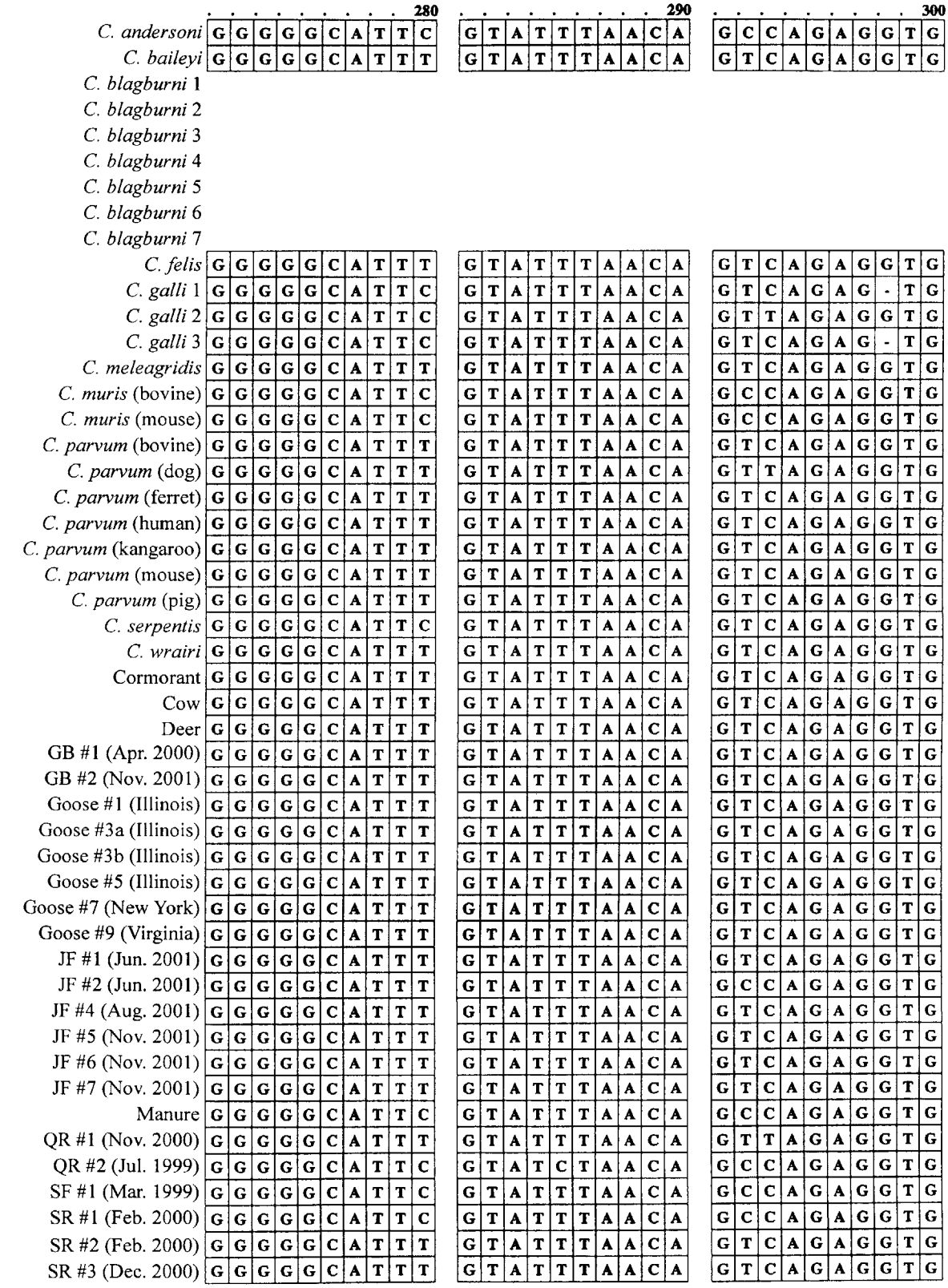

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