博士論文

Molecular analyses and epidemiology of *Cryptosporidium* and

the symbiotic virus of this parasite

(クリプトスポリジウム原虫及び本原虫内在性ウイルスの疫学と分子生物学的解析)

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Contents

Preface: Aims and scope of the thesis

The genus *Cryptosporidium*, which is an obligate intracellular parasite, infects various vertebrates and especially, *C. parvum* causes an acute diarrheal disease known as cryptosporidiosis. *C. parvum* has been reported to be the zoonotic species and their oocyst shedding from domestic animals have been reported to be a contamination source in human cryptosporidiosis outbreaks. However, there are no high-resolution genes for subtyping *C. parvum*. Moreover, for the treatment of *Cryptosporidium*, there are no effective therapy and drug. Nitazoxanide is the only approved drug but their effectiveness is limited. To solve these problems, in the present thesis, the author searched good subtyping gene for sensitive subtyping of *C. parvum* and drug candidate have positive effects in human and cattle.

In the **General Introduction**, the author described clinical diagnosis, life cycle, taxonomy and current status of research on *Cryptosporidium*. There are number of issues with the use of genotyping and subtyping in resolution. Moreover, there are a few information of molecular biology of this parasite because of few molecular tools. These problems are overviewed for the background of the present thesis.

In **Chaper 1**, the author did the epidemiological research in Ishikari District, Hokkaido, Japan. The author detected *C. parvum*, *C. ryanae* and *C. bovis*. Subtype of *C. parvum* was IIa. This is the common subtype found in humans and cattle in many countries. In Japan, many papers reported that IIa type was only subtype of *C. parvum* in Japan. To resolve this problem, in

Chapter 2, the author showed that *Cryspovirus* infected *Cryptosporidium parvum*. The dsRNA1 sequence of this virus is new candidates of *C. parvum* subtyping marker.

In **Chaper 3**, the author assessed the efficacy of lasalocid-Na in the treatment or prevention of cryptosporidiosis in calves. The author successfully identified an appropriate dose of lasalocid that did not cause toxic effects in the calves. Lasalocid-Na was protective during the dosing period and this data suggest that lasalocid-Na administration may delay oocyst excretion. However, lasalocid-Na is not suitable for applying to human because of side effects. To resolve this problem, in **Chapter 4**, the author focuses on histone modification of *Cryptosporidium*. In Apicomplexa, many papers reported the histone modification is also important in parasite virulence and control their life cycle of *Toxoplasma* and *Plasmodium* closely related with *Cryptosporidium*. The author confirmed presence of histone modification in *C. parvum* for the first time, additionally, concluded that the histone modification enzymes of this parasite may become drug targets.

In **General Conclusion**, the author summarized the data in the thesis collectively and remarked the conclusion.

General Introduction

1. Cryptosporidiosis in humans

Cryptosporidium parvum belonging to Apicomplexa causes acute watery diarrhea, therefore, the parasite is one of the most important enteric pathogens to public health. The pathogen infects mammals including human worldwide (O'Donoghue, 1995; Fayer, 2004), and their infection is generally restricted to the intestinal epithelium. In healthy adults or children, *Cryptosporidium* infection causes typically self-limiting disease. On the contrary, in immunocompromised individuals such as AIDS patients, their infection results in life-threatening diarrhea (Current *et al*., 1983), but no effective therapy has been established. *Cryptosporidium* is transmitted via the fecal-oral route by oocysts. Major transmission routes are human-to-human contact, food, during care of livestock, companion animals, drinking water and recreational waters. Prepatent period of this parasite is 3-6 days and watery diarrhea usually lasts 6 to 14 days. Oocyst stage of this parasite is resistant to chlorine, therefore, sometimes outbreaks are reported even in advanced country which has well-resourced waterworks.

2. Cryptosporidiosis in America and Japan

The most severe outbreak of *Cryptosporidium* occurred in Milwaukee, Wisconsin, the United States in 1993. More than 400,000 residents were infected with the parasite (Mac Kenzie *et al*., 1994). In the United States, the number of cases of cryptosporidiosis is increasing yearly. In 1999, 2,769

cases of cryptosporidiosis were reported, in recent years, the number of cryptosporidiosis case reports increased from 6,479 cases in 2006 to 10,500 cases in 2008 (Fig 1). Based on the data, more than 740,000 patients each year are estimated. Therefore, *C. parvum* is categorized as a category B biodefense agent by NIH.

In Japan, *Cryptosporidium* type I and II (*C. hominis* and *C. parvum*) were categorized as a category four by Infectious Diseases Control Law in 2003. Cryptosporidiosis in human is notifiable infectious disease and 233 cases were reported from 1999 to 2005 in Japan (Fig 2). Most cases were caused from outbreaks which happened from 1999 to 2005. Fifty-three cases were sporadic cases and 21/53 cases were overseas infection. Major transmission routes are through care of livestock, and human-to-human contact in Japan (Table 1). Up to now, two big outbreaks of cryptosporidiosis occurred in Japan. In 1994, an outbreak of diarrhea due to infection with *Cryptosporidium* occurred in Kanagawa Prefecture, Japan. About 400 residents were infected with *Cryptosporidium* (Kuroki *et al*., 1996). In 1996, an outbreak of cryptosporidiosis occurred in Saitama Prefecture, Japan. About 9,000 residents were infected with the parasite (Yamamoto *et al*., 2000). Other outbreaks of cryptosporidiosis also occurred in five cases in 2004 and one cases in 2002. Transmission routes were through care of livestock, drinking water and recreational waters. In Japan, case reports are less than other countries but there are some reports that *C. parvum* was detected from river, shellfish and non-treated drinking water (Tsushima *et al*., 2003; Izumi *et al*.,

2006; Ono *et al*., 2001). These data suggested that there are also high risks of transmission of *Cryptosporidium* in Japan.

3. Cryptosporidiosis in cattle

Cattle are major hosts of *Cryptosporidium* spp. (Santín and Trout, 2008). Oocyst shedding from cattle has been suggested to be a contamination source for human cryptosporidiosis outbreaks (Nichols e*t al*., 2006). Additionally, cryptosporidiosis is associated with neonatal diarrhea syndrome in calves, leading to direct and indirect economic losses (de Graaf *et al*., 1999). Therefore, studies of the species distribution and transmission routes of *Cryptosporidium* spp. are important in public health and food production.

4. Life cycle of *Cryptosporidium*

The life cycle for *Cyptosporidium* spp. is shown in Figure 3. All members of the genus *Cryptosporidium* are intracellular parasites. This parasite develops without passing through an intermediate host or vectors. Hosts are parasitized by the oral uptake of oocysts. Oocysts are one stage of life cycle of *Cryptosporidium* and show environmental tolerance. Oocysts have four infectious sporozoites and sporozoites can excyst from oocysts in warm aqueous solution; inside of the host's body. Sporozoites excysted from oocysts invade microvillar surface of intestinal epithelial cells. Sporozoites within the parasitophorous vacuole are intracellular, but in the host-cell cytoplasm it is called extracytoplasmic. Sporozoite develops to trophozoite

stage and trophozoite becomes Type I meront which has eight merozoites. Mature type I meronts release merozoites to infect another host cell. Some merozoites develop into Type II meronts which have four morozoites. Merozoites packaged in type II meront, infect another host cell and develop microgamont or macrogamont. After microgamont and macrogamont are fertilized, parasites develop into four 1N sporozoites packaged by oocysts. Finally, oocysts are excreted with feces.

5. Taxonomy

Cryptosporidium are eukaryotic protozoa that include in the phylum Apicomplexa. *Cryptosporidium* was first described by E. E. Tyzzer. In 1907, he found *C. muris* from gastric glands and found *C. parvum* from intestines of mice (Tyzzer, 1907, 1912) However, oocysts of this parasite are very small, therefore, could not distinguish species from oocysts morphology. Recently, small subunit ribosomal RNA gene data have been used to define new species. Over 150 species of mammals including humans are parasitized by genus *Cryptosporidium* (Fayer, 2004). There are 21 valid named species of *Cryptosporidium* (Fayer, 2010) (Table 2). *C. molnari* was reported to infect fish (Alvarez-Pellitero and Sitijà-Bobadilla, 2002). *C. serpentis* (Levine, 1980), *C. varanii* (Pavlásek *et al*., 1995) and *C. fragile* (Jirku *et al*., 2008) was found in reptile. *C. baileyi* (Current *et al*., 1986), *C. meleagridis* (Slavin, 1955), *C. galli* (Pavlásek, 1999) were found from birds. *C. felis* (Iseki, 1979) were found from cats. *C. muris* (Tyzzer, 1907) were found from mice. *C. wrairi* (Vetterling

et al., 1971) have been detected in guinea pigs. *C. andersoni* (Lindsay *et al*, 2000*), C. bovis* (Fayer *et al*., 2005), *C. ubiquitum* (Fayer *et al*., 2010), *C. xiaoi* (Fayer and Santin, 2009) and *C. ryanae* (Fayer *et al*., 2008) were found from cattle. *C. canis* (Fayer *et al*., 2001), *C. suis* (Ryan *et al*., 2004) and *C. macropodum* (Power and Ryan, 2008) have been identified in dogs, pigs and kangaroo, respectively. *C. hominis* (Morgan-Ryan *et al*., 2002) and *C. parvum* were major species which infect humans. *C. baileyi*, *C. canis*, *C. felis*, *C. meleagridis*, *C. muris* and *C. ubiquitum* were also detected in humans with diarrhea but it is thought to be rare case (Xiao and Feng, 2008). *C. hominis* is known to infect only humans. The 60-kDa glycoprotein (GP60) gene is the most major subtyping gene of *C. hominis* and *C. parvum* (Leave *et al*., 2002; Glaberman *et al*., 2002; Abe et al., 2006) because GP60 gene is the most polymorphic marker identified in the *Cryptosporidium* genome. Moreover, GP60 is located on the one of the dominant target for neutralizing antibody responses in humans (Cevallos *et al*., 2000). Based on this gene, *C. parvum* and *C. hominis* were subtyped in their sequence following designation (Ia, Ib, Id, Ie, If and Ig for *C. hominis*, and IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh, and IIi for *C. parvum*) (Table 2) and they have some trinucleotide repeat variation.

6. Chemotherapy

Researches on chemotherapy of cryptosporidiosis have been less advanced. The reason for the slower progress is their complex developed location; it is called extracytoplasmic. Nitazoxanide (NTZ), a 5-nitrothiazole

analogue, is only drug approved by U.S. Food and Drug Administration (FDA) for the treatment of *Cryptosporidium* infection in human adult and children (Fox and Saravolatz, 2005). *Cryptosporidium* have unique PNO (pyruvate-NADP oxidoreductase). NTZ enter the parasite by diffusion and then be reductively activated by the PNO. The free radical resulting from the one electron reduction of NTZ is toxic to the *Cryptosporidium* (Coombs and Müller, 2002). The study demonstrated that nitazoxanide had 81 % clinical cure rate and 67 % parasitologic cure rate at 7 days after the initial treatment (Rossignol *et al*., 2001). Therefore, this drug has mildly-effectiveness and for immune compromised person, there is no drug for the treatment of *Cryptosporidium* infection.

Figure legends

Figure 1. Cryptosporidiosis case report from 1999 to 2008 in the United States

Gray bar means outbreak cases and black bar means sporadic cases (Hlavsa *et al*., 2005; Yoder *et al*., 2007, 2008).

Figure 2. Cryptosporidiosis case report from 1999 to 2008 in Japan

The author referred to NIID data (http://idsc.nih.go.jp/idwr/ydata/report-Ja.html) for information about cryptosporidiosis in Japan.

Figure 3. Life cycle of *Cryptosporidium*

a. Oocyst   b. Sporozoite   c. Trophozoite   d. Type I Meront   e. Type II Meront   f. Microgamont   g. Macrogamont   h. Zygote

Figure 1.
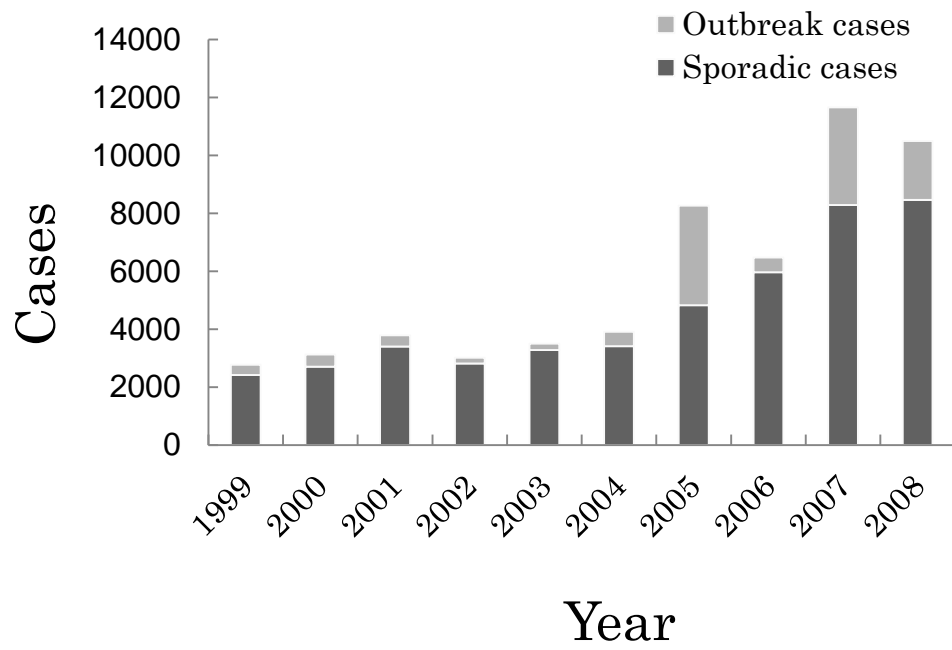
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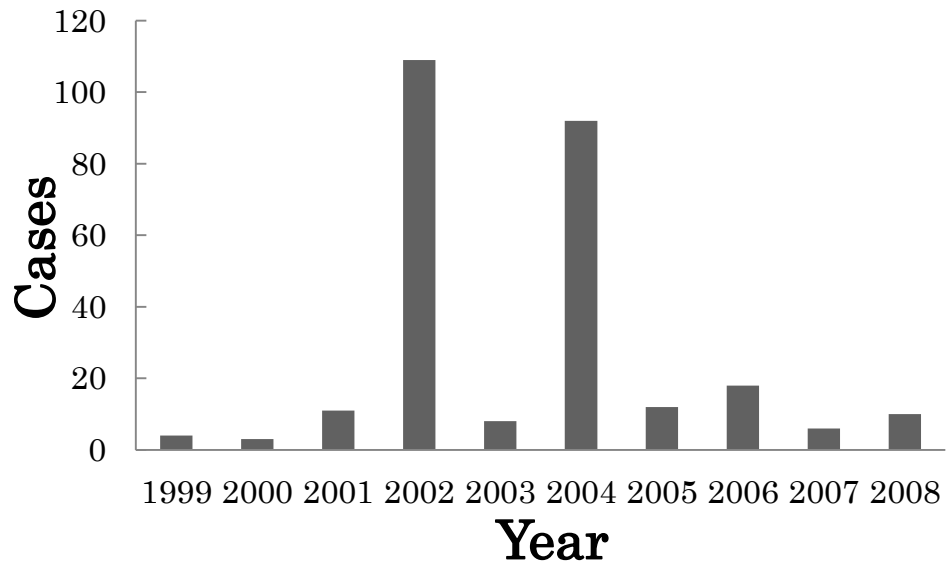
Figure 3.
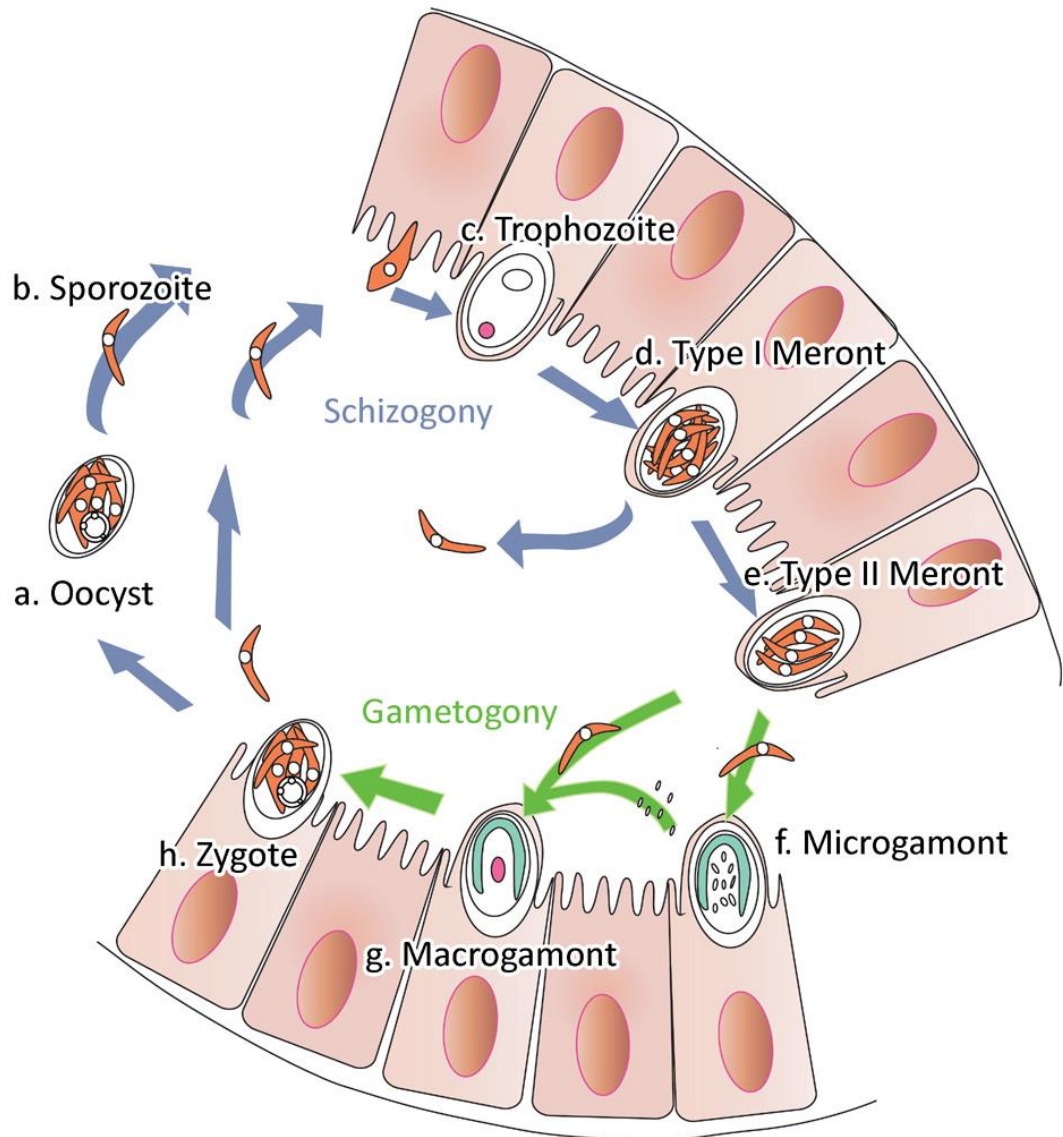
Table 1. Transmission routes of cryptosporidiosis in Japan from 1999 to 2005

