Studies on molecular epidemiology of cryptosporidiosis in poultry and young ruminants

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家禽及び幼若反芻動物におけるクリプトスポリジウム症の分子疫学に関する研究

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Contents

Abbreviations and Units Abbreviations

A	AIDS	acquired immunodeficieney syndrome
B	bp	base pair
	BLAST	Basic Local Alignment Search Tool
C	CI	confidence interval
	C. parvum	*Cryptosporidium parvum*
	C. bovis	*Cryptosporidium bovis*
	C. andersoni	*Cryptosporidium andersoni*
	C. ryanae	*Cryptosporidium ryanae*
	C.meleagridis	*Cryptosporidium meleagridis*
	C. galli	*Cryptosporidium galli*
	C. baileyi	*Cryptosporidium beylei*
	C. xiaoi	*Cryptosporidium xiaoi*
	C. ubiquitum	*Cryptosporidium ubiquitum*
	C. hominis	*Cryptosporidium hominis*
	C. suis	*Cryptosporidium suis*
	C. muris	*Cryptosporidium muris*
D	DNA	deoxyribonucleic acid
E	ELISA	enzyme linked immunosorbent assay
G	gp60	60-kDa glycoprotein
	g	grams
L	LBM	live bird markets

M	ML	maximum likelihood
	ml	milliliter
	mM	millimolar
	MZN	modified Ziehl-Neelson
	MAF	modified acid-fast
N	nested PCR	nested polymerase chain reactions
P	*P. falciparum*	*Plasmodium falciparum*
	PCR	polymerase chain reaction
R	RFLP	restriction fragment length polymorphism
	RNA	ribonucleic acid
S	SSU rRNA	small subunit ribosomal RNA
	18S rRNA	18S ribosomal RNA
	µl	microliter
	µM	micromolar

General introduction

1. Introduction

Cryptosporidiosis is an apicomplexan protozoan disease caused by *Cryptosporidium* spp. that has completed a complex life cycle on hosts. By the zoonotic and anthroponotic way the disease became transmitted globally [Putignani and Menichella, 2010]. *Cryptosporidium* protozoa is one of the coccidian infections that leads to diarrhea. *Cryptosporidium parvum* causes watery diarrhea in people, particularly children in young stage susceptible for this vital zoonotic disease [Thomson *et al.,* 2017]. *C. parvum* was identified for the first time in the gut of mice in 1907 by Edward Ernst Tyzzer [Thomson *et al.,* 2017]. Recurrent diarrhea and malabsorption are vulnerable to an individual health, especially in the patients suffering with acquired immunedeficiency syndrome (AIDS) [Leitch and He, 2011]. Most *Cryptosporidium* species and genotypes have host specificity. To date, *Cryptosporidium* has thus far 38 recognized species. The report of new *Cryptosporidium* species has enhanced in recent years [Feng *et al.,* 2018].

The *Cryptosporidium* thick-walled oocyst (5 μm) is resistant to many decontaminating agents like bleach at the time of isolation of oocysts from fecal samples, and also resistant to several sources of water in the sustained environment [Leitch and He, 2011]. The receptors on the surface of oocysts also show significant roles in settling close to the target tissue in the small intestine of the host [Leitch and He, 2011]. The functional damage of villi and microvilli, the large internal area for absorption in gut, is often observed in intestinal cryptosporidiosis [Fayer, 2008]. Globally, humans cryptosporidiosis has been found in surrounding environments with deprived hygienic situations predominantly in developing country [Thomson *et al.,* 2017]. Humans can get infected with *Cryptosporidium* by taking their water or food contaminated with infective oocysts,

and this could be spread to the community directly from animals or humans [Ibrahim *et al.,* 2016].

Cryptosporidium tends to cause self-limiting diarrhea while in infection with the immunocompetent people, cryptosporidiosis is thought to be a second-most reason for infant diarrhea and death in African and Asian continent after infection of rotavirus [Thomson *et al.,* 2017]. Hence, in developing countries*, Cryptosporidium* is responsible for increasing the number of deaths. Control of cryptosporidiosis is challenging because of its oocysts that is resistant to environment, highly infective, and extreme number of defecated oocysts, and the infection spread rapidly to a susceptible cluster of hosts [Thomson *et al.,* 2017]. For the people having immunocompromised and immunocompetent of cryptosporidiosis, *C. meleagridis* is considered the third-largest usual source and indicates that the birds are the major reservoir of this protozoan based on the epidemiological aspect [da Cunha *et al.,* 2018].

2. Distribution and life cycle

Cryptosporidium protozoa have a global distribution. There is also needs for a better understanding of the environmental issues associated with *Cryptosporidium* infection. A meta-analysis examining the effects of seasonality showed that both high ambient temperature (more important in temperate countries) and high rainfall (more important in the tropics) are related to an increased level of risk for cryptosporidiosis [Jagai *et al.,* 2009]. *Cryptosporidium* has asexual and sexual stages in its life cycle that occur in the same host. Although the parasite has its invasive free-living stages, propagation of the parasite takes place inside a parasitophorous vacuole beneath the host cell brush-border but outside the cytoplasm of the intestinal epithelium [Leitch and He, 2011]. The infection transmitted through environmentally resistant oocysts that mostly comes from water and rarely food sources, as a result, causes outbreaks of diarrhea in immunocompetent

patients that commonly takes fewer than 2 weeks [Leitch and He, 2011].

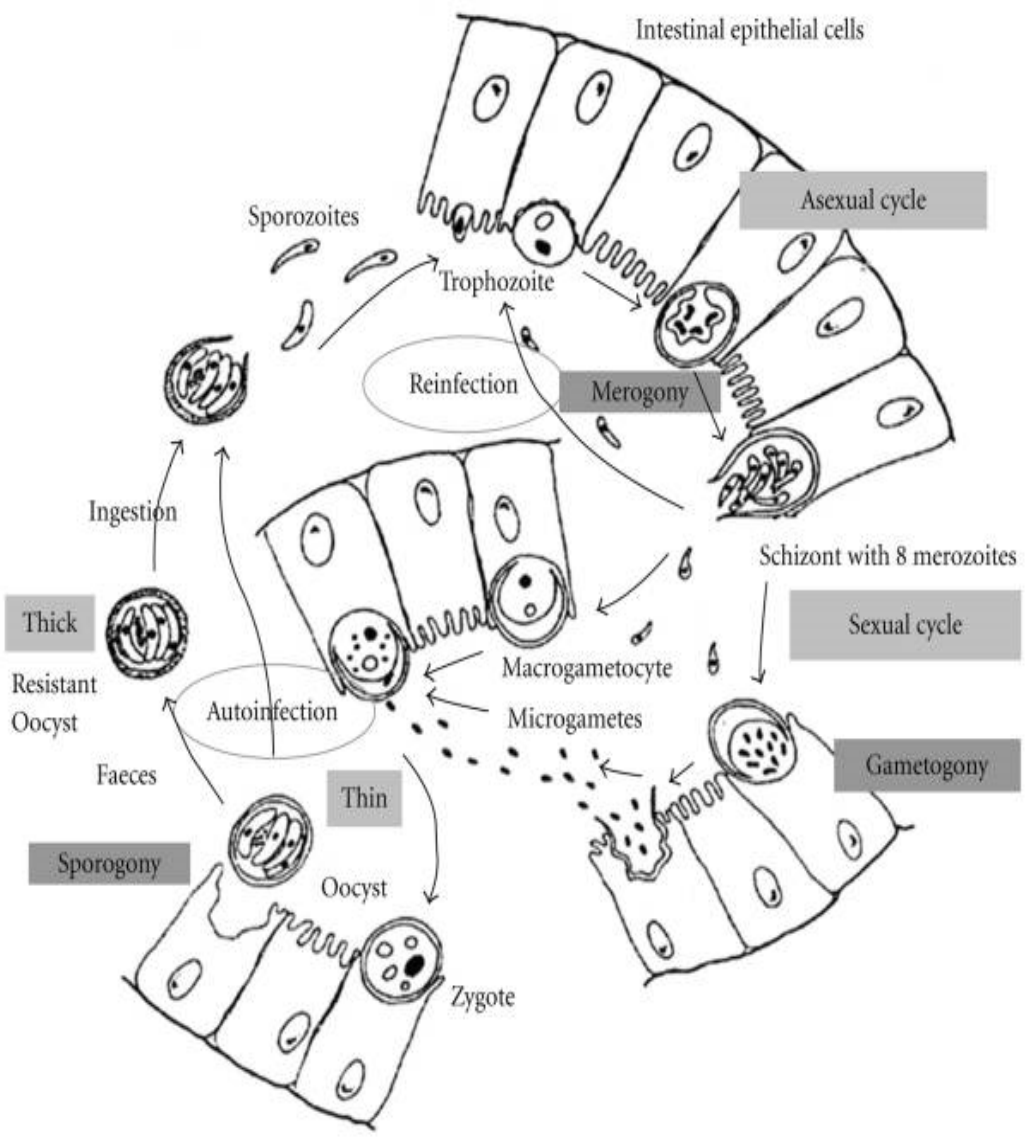

Fig. 1. *Cryptosporidium* life cycle in the enterocyte [Putignani and Menichella, 2010].

In general, the life cycle is that once ingestion of sporulated oocysts by the host, it excysts in the small intestine, then sporozoites release and penetrate to the intestinal epithelial cells and infect the enterocytes, forming a parasitophorous vacuole, which then differentiates into a

trophozoite. After that, the mitotic division of the parasite starts to type I merogony process and to produce schizont with 8 merozoites. They come out from the parasitophorous vacuole and reinfection to the cells. Then again, the merozoite infection initiates in a process of type II merogony, and produce 4 merozoites. The merozoites producing from both type I and type II merogony comes out and infect again the epithelial cells, as a result, forming of a macrogamont (female) or a microgamont (male). More than sixteen microgametes are produced from the microgamont and a diploid zygote is formed by fertilization of a macrogamont that distinguishes to produce an oocyst. Also, meiosis initiates to produce 4 sporozoites. After that, it starts the sexual cycle, and produce 2 types of oocysts. One is thin-walled sporulated oocysts (20%) which cause autoinfection within the host and another is thick-walled resistant oocysts (80%) that pass through feces into the environment (Fig. 1) [Leitch and He, 2011]. *Cryptosporidium* has several protein candidates like microneme, rhoptry, and dense granules that has a role in the attachment of the host cell [Leitch and He, 2011].

3. Poultry cryptosporidiosis

Most of the mammals, birds, reptiles, amphibians, and fish are found to be infected by *Cryptosporidium* species. Regarding increasing demand for free-range products and increasing the number of free-range poultry farms, in the north part of Iran, a notable *Cryptosporidium* infection rate was detected in the free-range chicks and commercial broiler chickens, which notify the role of this host as a reservoir and should more emphasize due to the economic and zoonotic importance [Shahbazi *et al.,* 2020]. Respiratory cryptosporidiosis is recognized as difficulty with birds for a long time [Sréter and Varga, 2000].

Birds could be a susceptible host for the infections of people because of the potentiality of

C. parvum transmission [Helmy *et al.,* 2017]. Besides, recent of the year, total 13 genotypes of *Cryptosporidium* have been reported in birds, for example avian genotypes (I to VI), black duck genotype, goose genotypes (I to V), and Eurasian woodcock genotype [Abe and Makino, 2010; Nakamura and Meireles, 2015; Xiao *et al.,* 2002]. *C. meleagridis* and *C. galli* infections cause watery diarrhea, enteritis, and death of birds [Santín, 2013], however, *C. baileyi* infections are mainly responsible for lung problems with severe illness and death [Sréter *et al.,* 1995]. Among the avian *Cryptosporidium*, only *C. meleagridis* was the potential zoonotic species [Chalmers and Giles, 2010].

For the children in Peru, the existence infection of *C. meleagridis* was almost as common as cryptosporidiosis due to *C. parvum* bovine genotype [Xiao *et al.,* 2001]. Regarding public and animal health issues, birds are important because they convey many infectious agents, including the parasites that have zoonotic potentiality under the *Cryptosporidium* [Laatamna *et al.,* 2017]. There is a huge lack of knowledge about the prevalence of avian *Cryptosporidium*, especially the proper information of genotypes from the various countries [Laatamna *et al.,* 2017].

4. Ruminant cryptosporidiosis

Cryptosporidiosis is a coccidian disease found in an extensive range of hosts including ruminants. *C. parvum* causes mainly gastrointestinal disease in newborn calves which considered significant infections of the early age of ruminant animals. Overall, cryptosporidiosis is currently recognized as the most widespread infections in cattle and the significant reasons for gastrointestinal infections in newborn calves [Cho and Yoon, 2014]. For the epidemiology of bovine cryptosporidiosis, the persistence of oocysts in the environment and contamination of oocyst are important issues. Several *Cryptosporidium* species were typically identified in

ruminants, including *C. parvum*, *C. bovis*, deer-like genotype, *C. ryanae,* and *C. andersoni* in cattle, however, specific *C. parvum* was related to the clinical infection in newborn calves [Fayer *et al.,*2005; Fayer *et al.,*2008] and aged animals (> 6 weeks) showing excretion of oocysts without any symptom. Moreover, *C. parvum*, *C. xiaoi,* and *C. ubiquitum* were also found in lambs and goat kids [Baroudi *et al.,* 2018; Ryan *et al.,* 2014].

Water plays a significant role in the spread of *Cryptosporidium* transmission via the fecal-oral pathway [Lee *et al.,* 2016]. *C. parvum* of the *Cryptosporidium* is considered for the spread of zoonotic diseases in people [Taylan-Ozkan *et al.,* 2016]. Clinically, the start of diarrhea generally happens near 3–4 days after taking of infectious oocysts and it persists for about 1–2 weeks. The excretion of oocysts happens within 2 weeks of post-infection, although it can fluctuate dependent on the primary dose [Zambriski *et al.,* 2013a], and oocyst shedding is not always related to diarrhea. A few oocysts *of C. parvum* is needed to initiate disease in animals, athough the difference depends on the isolate of parasite. Experimentally challenged dairy calves with *C. parvum* determined that about 17 oocysts were enough to start both diarrhea and oocysts excretion [Zambriski *et al.,* 2013b].

Animal age is very important at the sampling time. Early age of calves is probably more susceptible to the shedding of *C. parvum* oocysts whereas aged calves may excrete other *Cryptosporidium* species oocysts [Fayer *et al.,* 2005; Fayer *et al.,* 2008]. Some studies have revealed the association of age between host: *C. parvum* was highly prevalent in newborn calves, since *C. bovis* and *C. ryanae* were found in young calves, and *C. andersoni* was detected in aged calves and adult animals [Fayer *et al.,*2006; Robinson *et al.,*2006; Santin *et al.,*2004], while *C. bovis* and *C. ryanae* were found through all ages of animals [Feng *et al.,* 2007]. Two zoonotic subtype alleles, IIa and IId of *C. parvum* were revealed by the subtyping analysis in samples, and the contagious IIaA15G2R1 subtype was reported commonly and globally [Mammeri *et al.,* 2019].

The identical subtype is found in all the wild ruminants including the domestic animals in entire the Portugal [Alves *et al.,* 2006]. Moreover, it is reported that *Cryptosporidium* different genotypes are found in lambs like *C. suis*, *C. andersoni*, *C. bovis* like genotype, *C. parvum* deer genotype, pig genotype and marsupial genotype [Sari *et al.,* 2009]. Clinical progress of the *Cryptosporidium* infection in lambs and goat kids was similar to calves, and the period of disease development was about 3–4 days, and the outbreaks occurred in about 7 to 10-day of age having clinical signs continue for two weeks [Sari *et al.,* 2009].

Recently, the entire spreading of *Cryptosporidium* and a wide range of host variety with the cow are a major alarming issue for cryptosporidiosis [Xiao, 2010]. Moreover, *Cryptosporidium* might initiate an increase in the number of disease and death when coinfection with HIV [Amadi *et al.,* 2009]. Bovine cryptosporidiosis is of a key issue because of the many calves distress from clinical cryptosporidiosis, and face financial losses and the possible zoonotic implications [Feng *et al.,* 2007].

5. Objectives of the present study

In Bangladesh, there were no studies of poultry cryptosporidiosis based on sensitive diagnostic methods in the country. However, the widespread universal distribution of cryptosporidiosis in animals and humans show the importance of developing effective control measures against the disease. To explain the relations between infections in diverse hosts, it is first essential to recognize species and genotypes properly. To set up an efficient strategy for control of an infection or disease, it is important to offer accurate information on the disease circulation and information required about the current prevalence.

Accordingly, my study aims to discuss in the first chapter, describes the epidemiology of *Cryptosporidium* pathogen present in poultry in the live bird markets of Bangladesh and analyzed their genetic characterizations to estimate the current status of *Cryptosporidium* infection in Bangladesh. Next, in the second chapter, I investigated the bovine cryptosporidiosis detection expanded to cover several prefectures in Japan. Finally, in the third chapter, molecular evaluation of genotypes and subtypes of young ruminants in the Konya region of central Anatolia province in Turkey is described.

Chapter 1

Prevalence and molecular characterization of *Cryptosporidium* species in poultry in Bangladesh

1-1. Introduction

Cryptosporidiosis is an apicomplexan disease widely found in wild, domestic, and captive birds from several parts of the world [Sréter *et al.,* 2000]. *Cryptosporidium* was reported in over 30 avian species in global, with chickens, turkeys, ducks, geese, quails, pheasants, and peacocks [Ryan, 2010]. So far, four species: *C. baileyi*, *C. galli*, *C. meleagridis,* and *C. avium* generally triggered the infections in birds [Nakamura and Meireles, 2015]. *Cryptosporidium* spread over the fecal-oral path by environmentally resistant oocysts shedding in the feces, contaminating soil and water, and thus providing multiple pathways into the food chain [Samad, 2011]. Only *C. meleagridis* of the *Cryptosporidium* is familiar to infect both birds and mammals [Chappell *et al.,* 2011]. Therefore, humans can acquire cryptosporidiosis either by consumption of water and food contained infected oocysts or direct interaction with diseased people or animals [Xiao and Ryan, 2015]. In 2010, *C. baileyi* was described as the leading *Cryptosporidium* species found in all age groups of chickens [Wang *et al.,* 2010], and in 2017, *C. parvum* was the common leading observed species recognized in poultry in Germany [Helmy *et al.,* 2017]. In Peru, *C. meleagridis* cross-species transmission has been described between birds and humans [Wang *et al.,* 2014]. *C. meleagridis* is considered to be the third record observed species of this genus emerging in humans [Xiao, 2010]. *Cryptosporidium*-infected domestic pigeons have been mentioned in some countries,

like in Thailand [Koompapong *et al.,* 2014], China [Li *et al.,* 2015], and Brazil [Oliveira *et al.,* 2017]. The markets where poultry is purchased are crowded with people, and it is possible for *Cryptosporidium* to spread to the surrounding environment due to close contact with other species of birds or mammals in the markets [Bomfim *et al.,* 2013].

The poultry industry of Bangladesh is a promising sector for economic growth. In Bangladesh, documented studies of cryptosporidiosis in animals and poultry are limited. Moreover, no studies examining protozoan infections have been done in the open live bird markets of Bangladesh. A few studies of *Cryptosporidium* related to human disease have been done in Bangladesh, including reports of *C. parvum* and *C. meleagridis* in infant in Bangladesh [Hira *et al.,* 2011; Korpe *et al.,* 2019]. *C. meleagridis* was the vital species in rural areas, whereas in urban areas *C. meleagridis* was often identified as second record species in Bangladesh after *C. parvum* [Steiner *et al.,* 2018].

In Bangladesh, open live bird markets offer wholesale poultry. Peoples gather to buy the poultry for consumption and sellers slaughter and process the poultry under unhygienic conditions. The poultry excrete droppings, which are not regularly cleaned up, creating a high risk for transmission of *Cryptosporidium* infection from poultry to humans. So, my study sought to determine the epidemiology of *Cryptosporidium* spp. in poultry at open live bird markets in Bangladesh and to molecularly characterize the *Cryptosporidium* spp. identified.

1-2. Materials and methods

Ethics statement. All samples were collected from live bird markets. Since samples were collected after slaughter of the poultry, considering the animal protection law unnecessary to take permission. I got permission from the poultry sellers for collecting these samples. All the

experiments were carried out by strictly following the guidelines of the Care and Use of Laboratory Animals of Obihiro University of Agriculture and Veterinary Medicine, Japan. I also got permission for handling all of my experiments by the Ethics committee of animal or pathogen experiments in the Obihiro University of Agriculture and Veterinary Medicine, Japan (my approval numbers: 290156, 2019724)

Sample collection. A total of 197 intestinal colon samples from different poultry species including layer chickens (n = 12), broiler chickens (n=80), sonali chickens (n=93), native chickens (n=8) and pigeons (n=4) were obtained from 19 different live bird markets (LBM) in Dhaka, Bangladesh. The age of the poultry ranged from one month to two years. Poultry were kept together in cages for sale to customers. Fresh intestinal colon samples were collected after the slaughter of every bird from a cage, with consideration to collect only that part without contact of any materials to escape other contamination. All relevant data such as breed and age were recorded for further analysis. Individually sample was placed into a sterile polystyrene tube, labelled, and transferred in an isothermal box to the Environmental Biotechnology laboratory of Sher-e-Bangla Agricultural University (SAU), Dhaka, Bangladesh where it was kept at 4 °C until DNA extraction. After DNA extraction, the DNA samples were imported into Japan according to the rules and guidelines of the Care and Use of Laboratory Animals of Obihiro University of Agriculture and Veterinary Medicine, Japan.