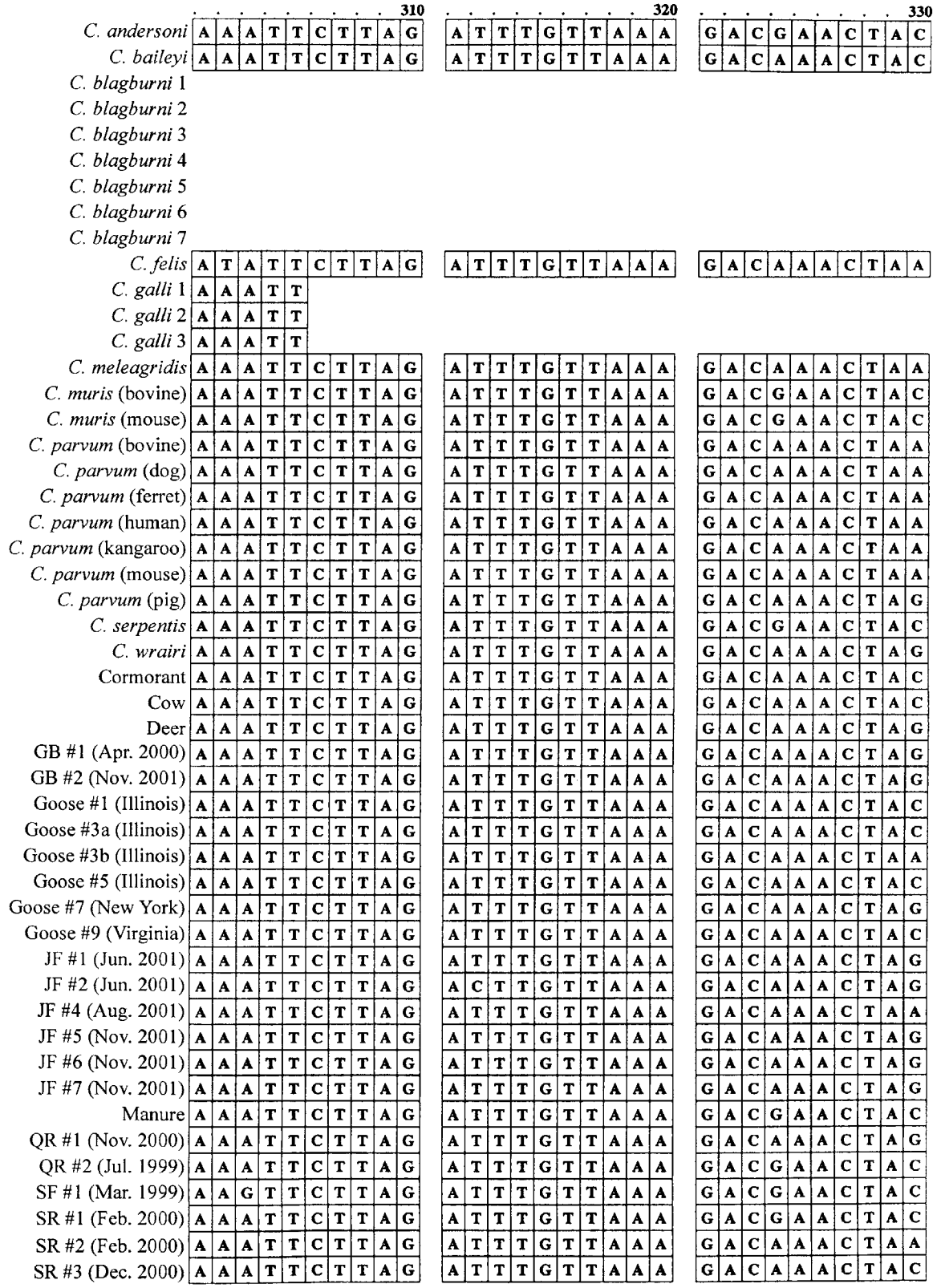

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