Transmission routes	cases
Care of livestock	7
Visiting farm	1
Human-to-human contact	7
HIV infection	1
Unknown	16
Sum	32

The author referred to NIID data (http://idsc.nih.go.jp/idwr/ydata/report-Ja.html) for information about cryptosporidiosis in Japan.

Table 2 Valid named species of *Cryptosporidium*

Species	Type host	Site of infection	Oocyst size (μm)	Author
Large oocysts type				
C. andersoni	*Bos taurus*	the gastro-intestinal tract	7.4×5.5	Lindsay *et al*. (2000)
C. muris	*Mus musculus*	the gastro-intestinal tract	8.4×6.3	Tyzzer (1907)
C. fragile	*Duttaphrynus melanostictus*	the gastro-intestinal tract	6.2×5.5	Jirku *et al*. (2008)
C. galli	*Gallus gallus*	the gastro-intestinal tract	8.3×6.3	Pavlásek (1999)
C. molnari	*Sparus auratus*	the gastro-intestinal tract	4.7×4.5	Alvarez-Pellitero and Sitjà-Bobadilla(2002)
	Dicentrarchus labrax			
C. serpentis	*Elaphe guttata*	the gastro-intestinal tract	6.2×5.3	Levine (1980)
	E. subocularis			
	Sanzinia madagascarensus			
Small oocysts type				
C. baileyi	*Gallus gallus*	intestine	6.2×4.6	Current *et al*. (1986)
C. bovis	*Bos taurus*	unknown	4.9×4.6	Fayer *et al*. (2005)
C. canis	*Canis familiaris*	intestine	5.0×4.7	Fayer *et al*. (2001)
C. fayeri	*Macropus rufus*	unknown	4.9×4.3	Ryan *et al*. (2008)
C. feris	*Felis catis*	intestine	4.6×4.0	Iseki (1979)
C. hominis	*Homo sapiens*	intestine	5.2×4.9	Ryan *et al*. (2002)
C. macropodum	*Macropus giganteus*	unknown	5.4×4.9	Power and Ryan (2008)
C. meleagridis	*Mereagris gallopavo*	intestine	5.2×4.6	Slavin (1955)
C. parvum	*Mus masuculus*	intestine	5.0×4.5	Tyzzer (1912)
C. ryanae	*Bos taurus*	unknown	3.2×3.7	Fayer *et al*. (2008)
C. suis	*Sus scrofa*	intestine	4.6×4.2	Ryan *et al*. (2004)
C. varanii	*Varanus prasinus*	the gastro-intestinal tract	4.8×4.7	Pavlásek *et al*. (1995)
C. wrairi	*Cavia porcellus*	intestine	5.4×4.6	Vetterling *et al*. (1971)
C. xiaoi	*Ovis aries*	unknown	3.9×3.4	Fayer and Santín (2009)
C. ubiquitum	*Bos taurus*	intestine	4.7×5.3	Fayer *et al*. (2010)

Table 2 GP60 Subtype family of *C. hominis and C. parvum*

Species	Subtype family	Accession number
C. hominis	Ia	AF164502
	Ib	AY262031
	Id	DQ665688
	Ie	AY262031
	If	AF440638
	Ig	EF208067
C. parvum	IIa	AY262034
	IIb	AF402285
	IIc	AF164491
	IId	AY738194
	IIe	AY382675
	IIf	AY738188
	IIg	AY873780
	IIh	AY873781
	IIi	AY873782

Chapter 1

Molecular characterization of *Cryptosporidium* isolates from calves in Ishikari District, Hokkaido, Japan

Abstract

Cattle are major hosts of *Cryptosporidium*. Cryptosporidiosis in neonatal calves is associated with retarded growth, weight loss and calf mortality, and zoonotic infections in humans. Fecal samples were collected from calves in Ishikari District, Hokkaido, Japan, and examined by PCR and sequence analyses. Among the 107 fecal samples collected in May and June 2012, 25 (23%) were positive for *Cryptosporidium*, including 8 samples (7%) having *C. parvum*, 10 (10%) having *C. bovis* and 7 (6%) having *C. ryanae*. This is the first time *C. ryanae* has been detected in Hokkaido. Furthermore, it is the first detection of *C. ryana*e from pre-weaned calves in Japan. Here, the zoonotic species, *C. parvum*, was also observed. Therefore, calves can be potential sources of cryptosporidial infections for humans and other animals. The detection of *C. parvum* was statistically correlated with diarrhea in calves.

Introduction

Cattle are major hosts of *Cryptosporidium* spp. (Santín and Trout, 2008). They are primarily infected with four *Cryptosporidium* species, namely *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosoridium ryanae* and *Cryptosporidium andersoni*. *C. parvum* has been reported to be the zoonotic species and the major cause of diarrhea in calves (Thompson *et al*., 2008; Xiao and Ryan *et al*., 2004). Oocyst shedding from cattle has been reported to be a contamination source in human cryptosporidiosis outbreaks (Nichols *et al*., 2006). Additionally, cryptosporidiosis is associated with neonatal diarrhea syndrome in calves leading to direct and indirect economic losses (de Graaf *et al*., 1999).

In Japan, there are very few studies on the epidemiology of *Cryptosporidium* in cattle. Additionally, most previous epidemiological studies of cryptosporidiosis in cattle were performed by only microscopic observations of oocyst shedding or microscopic observation used for initial screening of molecular epidemiology. However, these traditional screening methods might lead to low detection of *Cryptosporidium* species with low oocyst shedding intensity. There are differing results according to the type and age of host animals, area of study, detection methods and season, and because of this variation, more epidemiological studies are needed.

To assess the potential public health and production importance, *Cryptosporidium* infection in pre- and post-weaned calves in Ishikari District,

Hokkaido, Japan was examined. This study is an initial *Cryptosporidium* screening of Ishikari District. Specifically, we analyzed the relationships between the occurrence of diarrhea, genotype, *C. parvum* subtype and age using molecular diagnostic tools.

2. Materials and Methods

2.1. Collection of samples

Fecal samples were collected from 27 farms located in Ishikari District, Hokkaido, Japan. Samples were collected from 86 dairy, 17 Japanese black and 4 crossbred calves (*Bos taurus*) that ranged in age from 2 to 120 days. A total of 107 samples were randomly collected at each farm (1–8 calves per farm) in May and June, 2012. Specimens were stored at 4°C prior to DNA extraction and microscopic examination.

2.2. Sample analysis

DNA was extracted using easy beads (AMR, Gifu, Japan) and QIAamp® DNAstool minikit (Qiagen, Hilden, Germany). For DNA extraction, 0.3-0.4g of fecal specimen was used. *Cryptosporidium* spp. were detected and subtyped by nested polymerase chain reaction (PCR) amplification targeting a ~830 bp and ~850 bp fragments of the small subunit (SSU) rRNA and 60 kDa glycoprotein (GP60) genes, respectively, as described previously (Feng *et al*., 2007). Nested-PCR were performed following primers

18S_F1:5'-TTCTAGAGCTATACATGCG-3',

18S_R1:5'-CCCATTTCCTTCGAAACAGGA-3',

18S_F2:5'-GGAAGGGTTGTATTTATTAGATAAAG-3',

18S_R2:5'-CTCATAAGGTGCTGAAGGAGTA-3',

GP60_F1:5'-TTACTCTCCGTTATAGTCTCC-3',

GP60_R1:5'-GGAAGGAACGATGTATCTGA-3',

GP60_F2:5'-TCCGCTGTATTCTCAGCC-3',

GP60_R2:5'-GCAGAGGAACCAGCATC-3' and run the following PCR program: 94°C: 3 min, 35 cycles of: 94°C for 45", 50°C for 45" and 72°C for 1 min, 72 °C for 7 min, 4 °C soaking. GP60 gene was used to subtype *C. parvum*. For each sample, PCR detection was repeated independently at least two times. All secondary PCR products determined to be *Cryptosporidium* positive were sequenced in both directions using an ABI 3130 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan) with the secondary primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Japan). The sequences obtained were aligned using ClustalX 2 (Larkin *et al*., 2007), edited manually in BioEdit 7.0.5.3 (Hall et al., 1999) and compared to *C. parvum*, *C. bovis* and *C. ryanae* reference sequences in GenBank. Unique 18S rRNA and GP60 sequences acquired in this study were deposited in the GenBank database under accession numbers; AB746195–AB746198.

The sugar flotation method and microscopic examination were also performed for *Cryptosporidium* positive fecal samples. In brief, 1g of feces was suspended in sucrose (1.2 g/ml), and oocysts were floated to cover grass.

2.3. Statistical analyses

Data were compared using a Fisher's exact test with the use of R 2.13.0 software. Differences at $p < 0.05$ were considered significant.

3. Results

3.1. Genotype and analysis

Of the 107 fecal samples, 25 samples (23%) were positive for *Cryptosporidium* infection based on PCR-sequence analysis. Three *Cryptosporidium* species (*C. parvum*, *C. bovis* and *C. ryanae*) were detected; 8 (7%), 10 (10%) and 7 (6%) samples had *C. parvum*, *C. bovis* and *C. ryanae*, respectively. The percentage of each genotype of *Cryptosporidium* represented among the 25 *Cryptosporidium* positive specimens in the two age categories (pre-weaned; 1–8 weeks, and post-weaned; 3–12 months) (Table 1). The infection rate of pre-weaned calves was 21.5%; 10%, 6% and 5% of them had *C. parvum*, *C. bovis* and *C. ryanae*, respectively. The infection rate of post-weaned calves was 28.5%; 0%, 18% and 11% of the calves were infected with *C. parvum*, *C. bovis* and *C. ryanae*, respectively. Fragments of the small subunit (SSU) rRNA nucleotide sequences acquired in this study were deposited in the GenBank database under accession numbers; AB746195-AB746197. For *C. parvum* (AB746195), nucleotide sequences of the SSU rRNA gene derived from our fecal samples were identical to those of HQ009805 from dairy cattle from China. *C. bovis* (AB746197) was identical to JX515546 from dairy calves, China, and *C. ryanae* (AB746196) was identical to HQ179574 from dairy calves, China. Therefore, it is concluded that *C. parvum*, *C. bovis* and *C. ryanae* detected here were not geographically unique. The sugar flotation method and microscopic examination were also performed for all 25 *Cryptosporidium* PCR-positive specimens. All *C. bovis*

and *C. ryanae* PCR-positive specimens were negative by sugar flotation method. The *C. parvum* PCR-positive specimens were positive by sugar flotation method, except for only one specimen.

3.2. Correlation between diarrhea and detected genotype

Diarrhea rate was no statistically significant between pre-weaned and post-weaned calves (Table 2). *C. parvum* were only detected in pre-weaned calves, and only *C. parvum* was statistically correlated with diarrhea (p< 0.05) (Table 3). Among diarrheal *Cryptosporidium* positive samples, *C. parvum* was identified in 3 cases, but some calves with diarrhea were not infected with *C. parvum*.

3.3. Relationship between *Cryptosporidium* infection and animal age

The percentages of each *Cryptosporidium* genotype represented among the 25 *Cryptosporidium*-positive specimens in the two age categories are presented in Table 1. There were no significant correlations between *Cryptosporidium* infection and animal age. In post-weaned calves, the majority of infections were due to *C. bovis* and *C. ryanae*. *C. bovis* and *C. ryanae* were found in pre-weaned and post-weaned calves. The percentages of *C. parvum*, *C. bovis* and *C. ryanae* detected from pre-weaned calves were 47%, 29% and 24%, respectively, whereas those from post-weaned calves were 0%, 63% and 38%, respectively.

3.4. Subtyping of *C. parvum* by GP60 gene

Sequencing of GP60 gene PCR products was successful in all *C. parvum* isolates. DNA sequencing indicated that all GP60 nucleotide sequences were identical to each other and had a 100% similarity with *C. parvum* subtype IIa. GP60 nucleotide sequences acquired in this study were deposited in the GenBank database under accession numbers; AB746198.

4. Discussion

Of the 107 fecal samples, 25 samples (23%) were positive for *Cryptosporidium* infection based on PCR-sequence analyses. In Kenya, 20% of dairy cattle were PCR-positive for *Cryptosporidium* (Kang'ethe *et al*., 2012). In China, 18.82% positive samples were identified from dairy cattle (Chen *et al*., 2012). In India, 17.65–86.67% of calves were *Cryptosporidium* positive (Venu *et al*., 2012). Again for calves, 25%, 21.5% and 10.7% were *Cryptosporidium* positive in Romania, China and Brazil, respectively (Imre *et al*., 2011; Wang *et al* 2011; Meireles *et al*., 2011). Therefore, this study's results were consistent with those epidemiologic data in the world. Furthermore, in this study, we collected 1-8 samples per farm. Those from more than half of farms were *Cryptosporidium* positive. *Cryptosporidium* positive farms were widely distributed in Ishikari District.

Cryptosporidium species were detected; 8 (7%), 10 (10%) and 7 (6%) samples had *C. parvum*, *C. bovis* and *C. ryanae*, respectively. In Hyogo Prefecture, 93% of calves were reported to pass *C. parvum* oocysts (Uga *et al*., 2000). Oocysts of this species have previously been isolated from cattle in many areas in Japan (Yagita *et al*., 2001; Sakai *et al*., 2003; Abe *et al*., 2006; Amer *et al*., 2009; Karanis *et al*., 2010). Here, the zoonotic species, *C. parvum*, was observed. Therefore, calves can be potential sources of cryptosporidial infections for humans and animals in Hokkaido.

*C. bovi*s has been identified previously as *Cryptosporidium* genotype bovine B (Fayer *et al*., 2005), and *C. ryanae* has been identified previously as

the *Cryptosporidium* deer-like genotype (Fayer *et al*., 2008). These species have been detected all over the world (Feng *et al*., 2007). It is very difficult to distinguish *C. bovis* and *C. ryanae* from *C. parvum* morphologically, and molecular analysis is needed for the differentiation of three intestinal *Cryptosporidium* spp. in cattle. For this reason, in Japan, these species have been described in only three studies. In Japan, *C. ryanae* was first detected from adult dairy cattle in Miyagi Prefecture (Amer *et al*., 2009), and *C. bovis* was first detected from pre-weaned calves with diarrhea in Hokkaido (Karanis *et al*., 2010). These studies showed only a single case infected with these species. Subsequently, 15 samples of *C. bovis*, 6 of *C. ryanae* and 2 of mixed infection were detected from post-weaned and adult beef cattle in Miyagi Prefecture (Murakoshi *et al*., 2012). This is the first description of *C. ryanae* in Hokkaido. Furthermore, this is first detection of *C. ryana*e from pre-weaned calves in Japan. All *C. bovis* and *C. ryanae* PCR-positive specimens were negative by sugar flotation method. Therefore, the low detection frequency of *C. bovis* and *C. ryanae* in Japan might have been the result of the wide use of microscopy in the previous studies. *C. bovis* and *C. ryanae* in younger calves might be concealed by an overwhelming *C. parvum* infection (Feng *et al*., 2007). Using molecular diagnostic tools and examination of normal and calves with diarrhea, *C. bovis* and *C. ryanae* might be detected in other areas of Japan including Hokkaido.