Microscopic examinations. The samples were examined for *Cryptosporidium* oocysts by using the sucrose flotation technique. Briefly, 1 g of colon contents from the sample was suspended in 9 ml of saturated sucrose solution (Specific gravity 1.2) in a tube and centrifuged at, 1300g for 5 min. Then, sucrose solution was added up to the 15-ml tube, which was then left at normal temperature for 30 min. Then, the tube was filled to create a meniscus by adding drops of solution

and putting a coverslip over the tube for 5–10 min. After that, the coverslip was put on a glass slide and observed for oocysts under a light microscope [Fujino *et al.,* 2006]. Thereafter, oocysts of *Cryptosporidium*-positive samples were placed to a microscopic slide, air dried, and then fixed for 3-5 min in absolute methanol, already it stained with Ziehl–Neelsen carbol fuchsin for 20 min. Next wash with clean water, smears were decolorized with 5% acid alcohol for 20–30 s, washed again with clean water and counterstained with 0.4% methylene blue for 2 min, then cleaned again with water and air dried. After drying, the stained smears were observed for oocysts under light microscope using an oil immersion objective lens (×100) [Zaglool *et al.,* 2013]. The remaining samples were kept at 4 °C until DNA extraction [Elliot *et al.,* 1999].

DNA extraction. DNA was extracted directly from samples by using the QIAamp Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with minor modifications. Modifications included the addition of 0.2 g of E-Z zirconia beads to 0.2 g of feces and 1.4 ml of lysis buffer [McLauchlin *et al.,* 1999]. Then, the mixture was heated at 95 °C for 10 min followed by vigorous shaking using the Biomedicals Fast Prep® instrument (1620 r/min for 150 s) to facilitate oocyst rupture. Nucleic acid was eluted in 100 µl of AE buffer to increase the quantity of DNA recovered. After the DNA was extracted, it was stored at −20 °C.

Nested PCR analysis of the 18S rRNA and GP60 genes. For the primary PCR, a PCR product of, 1325 bp was first amplified using the primers (Table 1). The amplification was performed in a 25-µl volume with 2 µl of each DNA sample in 12.5 µl of 2 × PCR buffer, 5 µl of deoxynucleotide triphosphates (2 mM each), 0.25 µl of each primer (50 µM), 4.5 µl of double distilled water, and 0.5 µl of KOD- Fx Neo amplification enzyme (1 units/µl) (ToYoBo Co., Ltd., Japan). Then, for the nested-PCR, 2 µl of the primary PCR product was used with appropriate primers to amplify a ~830-bp fragment of the *Cryptosporidium* 18S rRNA gene as described

previously [Xiao *et al.,* 2001]. The PCR reaction consisted of an initial heating at 94 °C for 2 min, and 35 cycles of 98 °C for 10 s, 55 °C for 60 s, and 68 °C for 30 s (primary PCR) or 98 °C for 10 s, 60 °C for 30 s, and 68 °C for 1 min 30 s (nested PCR). The 60-kDa glycoprotein (GP60; ~ 830 bp; Alves *et al.,* 2003; Guo *et al.,* 2015; Li *et al.,* 2014]) was also amplified in positive samples on the basis of the results for the 18S rRNA. For subtyping of *C. meleagridis*, nested PCR with a specific set of primers was performed to amplify a 1100-bp and a 900-bp fragment of the GP60 gene [Stensvold *et al.,* 2014]. For subtyping of *C. parvum*, nested PCR with a specific set of primers was performed to amplify an 850-bp and an 800-bp fragment of the GP60 gene [Feng *et al.,* 2007]. Primary PCR was carried out in a total volume of 25 µl with 2 µl of each DNA sample in 12.5 µl of 2 × PCR buffer, 5 µl of deoxynucleotide triphosphates (2 mM each), 0.25 µl of each primer (50 µM), 4.5 µl of nuclease-free water, and 0.5 µl of KOD- Fx Neo amplification enzyme (1 units/µl). For the nested PCR, 2 µl of the primary PCR product was used. The PCR and cycling conditions were unique to the primary and nested PCR and consisted of an initial denaturation at 94 °C for 2 min, and 35 cycles of 98 °C for 10 s, 50 °C for 30 s, and 68 °C for 60 s. As positive and negative controls, *C. parvum* genomic DNA and ultrapure water, respectively, were used instead of sample DNA. Known positive standards were used during each PCR run. Optimization of PCR was achieved and different temperature and PCR run conditions were followed as described previously by other investigators who have used similar primer sets [Feng *et al.,* 2007] to identify and characterize *Cryptosporidium*. The amplified fragments were electrophoresed in 1.5% agarose, stained with GelRed® (Biotium), and visualized on an UV transilluminator by electrophoresis in the QIAxcel Advanced system (Qiagen, Valencia, USA).

Sequencing. PCR products were purified from 1.5% agarose gel by using NucleoSpin® gel and a PCR clean-up kit (MACHEREY-NAGEL, Germany). Amplicons of 18S rRNA and

GP60 genes were directly sequenced in both directions with the primers used for the secondary PCR by the ABI 3100 Genetic Analyzer and the BigDye Terminator v3.1 Cycle Sequencing Kit. DNA sequences were assembled with Codoncode Aligner version 7.1.1 software (CodonCode Corporation). The consensus sequences were assembled with homologous sequences published in GenBank using Clustal W [Thompson *et al.,* 1997] and BioEdit Sequence Alignment Editor [Hall, 1999]. The acquired sequences were submitted to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to initially define the species/genotypes and to confirm the high similarity and homology with other known sequences of *Cryptosporidium* spp. in GenBank. All sequences were multiple-aligned and analyzed by Bioedit and MEGA 7.0 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html and http://www.megasoftware.net/). Phylogenetic trees were generated using Maximum Likelihood (ML) analysis based on the T92+I model [Tamura, 1992] for 18S rRNA gene in MEGA7 [Kumar *et al.,* 2016] using *Plasmodium falciparum* as an out-group. Phylogenetic trees were also generated using ML analysis based on the Tamura-Nei model [Tamura and Nei, 1993] for the GP60 genes of the *Cryptosporidium* subtypes in MEGA7 [Kumar *et al.,*2016]. To assess the reliability of this tree, bootstrap analysis was done with 1000 replicates.

Nucleotide sequence accession number. The partial 18S rRNA nucleotide sequences and GP60 sequences obtained in this study have been deposited in the GenBank database under accession numbers (MN133966 - MN133996 and MN192414 - MN192426).

Statistical analyses. Data analysis was performed using Microsoft Office Excel 2010, and results were considered to be statistically significant when *P*-values were ≤ 0.05. Prevalence rates with 95% confidence intervals were calculated by using Wilson (score) intervals [Sergeant, 2017] obtained in OpenEpi software (http: //www. openepi.com/Menu/OE_Menu.htm).

1-3. Results

Positive rates of *Cryptosporidium* spp. in poultry. Microscopic investigations showed the occurrence of *Cryptosporidium* oocysts in 19.8% (39 of the 197) poultry samples (Fig. 2). The modified Ziehl-Neelsen (MZN) method indicated that *Cryptosporidium* was present in 50% (2/4) of the pigeon samples, 20.4% (19/93) of the sonali chicken samples, 18.8% (15/80) of the broiler chicken samples, 16.7% (2/12) of the layer chicken samples and 12.5% (1/8) of the native chicken samples. Not all microscope-positive samples were PCR-positive. The positive rate for *Cryptosporidium* was 15.7% (31/197) by nested PCR. The highest rate (50%) of *Cryptosporidium* was found in pigeons both microscopically and by PCR, whereas the lowest rate was found in the native chicken samples (12.5%) by the microscopic method and in broiler chickens (11.3%) by nested PCR (Table 2).

Detection of *Cryptosporidium* species in poultry. *C. baileyi*, *C. meleagridis*, and *C. parvum* were identified through sequencing; however, *C. baileyi* was the most predominant species. Of the 31 positive samples, 17 were for *C. baileyi* (8.7%), 12 for *C. meleagridis* (6.0%), and 2 for *C. parvum* (1.0%). *C. baileyi* was detected in 11.9% (11/93) of sonalis, 5% (4/80) of broilers, 8.4% (1/12) of layers, and 25% (1/4) of pigeons. *C. meleagridis* was also detected in this study in 5.4% of sonalis, 5% of broilers, 8.4% of layers, 12.5% of natives, and 25% of pigeons. Moreover, *C. parvum* was identified in 1% of sonali and 1.3% of broiler (Table 3). There were no statistical differences observed about the different species of poultry and the relationship between age and infection (Table 4).

Prevalence of *Cryptosporidium* spp. in different live bird markets (LBM). *Cryptosporidium* spp. were found in most of the live bird markets in different areas of Dhaka, Bangladesh. The prevalence of *Cryptosporidium* spp. was high among chickens (10%–40%) and

pigeons (50%) in Mirpur areas where LBM 4, 10, 12, and 13 were located and where *C. baileyi*, *C. meleagridis*, and *C. parvum* were detected. However, I found no *Cryptosporidium* in the areas of Kollyanpur, Mirpur new society, where LBM 6 and 11 were located (Table 5).

Sequence and phylogenetic analyses. All PCR-positive specimens were successfully sequenced and analysis of the nucleotide sequences of the 18S rRNA genes revealed the presence of three *Cryptosporidium* species: *C. baileyi*, *C. meleagridis*, and *C. parvum*. Direct sequencing of the 18S rRNA gene amplicons identified *C. baileyi* (8.7%; 17/197), *C. meleagridis* (6.0%; 12/197), and *C. parvum* (1.0%; 2/197). The sequences from *C. baileyi*, *C. meleagridis,* and *C. parvum* had 100% genetic similarity with sequences previously published in GenBank (JX548294, KT151550, KU744845, MK311146, HQ917077, KY448456, KY352486, MH062745, MF671870, MK491508 and LC270282).

ML analysis of 18S rRNA gene sequences showed three different clusters among the isolates of cryptosporidia from poultry in this study. Seventeen isolates assembled with *C. baileyi*, sharing 100% sequence identity with accession numbers (MN133967-MN133971, MN133976-MN133977, MN133980, MN133981, MN133983, MN133986, MN133987, MN133989-MN133991, MN133993 and MN133995). Two isolates assembled with *C. parvum* sharing 100% sequence identity with accession numbers (MN133979 and MN133984). The remaining 12 isolates assembled with *C. meleagridis*, sharing 100% sequence identity with accession numbers (MN133966, MN133972- MN133975, MN133978, MN133982, MN133985, MN133988, MN133992, MN133994 and MN133996) (Fig. 3). All 12 *C. meleagridis*-positive specimens generated the expected GP60 PCR product. However, only 11 of the 12 isolates were successfully sequenced. Nucleotide sequence analysis of the GP60 gene revealed that all of the subtypes belonged to the most common subtype, the IIIb family of *C. meleagridis*. I detected two novel

subtypes, IIIbA21G2R1, in sonali chickens (n = 4) and a broiler chicken (n=1) and IIIbA20G2R1, in a layer chicken (n=1), that had a different nucleotide sequence within the trinucleotide repeat region. I also identified two other subtypes (IIIbA21G1R1 and IIIbA23G1R1) found in broiler chickens (n=2), a native chicken (n=1), a sonali chicken (n=1) and a pigeon (n=1). The GP60 gene of *C. parvum* was also amplified by specific sets of primers to reveal two subtypes (IIaA11G2R1 and IIaA13G2R1) identified in a sonali chicken (n=1) and a broiler chicken (n=1) that were previously reported in calf (Fig. 4). Therefore, these subtypes were named based on the established GP60 nomenclature [Stensvold *et al.,* 2014; Sulaiman *et al.,* 2005].

1-4. Discussion

Only limited research has genotyped and subtyped *Cryptosporidium* in poultry all over the world. This study represents 19.7% of *Cryptosporidium* prevalence in different breeds of poultry based on microscopic results. In contrast, a report mentioned a high frequency (about 37%) of *Cryptosporidium* in broiler flocks in Morocco by using microscopy [Kichou *et al.,*1996]; however, in China, lower *Cryptosporidium* infection rates of 3.4% in broilers and 10.6% in layer chickens were identified from fecal samples by using bright-field microscopy [Wang *et al.,* 2010]. Furthermore, only 0.5% of chickens at poultry slaughterhouses in Iran were reported to be *Cryptosporidium*-positive [Hamidinejat *et al.,* 2014]. The quantity of oocysts was not evaluated in this study. However, in a positive sample, the sensitivity of the primer that I used, there should be at least 10^1 oocysts found under microscope [Jae-Ran *et al.,* 2009].

In my study, the positive rates of *Cryptosporidium* obtained by using molecular techniques were comparatively higher than those obtained by other investigators, such as 7.03% found in Germany [Helmy *et al.,* 2017] and 10% found in China [Wang *et al.,* 2014], although

the rate of 14.8% found in Brazil is similar to my findings [da Cunha *et al.,*2018]. In general, molecular technique is more sensitive than microscopy to detect infection. However, sometimes, microscopy showed high detection rate. It might be due to presence of PCR inhibitors in feces including bilirubin, bile salts, and complex polysaccharides, and thus PCR can be inhibited [Morgan *et al.,* 1998].

In the current study, *C. baileyi* was detected in sonali, broiler, and layer chickens and also in pigeons. These findings are similar by the work of Baroudi *et al.,* who described a similar prevalence (5.5%; 5/90) in broiler chickens in Algeria using molecular techniques [Baroudi *et al.,*2013]. Moreover, similar findings have been reported in Germany in broilers (5.7%; 9/158) and layers (8.3%; 1/12) [Helmy *et al.,* 2017]. Broiler chickens might act as sources of infection due to shedding of oocysts and may be responsible for transmission and infection [Silverlas *et al.,* 2012]. *C. baileyi* might be a major bird species of *Cryptosporidium* in global and has an extensive range of host [Nakamura and Meireles, 2015; Santana *et al.,* 2018]. In the present study, *C. baileyi* was the leading species and was identified in all age groups of chickens, although *C. meleagridis* was also observed. *C. baileyi* was detected in sonali, broiler, and layer chickens and in pigeons in this study, while *C. baileyi* described frequently in chickens and pigeons worldwide [Huber *et al.,* 2007; Liao *et al.,* 2018]. *C. meleagridis* was also detected in sonali, broiler, layer, and native chickens as well as in pigeons. Similarly, *C. meleagridis* was previously reported in 3.2% of chickens [Liao *et al.,* 2018], 9% of broilers in Algeria [Laatamna *et al.,* 2017], 5.3% of broilers in China [Wang *et al.,* 2014] and 10% of layer chickens in China [Wang *et al.,* 2010]; however, some reports have shown infection rates of *C. meleagridis* as high as 28.9% in chickens [Baroudi *et al.,* 2013]. *C. baileyi* was the predominant species and detected in chickens of all ages, while, *C. meleagridis* was found in 31 to 120-day-old layer chickens [Wang *et al.,* 2010]. Both *C. baileyi* and *C.*

meleagridis were found in more than 4 months of age of chicken [Liao *et al.,* 2018]. The breeds of poultry in this study has different life span and genetic variation. I considered that young poultry were at risk group because of less immunity. However, in this current study the infection of poultry was in adult group which might be the reason of stress factors during the time of production of egg and meat [Helmy *et al.,* 2017].

This study identified two novel subtypes of *C. meleagridis* (IIIbA21G2R1 and IIIbA20G2R1) in sonali chickens, in one broiler chicken and in one layer chicken. The remaining 2 subtypes, IIIbA21G1R1 and IIIbA23G1R1, were identified in chickens and pigeons, consistent with previous reports in humans and birds [Abal-Fabeiro *et al.,* 2013; Glaberman *et al.,* 2001; Stensvold *et al.,* 2014]. The presence of *C. meleagridis* in poultry in live bird markets in the current study makes a questions about potent zoonosis from poultry to humans. *C. meleagridis* is also a public health concern in humans, related to digestive problems in both immunocompetent and immunocompromised persons and mammals [Chappell *et al.,* 2011]. Two subtypes of *C. meleagridis* was identified in the patients of AIDS that spread by poultry (chicken, pigeon, or duck) in a similar place of Peru [Wang *et al.,* 2014]. The IIIbA22G1R1 subtype was detected in people from Sweden who went to India or Thailand before the infection [Stensvold *et al.,* 2014]. These variations in prevalence could be related to the different breeds of poultry which has various susceptibility and immunity due to genetic variation, and the situation of the location of LBM, and the improper hygienic condition in poultry cages of LBM. Environmental factors and differences in host species may also be responsible. Unhygienic conditions in cages, overpopulation, and keeping different birds together have contributed to the high infection rates of *Cryptosporidium* [Bomfim *et al.,* 2013].

C. parvum also has zoonotic potential and is sporadically found in birds [Nakamura and

Meireles, 2015]. In Germany, the major leading species found in chickens and turkeys were *C. parvum* [Helmy *et al.,* 2017], that promotes the possibility of poultry that might be an infection source and mechanical vector for other zoonotic *Cryptosporidium*, besides *C. meleagridis*. However, in my study, the sanitary conditions were characterized as poor due to the lack of periodic cage cleaning and overpopulation. Also, the presence of ruminants in LBMs might be infected by *C. parvum*, and have caused the spread of oocysts in the surroundings, favoring ingestion by poultry. In contrast, migratory birds would act as a source of transmission, mechanical passage of oocysts, and contamination of the environment, even though they have low-level infections [Majewska *et al.,* 2009].

Birds are considered as the natural definitive host for *C. meleagridis*, and zoonosis is pointed out for the transmission of poultry to humans [Silverlas *et al.,* 2012]. In urban areas of Bangladesh, the second most commonly recognized species was *C. meleagridis* (13%), and also *C. parvum* was identified in 2% infecting children without diarrhea. However, in remote areas, the major species was *C. meleagridis* (90%) while *C. parvum* was much less prevalent (4%) causing subclinical cryptosporidiosis [Steiner *et al.,* 2018]. *C. meleagridis* and *C. parvum* have been identified in infants in Mirpur, Bangladesh. This might be anthropozoonotic; transmitted from chickens kept in households [Korpe *et al.,* 2019]. Interestingly, my study also detected *C. meleagridis* and *C. parvum* in LBMs in Mirpur areas. It is not possible to say whether the *C. meleagridis* subtypes identified in this study are related to subtypes that have infected humans due to lack of identified GP60 gene subtyping data of *C. meleagridis* in humans in Bangladesh. The *C. parvum* subtype IIcA5G3R2 and subtype IImA7G1 were identified in children with diarrhea aged less than 2 and 5 years, respectively, in Bangladesh. This subtype family has not been identified in any animal thus far and is generally considered “anthroponotic” [Hira *et al.,* 2011; Korpe *et al.,*

2019]. In the current study, we detected two subtypes (IIaA11G2R1 and IIaA13G2R1) of *C. parvum* in broiler and sonali chickens that were previously reported as calf subtypes [Wielinga *et al.,* 2008]. In the current study, *Cryptosporidium* was identified in pigeons; however, a detection rate of 7% was found in pigeons in Brazil and 25% in pigeons in Thailand [Koompapong *et al.,* 2014; Oliveira *et al.,* 2017]. Pigeons could be infected by contact with animals or their owners could passively spread oocysts in the surroundings. Owners become at high risk of infection during the time of contact of oocysts discharged from pigeons [Oliveira *et al.,* 2017]. The occurrence of *C. meleagridis* in domestic pigeons in China might lead to zoonotic transmission, particularly both in handlers and the surrounding place due to mice spreading the oocysts in water and food [Li *et al.,* 2015]. Market workers and customers who were handling poultry in the markets might transmit oocysts in water, feed, and litter in poultry. It might be shed from the oocysts of mammalian/human origin due to lack of hygienic maintenance. Therefore, it is vital to think about this pathogen. People and other animals could be infected with *C. meleagridis* and *C. parvum* via potential zoonotic transmission from poultry carrying the pathogen. Therefore, it is important to consider *Cryptosporidium* as a risk for public health and the economy.

1-5. Summary

In conclusion, my research revealed that *Cryptosporidium* parasites are common among the live bird markets in Bangladesh. Subsequent nested PCR targeting the 18S rRNA gene revealed that 15.7% (31/197) of the samples were *Cryptosporidium* positive. Of these 31 samples, 17 were *C. baileyi* (8.7%), 12 were *C. meleagridis* (6.0%), and 2 were *C. parvum* (1.0%). Nucleotide sequence analysis of the GP60 gene of the *C. meleagridis* revealed that two subtypes (IIIbA21G1R1 and IIIbA23G1R1), which were found in the broiler, native and sonali chickens

and a pigeon, matched those previously reported in humans and poultry. I identified two novel subtypes (IIIbA21G2R1 and IIIbA20G2R1) in sonali chickens, a broiler chicken, and a layer chicken. I also amplified the GP60 gene of *C. parvum* and found two subtypes (IIaA11G2R1 and IIaA13G2R1) in a sonali and a broiler chicken that were previously reported in the calf. These results indicate that poultry might be a source of cryptosporidial infections for humans and animals in Bangladesh. This is the first molecular investigation of *Cryptosporidium* genotypes and subtypes in poultry at open live bird markets in Bangladesh.

Table 1. Primers used for amplifying 18S rRNA and GP60 genes for the detection of *Cryptosporidium* genotypes and subtypes in poultry.

Primers	Primers 5′-3′ sequences	Amplified product (bp)	References
18S rRNA F1	TTCTAGAGCTAATACATGCG	~ 1325	Xiao *et al.,* 2001
18S rRNA R1	CCCATTTCCTTCGAAACAGGA	~ 1325	
18S rRNA F2	GGAAGGGTTGTATTTATTAGATAAAG	~ 830	
18S rRNA R2	AAGGAGTAAGGAACAACCTCCA	~ 830	
GP60 F1	ATAGTCTCCGCTGTATTC	~ 850	Feng *et al.,* 2007
GP60 R1	GGAAGGAACGATGTATCT	~ 850	
GP60 F2	TCCGCTGTATTCTCAGCC	~ 800	
GP60 R2	GCAGAGGAACCAGCATC	~ 800	
CRSout115F	GATGAGATTGTCGCTCGTTATC	~ 1100	Stensvold *et al.,* 2014
CRSout1328R	AACCTGCGGAACCTGTG	~ 1100	
ATGFmod	GAGATTGTCGCTCGTTATCG	~ 900	
GATR2	GATTGCAAAAACGGAAGG	~ 900	

Table 2. Positive rates of *Cryptosporidium* species in poultry.