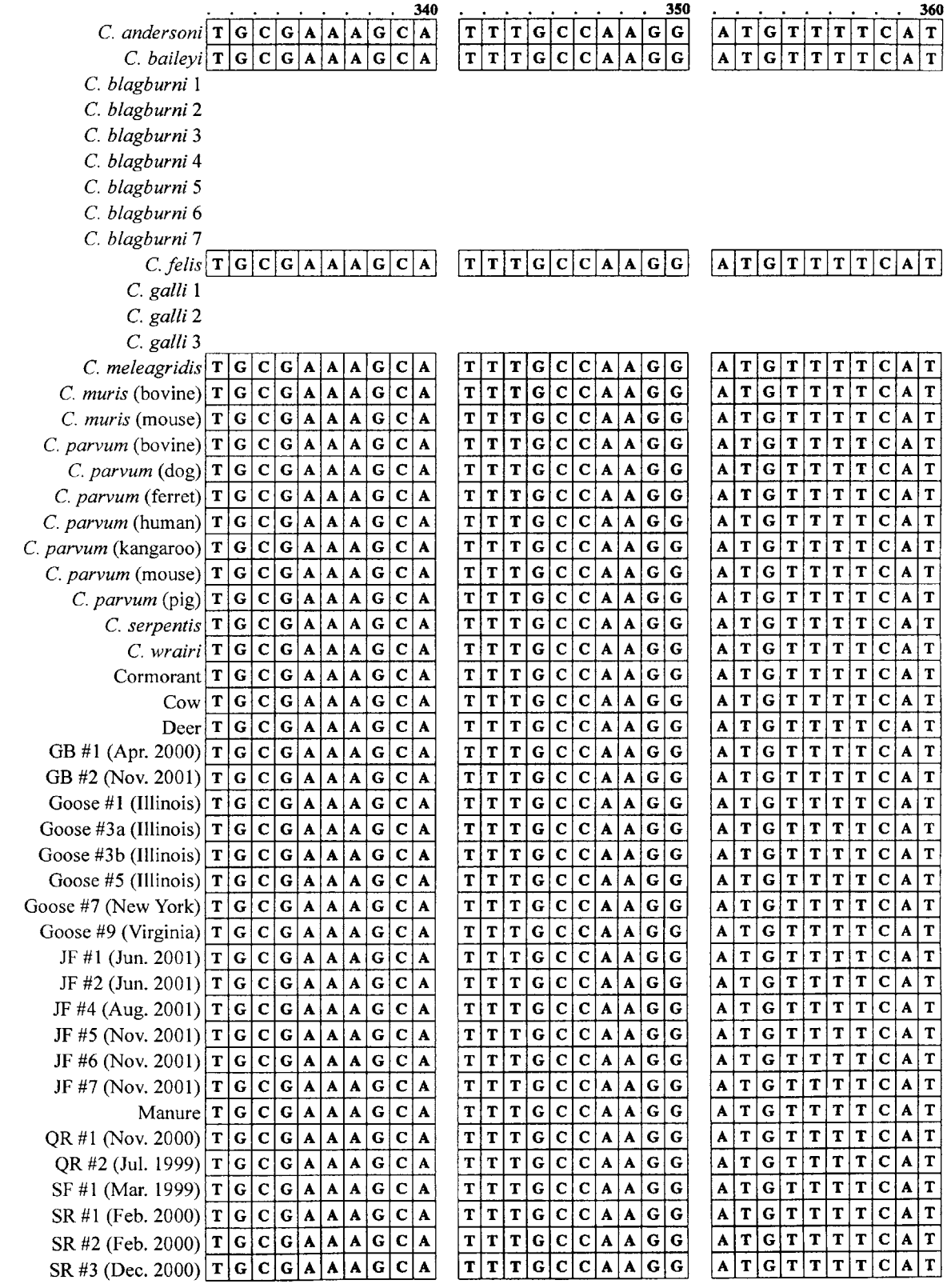