    C. andersoni is also a major species detected in cattle, especially post-weaned or adults (Santín *et al*., 2008). There are several studies on *C.*

andersoni detected in cattle in Japan (Kaneta *et al*., 1998; Satoh *et al*., 2003; Matsubayashi *et al*., 2004, 2008; Koyama *et al*., 2005; Nagano *et al*., 2007; Amer *et al*., 2009). Here, we did not detect this species, perhaps because we only sampled from calves.

C. parvum, *C. bovis* and *C. ryanae* were detected along with the occurrence of diarrhea, but only *C. parvum* was statistically correlated with diarrhea ($p<0.05$). Not all diarrheal symptoms were caused by *Cryptosporidium*, but cattle with diarrhea are associated with *C. parvum*. There were some diarrhea samples that were *C. bovis* or *C. ryanae* positive. In a previous study, none of the calves infected with *C. ryanae* and *C. bovis* had any signs of disease (Fayer *et al*., 2005, 2008). Therefore, there is a possibility that other pathogens or other environmental factors were related to diarrhea calves not infected with *C. parvum*. However, our data are not sufficient to address this question. It is noteworthy that one calf which was *C. parvum*-positive by PCR did not have diarrhea and was negative by sugar flotation method. This may come from recovering calves, which still discharge viable oocysts during their recovery. Therefore, asymptomatic infection of *C. parvum* probably results in transmission to other calves.

In pre-weaned calves, 47% versus 29% and 24% *Cryptosporidium*-positive calves were infected with *C. parvum* versus *C. bovis* and *C. ryanae.* In the United States, 85% versus 9% and 5% of

Cryptosporidium-positive dairy calves, which were 2 months of age and younger, were infected with *C. parvum* versus *C. bovis* and *C. ryanae* (Santín *et al*., 2004). When the percentage of each species/genotypes of *Cryptosporidium* is represented, *C. parvum* constituted 97% of the species in pre-weaned calves (Santín *et al*., 2008). Therefore, percentages of *C. bovis* and *C. ryanae* positive pre-weaned calves were higher than United States. The results are consistent with the report that *C. parvum* was most prevalent in pre-weaned calves (Fayer *et al*., 2007).

DNA sequencing showed that all *C. parvum* positive specimens had the *C. parvum* subtype IIa. This is the common subtype found in humans and cattle in many countries (Santín *et al*., 2008).

Tables

Table 1. Infection numbers of *Cryptosporidium* in fecal samples from calves

	C. parvum	*C. bovis*	*C. ryanae*	sum
pre weaned 1-8 weeks (n=79)	8	5	4	17
post weaned 3-12 months (n=28)	0	5	3	8
sum	8	10	7	25

Table 2. Correlation between diarrhea and age of cattle

	pre weaned	post weaned
diarrhea	8	4
normal	71	24

There were no statistically difference diarrhea rate between pre-weaned and post-weaned calves.

Table 3. Correlation between diarrhea and detected genotype

pre weaned	*C. parvum*	*C. bovis*	*C. ryanae*	Not detected
diarrhea	3*	0	1	4
normal	5*	5	3	58
post weaned				
diarrhea	0	1	0	3
normal	0	4	3	17

C. parvum was statistically correlated with diarrhea (p< 0.05)

Chapter 2

Molecular and epidemiological analyses of symbiotic virus of *Cryptosporidium parvum*

An article under submission

Abstract

Here, the author shows that *Cryptosporidium parvum virus 1* (CSpV1), a member of the family *Partitiviridae*, genus *Cryspovirus* that can infect *Cryptosporidium parvum*, is a new candidate for subtyping and could serve as a detection marker of *Cryptosporidium parvum*. CSpV1 was detected in all *C. parvum*-positive samples tested. Phylogenetic analysis of partial sequences of dsRNA1 from CSpV1 samples using the maximum likelihood method revealed that the Japanese samples belonged to a Japanese clade. Sequences detected in samples from Hokkaido (Ashoro and Ishikari), Honshu, and other islands (Iwate, Tanegashima, and Okinawa) belonged to a single clade. Samples from Iwate, Tanegashima, and Okinawa belonged to a single subclade. Therefore, the *Cryspovirus* dsRNA sequences reflect the regional distribution of their host and have potential as a high-resolution tool for *C. parvum* subtyping.

Recombinant CSpV1 capsid-GST protein was synthesized in *E. coli* and monoclonal antibodies were produced in mice. Anti-CSpV1 capsid protein antibody recognized *C. parvum* sporozoites and localized as dots in the cytosol. The cell biology of *Cryspovirus* infection and localization of other *C. parvum* lifecycle stages remain unknown. Therefore, this antibody has value for the detection of *C. parvum* and study of its cell biology.

1. Introduction

Cryptosporidium parvum belongs to the phylum Apicomplexa and has been reported to be a zoonotic species that causes severe diarrhea (Sulaiman *et al*., 2005). It can cause waterborne or foodborne outbreaks. In healthy adults and children, *Cryptosporidium* infection causes a typically self-limiting disease. In contrast, in immunocompromised individuals such as AIDS patients, infections can result in life-threatening diarrhea (Current *et al*., 1983), and no effective therapy has yet been established. Therefore, studies on the species/subspecies distribution and transmission routes of *C. parvum* are important for public health and food production. Some subtyping genes for *Cryptosporidium* spp. have been identified and have been used to investigate its distribution and transmission routes. The 60-kDa glycoprotein (GP60) gene is the major subtyping gene of *C. hominis* and *C. parvum* (Leave *et al*., 2002; Glaberman *et al*., 2002; Abe *et al*., 2006) because the GP60 gene is the most polymorphic marker identified to date in the *Cryptosporidium* genome. Moreover, GP60 is located on one of the dominant targets for neutralizing antibody responses in humans (Cevallos *et al*., 2000). On the basis of this gene, the sequences of *C. parvum* and *C. hominis* have been subtyped with following designations: Ia, Ib, Id, Ie, If, and Ig for *C. hominis*; and IIa, IIb, IIc, IId, IIe, IIf, IIg, IIh and IIi for *C. parvum*; some trinucleotide repeat variation has also been reported (Xiao, 2010; Valenzuela *et al*., 2014; Wang *et al*., 2014). The IIa subtype is the most common subtype found in humans and cattle in many countries (Santín *et al*., 2008). In fact, IIa subtype has been reported as

the predominant subtype of *C. parvum* worldwide (Trotz-Williams *et al.*, 2006; Glaberman *et al.*, 2002; Xiao *et al.*, 2004). In Japan, IIa subtype is the only subtype of *C. parvum* that has been detected to date (Wu *et al.*, 2003; Abe *et al.*, 2006; Murakoshi *et al.*, 2013, 2014; Ichikawa-Seki *et al.*, 2014), and trinucleotide variations are also similar across samples. Therefore, there are no tools that can trace infection and contamination sources in outbreaks.

Recently, double-stranded RNA (dsRNA) viruses, belonging to the family *Partitiviridae*, have been identified in *Cryptosporidium* and classified as the genus *Cryspovirus* (Nibert *et al.*, 2009). These viruses contain two unrelated, linear dsRNA segments of 1.7 kbp (dsRNA1) and 1.4 kbp (dsRNA2) that are encapsidated separately. dsRNA1 encodes the RNA-dependent RNA polymerase (RdRp) and dsRNA2 encodes the capsid protein (CP). The molecular weights of RdRp and CP are 62 kDa and 37 kDa, respectively (Khramtsov and Upton, 2000). *Cryspovirus* detected from *C. parvum* has been named *Cryptosporidium parvum virus 1* (CSpV1). This virus has also been detected from *C. felis*, *C. hominis*, and *C. meleagridis* (Leoni *et al.*, 2003, 2006). Most species classified with the family *Partitiviridae* infect fungi and plants and are transmitted from cell to cell during cell division. Therefore, CSpV1 likely transmits only by intracellular routes, although its exact mode of transmission remains unclear. Although there have been a few studies of the localization of this virus in *C. parvum*, its effects on host cells, and its virulence, only a correlation between *Cryspovirus* dsRNA2 levels and parasite fecundity of *C. parvum* has been reported (Jenkins *et al.*, 2008).

RNA viruses show higher mutation frequencies than their hosts because they lack proofreading enzymes (Holland *et al*., 1982). Consequently, we were interested in whether *Cryspovirus*: CspV1 dsRNA1 and dsRNA2 could serve as novel *C. parvum* subtyping markers. The results of this study show that CspV1 dsRNA sequences reflect the regional distribution of their host.

2. Materials and Methods

2.1. Collection of samples

From 2012 to 2014, bovine fecal samples were collected from farms located in Hokkaido (Ashoro and Ishikari), Iwate, Okinawa, and Kagoshima (Tanegashima) prefectures, Japan. Ashoro, Iwate, Okinawa, and Kagoshima samples were kindly provided by numerous scientists (Ichikawa-Seki *et al*., 2014). Sampling location information is summarized in Fig 1. The *C. parvum* HNJ-1 strain (kindly provided by Dr. Yagita) was first isolated from an infected woman in Japan (Satoh *et al*., 2005), and passaged by using nude mice (BALB/c Slc-nu/nu, SLC Japan). *C. parvum* was detected in the fecal samples using both direct microscopic observation and a molecular procedure. In brief, 1 g of feces was suspended in sucrose (1.2 g/ml) and oocysts were floated to a cover slip. For DNA extraction, 0.3–0.4g of fecal specimen was used. *Cryptosporidium* spp. were detected and subtyped by nested polymerase chain reaction (PCR) amplification targeting a ~830 bp and a ~850 bp fragment of the small subunit (SSU) rRNA and 60-kDa glycoprotein (GP60) genes, respectively, as described in the chapter 1.

2.2. Sample analysis

Oocysts were purified from *C. parvum*-positive feces by using the sucrose gradient method. Oocysts were excysted by modifying a method previously described (Rochelle *et al*., 2002). Briefly, the oocysts were suspended in acidified PBS (APBS) (pH 2.0) and incubated at 37°C for 30 min.

The suspension was then centrifuged for 5 min, the pellet was washed with PBS, then resuspended in a trypsin (Gibco) and sodium taurochenodeoxycholate (Sigma T0875) mixture (0.25% and 0.75%, respectively in phosphate buffer (PB) (pH8.0)) and incubated at 37°C for 90 min. Total RNAs were extracted from sporozoites with the TRIZOL reagent (Life Technologies) and total RNA was used for cDNA synthesis with a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions with the following primers: CPVS_ORF_R (5'-TCCCCCGGGATGGGAGCGATCTGCGCTACAC-3') and CPVL_ORF_R (5'-TCCCCCGGGTCCATAAATTTTGTGACTCCTG-3'). The resulting cDNA was amplified using KOD FX Neo (TOYOBO, Japan) with primers CPVL_ORF_F (5'-CGCGGATCCAAGTTTGTCAATATCTATGAGATAC-3') and CPVL_ORF_R (5'-TCCCCCGGGTCCATAAATTTTGTGACTCCTG-3') for CspV1dsRNA1, and CPVS_ORF_F (5'-CGCGGATCCATTACAAGTTTTGAATCAATAGAG-3') and CPVS_ORF_R (5'-TCCCCCGGGATGGGAGCGATCTGCGCTACAC-3') for CspV1dsRNA2. All PCR products and some of the cloning products were sequenced by using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

2.3. Phylogenetic analysis

The sequences were aligned using Clustal X2 (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 dsRNA1 and dsRNA2 sequences were used for the phylogenetic analysis. A maximum likelihood (ML) analysis was performed using MEGA 6.0.6 (Tamura *et al*., 2011) with the T92 substitution model (Tavaré, 1986) incorporating the invariable site and Gamma distribution (five categories) options. The used substitution model and optional parameter sets were evaluated by MEGA 6.0.6, and the most suitable sets were selected according to the Akaike information criterion (AIC). To calculate the bootstrap values, 500 ML trees were constructed using the same datasets.

2.4. Plasmid construction

The *E. coli* expression vector pGEX-6P2 was purchased (GE Healthcare). The resultant plasmid, designated CspV1capsid-GST, was used as a template for PCR using the following primers: CPVS_ORF_F: 5'-CGCGGATCCATTACAAGTTTTGAATCAATAGAG-3' (*Bam*HI site underlined) and CPVS_ORF_R: 5'-TCCCCCGGGATGGGAGCGATCTGCGCTACAC-3' (*Sma*I site underlined). The amplified fragment was cloned into the *Bam*HI/*Sma*I sites of pGEX-6P. The mammalian expression vector pCAGGS was kindly provided by Dr. Murakami and the resultant plasmid CspV1-capsid-Flag, was used as a template for PCR using the following primers: CPVSFlag_F:5'-CCGGAATTCGCCGCCATGGAATCAATAGAGAAAAAG

AATG-3' (*Eco*RI site underlined) and CPVSFlag_R:5'-CC<u>GTCGAG</u>CTACTTATCGTCGTCATCCTTGTAATCATG GGAGCGATCTGCGCTAC-3' (*Xho*I site underlined). The amplified fragment was cloned into the *Eco*RI/*Xho*I sites of the pCAGGS vector.

2.5. Antibodies

CspV1capsid-GST plasmids were transformed into *E. coli* strain BL21 (Takara). Recombinant capsid-GST protein was synthesized in *E. coli* after a 3-h induction with 0.1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside). Purified recombinant proteins were used to produce monoclonal antibodies in mice. Eight-week-old BALB/c mice (SLC, Japan) were immunized intraperitoneally (i.p.) with 50 μg of purified CspV1capsid-GST protein emulsified in GERBU ADJUVANT MM (GERBU Biotechnik GmbH) in 100 μl. Second, booster doses were given i.p. with 50 μg of the same antigens after a 21-day interval. Subsequently, 50 μg of the same antigens was boosted i.v. with GERBU ADJUVANT after a 21-day interval. Mouse spleen cells were collected three days after the last booster and used to obtain a monoclonal antibody, as described previously (Nakamura *et al*., 2003). All procedures were conducted in accordance with the University of Tokyo's guidelines on the care and use of laboratory animals.

2.6. Expression of Recombinant protein in 293T cells

pCAGGS-capsid-Flag plasmids were transfected into 293T cells

using TransIT-LT1 Reagent (Takara) according to the manufacturer's instructions. Three days after the transfection, the transformed cells were lysed and proteins were purified by using the Flag-tag.

2.7. Immunoblotting

C. parvum sporozoites were treated with SDS buffer (1% SDS, 50 mM Tris-HCL (pH 8.0), 10 mM EDTA). Capsid-Flag proteins and *C. parvum* sporozoites lysate were boiled for 5 min with 2×SDS gel-loading buffer and 10 μl of each sample was subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). After SDS-PAGE, the protein bands in the gel were transferred to a PVDF membrane (BIO-RAD). The membrane was blocked with 0.25% skim milk in PBST and then probed with an anti-Flag antibody conjugated with HRP or the anti-CspV1 capsid primary monoclonal antibody. After the membranes were washed three times with PBST, anti-mouse antibody conjugated with HRP was used as the secondary antibody.

2.8. Immunofluorescence assay

Oocysts of the *C. parvum* HNJ-1 strain were excysted to sporozoites as described in the Section 2.2. Sporozoites on coverslips were incubated at 37°C for 30 min and fixed with ice-cold methanol. Fixed sporozoites were blocked with 1% BSA in PBS for 10 min at room temperature and washed three times with PBS. The antibody reaction was performed in the blocking buffer. The anti-CspV1 capsid primary monoclonal antibody was used at a

dilution of 1:2; the anti-mouse secondary antibody conjugated with Alexa 488 and the anti-*Cryptosporidium* antibody Sporo-glo (Waterborne, Inc.) conjugated to Cy3, were also used. After each antibody reaction, coverslips were washed with PBS three times. Stained sporozoites were mounted with a fluorescence mounting medium (DAKO) and sealed with nail polish. Images were obtained with ELYRA (Zeiss).

3. Results

3.1. Detection of *Cryspovirus* and Subtyping *C. parvum*

On the basis of a Blast search with the *C. parvum* partial SSU rRNA gene sequences, the parasites in all of the *Cryptosporidium*-positive specimens were identified as *C. parvum*. The author then successfully sequenced the GP60 gene PCR products in all of the *C. parvum* isolates. DNA sequencing indicated that all of the GP60 nucleotide sequences were identical to each other and had similarity with *C. parvum* subtype IIa. In total, all 26 *C. parvum*-positive specimens were *Cryspovirus* dsRNA-positive by PCR. CSpV1 dsRNA1 and dsRNA2 sequences acquired in this study were deposited in the GenBank database under the following accession numbers: LC014992–LC015040.