Poultry spp.	No. sample	No. microscopy positive (%)	No. nested PCR positive (%)
Chicken (sonalis)	93	19 (20.4)	17 (18.3)
Chicken (broilers)	80	15 (18.8)	9 (11.3)
Chicken (layers)	12	2 (16.7)	2 (16.7)
Chicken (natives)	8	1 (12.5)	1 (12.5)
Pigeon	4	2 (50)	2 (50)
Total	197	39 (19.8)	31 (15.7)

Table 3. Detection of *Cryptosporidium* spp. in poultry.

Poultry spp.	No. sample	No. positive (%)			Total positive (%)	95% CI[a]
		C. baileyi	*C. meleagridis*	*C. parvum*		
Chicken (sonalis)	93	11 (11.9)	5 (5.4)	1 (1)	17 (18.3)	11.8-27.4
Chicken (broilers)	80	4 (5)	4 (5)	1 (1.3)	9 (11.3)	6.03-20.02
Chicken (layers)	12	1 (8.4)	1 (8.4)	0 (0)	2 (16.7)	4.7-44.8
Chicken (natives)	8	0 (0)	1 (12.5)	0 (0)	1 (12.5)	2.3-47.1
Pigeons	4	1 (25)	1 (25)	0 (0)	2 (50)	15-85
Total	197	17 (8.7)	12 (6.0)	2 (1.0)	31 (15.7)	11.4-21.5

[a] Confidence interval.

Table 4. Prevalence of *Cryptosporidium* spp. according to their age category in poultry.

Poultry spp.	No. sample	Age of poultry (days)	No. positive (%)	95% CI[a]
Chicken(sonali)	70	30-90	10 (10.8)	7.9-24.4
	23	>120	7 (7.6)	15.6-50.9
Chicken(broiler)	80	30	9 (11.3)	6.0-20.0
Chicken(layer)	12	>120	2 (16.7)	4.7-44.8
Chicken(native)	8	>120	1 (12.5)	2.2-47.0
Pigeon	4	>120	2 (50)	15-85
Total	197	-	31 (15.7)	11.3-21.5

[a] Confidence interval.

Table 5. Prevalence of *Cryptosporidium* spp. in different live bird markets in Bangladesh.

Location	Live Bird Market (LBM)	Poultry species	No. samples	No. *Cryptosporidium* positive (%)	*Cryptosporidium* spp. (n)
Taltala	LBM 1	Chicken (sonali)	10	1 (10)	*C. baileyi* (1)
Shewra para	LBM 2	Chicken (sonali)	6	1 (16.7)	*C. baileyi* (1)
		Chicken (broiler)	3	0	
		Pigeon	2	1 (50)	*C. meleagridis* (1)
Borobag	LBM 3	Chicken (sonali)	5	2 (40)	*C. meleagridis* (2)
		Chicken (broiler)	3	1 (33.4)	*C. baileyi* (1)
Mirpur-10	LBM 4	Chicken (sonali)	5	1 (20)	*C. meleagridis* (1)
		Chicken (broiler)	3	1 (33.4)	*C. meleagridis* (1)
		Chicken (layer)	2	0	
		Pigeon	2	1 (50)	*C. baileyi* (1)
West agargaon	LBM 5	Chicken (sonali)	5	2 (40)	*C. baileyi* (2)
Kollyanpur	LBM 6	Chicken (sonali)	8	0	
		Chicken (layer)	1	0	
Paikpara	LBM 7	Chicken (broiler)	4	0	
		Chicken (layer)	5	1 (20)	*C. meleagridis* (1)
Tolarbag	LBM 8	Chicken (broiler)	6	0	
		Chicken (sonali)	3	1 (33.4)	*C. meleagridis* (1)
New Market	LBM 9	Chicken (sonali)	9	2 (22.3)	*C. baileyi* (2)
		Chicken (native)	4	1 (25)	*C. meleagridis* (1)
Mirpur-7 masjid	LBM 10	Chicken (sonali)	18	2 (11.2)	*C. baileyi* (1), *C. parvum* (1)
Mirpur new society	LBM 11	Chicken (broiler)	10	0	
Mirpur-6	LBM 12	Chicken (broiler)	4	1 (25)	*C. parvum* (1)
		Chicken (sonali)	5	2 (40)	*C. baileyi* (1), *C. meleagridis* (1)
		Chicken (native)	2	0	
Mirpur-2	LBM 13	Chicken (broiler)	10	1 (10)	*C. baileyi* (1)
Kathal bagan	LBM 14	Chicken (broiler)	9	1 (11.2)	*C. meleagridis* (1)
		Chicken (sonali)	1	0	
Farmgate	LBM 15	Chicken (broiler)	11	0	
		Chicken (layer)	4	1 (25)	*C. baileyi* (1)
		Chicken (native)	2	0	
Mohammadpur Krishi	LBM 16	Chicken (sonali)	10	2 (20)	*C. baileyi* (2)
Kalshi kacha	LBM 17	Chicken (sonali)	8	1 (12.5)	*C. baileyi* (1)
Townhall kacha	LBM 18	Chicken (broiler)	10	2 (20)	*C. baileyi* (1), *C. meleagridis* (1)
Sukrabad bazar	LBM 19	Chicken (broiler)	7	2 (28.6)	*C. baileyi* (1), *C. meleagridis* (1)

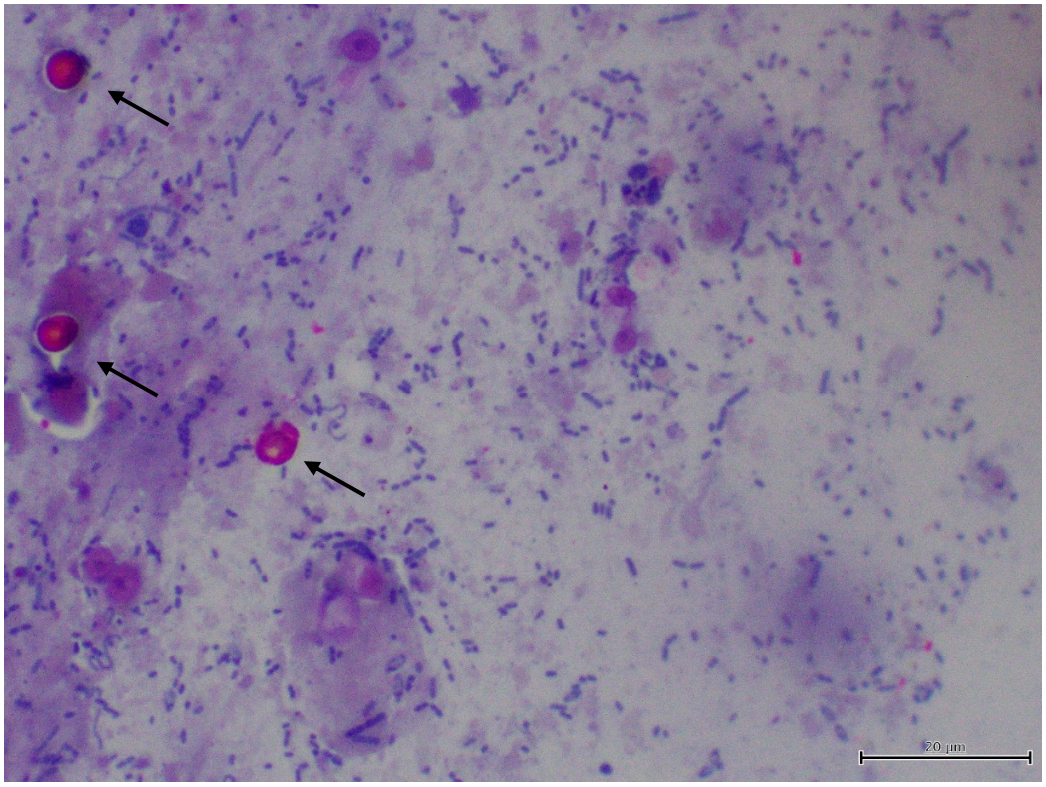

Fig. 2. Microscopic observation of *Cryptosporidium* oocysts. Oocysts were observed by using the MZN method under a microscope.

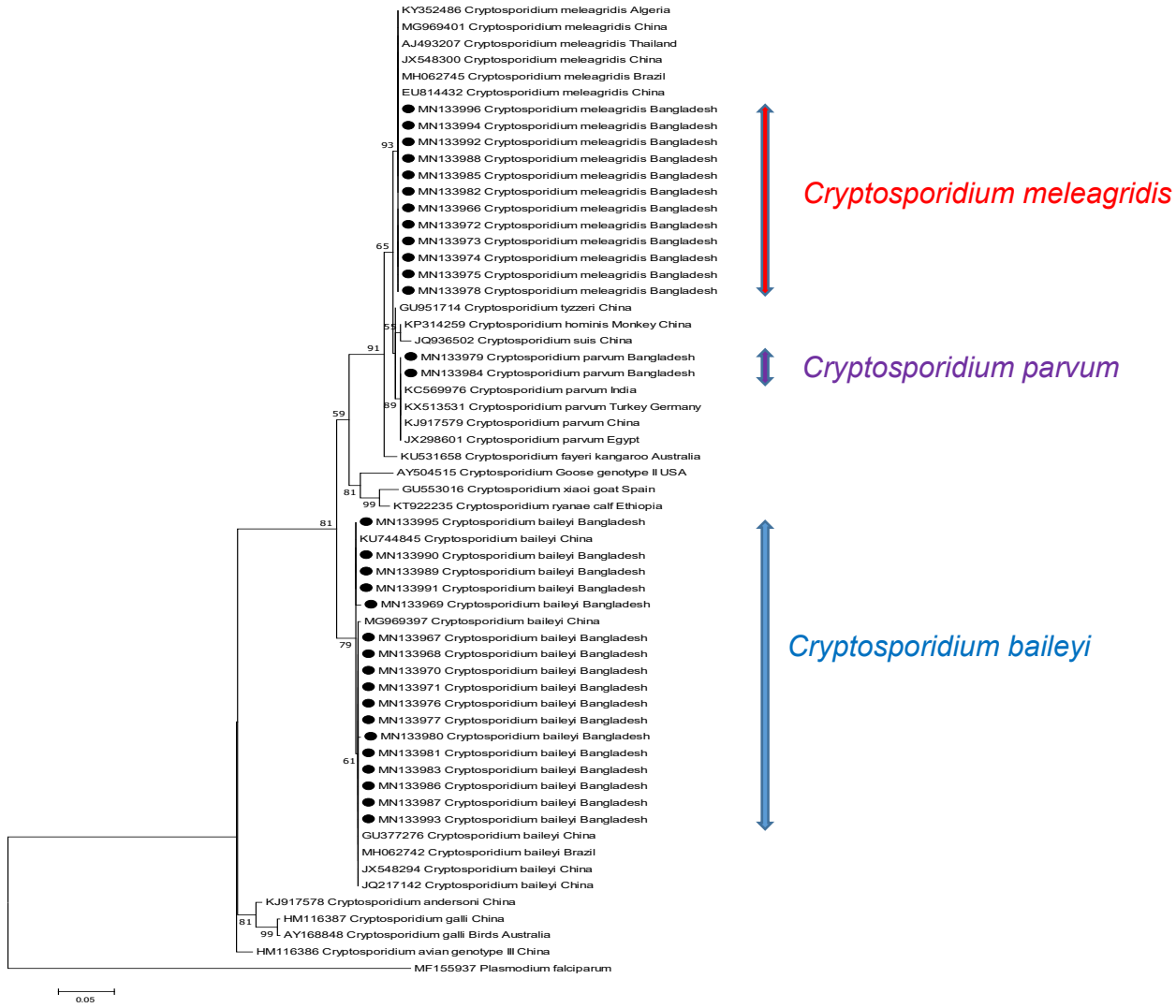

Fig. 3. Phylogenetic tree based on partial sequences of the 18S rRNA genes for *Cryptosporidium* spp. A Phylogenetic tree was constructed without nucleotide gaps using the Maximum Likelihood analysis with 1000 replicates based on the T92+I model [Tamura 1992]. Species, host, region of identification, and GenBank accession number are included. Newly obtained sequences are bolded. The *Plasmodium falciparum* sequence was used as an out-group. Only bootstrap values >50% from 1000 pseudo-replicates are shown. Evolutionary analyses were conducted in MEGA7 [Kumar et al. 2016].

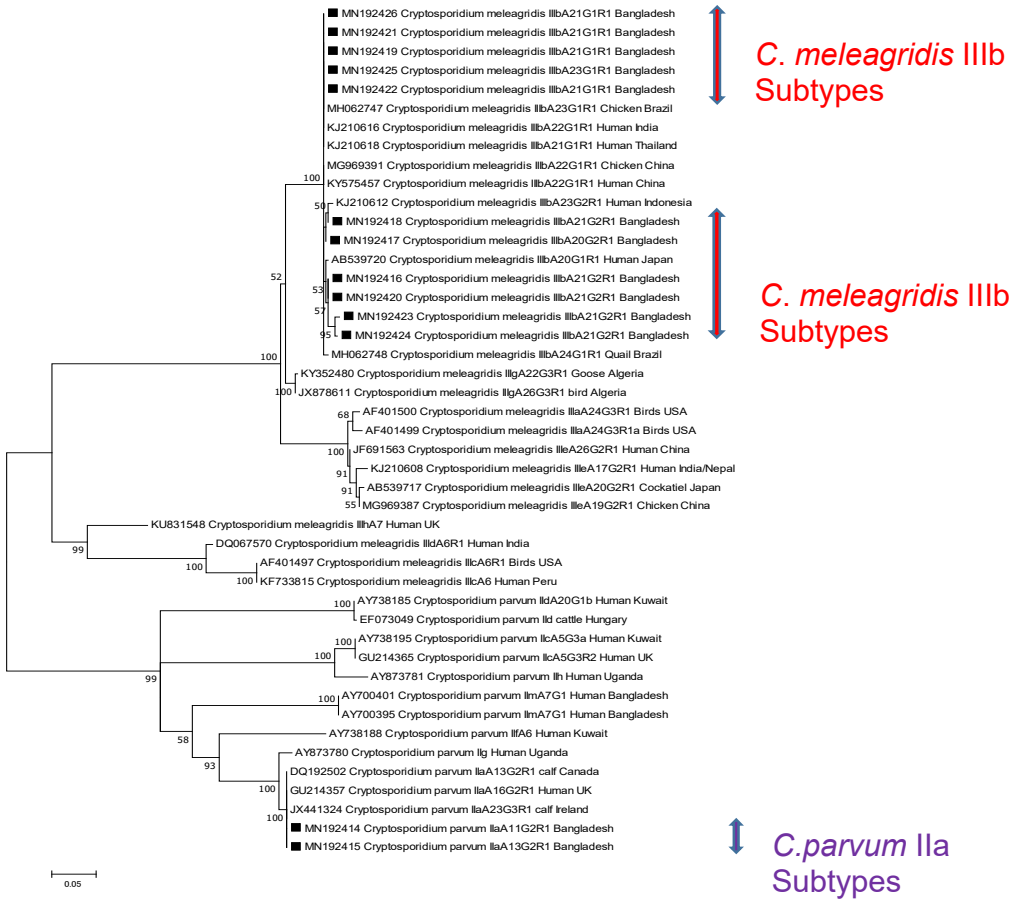

Fig. 4. Phylogenetic tree based on partial sequences of the GP60 genes for *Cryptosporidium* spp. Phylogenetic tree was constructed without nucleotide gaps using the Maximum Likelihood analysis with 1000 replicates based on the Tamura-Nei model [Tamura and Nei 1993]. Subtypes, host, region of identification, and GenBank accession number are included. Newly obtained sequences are bolded. *C. meleagridis and C. parvum* were subtyped in this study by use of reference sequences and observations. Only bootstrap values >50% from 1000 pseudo-replicates are shown. Evolutionary analyses were conducted in MEGA7 [Kumar et al. 2016].

Chapter 2

Distribution of *Cryptosporidium* species isolated from diarrhoeic calves in Japan

2-1. Introduction

Cryptosporidium spp. are common enteric protozoan and well-recognized reasons of diarrheal disease in humans, domestic animals, and wild vertebrates during waterborne epidemics [Checkley *et al.,* 2015]. Cattle are important reservoirs of *Cryptosporidium* spp., as they excrete a large number of oocysts into the environment [Xiao, 2010]. Many *Cryptosporidium* species, like *C. parvum*, *C. bovis*, *C. ryanae,* and *C. andersoni,* are usually observed in ruminants [Ryan *et al.,*2014]. *C. parvum* is the main reason of zoonotic infections in humans worldwide among *Cryptosporidium* [Chalmers *et al.,* 2010; Xiao, 2010].

Cryptosporidium is gradually a well-established reason for both newborn calf diarrhea and non-diarrhea complications, which can make a progressive dehydration, growth restriction, and perhaps even death [Silverlas *et al.,* 2010]. *Cryptosporidium* is spread through the fecal-oral route, and water contamination is responsible for the vital role in this way of spread [Baldursson and Karanis, 2011; Karanis *et al.,* 2007; Moon *et al.,* 2013]. *C. parvum* is dominant in diarrhoeic newborn calves and has been reported as a cause of cryptosporidial infections of humans and animals in Hokkaido [Karanis *et al.,* 2010]. *C. parvum* dominates in newborn calves, *C. bovis* and *C. ryanae* predominate in young calves, and *C. andersoni* is most prevalent in aged calves and adult animals [Fayer *et al.,*2006; Robinson *et al.,*2006; Santín *et al.,*2004], while *C. bovis* and *C.*

ryanae have been found by others through all ages [Feng *et al.,*2007]. *C. parvum* causes watery diarrhoea in infected cattle and in humans [Fayer *et al.,* 2005]. By use of the GP60 gene, the IIa, IId, and IIl subtype families have been detected in calves worldwide, and subtype IIaA15G2R1 is the most frequently reported zoonotic subtype of the IIa subtype family in cattle [Xiao, 2010]. Previous data on *C. parvum* GP60 sequences from Hokkaido [Murakoshi *et al.,* 2013], Gifu [Abe *et al.,* 2006], Hyogo [Satoh *et al.,* 2003], Okinawa [Ichikawa-Seki *et al.,* 2015], and Kagoshima prefectures [Aita *et al.,* 2015], in Japan, suggested that subtype IIaA15G2R1 of *C. parvum* was the only subtype present in these regions. However, the number and distribution of the locations analysed in those studies were insufficient to accurately define the genetic variation of *C. parvum* throughout Japan.

Accordingly, in this present study, I investigated *Cryptosporidium* infections in diarrhoeic pre-weaned calves from several locations and regions of Japan, and analysed the isolates to determine the distribution of *C. parvum* subtypes in calves throughout Japan and also that of other *Cryptosporidium* genotypes in infected calves in Japan.

2-2. Materials and methods

Specimen collection. A total of 80 diarrhoeic faecal specimens from pre-weaned calves were collected in between October 2018 to March 2019 from nine different prefectures in Japan (Fig. 5). All the specimens were collected from farm animals. The ages of calves were in between 3 to 35 days. The dairy calves were mostly Holstein Friesians and the beef calves were mostly the Japanese Black breed. Samples were collected from the rectum of animals or taken from the fresh feces defecated directly from the animals. Fresh faecal specimens were collected after observation of diarrhoea in each animal. For each animal, the sampling date, age, sex, and fecal consistency

were noted. Each specimen was placed into an individual sterile polystyrene tube, labelled, and transferred to an isothermal box, where it was kept at 4 °C prior to being DNA extraction. The Guide for the Care and Use of Field Animal samples of Obihiro University of Agriculture and Veterinary Medicine, Japan were strictly followed. I got approved for all of my experiments by the Ethics committee of animal or pathogen experiments in the Obihiro University of Agriculture and Veterinary Medicine, Japan just as it is followed by chapter 1 (My approval numbers: 290156, 2019724).

DNA extraction. DNA was extracted directly from specimens by using the QIAamp® Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with minor modifications. Briefly, 0. 2 g of E-Z zirconia beads was added to 0.3 g of faeces and 1.4 ml of lysis buffer [McLauchlin *et al.,* 1999]. Then, the mixture was heated at 70 °C for 10 min followed by vigorous shaking using a Biomedicals Fast Prep® instrument (1620 r/min for 150 s) to facilitate oocyst rupture. The nucleic acid was eluted in 100 µl of AE buffer to increase the quantity of DNA recovered. After extraction, the DNA was stored at −20 °C.

Specimen analysis by use of the 18S rRNA and GP60 genes. *Cryptosporidium* spp. were detected by using nested polymerase chain reaction (nested PCR) amplification targeting a ~830-bp fragment of the small subunit 18S rRNA as described previously [Xiao *et al.,* 2001]. For the primary PCR, a PCR product of 1325 bp was amplified using the primers 5'-TTCTAGAGCTAATACATGCG -3' and 5'-CCCATTTCCTTCGAAACAGGA-3'. Then, a nested PCR was done using the primers 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA -3' in order to amplify a ~830-bp fragment of the *Cryptosporidium* 18S rRNA gene. *C. baileyi* genomic DNA and ultrapure water, respectively were used as positive and negative controls. For each specimen, nested PCR detection was repeated

independently at least twice. Positive and negative controls were included in all PCR sets. The amplified fragments were electrophoresed through 1.5% agarose, stained with GelRed® (Biotium), and visualized on a UV transilluminator by using the QIAxcel Advanced system (Qiagen, Valencia, USA). The identity of the *Cryptosporidium* species was confirmed by sequencing the secondary PCR products from the specimens. For *C. parvum* subtyping, nested PCR was performed to amplify an 800-bp fragment of the 60 kDa glycoprotein (GP60) gene [Feng *et al.,* 2007]. Positive and negative controls were again included in all PCR sets.