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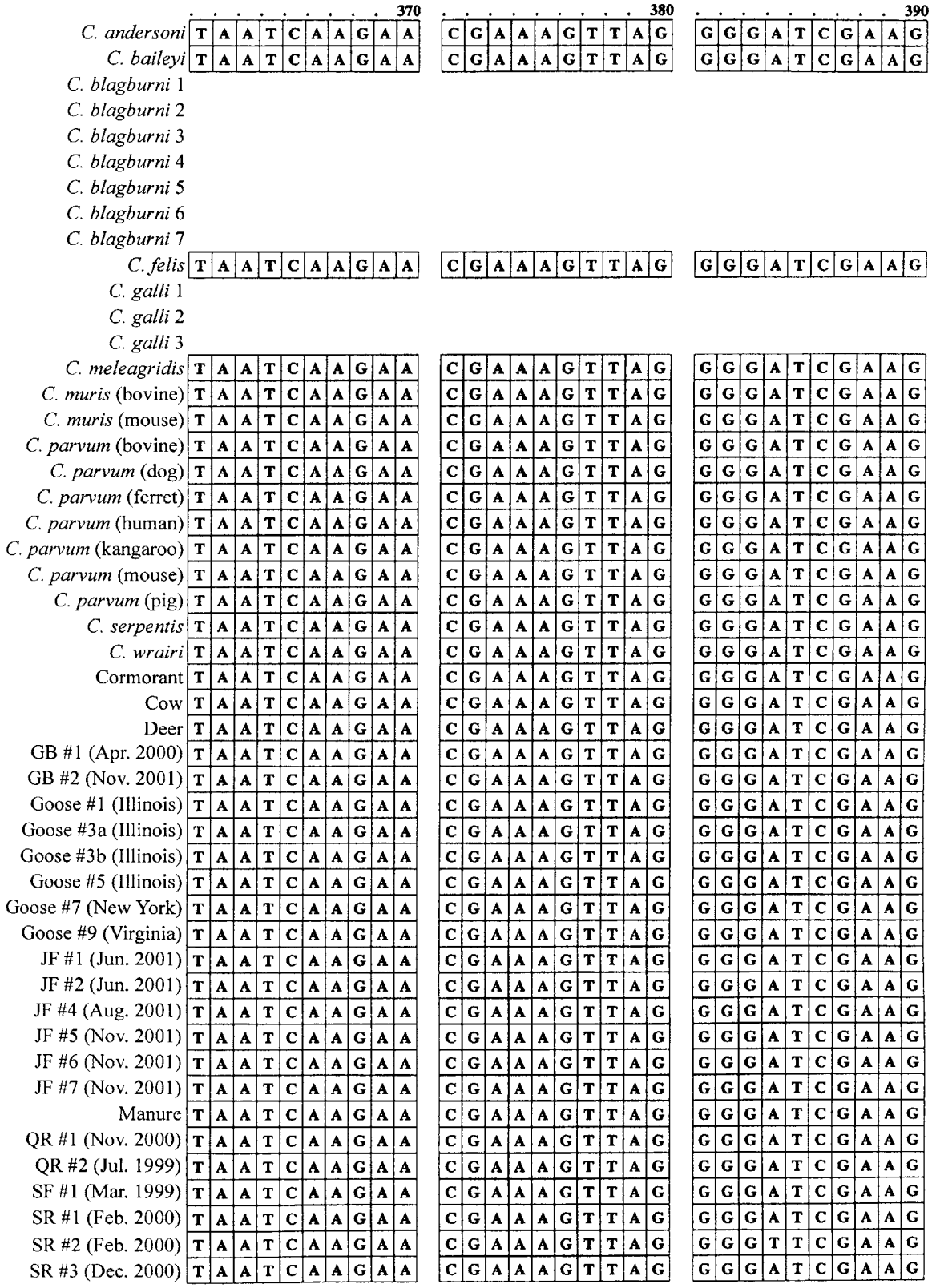