3.2. Phylogenetic analyses

Fig 2 shows the phylogenetic relationships based on partial dsRNA1 sequences (113 bp), obtained by using the ML method, among *Cryspovirus* collected from America, Australia, and Japan, and includes the samples analyzed in this study. Based on the dsRNA1 sequence, the Japanese samples formed a single Japanese clade. By contrast, based on the dsRNA2 sequence, there was no regional distribution of each clade. To acquire more detailed information, we constructed a phylogenetic tree based on the *Cryspovirus* dsRNA1 identified in Japan using longer sequences (1446 bp). The *Cryspovirus* dsRNA sequences detected from Hokkaido (Ashoro and Ishikari)

and other regions (Iwate, Tanegashima, and Okinawa) formed a single clade, and samples from Iwate, Tanegashima, and Okinawa formed a single subclade (Fig 3). The monophyly among these branches was clearly supported (50%− 100%). Sequences from the HNJ-1 strain (Japanese reference strain) were more closely related to those of samples from Hokkaido compared with samples from Iwate, Okinawa, and Tanegashima.

There were many nucleotide substitutions in the dsRNA1 sequences, however most of these substitutions were synonymous, preserving the amino acid sequence. Ten amino acid substitutions were detected when the partial dsRNA1 genes were compared with the *C. parvum* KSU strain, whereas four were detected when the partial dsRNA1 genes were compared to the Japanese reference strain (HNJ-1 strain) (Table 1).

3.3. Localizations of capsid protein

Western blotting of 293T/capsid-flag proteins purified by using the Flag-tag revealed a 37-kDa (CspV1-capsid protein = 36 kDa; Flag tag = 1 kDa) band, indicating the presence of the capsid-flag proteins. Therefore, immunoblotting analysis using the α-CSpV1-capsid antibody was specific for the CspV1-capsid protein (Fig 4a). Immunoblotting of parasite lysate revealed a band of approximately 36 kDa with the α-CSpV1-capsid antibody (Fig 4b). An immunofluorescence assay was performed with *C. parvum* sporozoites, which revealed that the capsid proteins localized as dots in the cytosol (Fig 4c).

4. Discussion

Double-stranded RNAs (dsRNAs) are very common in pathogenic protozoa such as *Giardia*, *Leishmania*, *Trichomonoas*, *Eimeria,* and *Babesia* (Wang and Wang, 1991). The presence of dsRNAs in *C. parvum* was first noted by Gallimore *et al.* (1995), has been observed continually, and characterized (Khramtsov *et al*., 1997, 2000; Khramtsov and Upton, 1998, 2000; Nibert *et al*., 2009) by using the *C. parvum* KSU-1 strain. This characterization revealed that these dsRNAs were the genomes of *Cryspovirus*. An attempt to subtype *C. parvum* using *Cryspovirus* dsRNA sequences has previously been reported by one research group, but their results indicated that *C. parvum* could not be distinguished from *C. hominis* (Xiao *et al*., 2001), probably due to the short size of the sequences analyzed.

In the present study, all 26 specimens were tested positive for *Cryspovirus* dsRNA. This extremely high prevalence is consistent with a previous report (Khramtosov *et al*., 2000). Together, these findings suggest that *C. parvum* infection with *Cryspoviruses* is not only prevalent in Japan but also in other countries and regions of the world. Based on the dsRNA1 sequence, the Japanese samples formed a distinct clade (Japanese clade); however, based on the dsRNA2 sequence, there was no such a convergence depending on the geographical origin of the parasites. Because the dsRNA1 and dsRNA2 sequences registered in database are short (~186 bp), longer sequences were necessary to trace strains using the *Cryspovirus* sequences as markers. The CSpV1 dsRNA1 sequences detected in the specimens from

Hokkaido and other regions (Iwate, Okinawa, and Tanegashima) clustered in a single clade, and specimens from Iwate, Okinawa, and Tanegashima clustered in a single subclade. As a result, the CSpV1 dsRNA1 sequences clearly reflected geographic origin. Therefore, *Cryspovirus* dsRNAs appear to have potential as a high-resolution tool for subtyping *C. parvum.* The *C. parvum* HNJ-1 strain was first isolated from an infected woman in Japan (Masuda *et al.*, 1991, Satoh *et al.*, 2005). She claimed to have had no close contact with pet animals or any person experiencing diarrhea. She had no recent history of traveling anywhere outside Tokyo. However, in this study, the *C. parvum* HNJ-1 strain was shown to belong to the Hokkaido clade. Therefore, it is possible that the HNJ-1 strain originated in Hokkaido. The number of sequences obtained in this study was limited, and additional molecular information will be needed to further address this issue. In this study, three sample sequences did not properly reflect the geographic regions; therefore, these specimens were removed from the phylogenetic tree. One possible explanation for this discrepancy may be that CSpV1-infected *C. parvum* are transferred throughout Japan depending on the movement of the host. Therefore, calf auctions may be one factor that influences the distribution of CSpV1.

The author found several amino acid substitutions in the coding region of the partial dsRNA1 gene when we compared the sequences of *C. parvum*-positive samples isolated in Japan with the sequence of the *C. parvum* KSU strain. Because dsRNA1 encode RdRp, these substitutions might affect

the transcription or replication of viral genomes. A correlation between *Cryspovirus* genome levels and parasite fecundity in terms of oocyst excretion has been reported (Jenkins *et al*., 2008). Some other *Partitiviridae* viruses also have relationships with their host (Nibert *et al*., 2014). For example, white clover cryptic virus 1 (WCCV1) capsid protein seems to play a role in regulating root-nodule formation by *Trifolium repens* (Suzuki *et al*, 2001, Boccardo and Candresse, 2005). Further *in vitro* and *in vivo* studies of the dsRNA1 and dsRNA2 sequences are warranted to address whether *Cryspovirus* has a direct effect on the virulence of *C. parvum*.

A previous report indicated that *Cryspovirus* capsid protein is a good target for the sensitive detection of *C. parvum* oocysts, and this protein is concentrated in the apical portion of *C. parvum* sporozoites (Kniel *et al*., 2004). However, in the present study, the capsid proteins localized as dots in the cytosol. The cell biology of *Cryspovirus* infection and the localization of other *C. parvum* lifecycle stages remain unknown; therefore, this antibody represents a useful tool to explore the cell biology of this virus.

Figure legends

Figure 1. Geographic origins of the calf fecal samples.

Geographic origins of the calf fecal samples infected with *C. parvum* used in this study. The author compared all of the CSpV1 sequences detected from the northernmost prefecture (Hokkaido), Okinawa, Iwate, and Tanegashima in Japan. The sample collection sites in Hokkaido were Ashoro and Ishikari.

Figure 2. Phylogenetic tree based on partial sequences of the *Cryspovirus* dsRNA1 sequence from around the world.

Phylogenetic tree based on partial sequences of the *Cryspovirus* dsRNA1 sequence constructed by using the maximum likelihood method for *Cryptosporidium* spp. using 178 nucleotides without gaps. Substitution model and optional parameters = T92+I. The numbers at the nodes are bootstrap values.

Figure 3. Phylogenetic tree based on partial sequences of the *Cryspovirus* dsRNA1 sequence from Japan.

Phylogenetic tree based on partial sequences of the *Cryspovirus* dsRNA1 sequence constructed by using the maximum likelihood method for *Cryptosporidium* spp. using 1446 nucleotides without gaps. Substitution model and optional parameters = T92+Γ+I. The numbers at the nodes are bootstrap values.

Figure 4. The α-CSpV1-capsid monoclonal antibody reacts with recombinant capsid-flag proteins and *C. parvum* sporozoites.

(a) Western blot of 293T/capsid-flag proteins purified by using Flag-tag. Anti-flag (left panel) and anti-CSpV1-capsid antibodies were used to detect protein expression. Molecular weight (kDa) is shown beside the panels.

(b) The α-CSpV1-capsid monoclonal antibody reacts with *C. parvum* sporozoite lysate.

(c) Immunofluorescence analysis of the *C. parvum* sporozoite. Red: Sporo-glo^{TM} (α-*Cryptosporidium* polyclonal antibody), Green: α-CSpV1-capsid monoclonal antibody conjugated with Alexa488. Scale bar = 2 μm.

Figure 1.
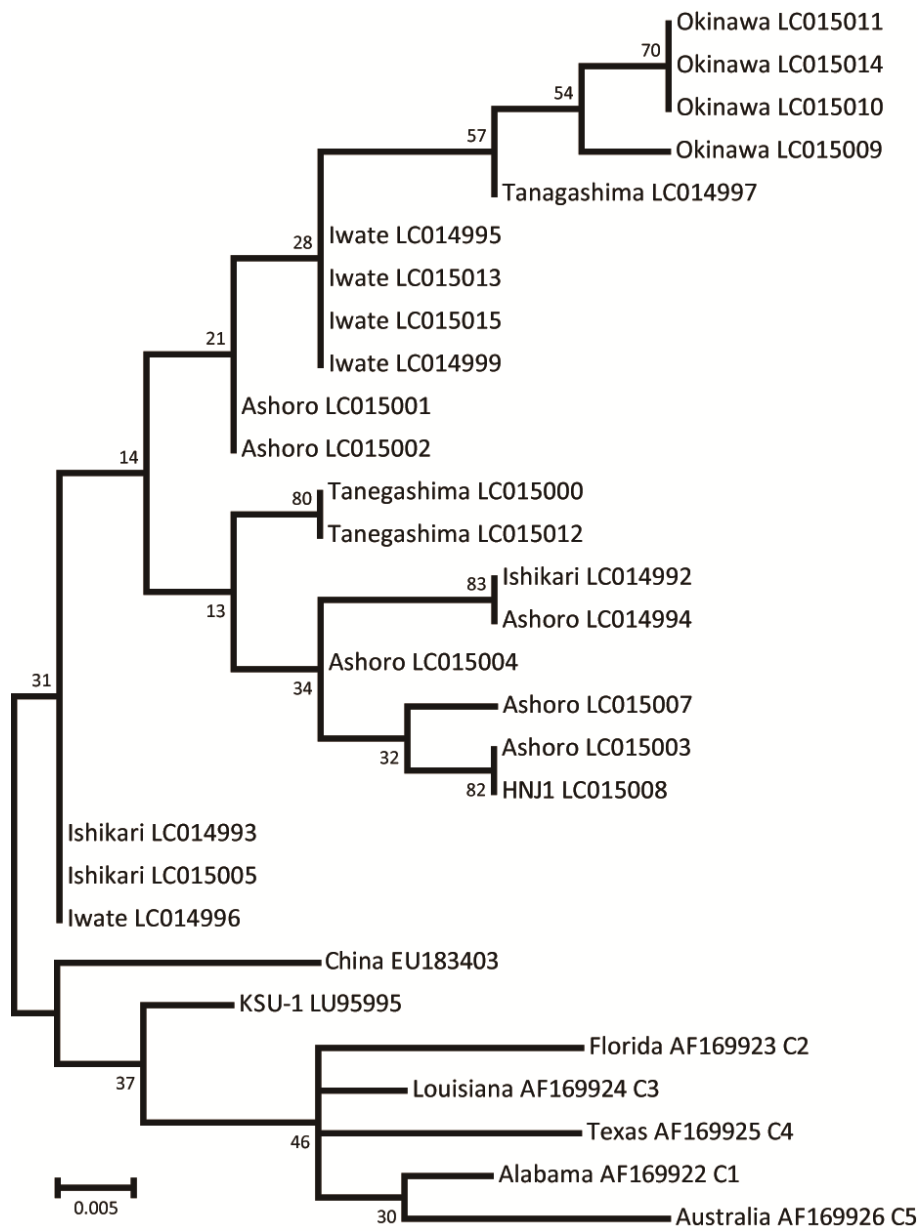

Ashoro

Ishikari

Iwate

Okinawa

Tanegashima
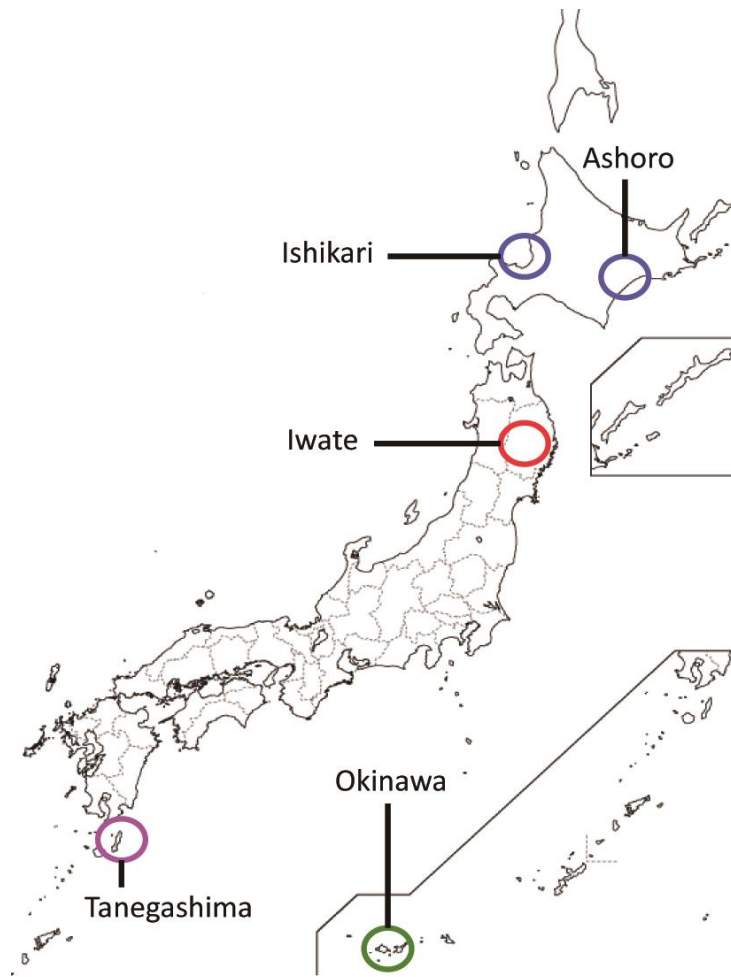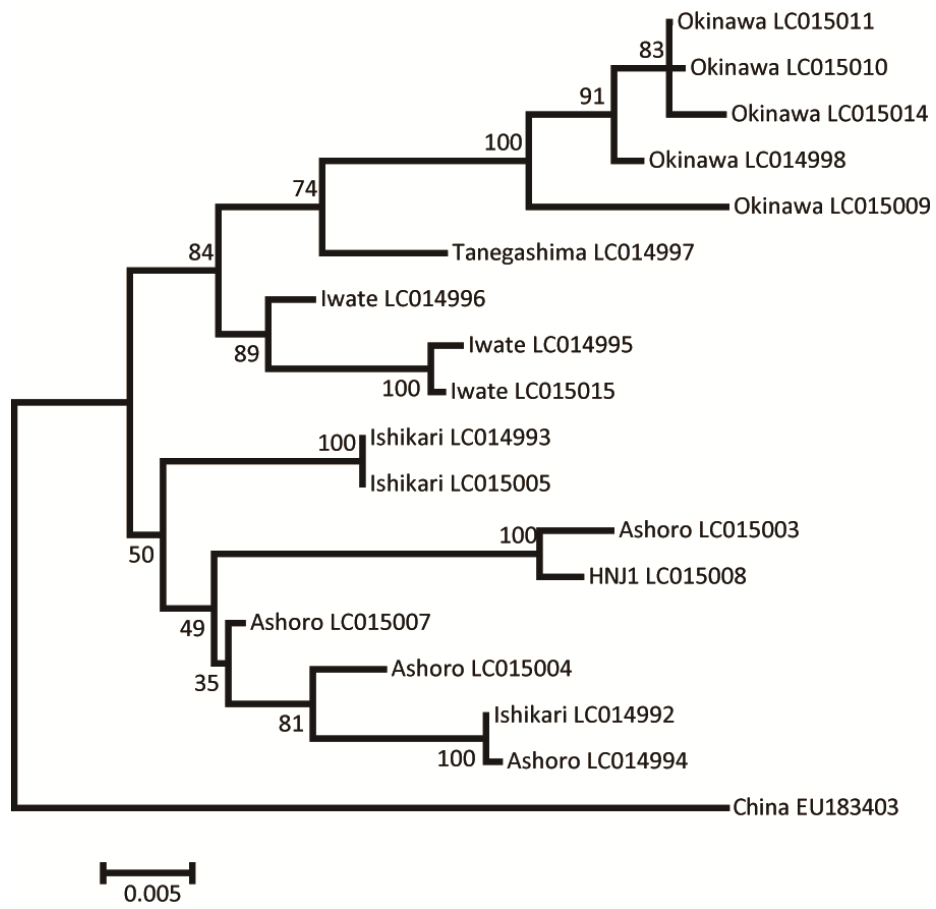
Figure 2.

Figure 3.