Sequencing and phylogenetic analyses. All secondary PCR products determined to be *Cryptosporidium*-positive were sequenced in both directions by using an ABI 3130 Genetic Analyzer (Applied Biosystems, Japan) with the secondary primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Japan); cloning was also done when needed. The sequences were assembled by using Codoncode Aligner version7.1.1 software (CodonCode Corporation). The consensus sequences were aligned with homologous sequences published in GenBank by using ClustalX (http://www.clustal.org/) and the BioEdit Sequence Alignment Editor [Hall, 1999]. The acquired sequences were subjected to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to initially define the species/genotypes and to confirm the high similarity and homology with other known sequences of *Cryptosporidium* spp. in GenBank (based on 18S rRNA sequences) and the established *C. parvum* subtype nomenclature system (based on GP60 sequences) [Sulaiman *et al.,*2005]. All sequences were multiple-aligned and analysed by using Bioedit and MEGA 7.0 software (http://www.mbio. ncsu.edu/BioEdit/bioedit.html and http://www.megasoftware. net/). Phylogenetic trees were constructed by using a Maximum Likelihood analysis based on the T92+G model [Tamura, 1992], in MEGA7 [Kumar *et al.,* 2016], using *P. falciparum* as an out-group. To assess the reliability of

this tree, a bootstrap analysis was done with 1000 replicates using the Kimura 3-parameter logarithm.

Nucleotide sequence accession number. The partial 18S rRNA nucleotide sequences and GP60 gene sequences obtained in this study were submitted to the GenBank database under the accession numbers: MN540724- MN540791, and MN561797-MN561843, respectively.

2-3. Results

Identification of *Cryptosporidium* spp. in different types of calves. A total of 83.8% (67/80) of the specimens were positive for *Cryptosporidium* by nested PCR. Of the 67 positives, 66 specimens were obtained in single species infection, and one specimen obtained was in mixed species infection. The genotyping results revealed the presence of three *Cryptosporidium* species, namely *C. parvum*, *C. bovis, and C. ryanae*. *C. parvum* was the leading dominant species detected in 77.5% (31/40) of beef calves and 80% (32/40) of dairy calves. *C. bovis* was also detected, but in only 5.0% (2/40) of dairy calves. Moreover, I detected *C. ryanae* in only 2.5% (1/40) of dairy calves. One mixed-species infection 2.5% (1/40) was found in a beef calf, detected in *C. parvum*, and *C. ryanae* (Table 6).

Distribution of *Cryptosporidium* spp. in different prefectures. This study found *Cryptosporidium* spp. in all nine different prefectures from the northern to the southern regions of Japan. In Fukuoka prefecture, I found the number of *Cryptosporidium*-positive at 27 among 29 specimens. Kagoshima, Hyogo, and Hokkaido prefectures had 13, 10, and 8 *Cryptosporidium*-positive among 14, 10, and 10 number of specimens, respectively. Both Miyazaki and Gifu prefectures had 3 *Cryptosporidium*-positive in each among 3 and 4 specimens, respectively. For Yamagata, Fukushima, and Aichi prefectures shared only 1 *Cryptosporidium*-positive among 1, 8, and 1 number of specimens. *C. parvum* was the most dominant species among these prefectures in

Japan. The *C. bovis* and *C. ryanae* identified for the first time in Gifu prefecture, and *C. ryanae* was detected for the first time in Miyazaki prefecture having mixed infections with *C. parvum*. (Table 7).

Sequencing and phylogenetic analyses. All PCR-positive specimens were sequenced, and analysis of the nucleotide sequences of the 18S rRNA genes revealed the presence of three *Cryptosporidium* species: *C. parvum*, *C. bovis,* and *C. ryanae.* The obtained 18S rRNA gene nucleotide sequences of the identified *Cryptosporidium* isolates were identical to that of *C. parvum*, *C. bovis*, and *C. ryanae* reference sequences available in GenBank, respectively, and they clustered with each of these three species when the 18S rRNA sequence data were analyzed by Maximum Likelihood method (Fig. 6).

Subtyping of *C. parvum*. All 63 *C. parvum*-positive single and one *C. parvum*-positive mixed infections isolates generated the expected GP60 locus. Only 47 isolates were positively sequenced, however, the rests of the 17 isolates are unable to be sequenced because of insufficient DNA in the template. Aligning the sequences obtained with reference sequences downloaded from GenBank showed that isolates belonged to the most common zoonotic subtype IIa family of *C. parvum*. Analysis of the GP60 gene of the *C. parvum* isolates revealed that the subtype IIaA15G2R1 was found in most specimens, and was by far the most prevalent subtype in calves. I also detected other subtypes, namely IIaA14G3R1, IIaA14G2R1, and IIaA13G1R1, which had nucleotide sequences that differed from each other within the trinucleotide TCA and/or TCG repeat region before the R repeat sequence ACATCA (Fig. 7).

2-4. Discussion

Cryptosporidiosis is a common disease among neonatal ruminants and is frequently observed in calves. The present study showed that watery diarrhoea in calves is often caused by

Cryptosporidium spp. In previous reports of 75% and 7% outbreaks of diarrhoea caused by *Cryptosporidium* spp. in calves in Japan [Karanis *et al.,*2010; Murakoshi *et al.,*2013]. In contrast, the occurrence of diarrhoea caused by rotavirus in calves in Japan has been reported to be 9.5% [Okada and Matsumoto, 2002] and 5.3% [Abe *et al.,* 2009]. Severe watery diarrhoea causes dehydration in neonatal calves, which may lead to restricted growth, weight loss, and sometimes death, which in turn, results in significant economic loss.

In this current study, *C. parvum, C. bovis,* and *C. ryanae* were detected in pre-weaned diarrhoeic calves by 18S rRNA gene sequences. Little information is available on clinical symptoms caused *C. bovis* and *C. ryanae*; however, there was a report of diarrhoea possibly due to C. *bovis* and *C. ryanae* infection in native calves reared using traditional husbandry practices [Ayinmode *et al.,*2010]. Here, I detected one mixed infections of *C. parvum* and *C. ryanae* in Miazaki prefecture, related to those described before in Hokkaido prefecture [Murakoshi *et al.,* 2012]. In Japan, only two reports of *C. bovis* have been published, with the first reported detection being in pre-weaned calves in Hokkaido prefecture [Karanis *et al.,* 2010; Murakoshi *et al.,* 2012]. The present study represent the first detection of *C. bovis* in the Gifu prefecture of Japan. Several studies in different countries around the world have reported that the distribution of *C. parvum* is the leading dominant species in pre-weaned calves [Geurden *et al.,* 2007; Imre *et al.,* 2011; Meireles *et al.,*2011; Plutzer and Karanis, 2007; Santín *et al.,*2008]. In the previous report of Hyogo prefecture in Japan, the excretion of *C. parvum* oocysts was detected through feces of 93% calves [Murakoshi *et al.,* 2016]. Moreover, *C. ryanae* was first identified both in adult cattle in Miyagi prefecture [Amer *et al.,* 2009], and in young calves in Hokkaido prefecture [Murakoshi *et al.,* 2012]. In present study, *C. ryanae* was detected for the first time in Miyazaki prefecture. This findings demonstrated that at 1–5 weeks of age, pre-weaned calves appear to be more susceptible

to *Cryptosporidium* infection. In Japan, the *Cryptosporidium* infection rate is reported to be 20% among beef calves [Murakoshi *et al.,* 2012]. In developing countries, particularly Asian countries, there are few reports concerning the distribution of *C. ryanae* and *C. bovis*. *C. bovis* and *C. ryanae* might have low levels of detection in cow-calf grazing beef cattle in Japan [Murakoshi *et al.,* 2012]. Therefore, calves may be potential sources of cryptosporidial infections for humans and other animals in Japan.

Nucleotide sequence analysis of the GP60 gene based on the Tamura-Nei model [Tamura and Nei, 1993], showed that most dominant subtype among the *C. parvum*-positive specimens was subtype IIaA15G2R1. This is the main subtype observed in cattle in several countries [Santín *et al.,* 2008]. The *C. parvum* subtype families can be distinguished based on variations in their gene *GP60* sequences and according to repetition of the trinucleotide region TCA and/or TCG [Sulaiman *et al.,* 2005]. Most of the GP60 nucleotide sequences detected from the different prefectures were identical to each other, and belonged to the IIaA15G2R1 subtype. Previous analyses of the GP60 subtype of *C. parvum* from Gifu, Kobe, Hokkaido, and Kagoshima also described the existence of the IIaA15G2R1 subtype alone [Abe *et al.,* 2006; Murakoshi *et al.,* 2013; Wu *et al.,* 2003]. Although the low number of specimens surveyed in this study, however, I also identified subtype IIaA14G3R1, subtype IIaA14G2R1, and subtype IIaA13G1R1, which have not previously been detected in Japan. The subtype IIaA14G3R1 found in Fukuoka and Kagoshima prefectures, while the subtype IIaA14G2R1 detected in Fukuoka and Miyazaki prefectures along with the subtype IIaA15G2R1. The subtype IIaA13G1R1 identified in Hyogo prefecture beside the subtype IIaA15G2R1. In this study, *C. parvum* genotype and subtypes were not detected in calves in Gifu prefecture. These results indicate that although *C. parvum* with the IIaA15G2R1 subtype is predominant, it is not the only subtype in Japan. Given the zoonotic nature of subtype

IIaA15G2R1 [Xiao, 2010], this is considered to think about the public health aspects of cryptosporodial infections in calves in Japan. This study revealed that *C. parvum is* a widely distributed *Cryptosporidium* species in newborn diarrhoeic calves in Japan. In addition, three different *C. parvum* subtypes, namely IIaA14G3R1, IIaA14G2R1, IIaA13G1R1, were found along with the subtype IIaA15G2R1. Considering the zoonotic significance of the *C. parvum* IIa subtypes, more intensive surveillance of *Cryptosporidium* species is necessary in Japan.

2-5. Summary

In conclusion, a nested PCR aiming the small subunit 18S rRNA and GP60 genes were used to detect the *Cryptosporidium* genotypes and subtypes in calves in Japan. 83.8% (67 out of 80) of the specimens were positive for *Cryptosporidium* spp.; *Cryptosporidium* was found in both beef and dairy calves. *C. parvum* was the major species, identified in 77.5% (31/40) of beef calves and 80% (32/40) of dairy calves. *C. bovis* was also detected, 5.0% (2/40) of dairy calves, and *C. ryanae* was also found 2.5% (1/40) of dairy calves. One mixed-species infection, 2.5% (1/40) was detected in a beef calf having *C. parvum*, and *C. ryanae*. I detected the most common subtype of *C. parvum* (i.e., IIaA15G2R1), as well as other subtypes (i.e., IIaA14G3R1, IIaA14G2R1, and IIaA13G1R1) that have not previously been detected in calves in Japan. My results demonstrate the wide diversity of *Cryptosporidium* infection in calves in Japan.

Table 6. Identification of *Cryptosporidium* spp. in different types of calves.

Types of calf	No. specimens	No. positive (%)			
		C. parvum	*C. bovis*	*C. ryanae*	Mixed[a] *C. parvum* + *C. ryanae*
Beef calves	40	31 (77.5)	0	0	1 (2.5)
Dairy calves	40	32 (80.0)	2 (5.0)	1 (2.5)	0
Total	80	63 (78.8)	2 (2.5)	1 (1.3)	1 (1.3)

[a] The *C. parvum* in mixed infections with *C. ryanae.*

Table 7. Distribution of *Cryptosporidium* spp. in different prefectures of Japan.

Prefectures	No. specimens tested	No. *Cryptosporidium*-positive specimens by species			
		C. parvum	*C. bovis*	*C. ryanae*	Mixed[a] *C. parvum*+ *C. ryanae*
Hokkaido	10	8	-	-	-
Yamagata	1	1	-	-	-
Fukushima	8	1	-	-	-
Gifu	4	-	2	1	-
Aichi	1	1	-	-	-
Hyogo	10	10	-	-	-
Fukuoka	29	27	-	-	-
Miyazaki	3	2	-	-	1
Kagoshima	14	13	-	-	-

[a] The *C. parvum* in mixed infections with *C. ryanae.*

Fig. 5. Sampling areas of Japan. A total of 80 diarrhoeic faecal specimens were collected from pre-weaned calves of nine different prefectures in Japan. The sampling areas in Japan are indicated in red pins.

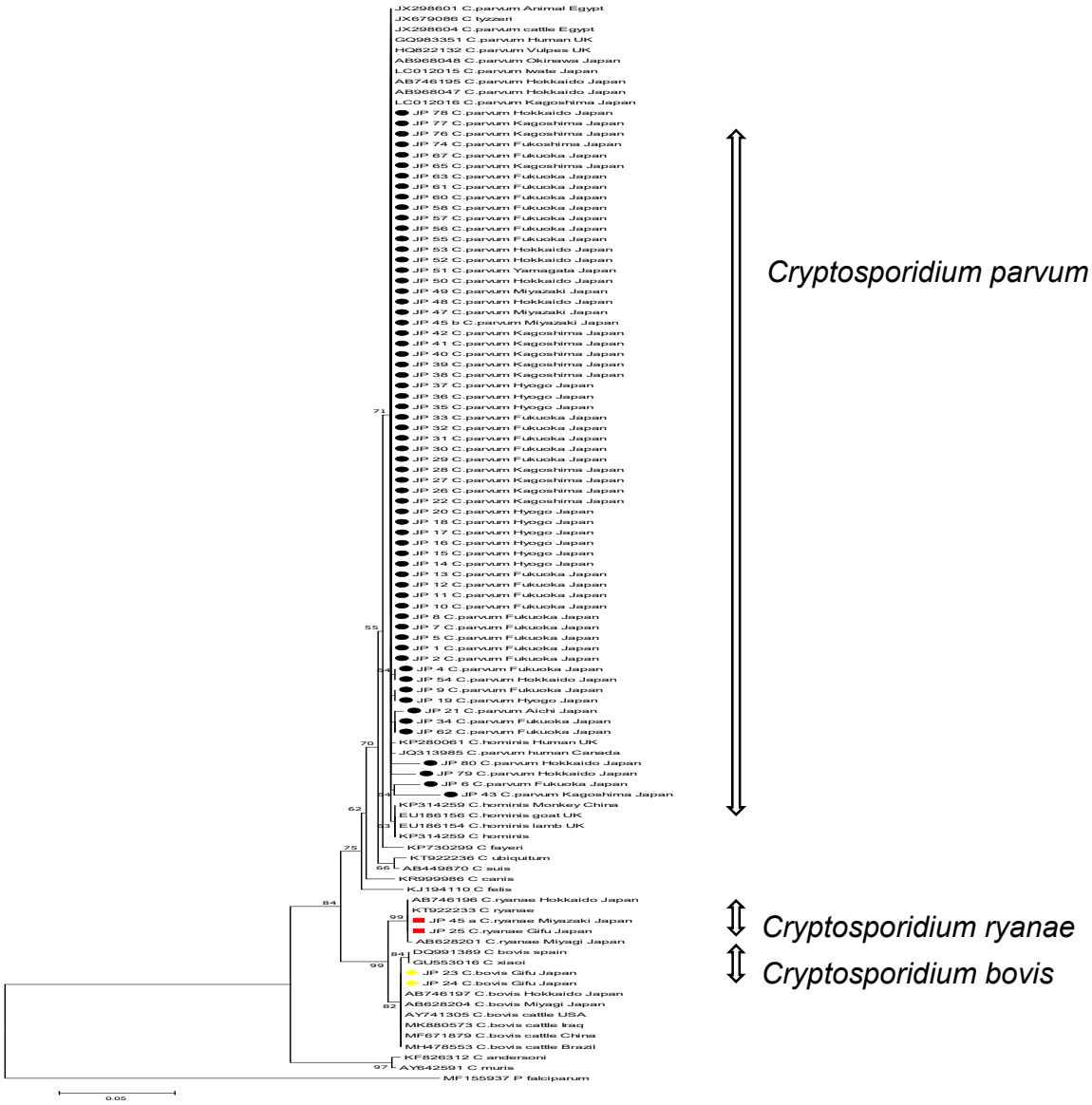

Fig. 6. Phylogenetic tree based on partial sequences of the 18S rRNA genes for *Cryptosporidium* spp. The tree was constructed without nucleotide gaps by using a Maximum Likelihood analysis with 1000 replicates based on the T92+G model. Only bootstrap values >50% from 1000 pseudo-replicates are shown. Evolutionary analyses were conducted in MEGA7.

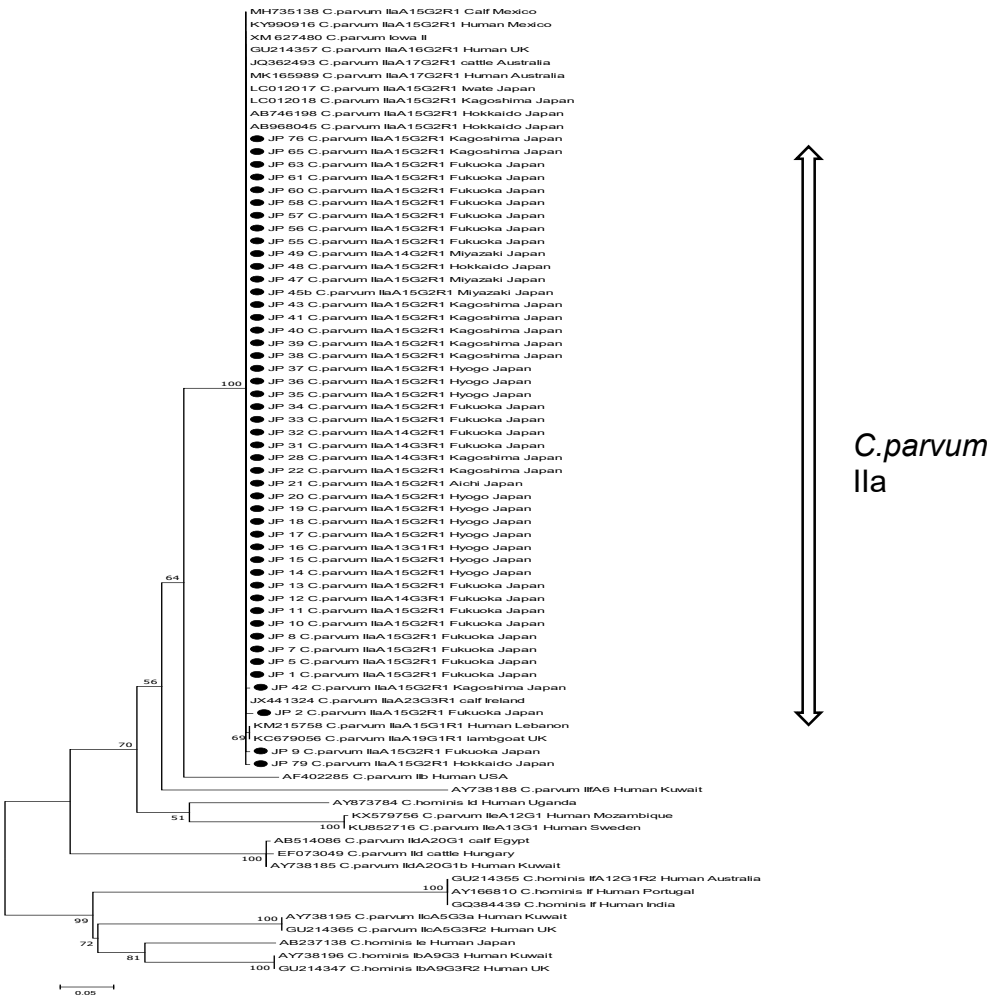

Fig. 7. Phylogenetic tree based on partial sequences of the GP60 genes for *Cryptosporidium* spp. The tree was constructed without nucleotide gaps by using a Maximum Likelihood analysis with 1000 replicates based on the Tamura-Nei model. Only bootstrap values >50% from 1000 pseudo-replicates are shown. Evolutionary analyses were conducted in MEGA7.

Chapter 3

Molecular detection of genotypes and subtypes of *Cryptosporidium* infection in diarrheic calves, lambs, and goat kids from Turkey

3-1. Introduction

Cryptosporidiosis is a primary disease playing a role in the causes of newborn diarrhea disorder of ruminants and causes severe illness or death in young animals [de Graaf *et al.,* 1999]. Healthy and diarrheic young calves may facilitate the spread of cryptosporidiosis in both humans and animals [Diaz *et al.,* 2018]. Other livestock are also potential reservoirs of this protozoan. In cattle herds, infected animals, particularly diarrheic calves, may cause to infect directly for other livestock [Olson *et al.,* 2004]. Also, secondary transmission by cattle sheds, bedding, pasture, and soil and contaminated drinking water from environmental oocysts, has been described as a major source of bovine transmission [Wells and Thompson, 2014]. Lambs and goat kids are similar to calves about the clinical progress of the disease.