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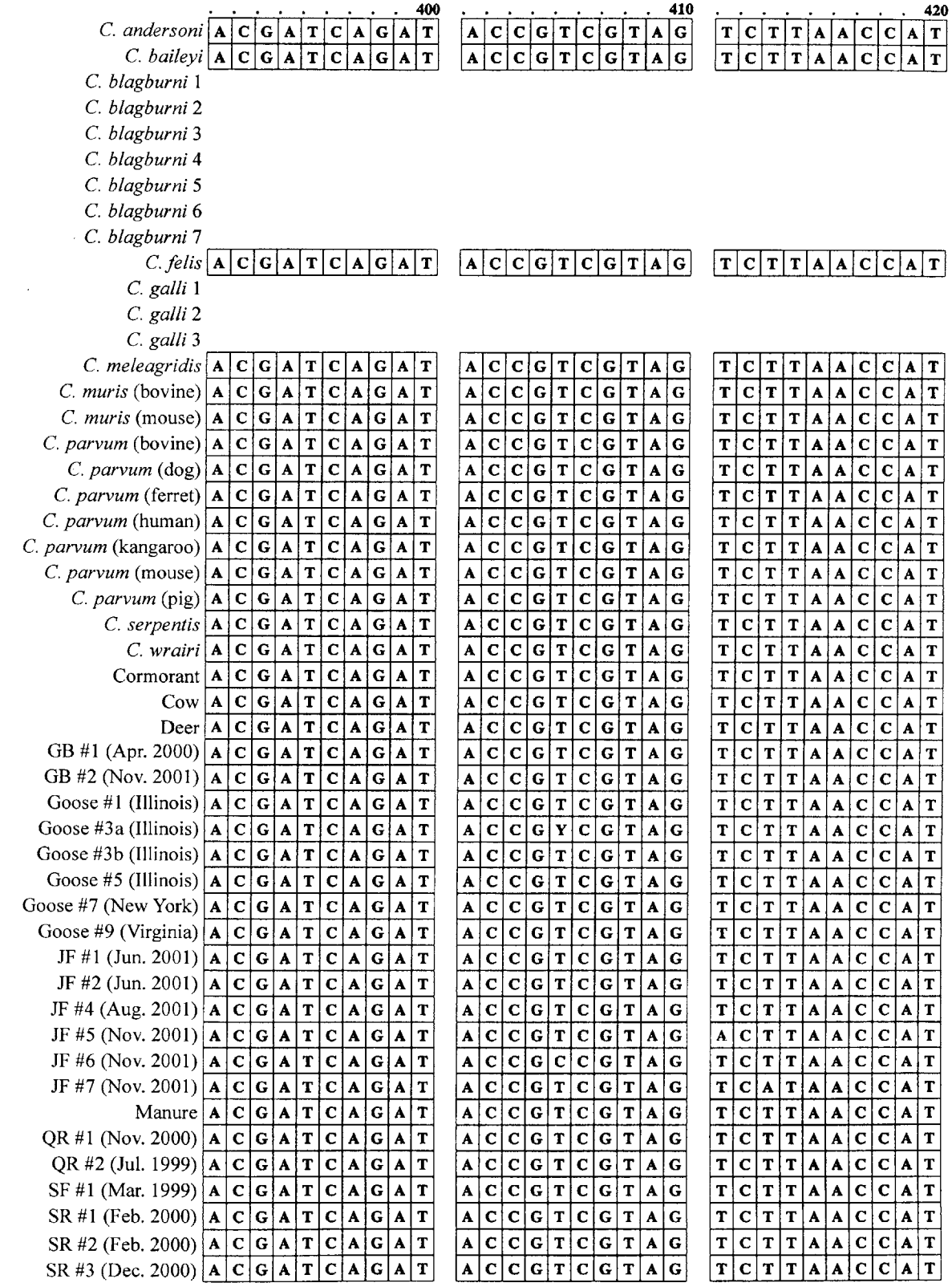