Figure 4.
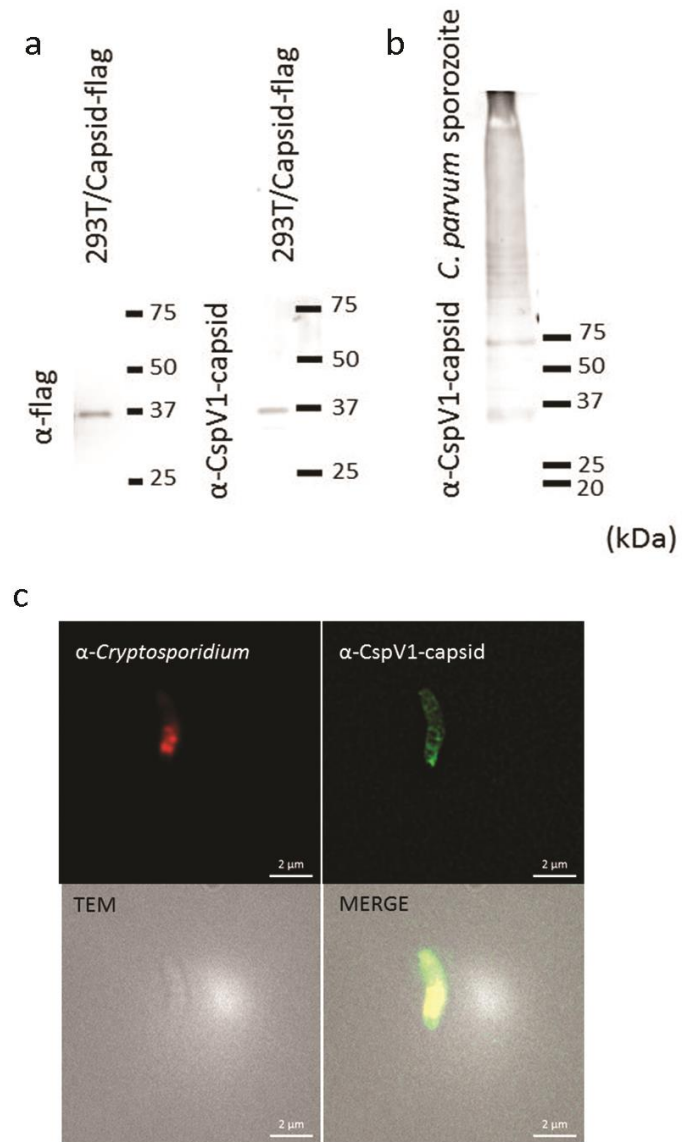
Table 1. Detection of amino acids substitution in the partial dsRNA1 gene sequence of *C. parvum*-positive samples isolated in Japan compared with that of the *C. parvum* KSU reference strain and a Japanese reference strain. The single letter codes for amino acids are used.

position	ref (KSU strain)	Substitution
19	A	V
99	F	L
110	E	G
111	L	S
120	L	S
168	T	I
175	K	R
423	E	V
443	Q	L
465	A	T
	ref (HNJ-1 strain)	
19	A	V
175	K	R
346	G	R
347	V	I

Chapter 3

Administration of lasalocid-NA is preventive against Cryptosporidiosis of newborn calves

Abstract

Lasalocid is an ionophorous, antiprotozoal agent that has been shown to prevent cryptosporidium growth *in vitro*. However, in calves, few studies have assessed the efficacy of lasalocid in the treatment or prevention of cryptosporidiosis because of its acute toxicity depending on the dose. Here, the author gave lasalocid-Na (3 mg/kg) to calves once daily from birth to 7 days of age to assess the preventive effects of lasalocid. The author successfully identified an appropriate dose of lasalocid that did not cause toxic effects in the calves. Compared to the untreated group, the treated group experienced no diarrhea during the dosing period. The positive rate of *Cryptosporidium* oocytes for the treated calves was significantly lower than that for the untreated calves at 7 days old; however, lasalocid was not protective after the dosing period. These data suggest that lasalocid administration may delay oocyst excretion.

1. Introduction

Cattle are major hosts of *Cryptosporidium* (Santín and Trout, 2008). Cryptosporidiosis in neonatal calves is associated with weight loss and mortality, as well as with zoonotic infections in humans (de Graaf *et al*., 1999). Although many chemotherapeutic antimicrobial compounds have been tested, there is no clearly effective compound currently available in cattle. Lasalocid is an ionophorous antibiotic and an antiprotozoal agent for the prevention of coccidiosis. It disrupts parasite membrane potential and stimulates ATPase activity in mitochondria (Schwingel *et al*., 1989).

In *Cryptosporidium* research, there have been some reports that lasalocid shows efficiency *in vitro* and in experimental animals (Leitch and He, 1994; Giacometti *et al*., 2000); however, few studies have examined the effectiveness of lasalocid against cryptosporidiosis in calves. There are several reasons for this: First, lasalocid can be acutely toxic to hosts depending on the dose used (Galitzer *et al*., 1986); second, at non-toxic doses, no clear effect of lasalocid has been demonstrated in calves with symptoms of severe diarrhea caused by *Cryptosporidium*; and third, the use of cattle in experimental infections can be problematic because of regulation. In previous studies, the anti-cryptosporidial activity of lasalocid was assessed from a treatment viewpoint in dairy calves infected with *Cryptosporidium*. In contrast, few preventive effects studies have been conducted.

To assess the preventive effects in lasalocid, here the author used calves naturally infected with *C. parvum*, and gave lasalocid-Na (3 mg/kg)

once daily from birth to 7 days of age. During this dosing period, the author found a significant difference in the positive rate of *Cryptosporidium* oocysts between the treated and untreated groups. Moreover, this dose of lasalocid did not produce cardiovascular symptoms such as atrial tachycardia and anorexia, which are typical side effects of lasalocid.

2. Materials and Methods

Twelve calves on two farms in the Aichi prefecture of Japan were used in this study. Cattle ID; #1, #2, #10 and #12 belonged to farm A, and others belonged to farm B (Table 1). All calves on such farms are naturally infected with *Cryptosporidium* in the first 7 days of life. Lasalocid-Na (3 mg/kg) was given by oral ingestion to 9 of the calves once daily from birth to 7 days of age; the other 3 calves served as the non-treated group. Dose of Lasalocid were determined by veterinarian of Aichi prefecture by pre-examination. Three mg/kg of lasalocid were high dose but did not produce any side effects. The author collected fecal samples daily from birth to 7 days and the every two days thereafter. The number of oocysts in the feces was counted on days 5 and 7 day after birth and then, every three days. The author used the sugar flotation method. Specifically, 1g of feces were diluted one-hundredfold in sucrose solution and oocysts were floated with plankton counting slides (Matsunami Glass IND., LTD) and determined the total number of oocysts per gram (OPG). The detection limit in this study was $< 4 \times 10^3$ OPG. DNA was also extracted from fecal samples (specifically, the 5, 7, 13, 22, and 28 day samples) and was sequenced as described chapter 1. The author estimated the positive rates for the 5, 7, 13, 22, and 28 day samples in the treated and control group, respectively, using the following calculations: Positive rate = the number of *Cryptosporidium* positive calves / the number of total used calves. Positive rates were compared by using a Fisher's exact test with R 3.0.2 software. Differences at $p < 0.01$ were considered significant.

Unique 18S rRNA and GP60 sequences were deposited in the GenBank database under the accession numbers AB909498 – AB909500.

3. Results

With the dosing regimen used (lasalocid 3 mg/kg), no side effects (cardiovascular symptoms and anorexia) were observed. Moreover, compared with the untreated group, the treated group did not experience diarrhea during the dosing period. Additionally, although *C. parvum* was detected in samples from the untreated group during the 0–7-day period, it was not detected in samples from the treated group over the same time period (i.e., the dosing period). The positive rate of *Cryptosporidium* oocysts for the treated calves was significantly lower than that for the untreated calves at 7 days old ($P < 0.01$) (Table 1) (Fig 1). However, lasalocid was not protective after the dosing period; the author detected both *C. parvum* and *C. bovis* in the calves. *C. parvum* was detected in samples from all treated and untreated calves during the experimental period (at 0 − 28 days old). *C. bovis* was detected in samples from three calves. GP60 gene subtype in all of the *C. parvum* isolates was identified as IIaA15G2R1. This is the most common subtype found in humans and cattle in many countries (Santín *et al*., 2008).

4. Discussion

To date, few studies have examined lasalocid as an anticryptosporidial agent in calves. Administration of lasalocid at 0.3 g/calf daily was effective but toxic, but 0.03 g/calf/day was not effective in up to 14-day-old calves (Moon *et al*., 1982). Göbel reported that 15 mg/kg for 3 days was effective in 4–5-day-old calves (Göbel, 1987a) and that administration of 6–8 mg/kg for 3–4 days was also effective in *C. parvum*-infected calves whose ages ranged from 2 days – 3 months (Göbel, 1987b). Another report showed that administration of 8 mg/kg of lasalocid was effective, however, some calves showed side effects (Sahal *et al*., 2005). These reports suggest that administration of 6–8 mg/kg of lasalocid is effective against cryptosporidiosis in calves, but may cause adverse effects and even death. In this study, lasalocid-Na (3 mg/kg) was given to calves once daily from birth to 7 days of age with the understanding that all newborn calves are naturally infected with *Cryptosporidium* in the first 7 days of life. No side effects (cardiovascular symptoms and anorexia) with this dosing regimen (lasalocid 3mg/kg) were observed. Moreover, during the dosing period, the author found a significant difference in the positive rate of *Cryptosporidium* oocytes between the treated and untreated groups (Table 1). These data suggested during treatment period, lasalocid is preventive against cryptosporidiosis. The finding that preventive effect of lasalocid supports a similar report regarding the administration of 120 mg/kg lasalocid to nude mice (Leitch and He, 1994). Lasalocid was not protective after the dosing period; however, this finding that lasalocid prevents oocyst excretion in calves

to be important because diarrhea in newborn calves can lead to a serious loss of growth, additionally, almost calves were infect with *C. parvum* by the age of 2 months and infections rates were dropped dramatically in post weaned calves (Santín *et al*., 2008). Lasalocid may affect microbes in lumen but is acceptable because lumen is not well-developed in pre weaned calves. The author recognizes that this study depends on natural infection and that there is no definite answer as to when the treatment group calves were exposed to *Cryptosporidium*. However, it is difficult to demonstrate anticryptosporidial effects using experimental infection of calves because of regulations. Nevertheless, this study using natural infection provides valuable insights and supports the need for further investigations to determine the optimum dosing period.

Figure legends

Figure 1. Average of oocysts shedding

Average OPG was counted on days 5 and 7 after birth and then every three days. The detection limit in this study was $< 4\times10^3$ oocyst per gram.

Figure 1.
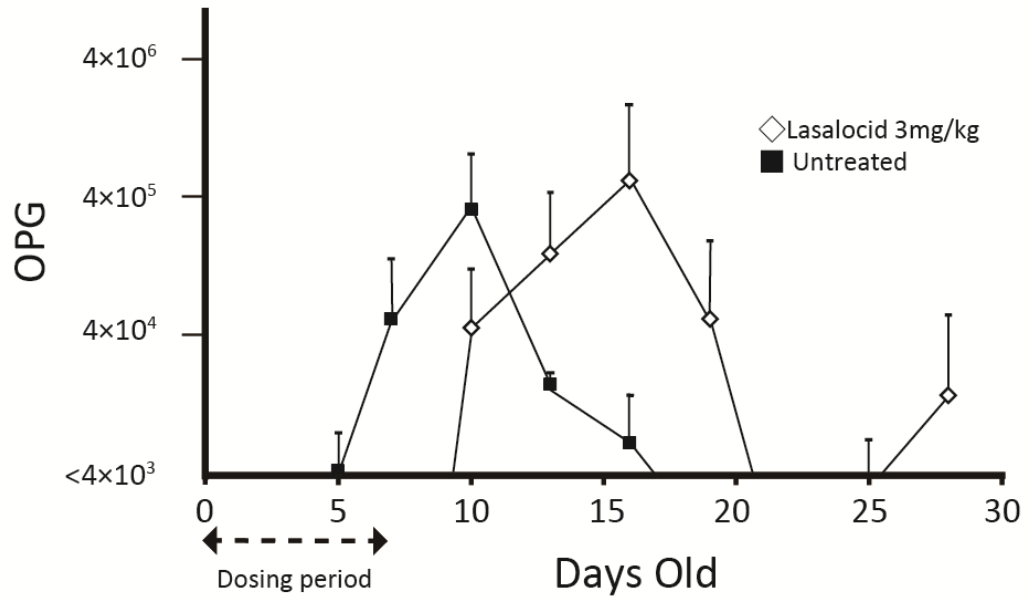
Table1. Genotyping of *Cryptosporidium* in feces at 7, 13, 22, and 28 days after birth

cattle ID	7*	13	22	28
		Days old		
1	N	*C. parvum*	N	N
2	N	*C. parvum*	N	*C. parvum*
3	N	*C. parvum*	*C. parvum*	*C. parvum*
4	N	*C. parvum*	N	*C. parvum*
5	N	*C. parvum*	N	N
6	N	*C. parvum*	N	N
7	N	*C. parvum*	N	*C. bovis*
8	N	*C. parvum*	*C. parvum*	N
9	N	Cp&Cb	*C. bovis*	*C. bovis*
10	*C. parvum*	*C. parvum*	N	N
11	N	*C. parvum*	N	N
12	*C. parvum*	*C. bovis*	*C. bovis*	*C. bovis*

N = not detected ID 1-9 were treated group, ID 10-12 were non treated group.

Positive rates were compared by using a Fisher's exact test with R 3.0.2 software. *Differences at $p < 0.01$ were considered to be significant.

Chapter 4

Analysis of histone modifications in *Cryptosporidium parvum*

Abstract

Cryptosporidium parvum belonging to Apicomplexa causes acute watery diarrhea. In Apicomplexa, many papers reported that histone modification is also important in the parasite virulence and controls their life cycle of *Toxoplasma* and *Plasmodium* closely related to *Cryptosporidium*. In *Cryptosporidium*, there is no information on histone modification. In this chapter, the author, therefore, attempted to find out whether the histone modification took place in *C. parvum*. Histone H3K9Ac, H3K4me1, H3K4me2, H3K4me3, and H4K20me3 signals were observed at the sporozoite stage. It was also observed that histone modification specific antibodies localized in the trophozoite / type I meront stages. Additionally, the author concludes that *C. parvum* histone methyltransferase may become new drug target.

1. Introduction

Cryptosporidium parvum belonging to Apicomplexa causes acute watery diarrhea. Therefore, the species is one of the most important enteric pathogen to public health. The pathogen can infect mammals including human worldwide (O'Donoghue, 1995; Fayer, 2004). However, there is no effective therapy, and, *in vitro* culture system, and transfection method have been established. Apicomplexa has a complex life cycle. To maintain this life cycle, the parasite may use various types of regulations, such as regulation of gene expression, translational repression, and posttranslational protein modification. The eukaryotic genome is packaged as chromatin with nucleosomes. Nucleosome, the nucleohistone subunits, of chromatin organization is critical for gene regulation for eukaryotes. Chromatin states are distinguished euchromatin (lightly packed form) and heterochromatin (heavy packed form) and these two variations control gene regulation. There are some elements of change nucleosome position, such as DNA methylation, histone variants, selectivity of DNA sequence and histone modification. DNA methylation is a highly stable silencing mark that extends over long chromosomal regions (Fazzari and Greally, 2004). Methylated cytosine mediates silencing of promoter activity. Histone variants distinct patterns of modifications of histone and histone tail binding proteins and contribute to establishment of construction of chromatin (Peterson and Laniel, 2004). Histone code is idea that histone modifications and remodeling of chromatin regulate the transcription and cell division (Strahl and Allis, 2000). Histone is composed of

two copies of the histone proteins H2A, H2B, H3 and H4. Methylation, acetylation, ubiquitinylation, Small Ubiquitinlike Modifier modification (SUMOylation), and phosphorylation. And these modification regulate transcriptional activation or suppression by the interaction with DNA and nuclear proteins (Li *et al*., 2007). In Apicomplexa, many researchers reported that histone modification is also important in the parasite virulence and controls their life cycle of *Toxoplasma* and *Plasmodium* closely related to *Cryptosporidium*. For example, in *P*. *falciparum*, histone modification trigger changes in the expression of *var* gene family, which encodes the antigen on the surface of expressed infected red blood cells (Jiang *et al*., 2013). Since the methylation of histone H4 is differentially redistributed in cell cycle in *T*. *gondii*, therefore, it might be expected that H4K20me3 was important in the stage conversion (Sautel *et al*., 2007). However, in *Cryptosporidium*, there is no information on histone modification. Moreover, there is no information on centromere sequence and centromere-specific histone H3 variant CenpA in *Cryptosporidium*. Centromere region is important for sister chromatid association and separation, chromosomal movement and the establishment of heterochromatin, and mitotic check-point control (Pluta *et al*., 1995; Cleveland *et al*., 2003; Morris and Moazed, 2007). CenpA is a defined marker for active centromeres (Marshall *et al*., 2008). These epigenetic systems are dramatically different between vertebrate and Apicomplexa. For example, histone methyltransferase inhibitors display the rapid antimalarial activity than mice (Malmquist *et al*., 2012). Unlike other organism, *Toxoplasma gondii*

centromeres show no nucleotide bias (Brooks *et al*., 2011). As described above, epigenetic mechanism may be different between host and *Cryptosporidium* in many points and become drug targets. In this chapter, the author, therefore, attempted to determine nucleosome state, and, whether histone modification took place in *C. parvum*. Additionally, the author tried to know if that *C. parvum* histone methyltransferase could become a new drug target.