The diversity of *Cryptosporidium* species like *C. parvum*, *C. xiaoi*, and *C. ubiquitum* are identical both in goat kids and lambs [Diaz *et al.,*2015; Kaupke *et al.,*2017; Papanikolopoulou *et al.,*2018; Quílez *et al.,*2008; Rieux *et al.,*2013; Tzanidakis *et al.,*2014]. However, geographical changes exist in the spreading of *Cryptosporidium* species in lambs. Mostly *C. parvum* is the main species in the European continent whereas *C. ubiquitum* in the American continent, and *C. xiaoi* in developing countries and all of them together found in Australia [Ryan *et al.,* 2014]. Further, European investigation revealed that *C. parvum* was mostly observed in clinically affected lambs,

where *C. ubiquitum* and *C. xiaoi* were usually observed in unaffected lambs [Mueller-Doblies *et al.,* 2008; Quílez *et al.,* 2008].

There is only limited research on *Cryptosporidium* infections in animals of Turkey; however, most of them rely on microscopic observation of oocysts and the antigens found in feces detected by the ELISA method. Since then, *Cryptosporidium* oocysts have been described in animals with or without diarrhea from Kars [Sari *et al.,* 2009], Erzurum [Sari *et al.,* 2008], and Van provinces of Turkey [Gul *et al.,* 2008]. Also, the distribution of *C. parvum* has been reported in unconfined newborn diarrheic calves and goat kids in five provinces of Turkey [Taylan-Ozkan *et al.,* 2016]. Recently, a report mentioned that *C. parvum* infection was dominant in newborn calves, however, *C. bovis* and *C. ryanae* were identified in aged calves and heifers in the Mediterranean and Central Anatolia regions of Turkey [Yildirim *et al.,* 2020]. Two studies subtyped *C. parvum*-positive isolates from newborn calves by using gp60 sequence analysis in Kars, Turkey [Arslan and Ekinci, 2012; Tanriverdi *et al.,* 2006].

Accordingly, the aim of this study was to evaluate the zoonotic potentiality of *Cryptosporidium* by identifying the genotypes and subtypes of *Cryptosporidium* spp. in the diarrheic calves, lambs, and goat kids in Turkey.

3-2. Materials and methods

Specimen collection. The hospitals, clinics, and mobile emergency clinics of Selcuk University, Faculty of Veterinary Medicine serve the duty to the local livestock in Konya province, Turkey. Konya is the largest city (38.873 km^2) located in the south central Anatolia region of Turkey, and cattle and small ruminant breeding are carried out intensely in this region. A total of 415 diarrheic fecal specimens from 333 calves, 67 lambs, and 15 goat kids were examined in this

study. In Konya, all the specimens were collected from diarrheic animals which were brought to the clinics for treatment with the main complaint of diarrhea between 2016 and 2018 (Fig. 8). Specimens were collected from fresh feces of the animals. Diarrhea was observed during the time of specimen collection. Ages of the animals ranged from 3 to 30 days. All relevant data were recorded during the sampling time for further analysis. Each specimen was placed into an individual sterile polystyrene tube with 2.5% potassium dichromate, labelled, and kept at 4 °C until DNA extraction. After DNA extraction, the DNA samples were imported into Japan according to the rules and guidelines of the Care and Use of Laboratory Animals of Obihiro University of Agriculture and Veterinary Medicine, Japan. I got approved number for all of my experiments by the Ethics committee of animal or pathogen experiments in the Obihiro University of Agriculture and Veterinary Medicine, Japan just as it is followed by chapter 1and 2 (My approval numbers: 290156, 2019724).

Specimen analyses. Specimens were microscopically examined for *Cryptosporidium* spp. oocysts by use of MZN method [Casemore, 1991]. DNA was extracted directly from the specimens by using the NucleoSpin®Tissue (MACHEREY-NAGEL, Germany) kits according to the manufacturer's instructions [McLauchlin *et al.,* 1999]. After extraction, DNA was stored at −20°C. For genotyping and subtyping of *Cryptosporidium* detection, an approximately 830-bp fragment of the SSU rRNA gene and the gp60 gene were amplified by using KOD FX Neo (TOYOBO, Japan) with primers as described previously [Feng *et al.,* 2007; Sulaiman *et al.,* 2002]. For the primary PCR, a product of 1325 bp was amplified using the following primers: 5'-TTCTAGAGCTAATACATGCG -3' and 5'-CCCATTTCCTTCGAAACAGGA-3'. Then, a nested PCR was done using the following primers: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'- AAGGAGTAAGGAACAACCTCCA -3' to amplify the ~830-bp fragment of the

Cryptosporidium SSU rRNA gene. *C. baileyi* genomic DNA and ultrapure water were used as positive and negative controls, respectively, in all PCR sets. The amplified fragments were electrophoresed in 1.5% agarose, stained with GelRed® (Biotium), and visualized on an UV transilluminator in the QIAxcel Advanced system (Qiagen, Valencia, USA). The identity of each *Cryptosporidium* species was confirmed by sequence analysis of the secondary PCR products from the specimens. The gp60 gene was used for subtyping *C. parvum.* The subtypes were named after sequencing of the gp60 gene of these isolates based on the number of trinucleotide repeats encoding the amino acid serine (TCA, TCG, TCT, and ACATCA) sequence [Sulaiman *et al.,*2005].

Sequencing and phylogenetic analyses. All secondary PCR products were sequenced in both directions by using an ABI 3130 Genetic Analyzer (Applied Biosystems, Japan) with the secondary primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Japan). The sequences were aligned by using Clustal X_2 [Larkin *et al.,* 2007], and the computed sequences were edited by hand with BioEdit 7.0.5.3 [Hall, 1999]. All gaps were eliminated, and SSU rRNA genes were used for the phylogenetic analysis. Maximum Likelihood analyses were performed by using MEGA version 7 software program [Kumar *et al.,* 2016]. Substitution models and optional parameter sets were selected according to the Akaike information criterion. To assess the reliability of the tree, bootstrap analysis was done with 1000 replicates using the same datasets. I constructed two phylogenetic trees: one for the SSU rRNA gene, in which the substitution model and optional parameters were also used on the Tamura 3-Parameter model [Tamura, 1992], and the other for the gp60 gene, in which the General-Time-Reversible model [Nei and Kumar, 2000] was used, incorporating the invariable site and Gamma distribution options.

3-3. Results

Prevalence of *Cryptosporidium* in the collected samples. Of the 415 fecal specimens investigated, 106 (25.6%) were confirmed to be infected with *Cryptosporidium* species by both microscopy and nested PCR. Two *Cryptosporidium* species, namely *C. parvum* and *C. bovis* were found in calves using SSU rRNA gene analyses, accounting for 27.1%, and 0.3%, respectively. Only *C. parvum* was found in lambs and goat kids using SSU rRNA gene analyses, accounting for 19.4%, and 13.4%, respectively (Table 8). I could not find any samples that was positive in PCR and negative in microscopic observation or vice versa. Moreover, I found the single-species infection in all individual infected animals, while no mixed species infection was detected. Among the specimens, *Cryptosporidium* was found more frequently in 3–7-day-old calves (30.8%) than in 8–15-day-old calves (26.3%) and 16–30-day-old calves (17.8%). *Cryptosporidium* prevalence was also higher in younger lambs: 23.1% in 3–7-day-old lambs compared with 14.2% in both 8–15-day-old lambs and 16–30-day-old lambs. However, in goat kids, 12.5% of specimens from 3–7-day-old animals were *Cryptosporidium*-positive compared with 16.7% of specimens from 8–15-day-old animals (Table 9).

Phylogenetic analyses. All PCR-positive specimens were directly sequenced; analyses of the nucleotide sequences of the SSU rRNA genes reported the presence of *C. parvum* in calves, lambs, and goat kids, and the presence of *C. bovis* in one calf in Turkey. Fragments of the SSU rRNA gene sequences of *Cryptosporidium* spp. acquired here were placed in GenBank (MN918153-MN918257 and MN918118). Based on a blast search, all of the sequences detected here were identified as *Cryptosporidium.* The nucleotide sequence of *C. parvum* had 99.5% to 100% genetic identity with the reference sequences previously published in GenBank (JX298604, KF533079, JQ313985, GQ983351, and MK731971). Among the *Cryptosporidium* partial SSU

rRNA gene sequences, only one sample had 100% identity with the *C. bovis* reference sequences in GenBank (AB777173, AB628204, EU408317, MK880573) (Fig. 9).

All of the *C. parvum*-positive specimens generated the expected gp60 PCR product. However, only 82 of the 105 isolates the gp60 gene were successfully sequenced and deposited in GenBank (MN962650-MN962718, MN998529-MN998541). However, the rests of the 23 isolates are unable to be sequenced because of insufficient amount of template DNA or poor-quality DNA in the template, and sometimes the template does not contain a sequence complementary to the primer. Inside the *C. parvum* IIa family, all sequences were same in the non-repeat region while three subtypes (IIaA13G4R1, IIaA12G3R1, and IIaA11G3R1) differed from reference sequences regarding the trinucleotide region of TCA and/or TCG repeats and were considered novel *C. parvum* subtypes. Eight subtypes were identified among the calf *C. parvum* specimens, including two novel (IIaA12G3R1 and IIaA11G3R1) subtypes and three other IIa subtype families (IIaA13G2R1, IIaA15G2R1, and IIaA11G2R1) and three IId subtype families (IIdA16G1, IIdA18G1, and IIdA22G1). The sequence difference from each subtype was mostly in the number serine-coding trinucleotide repeat region (i.e. had one copy of sequence ACATCA immediately after the trinucleotide repeats), containing 1, 2, 3 and 4 copies of the TCG repeat and 11, 12, 13, 14 and 15 copies of TCA within the IIa family, however, one copy of TCG and 16, 18 and 22 copies of TCA repeat region within the IId family.

Moreover, six subtypes were found in the lamb *C. parvum* samples, with two novel subtypes (IIaA13G4R1 and IIaA12G3R1) and four other IIa subtype family (IIaA15G2R1, IIaA13G2R1, IIaA14G3R1, and IIaA11G2R1). In addition, I identified two subtypes (IIaA14G1R1 and IIaA13G2R1) in the goat kid *C. parvum* specimens (Fig. 10 and 11).

3-4. Discussion

In this study, *Cryptosporidium* prevalence was found 25.6%, which is consistent with previous studies conducted in Turkey. Except for one calf which was infected with *C. bovis*, all of the *Cryptosporidium* from 90 calves, 13 lambs and 2 goat kids were of *C. parvum*. In Turkey, there is only one study reporting *C. bovis* in calves in the Mediterranean and Central Anatolia regions [Yildirim *et al.,* 2020]. In another report, *C. bovis* was detected in environmental water in Turkey [Koloren and Ayaz, 2016]. *C. parvum* was detected marginally in calves and cattle in northeastern Turkey [Gunduz and Arslan, 2017; Tanriverdi *et al.,* 2006]. *C. parvum* infections commonly cause profuse watery diarrhea which sometimes contains mucus or blood, dehydration, abdominal pain, loss of appetite, and weight loss. The disease causes severe illness and death in young ruminants. Therefore, *Cryptosporidium* infections drive to the real financial crisis [de Graaf *et al.,* 1999]. *C. parvum* is not host-specific; accordingly, an environment contaminated with oocysts during an outbreak in calves can give rise to infection in lambs and goat kids that subsequently use the same grazing area.

In Turkey, *Cryptosporidium* prevalence was observed as 38.8% in diarrheic lambs inspected by the modified acid-fast (MAF) method in the northeastern region of Anatolia in Turkey. Also, the excretion of oocysts was observed higher rates in 1st week age groups and lower rates in 3rd week age groups [Sari *et al.,* 2009]. However, similarly, the current study showed the *Cryptosporidium* infection positivity was higher in 3-7 days of age and lower in 16-30 days of age in calves, lambs, and goat kids. Another study was performed in the locality of Erzurum province in calves which were no more than three months old, and *Cryptosporidium* spp. was detected 22.8% examined under a microscope by using the MAF staining technique [Sari *et al.,* 2008]. Moreover, another report mentioned *Cryptosporidium* prevalence was determined as 13.19% in

older calves in Van province in Turkey by the MAF technique [Gul *et al.,* 2008]. Also, another study determined the *Cryptosporidium* prevalence infections in calves under the farm or rural community at the Kars Province by MAF and ELISA method. The incidence of *Cryptosporidium* in calves was 3.8% with the MAF method and 5.1% with ELISA. *Cryptosporidium* was observed 5.5% and 7.5% in 3 months aged diarrheic calves, and 3.3% in between 3-6 months aged calves [Gunduz and Arslan, 2017]. Even though the specificity of this staining technique is 100%, however, lack of its sensitivity is 44.0% comparison to nested PCR. The report mentioned that nested PCR is a more appropriate technique for understanding the causes of uncertain diarrheic cases [Sungur *et al.,* 2008]. Most of these studies in Turkey rely on microscopic examination of fecal specimens. Nested PCR protocol was more sensitive and specific for detecting *Cryptosporidium* oocysts than microscopic technique [Mirhashemi *et al.,* 2015]. However, both microscopy and nested PCR technique were used to identify the infections in the current study. Moreover, *Cryptosporidium* infection rates in Europe have been reported as follows: 38.8% of calves in Italy [Diaz *et al.,*2018], 37% and 12% of dairy and beef calves, respectively, in Belgium [Geurden *et al.,*2007], 36.7% of calves in Sweden [Bjorkman *et al.,*2015], 22.5% of calves in Poland [Kaupke and Rzeżutka, 2015], 19.2% of lambs and 37.1% of goats in Poland [Kaupke *et al.,*2017], 74.4% of lambs and 93.8% of goat kids in Spain [Diaz *et al.,*2015], 13.1% of lambs and 9.5% of goat kids in Belgium [Geurden *et al.,*2008], and 5.1% of lambs and 7.1% of goat kids in Greece [Tzanidakis *et al.,*2014]. Since the discussion above, clearly pointed the *Cryptosporidium* infections are widespread in calves, lambs, and goat kids in Europe, with Turkey.

Each gp60 subtype family has multiple subtypes, and first a subtype family is identified from the amino acid sequence which is notably different from those seen in other subtype families of a conserved region. Variation in a number of trinucleotide repeat region (counts of

TCA/TCG/TCT repeats after the subtype family name), then identifies subtypes within each family [Chalmers *et al.,* 2019]. Here, the results of gp60 sequence analysis pointed out the important genetic variability in the occurrence of two novel subtypes (IIaA12G3R1 and IIaA11G3R1) of the IIa subtype family of *C. parvum* in calves. Most of the calves were found to be infected with subtype IIaA13G2R1, which was common subtype in all three hosts and the subtype found firstly in lambs in Turkey. Subtype IIaA13G2R1 was previously identified in uncaged newborn diarrheic calves and goat kids in Turkey [Taylan-Ozkan *et al.,* 2016]. Moreover, IIaA13G2R1 was found the major subtype in pre-weaned diarrheic calves in the Central Anatolia region of Turkey [Yildirim *et al.,* 2020]. This uncommon zoonotic subtype was also reported in Algerian young calves, lambs, and goat kids [Baroudi *et al.,* 2018; Benhouda *et al.,* 2017]. Moreover, there are only a few reports of this uncommon subtype in a marginal quantity of calves in Belgium, Canada, and the Netherlands [Geurden *et al.,* 2007; Trotz-Williams *et al.,* 2006; Wielinga *et al.,* 2008]. The second-most common subtype identified in this study, IIaA15G2R1 which is the most common subtype found worldwide, has been reported previously in cattle and diarrheic calves in Kars province of Turkey [Arslan and Ekinci, 2012]. The occurrence of subtype IIaA15G2R1 in calves and lambs worldwide and its exposure in humans show that it spreads simply in animals and freely transmits to humans [Santín and Trout, 2007]. Subtype IIaA11G2R1 has not previously been reported in Turkey; however, it has been reported in humans in Slovenia and Slovakia [Hatalova *et al.,* 2019; Soba and Logar, 2008].

The IId subtype family (IIdA16G1, IIdA18G1 and IIdA22G1) of *C. parvum* was also reported in calves in my study. Subtypes IIdA18G1 and IIdA22G1 have previously been detected in calves and goat kids in Turkey [Taylan-Ozkan *et al.,* 2016]. In a report conducted in Greece, IIdA16G1 subtype was identified in diarrheic lambs and goat kids [Papanikolopoulou *et al.,* 2018].

Within the subtype IId family, IIdA16G1, IIdA18G1, and IIdA22G1 subtypes have been reported before in calves, lambs, and goat kids in Spain [Quílez *et al.,* 2008]. *C. parvum* IId subtypes were observed at low volume in calves in several European nations [Bjorkman *et al.,* 2015; Broglia *et al.,* 2008; Geurden *et al.,* 2007; Plutzer and Karanis, 2007]. The newly detected three subtypes (IIaA11G2R1, IIaA14G3R1, and IIdA16G1) were found in young ruminants for the first time in Konya province, Turkey. It is not confirmed to say how these new subtypes come, but probably it might be come through imported cattle from a neighbored country like Greece or contaminated water or food of these imported animals. Moreover, it can attribute to the lack of regulations on animal movements especially in the borderline of eastern and south-eastern Anatolia regions of Turkey.

It was elucidated that, the dominant *Cryptosporidium* species was *C. parvum* in the calves, lambs, and goat kids in the present study. Two novel subtypes (IIaA13G4R1 and IIaA12G3R1) were identified among the lambs. Here, the dominant subtype was IIa family found, either in lambs and or goat kids. IIa and IId subtypes were found previously in diarrheic goat kids in Turkey [Taylan-Ozkan *et al.,* 2016]. However, the IId subtype was dominant either in lambs and or goat kids in northeastern Spain [Quílez *et al.,* 2008]. Interestingly, subtype IIaA14G3R1, identified in lambs in this study was previously found in fresh molluscan shellfish in Italy [Giangaspero *et al.,* 2014]. The difference in subtype distribution might be due to the least cross-species transmission of *C. parvum* in calves, lambs, and goat kids. Importantly, *C. parvum* observed in pregnant women, children, and an infant in Turkey, however lacking subtype information [Dogan and Saglik, 2010; Hizli *et al.,* 2006; Tamer *et al.,* 2007]. The common available subtype alleles of *C. parvum* are IIa, IIc, IId, and IIe. In humans and animals, globally the major dominant subtype allele is IIa however in Asia, Australia, Europe, and North Africa, IId is the most zoonotic subtype family [Huang *et al.,*

2018]. Animals infected with these subtypes probably pose a threat to human health in Turkey. The zoonotic *C. parvum* subtype families (IIa, IId) identified here proposed that calves, lambs, and goat kids are probably a major reservoir of *C. parvum*. The present study showed the molecular characterization of diversified *Cryptosporidium* isolated in Turkey; *C. bovis* was identified in a calf, and three novel subtypes (IIaA13G4R1, IIaA11G3R1, and IIaA12G3R1) were found. Further, it would be better to go forward epidemiological analysis of cryptosporidiosis in young ruminants based on the molecular approach in different provinces in Turkey.

3-5. Summary

In conclusion, this current study revealed that 25.6% (106 of 415) of the specimens were positive for *Cryptosporidium* spp. infection. I identified 27.4% (91/333), 19.4% (13/67), and 13.4% (2/15) of positivity in calves, lambs and goat kids, respectively. Genotyping of the SSU rRNA indicated that almost all positive specimens were of *C. parvum*, except for one calf which was of *C. bovis*. Sequence analysis of the gp60 gene revealed the major zoonotic subtypes (IIa and IId) of *C. parvum.* I detected 11 subtypes (IIaA11G2R1, IIaA11G3R1, IIaA12G3R1, IIaA13G2R1, IIaA13G4R1, IIaA14G1R1, IIaA14G3R1, IIaA15G2R1, IIdA16G1, IIdA18G1, IIdA22G1); three of them (IIaA12G3R1, IIaA11G3R1 and IIaA13G4R1) was novel subtypes found in calves and lambs. Additionally, three subtypes (IIaA11G2R1, IIaA14G3R1, and IIdA16G1) were detected in young ruminants for the first time in Turkey. These results indicated the high infection of *Cryptosporidium* in Turkey and propose that young ruminants are likely a major carrier of *C. parvum* and a potential source of zoonotic transmission.

Table 8. Prevalence of *Cryptosporidium* spp. in calves, lambs, and goat kids in Turkey.

Animal	No. specimens tested	No. positive (%)		Total positive (%)
		C. parvum	*C. bovis*	
Calves	333	90 (27.1)	1 (0.3)	91 (27.4)
Lambs	67	13 (19.4)	-	13 (19.4)
Goat kids	15	2 (13.4)	-	2 (13.4)
Total	415	105 (25.3)	1 (0.3)	106 (25.6)

Table 9. Distribution of *Cryptosporidium* genotypes, subtypes and positive rates of *Cryptosporidium* spp. in calves, lambs, and goat kids by age.