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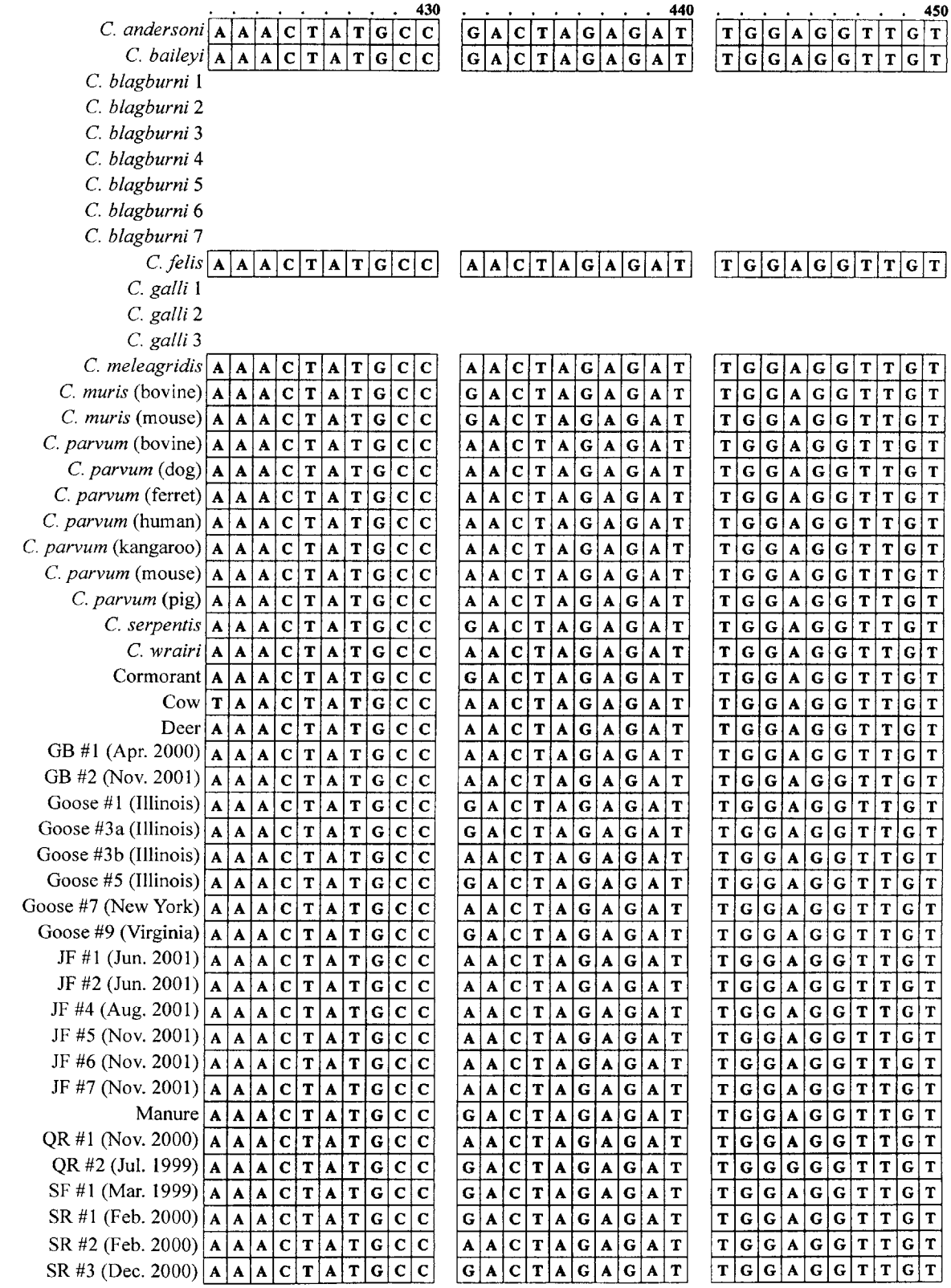
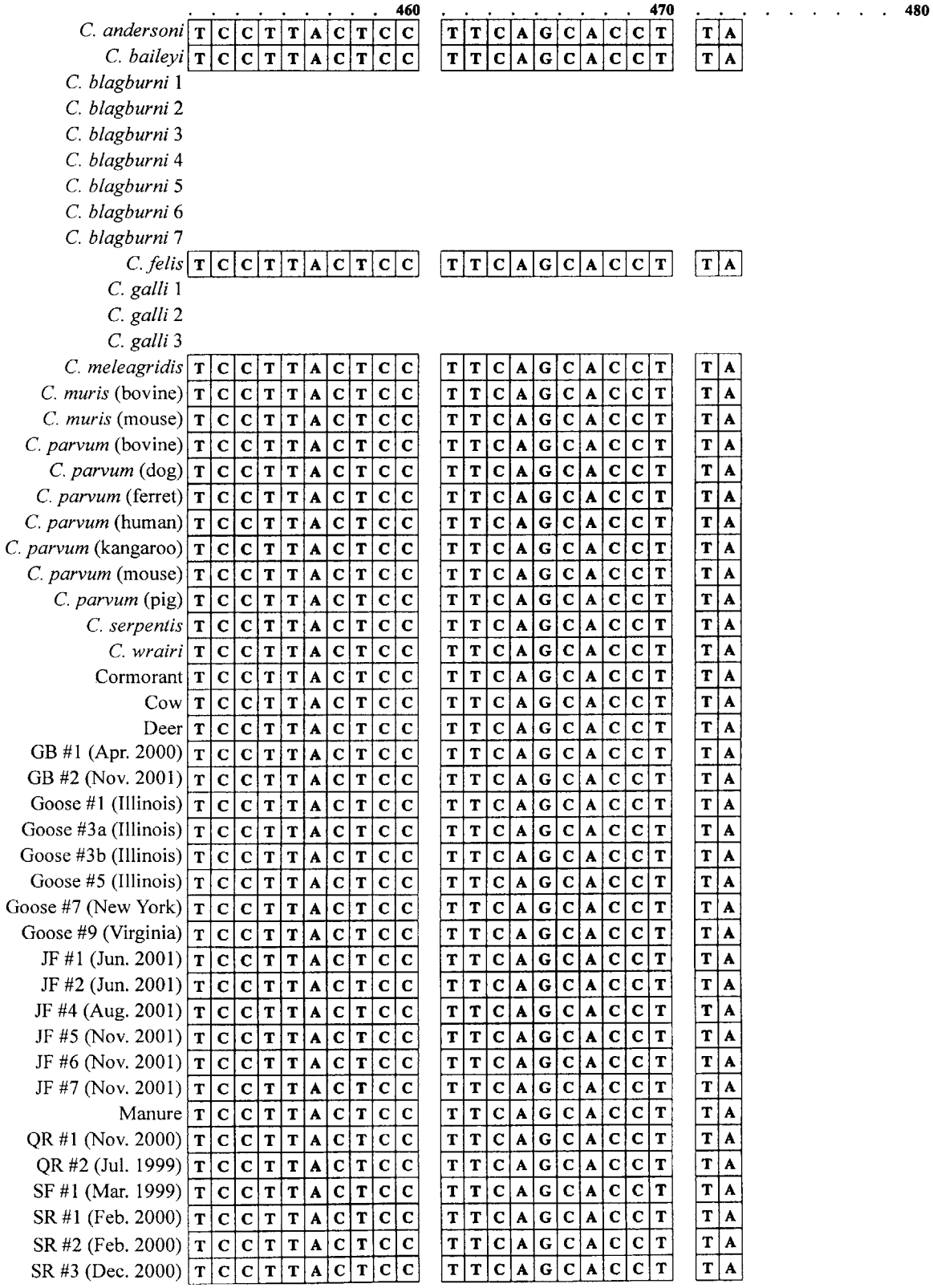
Figure 4 (cont.)