2. Materials and Methods

2.1. Analysis of histones in *C. parvum* and determined sequence of *C. parvum* centromere protein A (CenpA).

Protein sequences of histone H3, H2A, H2B and H4 of human, *Toxoplasma gondii* and *Cryptosporidium parvum* were aligned. The protein sequences of the human, *T. gondii* and *Cryptosporidium* histones were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and CryptoDB (http://cryptodb.org/cryptodb/) database. The sequences were aligned using Clustal X2 (Larkin *et al*. 2007), and visualized with BioEdit 7.0.5.3 (Hall, 1999).

To determine sequence of *C. parvum* centromere protein A (CenpA), the author obtained sequence of putative *C. parvum* CenpA (cgd4_2030) from CryptoDB database. *C. parvum* putative CenpA (cgd4_2030) have characteristic amino acid sequence in C-terminal regions, however, their N-terminal sequence was incomplete. The author designed CenpA_R primer (5'-TTAAATAAAGTCTCCATA-3') from C-terminal sequence and CenpA_F primer (5'-ATGGCTCGTTCTAAAACT-3') from *Cryptosporidium parvum* whole genome sequence. *C. parvum* oocysts were excysted to sporozoites as described in chapter 2. Total RNAs were extracted from sporozoites with the TRIZOL reagent (Life Technologies) and total RNA was used for cDNA synthesis with a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions with CenpA_R primer. The resulting cDNA was amplified using KOD FX Neo (TOYOBO,

Japan) with CenpA_F and CenpA_R primer. PCR products were sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Based on the determined sequence, anti-*C. parvum* CenpA peptide rabbit polyclonal antibody were made by Operon eurofins. Sequence information is shown in Figure. 6.

2.2. Analysis of histone modifications in *C. parvum*

C. parvum HNJ-1 strain (kindly provided by Dr. Yagita, NIID, Tokyo, Japan) was first isolated from infected woman in Japan (Satoh *et al*., 2005), and passaged using nude mice (BALB/c Slc-nu/nu, SLC Japan). Oocysts were purified from *C. parvum* positive feces by the sucrose gradients method. Oocysts were excysted to sporozoites as described in chapter 2. Histones of sporozoites were extracted by acid extraction methods (Shechter *et al*., 2007) using 4×10^8 sporozoites. Extracted histones were boiled for 5 min with $2 \times$SDS gel-loading buffer and each 10µl of sample was the subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). After SDS-PAGE, the protein bands in the gel were electrically transferred to a PVDF membrane (BIO-RAD). Membrane was blocked with 0.25% skim milk in PBST for 30min at room temperature. Then, membrane was probed with anti-histone modification antibodies (Table 1) of anti-*C. parvum* CenpA peptide antibody and anti-mouse or rabbit 2nd antibody conjugated with HRP. The antibody on the membrane was detected by development using ECL (GE healthcare

Bio-Sciences AB). The images were obtained with VersaDoc 5000 (BIO-RAD).

2.3. Immunofluorescence assay

Oocysts of *C. parvum* HNJ-1 strain were excysted to sporozoites as described in chapter 2. Sporozoites were maintained for 48h on monolayers of HFF (Human Foreskin Fibroblast) cells cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) containing 10% fetal calf serum (FCS) and 2 mM L-glutamine, streptomycin (50 mg/L), and penicillin (50,000 units/L). At 48h post parasites inoculation, infected HFF cells were washed with ice cold PBS three times and fixed with 4% paraformaldehyde in PBS for 20 min, followed by two washing with PBS. Fixed cells were permeabilized with 0.2 % Triton X-100 in PBS for 10 min and excess fixative was quenched with 0.25 M glycine in PBS for 5 min and washed twice with PBS. Cells were blocked with 1 % BSA in PBS for 10 min and washed three times with PBS. Anti-histone modification antibody (Anti- H3K4me1, H3K4me2, H3K4me3, H3K9Ac, and H4K20me3 antibodies: Table 1) was used at a dilution of 1:400 and anti-rabbit or mouse 2nd antibody conjugated to Alexa 488 (Invitrogen) and Sporo-glo^{TM} (Waterborne, Inc.) conjugated to Cy3 was used in a dilution of 1:1,000 and 1:3, respectively. After each antibody reaction, cells were washed with PBS three times. Stained cells were mounted with a fluorescence mounting medium (DAKO) and sealed with the nail polish. The images were obtained with confocal microscope (ELYRA, Zeiss.)

2.4. Phylogenetic analyses of SET domains in *Cryptosporidium*

SET domain amino acid sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/) were aligned using Clustal X2 (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 SET domain sequences were used for the phylogenetic analysis. A maximum likelihood *(*ML) analysis was performed using MEGA 6.0.6 (Tamura *et al*., 2011) with the LG substitution model (Le and Gascuel, 2008). Used substitution model and optional parameter sets were evaluated by MEGA 6.0.6, and the most suitable sets were selected according to the Akaike information criterion (AIC). To calculate the bootstrap value, 500 ML trees were constructed using the same datasets.

3. Results

3.1. Histone and histone modification of *C*. *parvum*

C. *parvum* histone H3, H2B and H4 were highly conserved compared with human and *T*. *gondii* histone sequence (Fig 1, 3 and 4). *C*. *parvum* have two variant of histone H2A. Based on sequence analysis, two variants regarded as H2AX and H2AZ. Histone H2 was not closely related to human histone H2 (Fig 2). Nucleotide sequence of *C*. *parvum* CenpA were determined and CenpA gene have two region of intron (Fig 5) Western blotting of parasite histone lysate showed a single band and molecular size was 15 kDa (Histone H3) or 11 kDa (Histone H4) with histone modification specific antibody (Fig 2). For example, 'H3K4me3' means that the fourth lysine of histone H3 (except methionine) were tri-methylated. In *Cryptosporidium* H3, H3K9Ac, H3K4me1, H3K4me2, H3K4me3 and H4K20me3 specific signals were observed (Fig 7). However, signals using other antibodies (specific for H3K9me, H3K27me, H3S10P, H3K36me and CenpA) signals were not observed (Table 1). Amino acid sequences of histone H3 are highly conserved and in *T*. *gondii*, clear signals were detected using the same antibody (Fig 8).

3.2. IFA

Immunofluorescence assay was performed with host cells infected with parasites at 48h post inoculation. The author observed fluorescence of histone modifications specific for the antibodies were localized in the parasite (Fig. 9). In 48h post inoculation, the parasites stage was trophozoite and type I

meront stages. H3K4me2, H3K4me3 and H3K9Ac signals were localized as dots in cytosol but H4K20me3 signals were localized in edge of the parasite.

3.3. Phylogenetic tree based on amino acid sequences of the SET domain sequence in the Apicomplexa, human and model organism.

Histone lysine methyltransferases (HMTases) have conserved domain and this domain is called SET domain. Previous studies showed invariant residues in the SET domain determined the methylation specificities. Figure 10 shows the phylogenetic relationships based on amino acids sequences of SET domain with ML method among *Cryptosporidium, Toxoplasma, Plasmodium,* human and model organism. Red letters show *Cryptosporidium* SET domains and Green show *Toxoplasma* and *Plasmodium*. Black letters show human and model organism as reference. Almost SET domain of Apicomplexa sequences were not classified with well-known clade. Additionally, there was no *Cryptosporidium* SET domain in the H3K9me clade.

4. Discussion

   C. parvum histone H3, H2B and H4 were highly conserved compared with other eukaryote histone sequence and WB analysis, histone H3 and H4 signals were clearly observed. Therefore, in histone study of *C. parvum*, most of commercial histone modification specific antibody is powerful tool to analyze histone modification pattern in *C. parvum* genome. *C. parvum* have two variant of histone H2A (H2AX and H2AZ). *C. parvum* H2AX and H2AZ were not closely related to human but closely related to *T. gondii*. Histone H2AX is phosphorylated in telomeres and elicits a DNA damage response in mammal and yeast (D'Adda di Fagagna et al., 2003; Kim et al., 2007). Histone H2AZ is involved in transcriptional regulation on a genomewide level (Madigan *et al*., 2002). Therefore, Apicomplexa may have different histone modification mechanism. In this study, the author demonstrated that some histone modifications were detected and colocalization with *Cryptosporidium parvum.* In the parasite, H3K9Ac, H3K4me1, H3K4me2 and H3K4me3 signals were observed at the sporozoite and trophozoite / type I meront. Therefore, *C. parvum* maintain various gene expressions by histone modification and have mechanism of histone acetylation and methylation. H3K9Ac and H3K4 have been implicated in gene activation in eukaryote. In *Plasmodium*, H3K4 are maintained through stages and spread equally across active and inactive genes in ring stage. In schizonts stage, H3K4me3 was associated with 5' end of active genes (Salcedo-Amaya *et al*., 2009). In *Toxoplasma*, H3K4me and H3K9ac existed at the 5' end of active genes in

tachyzoites stage (Gissot *et al.*, 2007). However, H3K9 repressive marks were not detected in *C. parvum* sporozoites. This data suggests that H3K9 methylation signal is very low or not enriched across genes in *C. parvum* sporozoites stage. Further analysis of other stages of *C. parvum* are needed to determine whether the parasite has H3K9 methylation or not. H3K9me was associated with heterochromatin regions near the centromeres in *T. gondii* (Brooks *et al.*, 2011), whereas it was associated with subtelomeric region in *Plasmodium* (Lopez-Rubio *et al.*, 2009). In repressive markers, only H4K20me3 was detected in *C. parvum* sporozoites and trophozoite / type I meront stage. H4K20me3 signals were localized in edge of the parasite. However, in *T. gondii*, H4K20me3 is associated with centromere and localize to an apical region (edge of the parasite) (Brooks *et al.*, 2011). Therefore, *Cryptosporidium* also may have unique elaboration of the nuclear envelope like *T. gondii*. Further immunofluorescence assay with nuclear staining is needed. Centromere sequence of *T. gondii* has no features such as AT-rich and repeated sequences in contrast to other eukaryote. Therefore, centromere region of this parasite was detected by the chromatin immunoprecipitation sequence (ChIP-seq) of H3K9me3 (Brooks *et al.*, 2011). On the other hand, in *P. falciparum*, H4K20me3 is spread across the genome at the ring stage (Lopez-Rubio *et al.*, 2009) and enriched at the telomeric foci in early schizont stage (Sautel *et al.*, 2007). Centromere region of *C. parvum* remains unknown. In bioinformatics analysis, there are no At-rich and long repeated sequence. Unfortunately, in this study, CenpA signals were not observed because of life

cycle timing or concentration. Threfore, ChIP-seq of H4K20me3 may be key of discover centromere regions in *C. parvum*.

This parasite has histone lysine methylation signals, however, the author did not find any histone lysine demethylase such as LSD (Lysine-specific demethylases) and JMJC (Jumonji C) type demethylase in database domain search. Next, the author searched histone methyl transferase in this parasites using CryptoDB domain search and found 10 putative methyl transferases. Previous studies showed invariant residues in the SET domain which determined the methylation specificities (Schubert *et al.*, 2003). Figure 4 shows the phylogenetic relationships based on amino acids sequences of SET domain with ML method among *Cryptosporidium, Toxoplasma, Plasmodium,* human and model organism. There was no *Cryptosporidium* SET domain in the H3K9me clade. This data suggested there were no methyl transferase that transfer mono-, di- and trimethyl H3K9 in *C. parvum* and was no conflict with the data of immunoblotting in this study. In *P. falciparum*, PfSET1, 3, 6, 7 and 9 genes could not be genetically disrupted, suggesting that these PfSET genes are essential (Jiang *et al.*, 2013) and histone methyltransferase inhibitors display the rapid antimalarial activity (Malmquist *et al.*, 2012). Additionally, in the study, most SET domain of Apicomplexa sequences were not assigned to well-known clade. These data suggest that *C. parvum* histone methyltransferase also may become new drug target.

Figure legends

Figure 1. Aliment of histone H3 of human and *Cryptosporidium parvum*.

Conserved amino acid sequences are represented by "*". The sequence of the human and *Cryptosporidium* histone H3 were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and CryptoDB (http://cryptodb.org/cryptodb/) database. GenBank accession number: CAB02546 (human histone H3). CryptoDB accession number: cgd3_2540 and cgd4_3220.

Figure 2. Aliment of histone H2AX and H2AZ of human, *Toxoplasma gondii* and *Cryptosporidium parvum*.

Conserved amino acid sequences are represented by "*". The sequence of the human and *Cryptosporidium* histone H2A were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and CryptoDB (http://cryptodb.org/cryptodb/) database. GenBank accession number: CAB02538, CAA47464 (human histone H2A), KFH15375 and AF502246 (*T. gondii* H2A). CryptoDB accession number: cgd8_2170 and cgd5_940.

Figure 3. Aliment of histone H2B of human, *Toxoplasma gondii* and *Cryptosporidium parvum*.

Conserved amino acid sequences are represented by "*". The sequence of the human and *Cryptosporidium* histone H2B were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and CryptoDB

(http://cryptodb.org/cryptodb/) database. GenBank accession number: CAB02543 (human histone H2B) and XP_002369740 (*T. gondii* H2B). CryptoDB accession number: cgd5_3170 and cgd7_1700.

Figure 4. Aliment of histone H4 of human and *Cryptosporidium parvum*

Conserved amino acid sequences are represented by "*". The sequence of the human and *Cryptosporidium* histone H4 were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and CryptoDB (http://cryptodb.org/cryptodb/) database. GenBank accession number: NP_778224 (human histone H4). CryptoDB accession number: cgd8_5230.

Figure 5. Genome and mRNA sequence of *Cryptosporidium parvum* centromere protein A (CenpA).

The mRNA sequence of the *C. parvum* CenpA is shown on top and genome sequence is shown on bottom. Color box shows intron region.

Figure 6. Protein sequence of *Cryptosporidium parvum* centromere protein A (CenpA).

Solid box shows specific sequence of CenpA compared with *C. parvum* histone H3. Gray sequence used to generate polyclonal anti-peptide antibody.

Figure 7. Histone modification of *C. parvum*.

Western blot of *C. parvum* histone proteins purified by acid extraction methods. Anti- H3K4me1, H3K4me2, H3K4me3, H3K9Ac and H4K20me3 antibodies were used to detect histone modification. Molecular weight (kDa) is shown beside the panels. Histone H3 monoclonal antibody is used as a reference.

Figure 8. Histone modification of *T. gondii*.

Western blot of *T. gondii* histone proteins purified by acid extraction methods. Anti- H3K9me2 and H3K9me3 antibodies were used to detect histone modification. Molecular weight (kDa) is shown beside the panels.

Figure 9. Histone modification specific antibodies can be reacted with *C. parvum* torophozoites.

Immunofluorescence analysis of the *C. parvum* torophozoites. Red: Sporo-glo[TM] (anti-*Cryptosporidium* polyclonal antibody), Green: anti-human Histone H3/H4 modification antibodies, Scale bar = 2μm.

Figure 10. Phylogenetic tree based on amino acid sequences of the SET domain sequence in the Apicomplexa, human and model organism.

Phylogenetic tree based on partial amino acids sequences of the SET domain sequence constructed by ML, using 82 sequences without gaps. Substitution model and optional parameters = LG. The numbers at the nodes are bootstrap values. Red letters show *Cryptosporidium* SET domains and

Green letters show those of *Toxoplasma* and *Plasmodium*. Black letters show those of human and model organism as reference.

Figure 1.

```
          ....|....| ....|....| ....|....| ....|....| ....|....|
                  10        20        30        40        50
cgd3_2540     MARTKQTARK STGGKAPRKQ LASKGARKSA PVTGGVKKPR RYRPGTVALR
cgd4_3220     MARTKQTARK STGGKAPRKQ LASKGARKSA PVTGGVKKPR RYRPGTVALR
humanH3_CA    MARTKQTARK STGGKAPRKQ LATKAARKSA PATGGVKKPH RYRPGTVALR
Clustal Co    ********** ********** **:*.***** *.*******: **********


          ....|....| ....|....| ....|....| ....|....| ....|....|
                  60        70        80        90       100
cgd3_2540     EIRRFQRSTE LLIRKLPFQR LVREIAQDFK TDLRFQSQAV MALQEAAEAY
cgd4_3220     EIRRYQRSTE LLIRKLPFQR LVREIAQDFK TDLRFQSSAV MALQEAAEAY
humanH3_CA    EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQSSAV MALQEACEAT
Clustal Co    ****:*:*** ********** ********** *******.** ******.**


          ....|....| ....|....| ....|....| ....|.
                 110       120       130
cgd3_2540     LVGLFEDTNL CAIHAHRVTI MPKDIQLARR IRGER-
cgd4_3220     LVGLFEDTNL CAIHAHRVTI MPKDVQLARR IRGER-
humanH3_CA    LVGLFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA
Clustal Co    ********** *****:**** ****:***** *****
```

Figure 2.