Animal	Age (days)	No. specimens	No. positive (%) / *Cryptosporidium* spp.	Detection of *C. parvum* gp60 subtypes (No.)
Calves	3–7	159	49 (30.8) / *C. parvum*	IIaA13G2R1 (35) IIaA15G2R1 (4) IIaA11G2R1 (2)
	8–15	129	34 (26.3) / *C. parvum*	IIaA13G2R1 (15) IIaA15G2R1 (2) IIaA12G3R1 (5) IIdA18G1 IIdA16G1 IIdA22G1
	16–30	45	7 (15.6) / *C. parvum*	IIaA12G3R1 (2) IIdA22G1 IIaA11G3R1
			1 (2.2) / *C. bovis*	-
Lambs	3–7	39	9 (23.1) / *C. parvum*	IIaA15G2R1 (3) IIaA13G2R1 (3) IIaA11G2R1
	8–15	21	3 (14.2) / *C. parvum*	IIaA12G3R1 IIaA14G3R1
	16–30	7	1 (14.2) / *C. parvum*	IIaA13G4R1
Goat kids	3–7	8	1 (12.5) / *C. parvum*	IIaA14G1R1
	8–15	6	1 (16.7) / *C. parvum*	IIaA13G2R1
	16–30	1	-	-

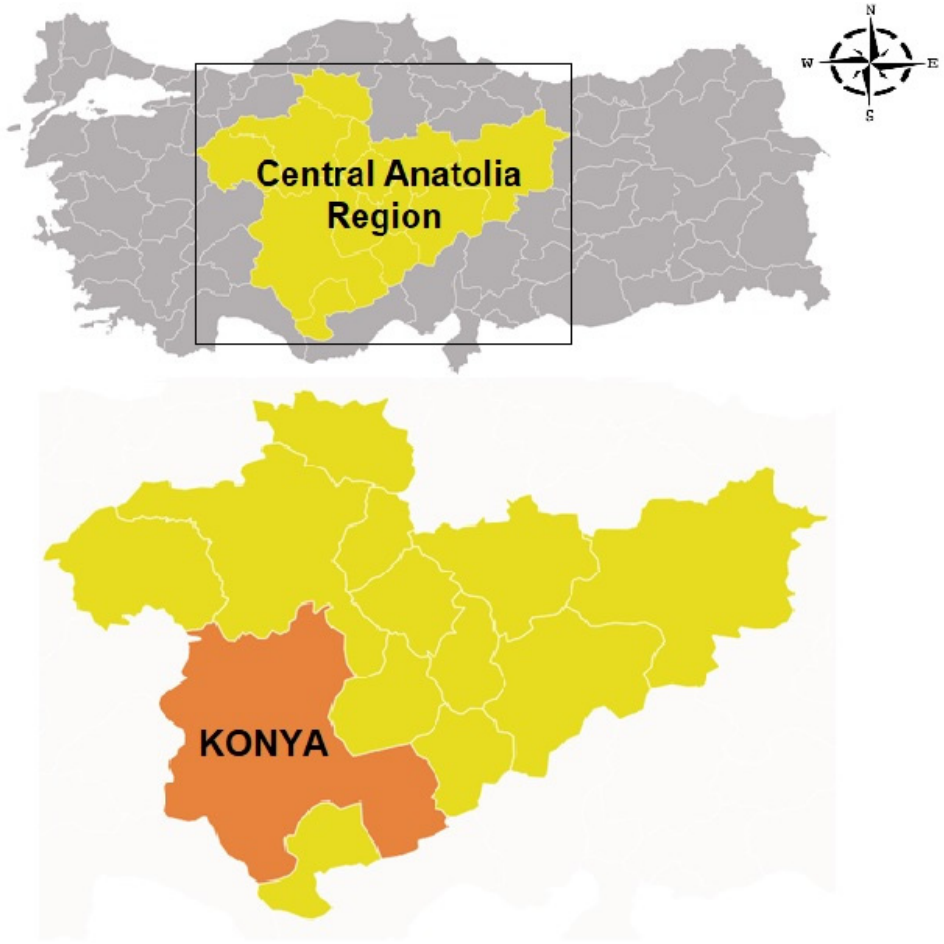

Fig. 8. Map of sampling location in Turkey.

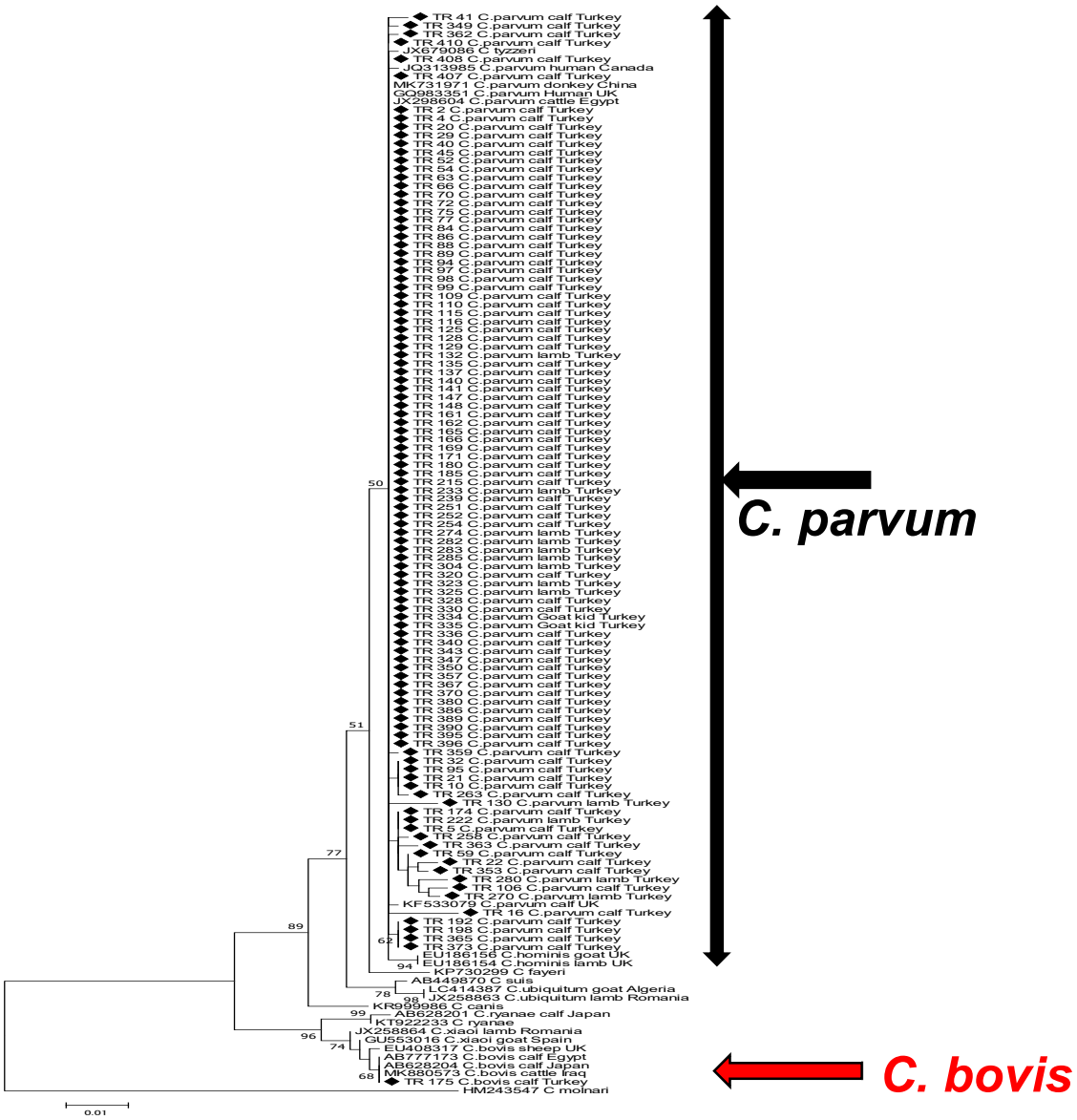

Fig. 9. Phylogenetic tree based on partial sequences of the SSU rRNA genes for *Cryptosporidium* spp. The phylogenetic tree was constructed without nucleotide gaps by using a Maximum Likelihood analysis with 1000 replicates based on the T92+G model. *Cryptosporidium molnari* sequence was used as the out-group. Only bootstrap values >50% from 1000 replicates are shown at the nodes. Black filled bold indicate sequences generated in the present study.

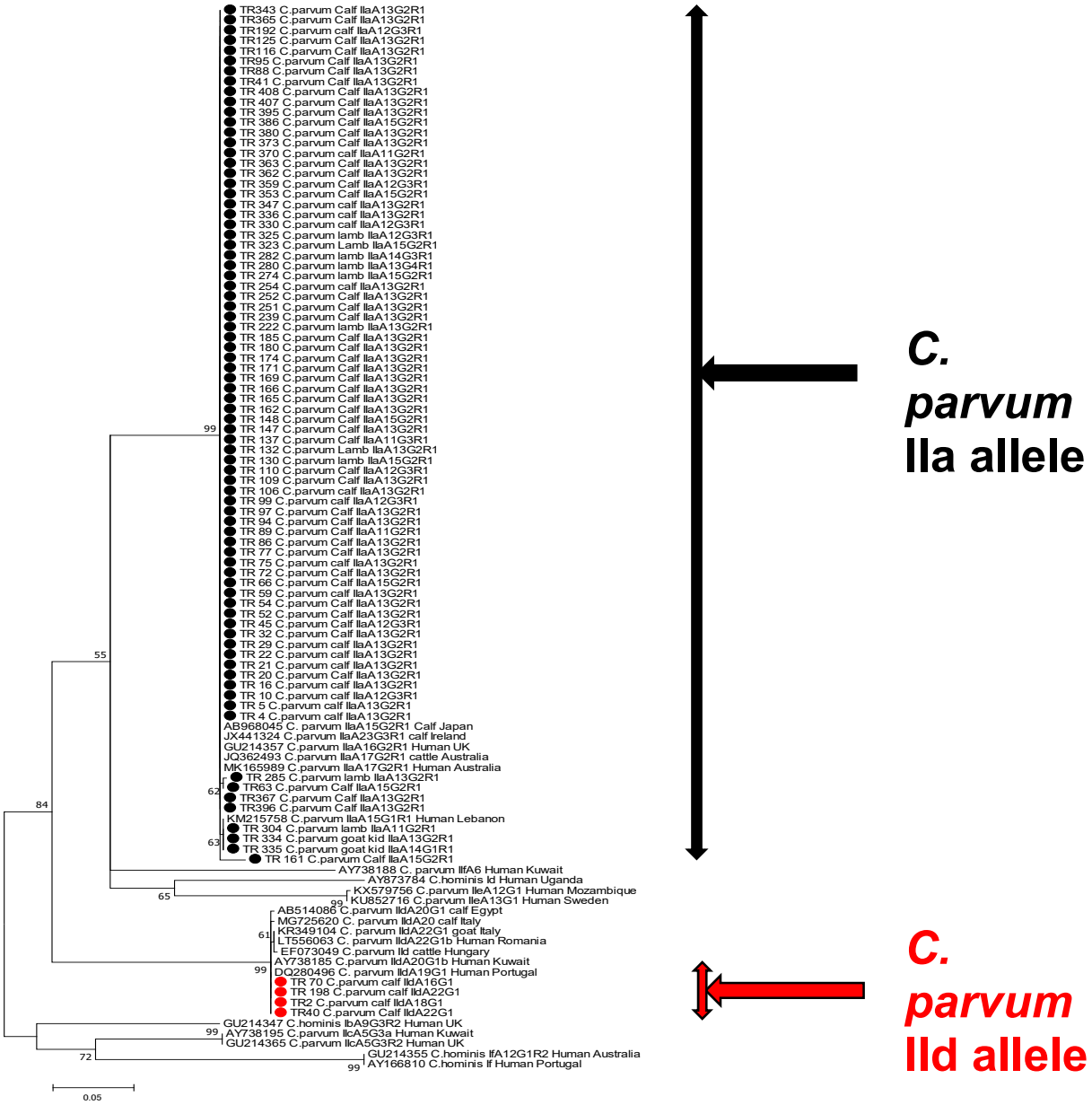

Fig. 10. Phylogenetic tree based on partial sequences of the gp60 genes for *Cryptosporidium parvum*. The phylogenetic tree was constructed without nucleotide gaps by using a Maximum Likelihood analysis with 1000 replicates based on the Hasegawa-Kishino-Yano model. Only bootstrap values >50% from 1000 replicates are shown at the nodes. Black and Red filled circles represent sequences in two different subtype families generated in this study.

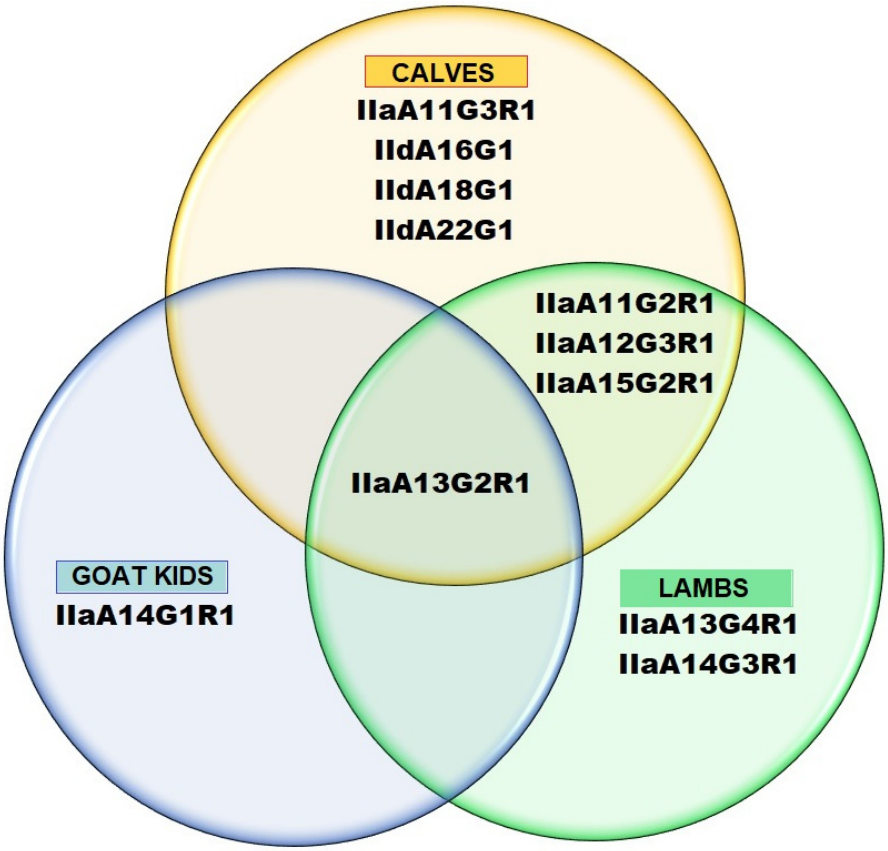

Fig. 11. Distribution of subtypes in the diarrheic calves, lambs and goat kids.

General Discussion

Overall, this study figures out the genetic diversity *Cryptosporidium* genotypes and subtypes family in different hosts and geographically different locations. The first chapter established the detection of poultry cryptosporidiosis in the live bird markets in Bangladesh. In this study, *C. baileyi* was found in chickens and pigeons. *C. baileyi* is responsible for the damage, loss of meat and egg production and respiratory diseases in poultry, especially broiler chickens [Sréter and Varga, 2000]. In the abattoirs, the infection of *C. baileyi* are associated with reduced weight gain, developed lower respiratory problems, enlarged death, and high rate of carcass condemnation found in broiler chickens [Gorham *et al.,* 1988]. *C. meleagridis* was also identified in the current study. *C. meleagridis* infection in native birds increases the chances of possible zoonosis from poultry to humans in this study. The genetic variation of the *C. meleagridis* is essential to illustrate its origin of transmission and infections. For those subtype alleles, anthroponotic and zoonotic pathways exposed were detected both in humans and birds [Stensvold *et al.,* 2014]. The *C. meleagridis* isolates reported in this study is under the family IIIb, few of them have been detected in birds and humans before [Abe and Makino, 2010]. There was no effect of age and breeds on host susceptibility to *Cryptosporidium* infection in this study. Published research explained that the early age of birds is more often susceptible to *Cryptosporidium* than adult birds [Helmy *et al.,* 2017; Wang *et al.,* 2010]. These infections could be the reason for the financial losses of poultry shopkeepers in the live bird markets due to weight loss, even the death of birds. In this study, *Cryptosporidium* infection occurs in all ages of poultry, it might be due to the stress condition during the time of egg and meat growth.

In Germany, the common species recorded in chickens and turkeys were *C. parvum* without

any contamination of feces from mammals [Helmy *et al.,* 2017], which is responsible because poultry might be a carrier for the infection sources and mechanical vectors for other zoonotic *Cryptosporidium*, in addition to *C. meleagridis* [Majewska *et al.,* 2009]. In Bangladesh, *C. parvum* and *C. meleagridis* were identified in children under 2 years of age. Probably, it might be anthropozoonotic; transmitted from chickens that kept in their family house in rural areas [Korpe *et al.,* 2019]. *C. parvu*m infections rate was higher in the people of rural areas, particularly in children than in municipal zones where *C. hominis* dominates [Essid *et al.,* 2008; Llorente *et al.,* 2007]. Some topographical deviations occur in the contamination caused from the diverse *Cryptosporidium* species. For example, there is higher occurrence of *C. meleagridis* contamination than somewhere else in Peru [Cama *et al.,* 2007]. While *C. parvum* is not a common pathogen of poultry, it would be transmitted from sources of contaminated water or shopkeepers who handle this poultry regularly. In Sweden, probably a case of humans got infected with *C. meleagridis* infection through directly touch with infected poultry on a farm [Silverlas *et al.,* 2012]. Here, the *C. parvum* and *C. meleagridis* were identified which were reported earlier as a zoonotic infection. The children of dwellings from the surroundings of LBM are probably more susceptible to get this infection due to unhygienic situation in the LBM and lacking knowledge of infection transmission. It would be a matter of concern that zoonotic *C. parvum* and its IIa subtype found in poultry in the LBM might be a potential risk for the people who regularly deal with.

The second chapter described the region of the study was extended to more prefectures of Japan. Although the constraints of this research on a low number of samples, however, the results were likely to show the recent distribution of bovine cryptosporidiosis in the country, besides, to reveal a high exposure of *C. parvum* infections both in beef and dairy calves. My reports supported the former explanations that *C. parvum* was an extremely dominant pathogen in calves with

diarrhea [Karanis *et al.,* 2010; Murakoshi *et al.,* 2013]. *C. bovis* and *C. ryanae* were also found in calves in low levels of infection in this study. In the developing countries, mostly Asian countries, only a few records about the prevalence of *C. ryanae* and *C. bovis*. *C. ryanae* and *C. bovis* might have low levels of detection in the rearing of cow-calf with browsing beef type cattle in Japan [Murakoshi *et al.,* 2012]. In a developed country, subtype IIaA15G2R1 is overwhelmingly the leading subtype in calves, however, in the United Kingdom, Ireland, and Australia, subtype IIaA18G3R1 is the most recorded subtype [Xiao, 2010]. Most of the GP60 nucleotide sequences detected from the different prefectures were identical to each other and belonged to the IIaA15G2R1 subtype. However, I also identified subtype IIaA14G3R1, subtype IIaA13G1R1, and subtype IIaA14G2R1, in newborn calves in Japan. Here, I identified *C. bovis* and *C. ryanae* in the newborn calf that is unusual for the early age of hosts. Though the early age of calves is probably more susceptible to the *C. parvum* infection while earlier studies supported *C. bovis* and *C. ryanae* were found through all ages of animals [Feng *et al.,* 2007]. These studies demonstrated the *C. parvum* with the IIaA15G2R1 subtype is predominant, however, it is not the only subtype, three more *C. parvum* subtypes, namely IIaA14G3R1, IIaA14G2R1, IIaA13G1R1, were detected in calves for the first time in Japan. It was probably because the study area was extended in more prefecture of Japan. This study identified *Cryptosporidium* infection in both dairy and beef calves. It would need to take careful precautions in the early period of the calves and keep up proper hygiene to control this infection as well as to save the economic losses of farmer.

In chapter 3, the molecular evaluation for the detection of *Cryptosporidium* agents in calves, lambs, and goat kids having diarrhea is discussed. The frequency of *C. parvum* infection differs in between country to country and also the studies. This current study revealed the higher prevalence of *C. parvum* and also detected two zoonotic subtypes of *C. parvum* IIa and IId in young ruminants

in Konya, Turkey. The variability in the earlier prevalence of *C. parvum* reported most likely reflects differences in the strategy of the studies. One study in Australia was carried out to decide the effects of cryptosporidiosis on growth and found lambs that infected with *Cryptosporidium* were lose weight up to 1.65 kg lighter than healthy lambs at slaughter [Sweeny *et al.,* 2011]. Since its detection in cattle in 1971, *Cryptosporidium* is progressively a well-recognized reason for diarrhea in the newborn calf [Silverlas *et al.,* 2010], which leads to progressive dehydration, growth hamper, and perhaps death. The occurrence of cryptosporidiosis relied either on the *Cryptosporidium* species and subtypes or the environmental exposure which determined by the elements such as season, the convenience of water supply and hygiene applies [Leitch and Qing, 2011]. Here, I identified 11 subtypes of *C. parvum* in young ruminants in their early stage of life. Three of them (IIaA12G3R1, IIaA11G3R1, and IIaA13G4R1) were novel subtypes found in calves and lambs. Among the causes of human cryptosporidiosis by these protozoans, direct contact between humans and animals is of great importance or contaminating oocysts of water or food, and this situation poses a risk especially for children, veterinarians, and other people who handle daily and take care of animals. These results indicate the high chances of potential sources of zoonotic transmission from young ruminants to children.

In brief, my study emphasizes the molecular characterization of *Cryptosporidium* parasites. Here, the zoonotic subtype IIa of *C. parvum* was identified in poultry and young ruminants, and another zoonotic subtype IId of *C. parvum* was identified in calves. People and other animals could be infected with this potential zoonotic pathogen. Therefore, it is important to consider *Cryptosporidium* as a risk for both human and animal health, and the economy. It would need to take precautions, growing awareness, and practice of good hygiene everywhere for the people to prevent this zoonotic infection.

General Summary

Cryptosporidium parasites cause an important economic problem in the animal industry sector. Outbreaks of cryptosporidiosis in hosts facilitate by the number of high shedding of environmentally resistant oocysts. The protozoa multiply in the intestine and again form oocysts, which are released from the body along with feces and become a new source of infection. Infection may lead to severe diarrhea with abdominal pain, inability to absorb nutrients from the intestinal tract, and lead to death in young livestock animals with insufficient immunity. Moreover, chlorine disinfection does not kill the oocysts shell. So, *Cryptosporidium* cannot be completely removed at the water purification plant. For this reason, large-scale outbreaks of waterborne infections have been reported all over the world. Moreover, studies of the *Cryptosporidium* species based on the dissemination and transmission pathway are significant in the aspect of public health and food production. The present study performed to evaluate an epidemiological analysis for molecular detection of *Cryptosporidium* samples in poultry and young ruminants. Because of medical and veterinary importance, *Cryptosporidium* is considered one of the recorded vital studied pathogens.

In chapter 1, the first molecular investigation for the identification of *Cryptosporidium* genotypes and subtypes in poultry at open live bird markets in Bangladesh was performed. *C. baileyi*, *C. meleagridis*, and *C. parvum* were found in poultry. Nucleotide sequence analyses of the GP60 gene of the *C. meleagridis* revealed that two subtypes (IIIbA21G1R1 and IIIbA23G1R1) were found in broiler, native and sonali chickens, and a pigeon. Here, I also identified two novel subtypes (IIIbA21G2R1 and IIIbA20G2R1) of *C. meleagridis* in sonali chickens, a broiler chicken, and a layer chicken. Moreover, this study found *C. parvum* IIa subtypes (IIaA11G2R1 and

IIaA13G2R1) in a sonali and a broiler chicken. I can assume that poultry might act as an important source of contaminations of *Cryptosporidium* for human beings and other animals. Besides, the information revealed the recent status of cryptosporidiosis in the live bird market in Bangladesh, and effective control methods were required to decrease financial losses due to the infection.

In chapter 2, the evaluation of *Cryptosporidium* molecular study in many prefectures of Japan was conducted. Consequently, *Cryptosporidium* was found both in beef and dairy calves. The recent study revealed *C. parvum* is widely distributed in the early age of diarrhoeic calves in Japan. *C. bovis* and *C. ryanae* were also detected in dairy calves. One mixed-species infection was detected in a beef calf having *C. parvum*, *and C. ryanae*. I detected the most common subtype of *C. parvum* (i.e., IIaA15G2R1), as well as other subtypes (i.e., IIaA14G3R1, IIaA14G2R1, and IIaA13G1R1) that have not previously been detected in calves in Japan. These records showing the existing distribution of *C. bovis* and *C. ryanae* infection in cattle in Japan was not found in a single prefecture rather extent it to different prefectures. The data demonstrate the wide diversity of *Cryptosporidium* infection in calves in Japan.

In chapter 3, a successful detection system was used to demonstrate the specific occurrence of zoonotic *C. parvum* subtype families (IIa and IId) in Turkey. The recent study demonstrates that calves, lambs, and goat kids are likely a major susceptible of *C. parvum*. *C. bovis* was identified in a calf, and sequence analyses of the gp60 genes presented the most common zoonotic subtypes (IIa and IId) of *C. parvum.* This study detected 11 subtypes (IIaA11G2R1, IIaA11G3R1, IIaA12G3R1, IIaA13G2R1, IIaA13G4R1, IIaA14G1R1, IIaA14G3R1, IIaA15G2R1, IIdA16G1, IIdA18G1, IIdA22G1); three of them (IIaA12G3R1, IIaA11G3R1 and IIaA13G4R1) were novel subtypes found in calves and lambs. Additionally, three subtypes (IIaA11G2R1, IIaA14G3R1, and IIdA16G1) were detected in young ruminants for the first time in Turkey. These results indicated

the considerable occurrence of *C. parvum* infection in Turkey and implied such infection as a national challenge. This study proposed the development of an effective control strategy for cryptosporidiosis via assessing the current situation of the occurrence of *C. parvum* in Turkey.

Overall, all of my studies emphasize the molecular characterization of *Cryptosporidium* parasites. To prevent and control this disease, some steps need to be taken as much as possible. Firstly, the development of vaccines and drugs has been, however, needed a long time. Secondly, it is needed to improve hygiene and sanitation from top to the bottom label of the society because the people are directly or indirectly involved with the animals and the environment. Finally, based on the One Health concept, the authority of the respective region or country should take initiatives to stop the infectious environmental exposure and to control the animal movements especially in the borderline areas, and to practice good hygiene. The government should also take precautions on the management system and publicity for growing awareness to the people in the country to prevent this *Cryptosporidium* infection by proper implementation of laws, hygienic rules, and efficient control approach for cryptosporidiosis.

和文要約

クリプトスポリジウムは、ヒトや家畜を含めた哺乳動物の他に鳥類、魚類などにも寄生する。コクシジウム類に属する腸管寄生原虫で、通常の浄水システムではろ過できない。水や食べ物の中では、殻に覆われたオーシストの形で存在し、体内に入ると小腸の中でオーシストの中にいたスポロゾイドが腸粘膜上皮細胞に侵入する。原虫は腸の中で増殖し、再びオーシストを形成し、これが糞便とともに体外に放出され、新たな感染源となる。感染すると、腹痛を伴う激しい下痢症状が続き、腸管からの栄養吸収ができず、免疫力がまだ十分ではない幼若な畜産動物においては斃死に至ることがある。このことから、クリプトスポリジウム症は世界の畜産業に大きな経済的打撃を与えている。オーシストの殻は塩素消毒でも死滅しない。従って、浄水場ではクリプトスポリジウムを除去しきれない。このことから、世界中で水系感染による大規模な集団感染が報告されてきた。本研究では、家禽類と幼若な反芻類を宿主としてクリプトスポリジウムの分子疫学解析を行った。獣医学分野や医学分野において、クリプトスポリジウムは重要な病原体と考えられる。

第一章では、バングラデシュの鳥肉市場で販売されている鳥肉からクリプトスポリジウムの分子疫学解析を行った。結果として、*Cryptosporidium baileyi*, *C. meleagridis*, *C. parvum* の同定に成功した。次に、*C. meleagridis* の GP60 の遺伝子解析によって、IIIbA21G1R1 と IIIbA23G1R1 のサブタイプに分けられることを明らかとした。両サブタイプは、ブロイラー、地鶏、ソナリ（交雑鶏）、ハトの腸管から同定された。また、*C. meleagridis* の新しいサブタイプとして、IIIbA21G2R1 と IIIbA20G2R1 をソナリ、ブロイラー、産卵鶏から同定した。さらに、*C. parvum* IIa サブタイプ（IIaA11G2R1, IIaA13G2R1）をソナリとブロイラーから同定した。このことは、家禽類がヒトを含めた動物へのクリプトスポリジウムの感染源となり得ることを示唆している。さらに、現状のバングラデシュの鳥肉市場においてはクリプトスポリジウム症蔓延による経済的な損失を防ぐため、公衆衛生上の対策を講ずる必要がある。

第二章では、日本の仔ウシの下痢便を採取し、前例の無い規模でのクリプトスポリジウムの分子疫学調査を行った。*C. parvum* は肉牛と乳牛の両方から同定されたことから、日本の離乳前の仔ウシに *C. parvum* が広く蔓延し、下痢症を起こしていることがわかる。乳牛については *C. bovis*, *C. ryanae* も同定された。一頭の肉牛において、*C. parvum* と *C. ryanae* の混合感染が同定された。*C. parvum* については、最も高頻度で同定されるサブタイプである IIaA15G2R1 とともに、これまで日本のウシでは見られなかったサブタイプである IIaA14G3R1, IIaA14G2R1, IIaA13G1R1 が同定された。これらの結果から、*C. bovis*, *C. ryanae* の日本のウシへの現在の感染状況が明らかとなり、１つの道県に留まらず、複数の県まで感染が広がっていることがわかる。本研究は、日本のウシでのクリプトスポリジウム症の広範な蔓延を示唆するものである。

第三章では、これまで構築したクリプトスポリジウムの分子疫学系を用いて、トルコの仔ウシ、仔ヒツジ、仔ヤギの糞便サンプルから人獣共通感染性である *C. parvum* のサブタイプファミリーである IIa, IId の同定に成功した。本研究の結果から、仔ウシ、仔ヒツジ、仔ヤギが *C.*

parvum の媒介動物になっていることが示唆された。*C. parvum* IIa, IId の同定には、GP60 遺伝子の分子系統樹解析を用いた。一方で、*C. bovis* も仔ウシから同定された。さらに、仔ウシと仔ヒツジの糞便サンプルから、IIaA11G2R1, IIaA11G3R1, IIaA12G3R1, IIaA13G2R1, IIaA13G4R1, IIaA14G1R1, IIaA14G3R1, IIaA15G2R1, IIdA16G1, IIdA18G1, IIdA22G1 の 11 のサブタイプを同定した。この中の３サブタイプ（IIaA12G3R1, IIaA11G3R1, IIaA13G4R1）は新しいものである。さらに、３サブタイプ（IIaA11G2R1, IIaA14G3R1, IIdA16G1）はトルコの幼若反芻類からは初めて同定されたものである。以上の結果から、トルコにおいてクリプトスポリジウムの感染は頻繁に起こっており、国の公衆衛生上の対策が求められる。特に、*C. parvum* の発生状況については現在の状況を適切に把握していくことが感染対策につながると考える。

以上のことから、本研究ではクリプトスポリジウム症の同定と遺伝子解析に焦点を当て、解析を行った。バングラデシュにおいては、クリプトスポリジウム症の爆発的な感染を避けるためにも、疫学解析と公衆衛生上の監視を徹底する必要がある。また、日本、トルコにおいても反芻類におけるクリプトスポリジウム症の効率的な感染制御対策の構築を行っていく必要がある。その際に、本研究のデータは大きく貢献することが期待できる。一方で、この重要な原虫病に対抗するため、さらなる解析を行っていく必要がある。

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References

Abal-Fabeiro JL, Maside X, Bello X, Llovo J, Bartolome C. 2013. Multilocus patterns of genetic variation across *Cryptosporidium* species suggest balancing selection at the gp60 locus. Mol. Ecol. 22, 4723–4732.

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

Abe M, Ito N, Morikawa S, Takasu M, Murase T, Kawashima T, Kawai Y, Kohara J, Sugiyama M. 2009. Molecular epidemiology of rotaviruses among healthy calves in Japan: isolation of a novel bovine rotavirus bearing new P and G genotypes. Virus Res. 144, 250–257.

Abe N, Matsubayashi M, Kimata I, Iseki M. 2006. Sub genotype analysis of *Cryptosporidium parvum* isolates from humans and animals in Japan using the 60-kDa glycoprotein gene sequences. Parasitol. Res. 99, 303–305.

Aita J, Ichikawa-Seki M, Kinami A, Yaita S, Kumagai Y, Nishikawa Y, Itagaki T. 2015. Molecular characterization of *Cryptosporidium parvum* detected in Japanese black and Holstein calves in Iwate Prefecture and Tanegashima Island, Kagoshima Prefecture, Japan. J. Vet. Med. Sci. 77, 997–999.

Alves M, Xiao L, Sulaiman I, Lal AA, Matos O, Antunes F. 2003. Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. J. Clin. Microbiol. 41, 2744–2747.

Alves M, Xiao L, Antunes F, Matos O. 2006. Distribution of *Cryptosporidium* subtypes in humans and domestic and wild ruminants in Portugal. Parasitol. Res. 99, 287–92.

Amadi B, Mwiya M, Sianongo S, Payne L, Watuka A, Katubulushi M, Kelly P. 2009. High dose prolonged treatment with nitazoxanide is not effective for cryptosporidiosis in HIV positive

zambian children: A randomised controlled trial. BMC Infect. Dis. 9, 195.

Amer S, Honma H, Ikarashi M, Oishi R, Endo M, Otawa K, Nakai Y. 2009. The first detection of *Cryptosporidium* deer-like genotype in cattle in Japan. Parasitol. Res. 104, 745–752.

Arslan MO, Ekinci AI. 2012. Determination of *Cryptosporidium parvum* subtypes in cattle in Kars province of Turkey. Kafkas Univ. Vet. Fak. Derg. 18, A221–A226.

Ayinmode AB, Olakunle FB, Xiao L. 2010. Molecular characterization of *Cryptosporidium* spp. in native calves in Nigeria. Parasitol. Res. 107, 1019–1021.

Baldursson S, Karanis P. 2011. Waterborne transmission of protozoan parasites: review of worldwide outbreaks—an update 2004–2010. Water Res. 45, 6603–6614.

Baroudi D, Hakem A, Adamu H, Amer S, Khelef D, Adjou K, Dahmani H, Chen X, Roellig D, Feng Y, Xiao L. 2018. Zoonotic *Cryptosporidium* species and subtypes in lambs and goat kids in Algeria. Parasit. Vectors. 11, 582.

Baroudi D, Khelef D, Goucem R, Adjou KT, Adamu H, Zhang H, Xiao L. 2013. Common occurrence of zoonotic pathogen *Cryptosporidium meleagridis* in broiler chickens and turkeys in Algeria. Vet. Parasitol. 196, 334–340.

Benhouda D, Hakem A, Sannella AR, Benhouda A, Cacciò SM. 2017. First molecular investigation of *Cryptosporidium* spp. in young calves in Algeria. Parasite. 24, 15.

Bjorkman C, Lindström L, Oweson C, Ahola H, Troell K, Axén C. 2015. *Cryptosporidium* infections in suckler herd beef calves. Parasitology. 142, 1108–1114.

Bomfim TCB, Gomes RS, Huber F, Couto MCM. 2013. The importance of poultry in environmental dissemination of *Cryptosporidium* spp. Open Vet. Sci. J. 7, 12–17.

Broglia A, Reckinger S, Caccio SM, Nockler K. 2008. Distribution of *Cryptosporidium parvum* subtypes in calves in Germany. Vet. Parasitol. 154, 8–13.

Cama VA, Ross JM, Crawford S, Kawai V, Chavez-Valdez R, Vargas D. 2007. Differences in clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons. J. Infect. Dis. 196, 684–691.

Casemore DP. 1991. Laboratory methods for diagnosing cryptosporidiosis, ACP Broadsheet. J. Clin. Pathol. 44, 445–451.

Chalmers RM, Giles M. 2010. Zoonotic cryptosporidiosis in the UK - challenges for control. J. Appl. Microbiol. 109, 1487–1497.

Chalmers R, Smith R, Hadfield S, Elwin K, Giles M. 2010. Zoonotic linkage and variation in *Cryptosporidium parvum* from patients in the United Kingdom. Parasitol. Res. 108, 1321–1325.

Chalmers RM, Robinson G, Elwin K, Elson R. 2019. Analysis of the *Cryptosporidium* spp. and gp60 subtypes linked to human outbreaks of cryptosporidiosis in England and Wales, 2009 to 2017, Parasit. Vectors. 12, 95.

Chappell CL, Okhuysen PC, Langer-Curry RC, Akiyoshi DE, Widmer G, Tzipori S. 2011. *Cryptosporidium meleagridis*: infectivity in healthy adult volunteers. Am. J. Trop. Med. Hyg. 85, 238–242.

Checkley W, White AC Jr, Jaganath D, Arrowood MJ, Chalmers RM, Chen XM, Fayer R, Griffiths JK, Guerrant RL, Hedstrom L, Huston CD, Kotloff KL, Kang G, Mead JR, Miller M, Petri WA Jr, Priest JW, Roos DS, Striepen B, Thompson RC, Ward HD, Van Voorhis WA, Xiao L, Zhu G, Houpt ER. 2015. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for cryptosporidium. Lancet Infect. Dis. 15, 85–94.

Cho YI, Yoon KJ. 2014. An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. J. Vet. Sci. 15, 1–17.

da Cunha MJR, Cury MC, Santín M. 2018. Molecular characterization of *Cryptosporidium* spp. in poultry from Brazil. Res. Vet. Sci. 118, 331–335.

de Graaf DC, Vanopdenbosch E, Ortega-Mora LM, Abbassi H, Peeters JE. 1999. A review of the importance of cryptosporidiosis in farm animals. Int. J. Parasitol. 29, 1269–1287.

Diaz P, Quilez J, Prieto A, Navarro E, Pèrez-Creo A, Fernández G, Panadero R, López C, Díez-Baños P, Morrondo P. 2015. *Cryptosporidium* species and subtype analysis in diarrhoeic pre-weaned lambs and goat kids from north-western Spain. Parasitol. Res. 114, 4099–4105.

Diaz P, Varcasia A, Pipia AP, Tamponi C, Sanna G, Prieto A, Ruiu A, Spissu P, Díez-Baños P, Morrondo P, Scala A. 2018. Molecular characterization and risk factor analysis of *Cryptosporidium* spp. in calves from Italy. Parasitol. Res. 117, 3081–3090.

Dogan N, Saglik I. 2010. *Cyclospora cayetanensis* and *Cryptosporidium parvum* coinfection in a pregnant women with prolonged diarrhea. Mikrobiyol. Bul. 44, 155–159.

Elliot A, Morgan UM, Thompsom RCA. 1999. Improved staining method for detecting *Cryptosporidium* oocysts in stools using malachite green. J. Gen. Appl. Microbiol. 45, 139–142.

Essid R, Mousli M, Aoun K, Abdelmalek R, Mellouli F, Kanoun F. 2008. Identification of *Cryptosporidium* species infecting humans in Tunisia. Am. J. Trop. Med. Hyg. 79, 702–705.

Fayer R, Santín M, Trout JM, Greiner E. 2006. Prevalence of species and genotypes of *Cryptosporidium* found in 1-2-year-old dairy cattle in the eastern United States. Vet. Parasitol. 135, 105–112.

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

Fayer R, Santín M, Xiao L. 2005. *Cryptosporidium bovis* n. sp. (Apicomplexa: Cryptosporidiidae)

in cattle (Bos taurus). J. Parasitol. 91, 624–629.

Fayer R. 2008. General Biology, in: Fayer R, Xiao L. (Eds.), *Cryptosporidium* and Cryptosporidiosis. CRC Press, Boca Raton. 1–42.

Feng Y, Ortega Y, He G, Das P, Xu M, Zhang X, Fayer R, Gatei W, Cama V, Xiao L. 2007. Wide geographic distribution of *Cryptosporidium bovis* and the deer like genotype in bovines. Vet. Parasitol. 144, 1–9.

Feng Y, Ryan UM, Xiao L. 2018. Genetic diversity and population structure of *Cryptosporidium*. Trends Parasitol. 34, 997–1011

Fujino T, Matsuo T, Okada M, Matsui T. 2006. Detection of a small number of *Cryptosporidium parvum* oocysts by sugar flotation and sugar centrifugation methods. J. Vet. Med. Sci. 68, 1191–1193.

Geurden T, Berkvens D, Martens C, Casaert S, Vercruysse J, Claerebout E. 2007. Molecular epidemiology with subtype analysis of *Cryptosporidium* in calves in Belgium. Parasitology. 134, 1981–1987.

Geurden T, Thomas P, Casaert S, Vercruysse J, Claerebout E. 2008. Prevalence and molecular characterization of *Cryptosporidium* and *Giardia* in lambs and goat kids in Belgium. Vet. Parasitol. 155, 142–145.

Giangaspero A, Papini R, Marangi M, Koehler AV, Gasser RB. 2014. *Cryptosporidium parvum* genotype IIa and *Giardia duodenalis* assemblage A in *Mytilus galloprovincialis* on sale at local food markets. Int. J. Food Microbiol. 171, 62–67.

Glaberman S, Sulaiman IM, Bern C, Limor J, Peng MM, Morgan U, Gilman R, Lal AA, Xiao L. 2001. A multilocus genotypic analysis of *Cryptosporidium meleagridis*. J. Eukaryot. Microbiol. 48, 19S–22S.

Gorham SL, Mallinson ET, Marquardt WW, Savage PK. 1988. Serologic incidence of *Cryptosporidium* in Delmarva broiler flocks. Poult. Sci. 67, 730–735.

Gul A, Cicek M, Kilinc O. 2008. Prevalence of *Eimeria* spp., *Cryptosporidium* spp. and *Giardia* spp. in calves in the Van province. Turkiye Parazitol. Derg. 32, 202–204.

Gunduz N, Arslan MO. 2017. Determining the prevalence of *Cryptosporidium* infections with acid fast staining and ELISA in calves at the Kars province of Turkey. Turkiye. Parazitol. Derg. 41, 5–8.

Guo Y, Cebelinski E, Matusevich C, Alderisio KA, Lebbad M, McEvoy J, Roellig DM, Yang C, Feng Y, Xiao L. 2015. Subtyping novel zoonotic pathogen *Cryptosporidium chipmunk* genotype I. J. Clin. Microbiol. 53, 1648–1654.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Hamidinejat H, Jalali MH, Jafari RA, Nourmohammadi K. 2014. Molecular determination and genotyping of *Cryptosporidium* spp. in fecal and respiratory samples of industrial poultry in Iran. Asian Pac. J. Trop. Med. 7, 517–520.

Hatalova E, Valencakova A, Luptakova L, Spalkova M, Kalinova J, Halanova M, Bednarova V, Gabzdilova J, Dedinska K, Ondriska F, Boldis V. 2019. The first report of animal genotypes of *Cryptosporidium parvum* in immunosuppressed and immunocompetent humans in Slovakia. Transbound. Emerg. Dis. 66, 243–249.

Helmy YA, Krücken J, Abdelwhab EM, Von Samson-Himmelstjerna G, Hafez HM. 2017. Molecular diagnosis and characterization of *Cryptosporidium* spp. in turkeys and chickens in Germany reveals evidence for previously undetected parasite species. PLoS One. 12, e0177150.

Hira KG, Mackay MR, Hempstead AD, Ahmed S, Karim MM, O'Connor RM, Hibberd PL, Calderwood SB, Ryan ET, Khan WA, Ward HD. 2011. Genetic diversity of *Cryptosporidium* spp. from Bangladeshi children. J. Clin. Microbiol. 49, 2307–2310.