H2AX

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                     10         20         30         40         50
cgd8_2170    MSGKVTSSGG RGGGKKTTRK TMSNSAKAGL QFPVGRVARY LKKGRYAKRI
Tg_KFH1537   MSAKG--AGG RK--KTSSGK KVSRSAKAGL QFPVSRIGRY LKKGRYAKRV
human_CAB0   MSGRGK-QGG KA-----RAK AKTRSSRAGL QFPVGRVHRL LRKGNYSERV
Clustal Co   **.:    ** :        *    :.*::*** ****.*: *  *:**.*::*:


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     60         70         80         90        100
cgd8_2170    GAAAPVYLAA VLEYLCAELL ELAGNAARDA KKTRITPRQI QLAVRNDEEL
Tg_KFH1537   GVGAPVYLAA VLEYLCAEIL ELAGNAARDH KKTRIIPRHI QLAVRNDEEL
human_CAB0   GAGAPVYLAA VLEYLTAEIL ELAGNAARDN KKTRIIPRHL QLAIRNDEEL
Clustal Co   *..******* ***** **:* ********* ***** **:: ***:******


             ....|....| ....|....| ....|....| ....|....
                    110        120        130
cgd8_2170    SKFLGNVTIA SGGVLPNIPT VLLPKKSKSK --QGNSQEF
Tg_KFH1537   SKFLGGVTIA NGGVMPHVHA VLLPKHSKSK GKHGVSQEF
human_CAB0   NKLLGKVTIA QGGVLPNIQA VLLPKKTESH --HKAKGK-
Clustal Co   .*:** **** .***:*:: : *****:::*:    :  . :
```

H2AZ

```
             ....|....| ....|....| ....|....| ....|....| ....|....|
                     10         20         30         40         50
cgd5_940     MDGATSSG-K IGGKVGGKVG -----GKGKA GSGKGSKKQP TSRAARAGLQ
Tg_AF50224   MDGAGKVGGK VGGKVGGKVG GMGKGGKGKS GSGKG-KKAP LSRAARAGLQ
human_CAA4   MSGR------ ---------- -----GKGGK GLGKGGAKRH R-KVLRDNIQ
Clustal Co   *.*                    ***  * *** *        :. * .:*


             ....|....| ....|....| ....|....| ....|....| ....|....|
                     60         70         80         90        100
cgd5_940     FPVGRIQRML KHRIPGDCRV GSTASVYAAA ILEYLTAEVL ELAGNASKDL
Tg_AF50224   FPVGRVHRML KSRISSEGRV GSTAAVYASA ILEYLTAEVL ELAGNASKDL
human_CAA4   GITKPAIRRL ARRG-GVKRI SGLIYEETRG VLKVFLENVI RDAVTYTEHA
Clustal Co   .     * *    *   .  *: ..       : . :*: :  :*: . *  . ::.


             ....|....| ....|....| ....|....| ....|....| ....|....|
                    110        120        130        140        150
cgd5_940     KVKRITPRHL QLAIRGDEEL DSLIKATIAG GGVIPHIEKS LMGKALIGKK
Tg_AF50224   KVKRITPRHL QLAIRGDEEL DTLIKATIAG GGVIPHIHKS LMTKGPSTQP
human_CAA4   KRKTVTAMDV VYALKRQG-- ----RTLYGF GG-------- ----------
Clustal Co   * * :*. .:   *:: :        ::  .  **


             ....|...

cgd5_940     GKKGNMSP
Tg_AF50224   MKKAKK--
human_CAA4   --------
Clustal Co
```

Figure 3.

```
          ....|....| ....|....| ....|....| ....|....| ....|....|
                   10        20        30        40        50
cgd7_1700  ---------- --MSGKSGKS IKGPAQKQ-- -------QAA KKTAGKSPAD
TgXP_00236 ---------- --MSGK---- --GPAQKS-- -------QAA KKTAGKS--L
human_CAB0 ---------- --MPDP---A KSAPAPK--- -------KGS KKAVTKAQKK
cgd5_3170  GVDIKWISLQ FQLCEKKGQS QSAVLHELGK LSTNSIKMAP KMSSKNNKGA
Clustal Co                 :          .  :            .. * :  :


          ....|....| ....|....| ....|....| ....|....| ....|....|
                   60        70        80        90       100
cgd7_1700  GGKRKRRKRT ESFALYIYKV LKQVHPETGI SKKSMSIMNS FINDVFDRLS
TgXP_00236 GPRYRRRKRT ESFALYIYKV LKQVHPETGV SKKSMSIMNS FINDIFDRLA
human_CAB0 DGKKRKRSRK ESYSVYVYKV LKQVHPDTGI SSKAMGIMNS FVNDIFERIA
cgd5_3170  APKKIHKKKK ESYSTYIYKV LKQVHPETGI SKKSMMIMNS YISDTFEKIA
Clustal Co    :  ::.:. **:: *:*** ******:**: *.*:* **** ::.* *::::


          ....|....| ....|....| ....|....| ....|....| ....|....|
                  110       120       130       140       150
cgd7_1700  AEAVKLVQYN KKRTLSSREV QTSVRLMLPG ELSKHAVSEG TKAVTKYTSA
TgXP_00236 DEAVRLIRYN KKRTLSSREI QTAVRLLLPG ELSKHAVSEG TKAVTKYTTS
human_CAB0 GEASRLAHYN KRSTITSREI QTAVRLLLPG ELAKHAVSEG TKAVTKYTSS
cgd5_3170  QQAAQLCQTT KKDTIASREI QTAVRLVLPG ELAKHAVSEG TKAVTKFTGG
Clustal Co  :* :* : . *: *::***: **:***:*** **:****** ******:* .


           ..

cgd7_1700  SA
TgXP_00236 GA
human_CAB0 K-
cgd5_3170  QK
Clustal Co
```

Figure 4.

```
         ....|....| ....|....| ....|....| ....|....| ....|....|
              10        20        30        40        50
cgd8_5230   MSGRGKGGKG LGKGGAKRHR KVLRDNIQGI TKPAIRRLAR RGGVKRISAL
human_H4_N  MSGRGKGGKG LGKGGAKRHR KVLRDNIQGI TKPAIRRLAR RGGVKRISGL
Clustal Co  ********** ********** ********** ********** *******.*


         ....|....| ....|....| ....|....| ....|....| ....|....|
              60        70        80        90        100
cgd8_5230   IYEEVRGVLK AFLETVIKDA VTYTEYARRK TVTAMDVVHA LKRQGKTLYG
human_H4_N  IYEETRGVLK VFLENVIRDA VTYTEHAKRK TVTAMDVVYA LKRQGRTLYG
Clustal Co  ****.***** .***.**:** *****:*:** ********:* *****:****


            ...

cgd8_5230   FGG
human_H4_N  FGG
Clustal Co  ***
```

Figure 5.

```
   ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
        10          20          30          40          50          60          70          80
ATGGCTCGTT CTAAAACTTC AAGCAAGCGA ATTGCAAAAT ATCCGTCAAA A--------- ---------- ----------
ATGGCTCGTT CTAAAACTTC AAGCAAGCGA ATTGCAAAAT ATCCGTCAAA A|GTAAGTTGA TTAATTCCAT TTATGCAACA|
********** ********** ********** ********** ********** *

   ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
        90         100         110         120         130         140         150         160
---------- --------AC TCCTATTCTT CACCAATCAA CGAATAATAG AGCAATTAGT AGTAGCCCCA TTAGCCCAAG
|TATTCATTAG TTCTCTAG|AC TCCTATTCTT CACCAATCAA CGAATAATAG AGCAATTAGT AGTAGCCCCA TTAGCCCAAG
           ** ********** ********** ********** ********** ********** ********** **********

   ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
       170         180         190         200         210         220         230         240
GAGATATAGA AGAAGACCAG G--------- ---------- ---------- ---------- ------AACG GTTGCATTAA
GAGATATAGA AGAAGACCAG G|TAAGCTAAA TAATTTATTC TGTGTTCAAA TTATATTTAT TTAAGG|AACG GTTGCATTAA
********** ********** *                                             **** **********

   ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
       250         260         270         280         290         300         310         320
GAGAAATTAG AAAATATCAA GCCTCAACTG ATTTACTTAT TGCTAAACTA CCTTTTGCTC GCGTTGTTAG AGAGGTTACA
GAGAAATTAG AAAATATCAA GCCTCAACTG ATTTACTTAT TGCTAAACTA CCTTTTGCTC GCGTTGTTAG AGAGGTTACA
********** ********** ********** ********** ********** ********** ********** **********

   ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
       330         340         350         360         370         380         390         400
TTAAAATTTG TGCCACATGG CGAAATGTGG AGATGGAATG CAGAGGCTTT GCATGCTATT CAATGCGCAG CAGAAGCATT
TTAAAATTTG TGCCACATGG CGAAATGTGG AGATGGAATG CAGAGGCTTT GCATGCTATT CAATGCGCAG CAGAAGCATT
********** ********** ********** ********** ********** ********** ********** **********

   ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
       410         420         430         440         450         460         470         480
TTTACAAGGG TTATTCGAGG ATGCATATTT ATGCACATTG CACTCCAAAA GAGTTACATT ACTACCAAGA GATATAAGAT
TTTACAAGGG TTATTCGAGG ATGCATATTT ATGCACATTG CACTCCAAAA GAGTTACATT ACTACCAAGA GATATAAGAT
********** ********** ********** ********** ********** ********** ********** **********