Hizli S, Aksoy U, Arslan N, Usluca S, Bekem O, Yalcin G, Ozturk Y. 2006. *Cryptosporidium parvum* diarrhea in an infant with short bowel syndrome. J. Pediatr. Surg. 41, e5–e6.

Huber F, da Silva S, Bomfim TCB, Teixeira KR, Bello AR. 2007. Genotypic characterization and phylogenetic analysis of *Cryptosporidium* sp. from domestic animals in Brazil. Vet. Parasitol. 150, 65–74.

Huang J, Zhang Z, Zhang Y, Yang Y, Zhao J, Wang R, Jian F, Ning C, Zhang W, Zhang L. 2018. Prevalence and molecular characterization of *Cryptosporidium* spp. and *Giardia duodenalis* in deer in Henan and Jilin, China. Parasit. Vectors. 11, 239.

Ibrahim MA, Abdel-Ghany AE, Abdel-Latef GK, Abdel-Aziz SA, Aboelhadid SM. 2016. Epidemiology and public health significance of *Cryptosporidium* isolated from cattle, buffaloes, and humans in Egypt. Parasitol. Res. 115, 2439–2448.

Ichikawa-Seki M, Aita J, Masatani T, Suzuki M, Nitta Y, Tamayose G, Iso T, Suganuma K, Fujiwara T, Matsuyama K, Niikura T, Yokoyama N, Suzuki H, Yamakawa K, Inokuma H, Itagaki T, Zakimi S, Nishikawa Y. 2015. Molecular characterization of *Cryptosporidium parvum* from two different Japanese prefectures, Okinawa and Hokkaido. Parasitol. Int. 64, 161–166.

Imre K, Lobo LM, Matos O, Popescu C, Genchi C, Darabus G. 2011. Molecular characterization of *Cryptosporidium* isolates from pre-weaned calves in Romania: is there an actual risk of zoonotic infections? Vet. Parasitol. 181, 321–324.

Jae-Ran Y, Soo-Ung L, Woo-Yoon P. 2009. Comparative sensitivity of PCR primer sets for

detection of *Cryptosporidium parvum*. Korean J. Parasitol. 47, 293–297.

Jagai JS, Castronovo DA, Monchak J, Naumova EN. 2009. Seasonality of cryptosporidiosis: A meta-analysis approach. Environ. Res. 109, 465–478.

Karanis P, Eiji T, Palomino L, Boonrod K, Plutzer J, Ongerth J, Igarashi I. 2010. First description of *Cryptosporidium bovis* in Japan and diagnosis and genotyping of *Cryptosporidium* spp. in diarrhoeic pre-weaned calves in Hokkaido. Vet. Parasitol. 169, 387–390.

Karanis P, Kourenti C, Smith H. 2007. Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J. Water Health. 5, 1–38.

Kaupke A, Michalski MM, Rzezutka A. 2017. Diversity of *Cryptosporidium* species occurring in sheep and goat breeds reared in Poland. Parasitol. Res. 116, 871–879.

Kaupke A, Rzeżutka A. 2015. Emergence of novel subtypes of *Cryptosporidium parvum* in calves in Poland. Parasitol. Res. 114, 4709–4716.

Kichou F, Saghir F, El Hamidi M. 1996. Natural *Cryptosporidium* sp. infection in broiler chickens in Morocco. Avian. Pathol. 25, 103–111.

Koloren Z, Ayaz E. 2016. Genotyping of *Cryptosporidium* spp. in environmental water in Turkey. Acta Parasitol. 61, 671–679.

Koompapong K, Mori H, Thammasonthijarern N, Prasertbun R, Pintong AR, Popruk S, Rojekittikhun W, Chaisiri K, Sukthana Y, Mahittikorn A. 2014. Molecular identification of *Cryptosporidium* spp. in seagulls, pigeons, dogs, and cats in Thailand. Parasite. 21, 52.

Korpe PS, Gilchrist C, Burkey C, Taniuchi M, Ahmed E, Madan V, Castillo R, Ahmed S, Arju T, Alam M, Kabir M, Ahmed T, Petri WA, Haque R, Faruque ASG, Duggal P. 2019. Case-control study of *Cryptosporidium* transmission in Bangladeshi households. Clin. Infect. Dis. 68, 1073–1079.

Kumar S, Stecher G, Tamura K. 2016. Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Laatamna AE, Holubova N, Sak B, Kvac M. 2017. *Cryptosporidium meleagridis* and *C. baileyi* (Apicomplexa) in domestic and wild birds in Algeria. Folia Parasitol (Praha). 64, 018.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X Version 2.0. Bioinformatics. 23, 2947–2948.

Leitch GL, He Q. 2011. Cryptosporidiosis-an overview. J. Biomed. Res. 25, 1–16.

Lee SH, VanBik D, Kim HY, Lee YR, Kim JW, Chae M, Oh SI, Goo YK, Kwon OD, Kwak D. 2016. Multilocus typing of *Cryptosporidium* spp. in young calves with diarrhea in Korea. Vet. Parasitol. 229, 81–89.

Li J, Lin X, Zhang L, Qi N, Liao S, Lv M, Wu C, Sun M. 2015. Molecular characterization of *Cryptosporidium* spp. in domestic pigeons (*Columba livia domestica*) in Guangdong province, southern China. Parasitol. Res. 114, 2237–2241.

Li N, Xiao L, Alderisio K, Elwin K, Cebelinski E, Chalmers R, Santin M, Fayer R, Kváč M, Ryan U, Sak B, Stanko M, Guo Y, Wang L, Zhang L, Cai J, Roellig D, Feng Y. 2014. Subtyping *Cryptosporidium ubiquitum*, a zoonotic pathogen emerging in humans. Emerg. Infect. Dis. 20, 217–224.

Liao C, Wang T, Koehler AV, Fan Y, Hu M, Gasser RB. 2018. Molecular investigation of *Cryptosporidium* in farmed chickens in Hubei province, China, identifies 'zoonotic' subtypes of *C. meleagridis*. Parasit. Vectors. 11, 484.

Llorente MT, Clavel A, Goñi MP, Varea M, Seral C, Becerril R, Suarez L, Gómez-Lus R. 2007. Genetic characterization of *Cryptosporidium* species from humans in Spain. Parasitol. Int. 56,

201–205.

Majewska AC, Graczyk TK, Slodkowicz-Kowalska A, Tamang L, Jedrzejewski S, Zduniak P. 2009. The role of free-ranging, captive, and domestic birds of western Poland in environmental contamination with *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts. Parasitol. Res. 104, 1093–1099.

Mammeri M, Chevillot A, Chenafi I, Thomas M, Julien C, Vallée I, Polack B, Follet J, Adjou KT. 2019. Molecular characterization of *Cryptosporidium* isolates from diarrheal dairy calves in France. Vet. Parasitol. Reg. Stud. Reports. 18, 100323.

McLauchlin J, Pedraza-Díaz S, Amar-Hoetzeneder C, Nichols GL. 1999. Genetic characterization of *Cryptosporidium* strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis. J. Clin. Microbiol. 37, 3153–3158.

Meireles MV, de Oliveira FP, Teixeira WF, Coelho WM, Mendes LC. 2011. Molecular characterization of *Cryptosporidium* spp. in dairy calves from the state of São Paulo, Brazil. Parasitol. Res. 109, 949–951.

Mirhashemi ME, Zintl A, Grant T, Lucy FE, Mulcahy G, Waal TD. 2015. Comparison of diagnostic techniques for the detection of *Cryptosporidium* oocysts in animal samples. Exp. Parasitol. 151-152, 14–20.

Moon S, Kwak W, Lee S, Kim W, Oh J, Youn SK. 2013. Epidemiological characteristics of the first water-borne outbreak of cryptosporidiosis in Seoul, Korea. J. Korean Med. Sci. 28, 983–989.

Morgan UM, Pallant L, Dwyer BW, Forbes DA, Rich G, Thompson RCA. 1998. Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: clinical trial. J. Clin. Microbiol. 36, 995–998.

Mueller-Doblies D, Giles M, Elwin K, Smith RP, Clifton-Hadley FA, Chalmers RM. 2008. Distribution of *Cryptosporidium* species in sheep in the UK. Vet. Parasitol. 154, 214–219.

Murakoshi F, Tozawa Y, Inomata A, Horimoto T, Wada Y, Kato K. 2013. Molecular characterization of *Cryptosporidium* isolates from calves in Ishikari district, Hokkaido, Japan. J. Vet. Med. Sci. 75, 837–840.

Murakoshi F, Ichikawa-Seki M, Aita J, Yaita S, Kinami A, Fujimoto K, Nishikawa Y, Murakami S, Horimoto T, Kato K. 2016. Molecular epidemiological analyses of *Cryptosporidium parvum* virus 1 (CSpV1), a symbiotic virus of *Cryptosporidium parvum*, in Japan. Virus Res. 211, 69–72.

Murakoshi F, Xiao L, Matsubara R, Sato R, Kato Y, Sasaki T, Fukuda Y, Tada C, Nakai Y. 2012. Molecular characterization of *Cryptosporidium* spp. in grazing beef cattle in Japan. Vet. Parasitol. 187, 123–128.

Nakamura AA, Meireles MV. 2015. *Cryptosporidium* infections in birds-a review. Rev. Bras. Parasitol. Vet. 24, 253–267.

Nei M, Kumar S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York. 333.

Okada N, Matsumoto Y. 2002. Bovine rotavirus G and P types and sequence analysis of the VP7 gene of two G8 bovine rotaviruses from Japan. Vet. Parasitol. 84, 297–305.

Oliveira BCM, Ferrari ED, Panegossi MFC, Nakamura AA, Corbucci FS, Nagata WB, Santos BM, Gomes JF, Meireles MV, Widmer G, Brescian KDS. 2017. First description of *Cryptosporidium parvum* in carrier pigeons (*Columba livia*). Vet. Parasitol. 243, 148–150.

Olson ME, O'Handley RM, Ralston BJ, McAllister TA, Thompson RC. 2004. Update on *Cryptosporidium* and *Giardia* infections in cattle. Trends Parasitol. 20, 185–191.

Papanikolopoulou V, Baroudi D, Guo Y, Wang Y, Papadopoulos E, Lafi SQ, Abd El-Tawab MM, Diakou A, Giadinis ND, Feng Y, Xiao L. 2018. Genotypes and subtypes of *Cryptosporidium* spp. in diarrheic lambs and goat kids in northern Greece. Parasitol. Int. 67, 472–475.

Plutzer J, Karanis P. 2007. Genotype and subtype analyses of *Cryptosporidium* isolates from cattle in Hungary. Vet. Parasitol. 146, 357–362.

Putignani L, Menichella D. 2010. Global distribution, public health and clinical impact of the protozoan pathogen *Cryptosporidium.* Interdiscip. Perspect. Infect. Dis. 2010, 753512.

Quílez J, Torres E, Chalmers RM, Hadfield SJ, Del Cacho E, Sánchez- Acedo C. 2008. *Cryptosporidium* genotypes and subtypes in lambs and goat kids in Spain. Appl. Environ. Microbiol. 74, 6026–6031.

Rieux A, Paraud C, Pors I, Chartier C. 2013. Molecular characterization of *Cryptosporidium* spp. in pre-weaned kids in a dairy goat farm in western France. Vet. Parasitol. 192, 268–272.

Robinson G, Thomas AL, Daniel RG, Hadfield SJ, Elwin K, Chalmers RM. 2006. Sample prevalence and molecular characterization of *Cryptosporidium andersoni* within a dairy herd in the United Kingdom. Vet. Parasitol. 142, 163–167.

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

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

Samad MA. 2011. Public health threat caused by zoonotic diseases in Bangladesh. Bangl. J. Vet. Med. 9, 95–120.

Santana BN, Kurahara B, Nakamura AA, da Silva CV, Ferrari ED, da Silva GS. 2018. Detection and characterization of *Cryptosporidium* species and genotypes in three chicken production systems in Brazil using different molecular diagnosis protocols. Prev. Vet. Med. 151, 73–78.

Santín M, and Trout JM. 2007. Livestock, in: Fayer R, Xiao L. (Eds.), *Cryptosporidium* and Cryptosporidiosis. CRC Press, Boca Raton. 18, 34.

Santín M, Trout JM, Fayer R. 2008. A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. Vet. Parasitol. 155, 15–23.

Santín M, Trout JM, Xiao L, Zhou L, Greiner E, Fayer R. 2004. Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves. Vet. Parasitol. 122, 103–117.

Santín M. 2013. Clinical and subclinical infections with *Cryptosporidium* in animals. N. Z. Vet. J. 61, 1–10.

Sari B, Aktas MS, Arslan MO. 2008. The prevalence of *Cryptosporidium* spp. in calves in Erzurum province. Turkiye Parazitol. Derg. 32, 116–119.

Sari B, Arslan MO, Gicik Y, Kara M, Tasci GT. 2009. The prevalence of *Cryptosporidium* species in diarrhoeic lambs in Kars province and potential risk factors. Trop. Anim. Health Prod. 41, 819–826.

Satoh M, Hikosaka K, Sasaki T, Suyama Y, Yanai T, Ohta M, Nakai Y. 2003. Characteristics of a novel type of bovine *Cryptosporidium andersoni*. Appl. Environ. Microbiol. 69, 691–692.

Sergeant ESG. 2017. Epitools Epidemiological Calculators. Ausvet Pty Ltd. Available at http://epitools.ausvet.com.au. Accessed 20 June 2019.

Shahbazi P, Aligolzadeh A, Khordadmehr M, Hashemzadeh FH, Katiraee F. 2020. Molecular study and genotyping of *Cryptosporidium baileyi* and *Cryptosporidium parvum* from free-range and commercial broiler chickens in Guilan province, Iran. Comp. Immunol. Microbiol. Infect. Dis. 69, 101411.

Silverlas C, De Verdier K, Emanuelson U, Mattsson JG, Björkman C. 2010. *Cryptosporidium*

infection in herds with and without calf diarrheal problems. Parasitol. Res. 107, 1435–1444.

Silverlas C, Mattsson JG, Insulander M, Lebbad M. 2012. Zoonotic transmission of *Cryptosporidium meleagridis* on an organic swedish farm. Int. J. Parasitol. 42, 963–967.

Soba B, Logar J. 2008. Genetic classification of *Cryptosporidium* isolates from humans and calves in Slovenia. Parasitology. 135, 1263–1270.

Sréter T, Varga I, Békési L. 1995. Age-dependent resistance to *Cryptosporidium baileyi* infection in chickens. J. Parasitol. 81, 827–829.

Sréter T, Kovacs G, Da Silva AJ, Pieniazek NJ, Szell Z, Dobos-Kovacs M, Marialigeti K, Varga I. 2000. Morphologic, host specificity, and molecular characterization of a Hungarian *Cryptosporidium meleagridis* isolate. Appl. Environ. Microbiol. 66, 735–738.

Sréter T, Varga I. 2000. Cryptosporidiosis in birds--a review. Vet. Parasitol. 87, 261–279.

Steiner KL, Ahmed S, Gilchrist C. 2018. Species of cryptosporidia causing subclinical infection associated with growth faltering in rural and urban Bangladesh: A birth cohort study. Clin. Infect. Dis. 67, 1347–1355.

Stensvold CR, Beser J, Axén C, Lebbad M. 2014. High applicability of a novel method for gp60-based subtyping of *Cryptosporidium meleagridis*. J. Clin. Microbiol. 52, 2311–2319.

Sulaiman IM, Hira PR, Zhou L, Al-Ali FM, Al-Shelahi FA, Shweiki HM, Iqbal J, Khalid N, Xiao L. 2005. Unique endemicity of cryptosporidiosis in children in Kuwait. J. Clin. Microbiol. 43, 2805–2809.

Sulaiman IM, Lal AA, Xiao L. 2002. Molecular phylogeny and evolutionary relationships of *Cryptosporidium* parasites at the actin locus. J. Parasitol. 88, 388–394.

Sungur T, Kar S, Guven E, Aktaş M, Karaer Z, Vatansever Z. 2008. Detection of *Cryptosporidium* spp. in feces with nested PCR and carbol fuchsin staining method. Turkiye Parazitol. Derg.

32, 305–308.

Sweeny JP, Ryan UM, Robertson ID, Jacobson C. 2011. *Cryptosporidium* and *Giardia* associated with reduced lamb carcase productivity. Vet. Parasitol. 182, 127–139.

Tamer GS, Turk M, Dagci H, Pektas B, Guy EC, Guruz AY, Uner A. 2007. The prevalence of cryptosporidiosis in Turkish children, and genotyping of isolates by nested polymerase chain reaction-restriction fragment length polymorphism. Saudi. Med. J. 28, 1243–1246.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10, 512–526.

Tamura K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Mol. Biol. Evol. 9, 678–687.

Tanriverdi S, Markovics A, Arslan MO, Itik A, Shkap V, Widmer G. 2006. Emergence of distinct genotypes of *Cryptosporidium parvum* in structured host populations. Appl. Environ. Microbiol. 72, 2507–2513.

Taylan-Ozkan A, Yasa-Duru S, Usluca S, Lysen C, Ye J, Roellig DM, Feng Y, Xiao L. 2016. *Cryptosporidium* species and *C. parvum* subtypes in dairy calves and goat kids reared under traditional farming systems in Turkey. Exp. Parasitol. 170, 16–20.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25, 4876–4882.

Thomson S, Hamilton CA, Hope JC, Katzer F, Mabbott NA, Morrison LJ, Innes EA. 2017. Bovine cryptosporidiosis: impact, host-parasite interaction and control strategies. Vet. Res. 48, 42.

Trotz-Williams LA, Martin DS, Gatei W, Cama V, Peregrine AS, Martin SW, Nydam DV, Jamieson F, Xiao L. 2006. Genotype and subtype analyses of *Cryptosporidium* isolates from dairy

calves and humans in Ontario. Parasitol. Res. 99, 346–352.

Tzanidakis N, Sotiraki S, Claerebout E, Ehsan A, Voutzourakis N, Kostopoulou D, Stijn C, Vercruysse J, Geurden T. 2014. Occurrence and molecular characterization of *Giardia duodenalis* and *Cryptosporidium* spp. in sheep and goats reared under dairy husbandry systems in Greece. Parasite. 21, 45.

Wang L, Xue X, Li J, Zhou Q, Yu Y, Du A. 2014. Cryptosporidiosis in broiler chickens in Zhejiang province, China: molecular characterization of oocysts detected in fecal samples. Parasite. 21, 36.

Wang R, Jian F, Sun Y, Hu Q, Zhu J, Wang F. 2010. Large-scale survey of *Cryptosporidium* spp. in chickens and pekin ducks (*Anas platyrhynchos*) in Henan, China: prevalence and molecular characterization. Avian. Pathol. 39, 447–451.

Wang R, Zhang L, Axen C, Bjorkman C, Jian F, Amer S. 2014. *Cryptosporidium parvum* IId family: clonal population and dispersal from western asia to other geographical regions. Sci. Rep. 4, 4208.

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

Wells B, Thompson S. 2014. Cryptosporidiosis in cattle. The Moredun Foundation News Sheet. 6, 1.

Wielinga PR, de Vries A, van der Goot TH, Mank T, Mars MH, Kortbeek LM, van der Giessen JW. 2008. Molecular epidemiology of *Cryptosporidium* in humans and cattle in the Netherlands. Int. J. Parasitol. 38, 809–817.

Wu Z, Nagano I, Boonmars T, Nakada T, Takahashi Y. 2003. Intraspecies polymorphism of

Cryptosporidium parvum revealed by PCR-restriction fragment length polymorphism (RFLP) and RFLP-single-strand conformational polymorphism analyses. Appl. Environ. Microbiol. 69, 4720–4726.

Xiao L, Bern C, Limor J, Sulaiman I, Roberts J, Checkley W, Cabrera L, Gilman RH, Lal AA. 2001. Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. J. Infect. Dis. 183, 492–497.

Xiao L, Ryan U. 2015. *Cryptosporidium*, in: Xiao L, Ryan U. (Eds.), Biology of Foodborne Parasites. CRC Press, Boca Raton. 77–95.

Xiao L, Sulaiman IM, Ryan UM, Zhou L, Atwill ER, Tischler ML, Zhang X, Fayer R, Lal AA. 2002. Host adaptation and host-parasite co-evolution in *Cryptosporidium*: implications for taxonomy and public health. Int. J. Parasitol. 32, 1773–1785.

Xiao L. 2010. Molecular epidemiology of cryptosporidiosis: an update, Exp. Parasitol. 124, 80–89.

Yildirim A, Adanir R, Inci A, Yukari BA, Duzlu O, Onder Z, Ciloglu A, Simsek E. 2020. Prevalence and genotyping of bovine *Cryptosporidium* species in the mediterranean and central Anatolia region of Turkey. Comp. Immunol. Microbiol. Infect. Dis. 69, 101425.

Zaglool DA, Mohamed A, Khodari YA, Farooq MU. 2013. Crypto-Giardia antigen rapid test versus conventional modified Ziehl-Neelsen acid fast staining method for diagnosis of cryptosporidiosis. Asian Pac. J. Trop. Med. 6, 212–215

Zambriski JA, Nydam DV, Wilcox ZJ, Bowman DD, Mohammed HO, Liotta JL. 2013a. *Cryptosporidium parvum*: determination of ID_{50} and the dose-response relationship in experimentally challenged dairy calves. Vet. Parasitol. 197, 104–112.

Zambriski JA, Nydam DV, Bowman DD, Bellosa ML, Burton AJ, Linden TC, Liotta JL, Ollivett

TL, Tondello-Martins L, Mohammed HO. 2013b. Description of fecal shedding of *Cryptosporidium parvum* oocysts in experimentally challenged dairy calves. Parasitol. Res. 112, 1247–1254.