   ....|....|  ....|....|  ....|....|  ....|....|  .
       490         500         510         520
TAGCTAGACA ATTAAGAGGC AGGTATGGAG ACTTTATTTA A
TAGCTAGACA ATTAAGAGGC AGGTATGGAG ACTTTATTTA A
********** ********** ********** ********** *
```
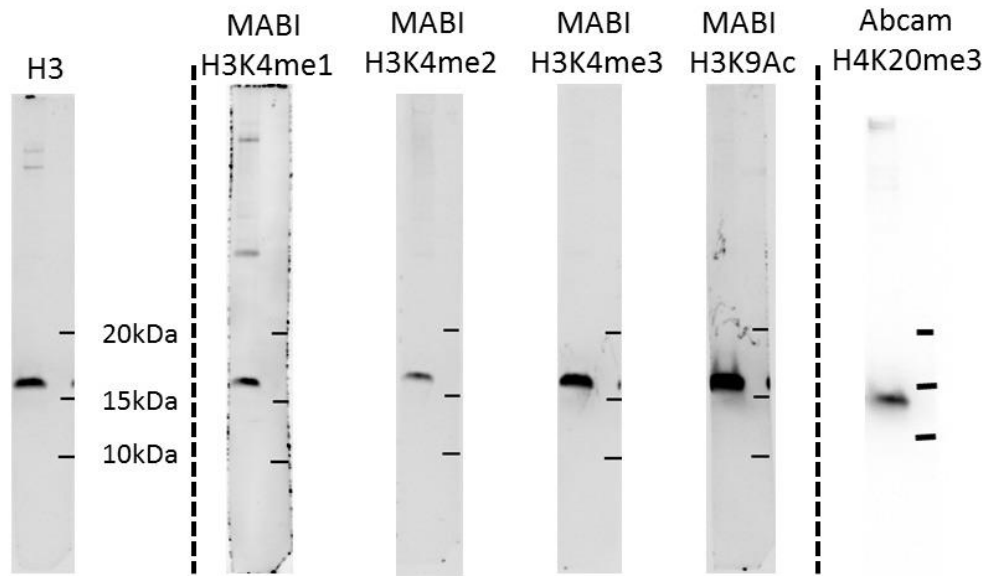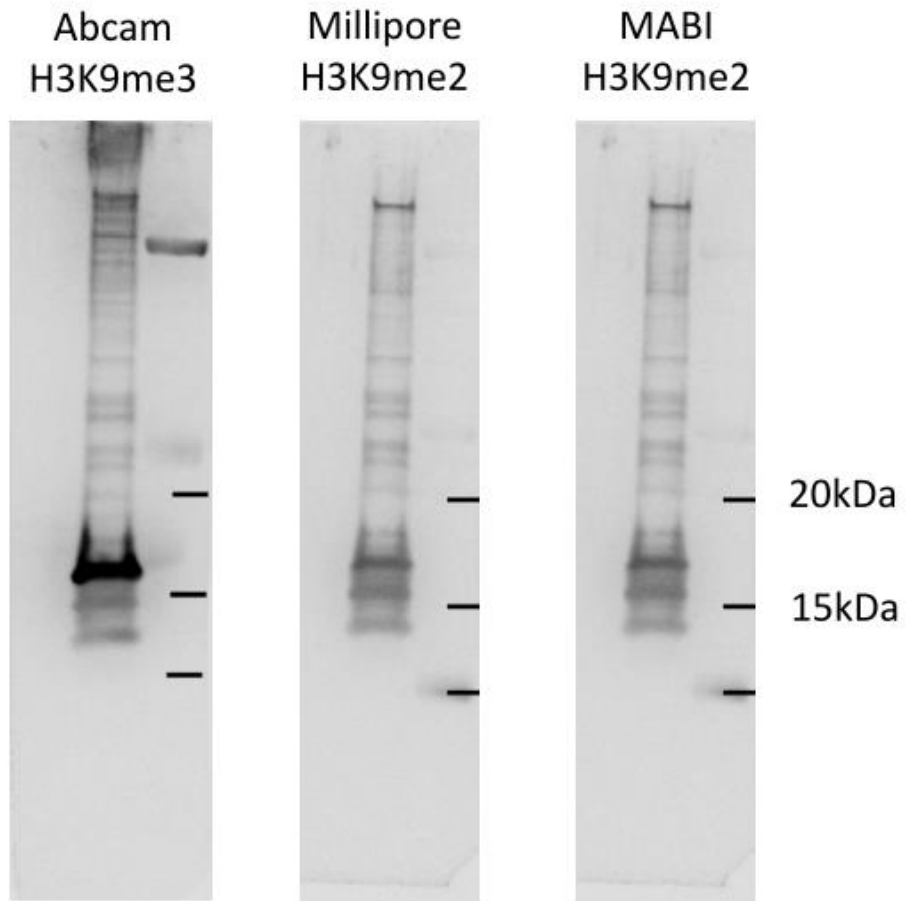
Figure 6.

MAR SKTSSKRIAKYPSK TPILHQSTNNRAISSSPIS PRRYRRRPGTVALREIRKYQA

STDLLIAKLPFARVVRE VTLKFVPHGEMWRWNAEALH AIQCAAEAFLQGLFEDA

YLCTLHSKRVTLLPRDIRLARQLRGRY GDFI
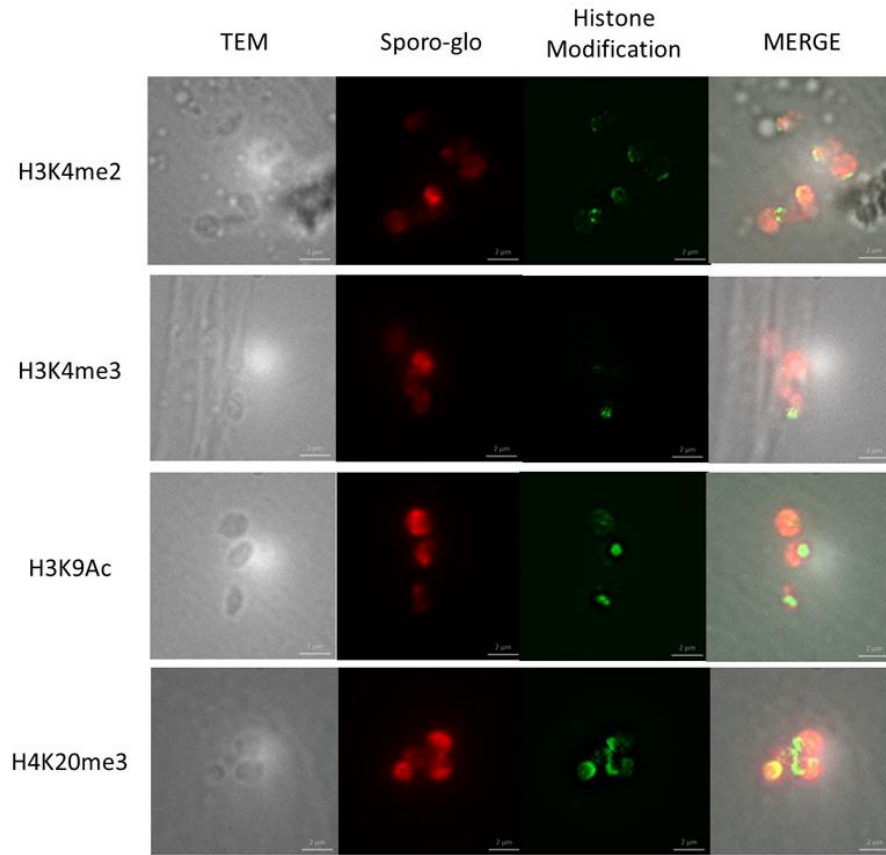
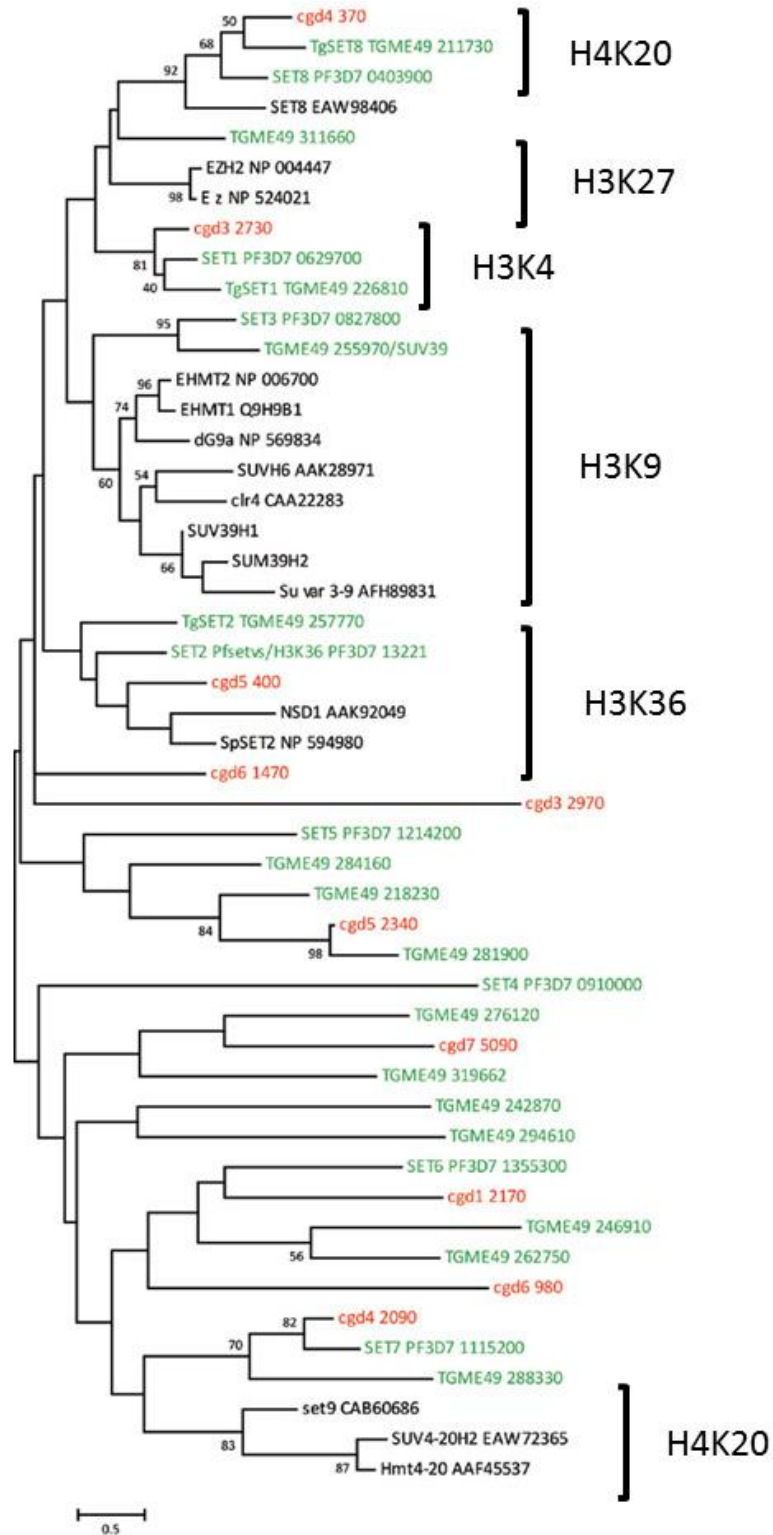**Figure 7.**

Figure 8.

Figure 9.

Figure 10.

Table 1. Information of antibodies

	Company	Number	Signal
Suppression			
H3K9me2	MABI	MABI 0307	×
H3K9me3	MABI	MABI 0308	×
	abcam	ab8898	×
	Millipore	07-441	×
H3K27me1	MABI	MABI 0321	×
H3K27me3	MABI	MABI 0323	×
H4K20me1	abcam	ab9051	×
H4K20me2	Active Motif	39174	×
H4K20me3	Active Motif	39672	○
	abcam	ab9053	○
Activation			
H3K4me1	MABI	MABI 0302	○
H3K4me2	MABI	MABI 0303	○
H3K4me3	MABI	MABI 0304	○
H3K9Ac	MABI	MABI 0305	○
H3S10P	MABI	MABI 0312	×
H3K36me1	MABI	MABI 0331	×
H3K36me2	MABI	MABI 0332	×
Control			
Histone H3	MABI	MABI 0301	
	Cell Signaling	4499	
Histone H4	abcam	ab9051	

General Conclusion

The genus *Cryptosporidium* is a causative pathogen of cryptosporidiosis. Especially, *C. parvum* causes an acute diarrheal disease, and, *in vitro* culture system and transfection methods have not been established. There are a number of issues with the use of genotyping and subtyping in resolution. First, most previous epidemiological studies of cryptosporidiosis in cattle were performed by only microscopic observations of oocyst shedding or microscopic observation used for initial screening of molecular epidemiology. However, these traditional screening methods might lead to low detection of *Cryptosporidium* species with low oocyst shedding intensity. Second, In Japan, IIa, which is the common subtype, found in many countries, is the only subtype of *C. parvum*, therefore, we can't track infection and contamination sources in outbreaks. For the treatment of cryptosporidiosis, Nitazoxanide is the only drug approved in U. S. A. However, this drug has the limited effectiveness against cryptosporidiosis. Therefore, new drug and drug target are needed for the cryptosporidiosis.

In this thesis, the author focused on epidemiology of *Cryptosporidium* and the symbiotic virus of this parasite. The author showed that *Cryspovirus* is new candidate of *C. parvum* subtyping marker. The author also assessed the efficacy of lasalocid-Na and explore new drug target based on histone modification in *C. parvum*.

For a start, in chapter 1, the author did epidemiological research in Ishikari District, Hokkaido, Japan. There are number of issues with the use of

typing and subtyping in resolution. In Japan, Microscopic observation with the flotation method is powerful and traditional tool for screening for *Cryptosporidium* species, but it sometimes leads to low detection of *Cryptosporidium* with low oocyst shedding intensity and it is very difficult to distinguish *Cryptosporidium* species by morphologically. Therefore, the author used molecular diagnostic tools. As a result, the author detected *C. parvum*, *C. bovis*, and *C. ryanae* in calves. This is the first time that *C. ryanae* has been detected in Hokkaido. Furthermore, it is the first detection of *C. ryana*e from pre-weaned calves in Japan. DNA sequencing showed that all *C. parvum* positive specimens had the *C. parvum* subtype IIa. This is the common subtype found in humans and cattle in many countries.

In Japan, many papers reported that IIa subtype was only subtype of *C. parvum* in Japan. In chapters 1, detected subtype of *C. parvum* was only IIa subtype. Therefore, we can't track infection and contamination sources in outbreaks. To resolve this problem, in chapter 2, the author showed that dsRNA of CSpV1 sequences reflected regional distributions of their host. In the phylogenetic relationships based on partial sequences of dsRNA1 and dsRNA2 with ML method among *Cryspovirus,* Japanese samples made a Japanese clade. The *Cryspovirus* dsRNA sequences detected from Hokkaido (Ashoro and Ishikari) and other region (Iwate, Tanegashima and Okinawa) made a single clade. Iwate, Tanegashima and Okinawa specimens made a single subclade. Therefore, *Cryspovirus* dsRNAs has potential as a high-resolution tool for subtyping *C. parvum*.

In chapter 3, the author focused on the efficiency of Lasalocid-Na. Lasalocid is an ionophorous antibiotic and an antiprotozoal agent for the prevention of coccidiosis. Few studies have examined the effectiveness of lasalocid against cryptosporidiosis in calves. In previous studies, the anti-cryptosporidial activity of lasalocid was assessed from a treatment viewpoint in dairy calves infected with *Cryptosporidium*. In contrast, the author assessed preventive effects used for the farm calves which are naturally infected with *Cryptosporidium* in the first 7 days of life. The author successfully identified an appropriate dose of lasalocid that did not cause toxic effects in the calves. Lasalocid-Na was protective during the dosing period and this data suggest that lasalocid-Na administration may delay oocyst excretion.

However, lasalocid-Na is not suitable for applying to human because of side effects. To resolve this problem, in chapter 4, the author focuses on histone modification of *Cryptosporidium*. There were a few data of molecular biology in *C. parvum*. In Apicomplexa, many papers reported histone modification is also important in parasite virulence and control of their life cycle of *Toxoplasma* and *Plasmodium* closely related with *Cryptosporidium*. The author found the presence of histone modification in *C. parvum* for the first time, and additionally, discussed that this histone modification enzymes of this parasites may become drug target.

In conclusion, the author elucidates that *Cryspovirus*; CSpV1 dsRNA1 sequence is new candidates of *C. parvum* subtyping marker. Further *in vivo* analyses of relationship between dsRNA1 sequence and virulence of *C.*

parvum will contribute to *Cryptosporidium* studies. Further *in vivo* studies associated with dsRNA1 sequence was needed to address that *Cryspovirus* has a direct effect on the virulence of *C. parvum*. Additionally, the author elucidates that lasalocid-Na administration delays oocyst excretion in calves and histone modification enzymes of this parasite may become drug target. This study provides valuable insights and supports further investigations to drug development for cryptosporidiosis.

Acknowledgement

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List of publications

1. F. C. Recuenco, R. Takano, S. Chiba, T. Sugi, H. Takemae, **F. Murakoshi**, A. Ishiwa, A. Inomata, T. Horimoto, Y. Kobayashi, N. Horiuchi, K. Kato. Lambda-carrageenan treatment exacerbates the severity of cerebral malaria caused by *Plasmodium berghei* ANKA in BALB/c mice. *Malar J*., 13:487. (2014).

2. **F. Murakoshi**, M. Takeuchi, A. Inomata, T. Horimoto, M. Ito, Y. Suzuki, K. Kato. Administration of lasalocid-NA is preventive against Cryptosporidiosis of newborn calves. *Vet Rec*., 175:353. (2014)

3. T. Sugi, T. Masatani, **F. Murakoshi**, S. Kawazu, K. Kato. Microplate assay for screening *Toxoplasma gondii* bradyzoite differentiation with DUAL luciferase assay. *Anal Biochem*., 464:9-11. (2014)

4. H. Takemae H, T. Sugi, K. Kobayashi, **F. Murakoshi**, H. Gong, F. C. Recuenco, A. Ishiwa, A. Inomata, T. Horimoto, N. Yokoyama, K. Kato. Interaction between *Theileria orientalis* 23-kDa piroplasm membrane protein and heparin. *Jpn J Vet Res*., 62:17-24. (2014)

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Int J Parasitol Drugs Drug Resist., 3:93-101. (2013)

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8. **F. Murakoshi**, Y. Fukuda, R. Matsubara, Y. Kato, R. Sato, T. Sasaki, C. Tada, Y. Nakai. Detection and genotyping of *Cryptosporidium* spp. in large Japanese field mice, *Apodemus speciosus*. *Vet Parasitol*., 196:184-188. (2013)

9. **F. Murakoshi**, Y. Tozawa, A. Inomata, H. Taisuke, Y. Wada, K. Kato. Molecular characterization of *Cryptosporidium* isolates from calves in Ishikari District, Hokkaido, Japan. *J Vet Med Sci*., 75:837-840. (2013)

10. H. Takemae, T. Sugi, K. Kobayashi, H. Gong, A. Ishiwa, F. C. Recuenco, **F. Murakoshi**, T. Iwanaga, A. Inomata, T. Horimoto, H. Akashi, K. Kato. Characterization of the interaction between *Toxoplasma gondii* rhoptry neck protein 4 and host cellular β-tubulin. *Sci Rep*., 3:3199. (2013)

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学術雑誌等又は商業誌における解説、総説

1. **村越ふみ**、加藤健太郎.「クリプトスポリジウム原虫における培養細胞を用いた感染・増殖阻害アッセイ」『寄生虫学研究　材料と方法 2014 年度版』 *in press.*

2. **村越ふみ**、猪又敦子、加藤健太郎.「クリプトスポリジウム原虫の分子疫学」『寄生虫学研究　材料と方法 2013 年度版』三恵社 pp101-103、2013.

Summary in Japanese

<div align="center">論文の内容の要旨</div>

<div align="right">獣医学　専攻</div>

<div align="right">平成 22 年度博士課程　入学</div>

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論文題目　Molecular analyses and epidemiology of *Cryptosporidium* and the symbiotic virus of this parasite

（クリプトスポリジウム原虫及び本原虫内在性ウイルスの疫学と分子生物学的解析）

　Cryptosporidium の中でも、特に *Cryptosporidium parvum* (以下 *C. parvum*)は、ヒトで重篤な下痢症を呈し、家畜に対し生産性の低下および斃死を引き起こす。これはアピコンプレクサに属する人獣共通感染原虫であり、獣医・畜産学領域で極めて重要である。新興感染症である本原虫症は発展途上国のみならず先進国でも深刻な問題であり、米国でのヒトへのクリプトスポリジウム感染推定件数は年間 740,000 件にも上る。日本でも、1996 年に埼玉県で 9,140 人の集団感染が発生している。本原虫は形態学的な分類が不可能であるため、疫学や病原

性などの基礎データの蓄積が不十分であること、有効な治療薬が存在しないことが大きな課題である。そこで、本研究ではクリプトスポリジウム原虫及び本原虫共生ウイルスの疫学と、薬剤探索に向けた分子生物学的解析を行うことを目的とし、解析を行った。

　第一章では北海道石狩地区の仔牛の疫学調査を行った。結果として *C. parvum, C. bovis, C. ryanae* の三種のクリプトスポリジウムを検出した。筆者は糞便から直接原虫の DNA を抽出し、精度の高い検出系を用いることにより、北海道で初めて *C. ryanae* を検出した。*C. bovis* および *C. ryanae* と下痢には相関がみられず、二種の仔牛に対する病原性は低いことが示唆された。*C. parvum* のサブタイプは IIa サブタイプであった。*C. parvum* のサブタイピングは、60 kDa glycoprotein (GP60)遺伝子によって行われる。これは *C. parvum* の病原性と関連性があると言われているが、本邦においては全て IIa サブタイプであることが知られているため、感染源の推定や病原性、進化や宿主の移動を明らかにするために、新たなサブタイピング遺伝子が求められている。そこで第二章では、本原虫に感染している dsRNA virus として報告されており、病原性とも関係があると報告される *Cryspovirus (Cryptosporidium virus 1*: CSpV1)に注目した。本原虫ウイルスはポリメラーゼをコードする dsRNA1 とカプシドタンパク質をコードする dsRNA2 を持つウイルスである。疫学調査の結果として、*C. parvum* 陽性サンプル全てにおいて CSpV1 が検出され、本ウイルスが原虫の疫学に用いることができることが示唆された。そこで、日本の標準株である HNJ-1 株および、ウシ糞便から採取した北海道、岩手、種子島、沖縄由来の *C. parvum* 陽性検体における CSpV1 の dsRNA 配列から ML 系統樹を作成した。

日本のサンプルはクレードを形成し、本ウイルス配列によって、由来国が明らかになることが示唆された。更に細かく解析を行うと、系統樹は大きく北海道、北海道以外のクレードに分かれた。更に、北海道以外のクレードは、岩手および沖縄・種子島サブクレードに分かれ、沖縄サンプルもまとまったクレードを形成した。以上から、CSpV1 dsRNA を用い、*C. parvum* 感染地の推定が行えることが明らかとなった。スポロゾイトにおいて、カプシドタンパク質は原虫細胞質全域にドット状に存在していた。従って、疫学解析に用いることができることが示唆された。

　第三章では、本原虫の治療を目的とし、近縁のコクシジウム治療に使用されている薬剤であるラサロシドの仔牛への投与試験を行った。クリプトスポリジウムが蔓延している農場において、生後間もない仔牛に本薬剤の予防的投与を行い、本原虫に与える影響を調査した。その結果、本薬剤を生後間もない仔牛に投与すると、投与期間中はクリプトスポリジウム排出を抑えることが明らかとなった。従って、本薬剤は、本原虫に感受性の高い仔牛の予防薬に成りうる。しかしながら、本薬剤をヒトに用いることは現実的ではないため、第四章ではエピジェネティクス阻害剤に注目した。本原虫に近縁であるマラリア原虫やトキソプラズマにおいて、ヒストン修飾が病原性にかかわる因子の発現制御や伝播における形態の変換に密接に関わっており、エピジェネティクス阻害剤は原虫の生育を抑えるという報告が存在する。そこで筆者は本原虫のヒストン修飾およびヒストン修飾酵素の解析を行った。その結果、本原虫にヒストン修飾が存在することがはじめて明らかになった。原虫の侵入形態であるスポロゾイトステージにおいて、遺伝子発現抑制的なヒストン修飾は検出されないこと、ス

ポロゾイトおよび細胞内ステージにおいては遺伝子発現を活性化すると考えられる修飾が検出されること、H4K20me3 の修飾を今後調べることにより、本原虫のセントロメア領域が明らかになることが示唆された。更に、本原虫のメチル化修飾酵素が薬剤ターゲットとなりうることが明らかとなった。

　以上のように、本研究はクリプトスポリジウムにおいて有用なサブタイピング遺伝子、薬剤候補を明らかにすることに成功しており、これらの研究成果は、学術上応用上寄与するところが少なくない。今後、本データが本原虫の薬剤開発および病原性の解明に寄与することが期待される。