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Report of fatal mixed infection with *Cryptosporidium parvum* and *Giardia intestinalis* in neonatal calves

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Abstract

In the production and management of beef and dairy cattle, controlling diarrhea is one of the important concerns. Pathogenic agents of the disease, protozoan parasites including *Cryptosporidium* spp., are difficult to control, making prevention, diagnoses, and treatment of diarrhea. In the present study, we investigated a farm with a history of calf deaths over a period of 10 years in order to determine the cause of disease and to clarify the detailed distribution of the pathogens. In four examined calves that were reared in calf pens, all were positive with *Cryptosporidium* and/or *Giardia*, while the other breeding stock and adult cattle were negative. Molecular analyses revealed that the isolates from calves were *C. parvum* subtype IIaA15G2R1 as a zoonotic and *G. intestinalis* assemblage E. Other pathogenic bacteria and diarrhea-causing viruses were not detected. After treating the calf pens with boiling water and milk of lime (Ca[OH]$_2$), oocysts of *C. parvum* and cysts of *G. intestinalis* were not found and no additional calves died. This is the first report to describe the mixed infection of both parasites in Japan.

Keywords

Cryptosporidium parvum, genotyping, *Giardia intestinalis***,** neonatal calves

Introduction

Controlling diarrhea is one of the most important concerns in animal husbandry. The occurrence of disease can markedly affect the weight gain of livestock, resulting in economic loss due to reduced productivity or the need to administer therapeutic treatments (Cho and Yoon 2014). Diarrhea is mainly caused by etiologic agents, such as bacteria, viruses, and parasites, and rapid diagnosis of the cause followed by successful treatment is required (Meganck *et al.* 2014). Namely, diarrhea can be the leading cause of mortality, especially before weaning, in both beef and dairy calves, although enteric diseases are often common in both adult and neonatal cattle

(Lorenz *et al.* 2011). Consequently, veterinarians and the farmers make great efforts to design rational and effective protocols for prevention as well as treatment of diarrhea (Smith 2015).

Among the diseases affecting cattle, cryptosporidiosis in breeding stock poses difficulties in terms of prevention, diagnoses, and treatment. Cryptosporidiosis is caused by protozoan parasites of *Cryptosporidium* spp. that belong to phylum Apicomplexan and are characterized as a zoonotic disease causing watery diarrhea in humans, domestic, and companion animals, as well as cattle (Esch and Petersen 2013). To date, cattle are known to be infected by at least five species of *Cryptosporidium* spp., namely, *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium ryanae*, and *Cryptosporidium ubiqui-*

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tum, which parasitize the small intestine, and *Cryptosporidium andersoni*, which parasitizes the abomasums (Ryan *et al.* 2014). Transmission of the parasites is through fecal-oral routes and a large number of oocysts are shed in feces of infected hosts. These oocysts are resistant to disinfectants like chlorine bleach, and they can survive in environments for a long period of time (Chauret *et al.* 2001). Currently, no effective treatments are available, and calves are the most susceptible to infection, with infection often leading to death.

Another protozoan parasite having disease symptoms similar to *Cryptosporidium* spp. is *Giardia intestinalis*, which can infect cattle and is implicated in causing diarrhea (Barigye *et al.* 2008). This parasite is a binucleated flagellated protozoan, and it consists two different stages, the trophozoite (vegetative form), which colonizes the host intestine, and the cyst (infective form), which is tolerant to environmental conditions (Carranza and Lujan 2010). While epidemiological studies have shown the infectious agents to have a prevalence of 100% in beef and dairy cattle in some countries (O'Handley *et al.* 1999; Ralston *et al.* 2003), distributions vary in other countries depending on management practices, climate and also the study design of the surveys. *G. intestinalis* genetically comprises eight genotypes (assemblages A-H, including A-I to A-IV of subtypes); assemblages A-I and B, a zoonotic genotype, and assemblage E, which is specific to livestock, are known to infect cattle (Ballweber *et al.* 2010; Heyworth 2016). In most cases, cattle have been reported to harbor the infections caused by the non-zoonotic livestock genotype (assemblage E) (Appelbee *et al.* 2003).

Despite the large number of surveys conducted on *Cryptosporidium* spp. and *G. intestinalis* in cattle, the occurrence of lethal disease on farms and measures for preventing infections have rarely been reported. In Japan, only two cases involving *G. intestinalis* have been identified by molecular methods (Itagaki *et al.* 2005; Matsubayashi *et al.* 2005), and mortality cases due to *Cryptosporidium* spp. and *G. intestinalis* have never been documented.

Here, we examined a dairy farm with a history of calf deaths over a period of 10 years for the presence of oocysts of *Cryptosporidium* spp. and cysts of *G. intestinalis*, in order to clarify these distributions at the farm and to identify the cause of disease. Furthermore, we tested the following treatment for the housing facilities: pouring boiling water based on the report that *Cryptosporidium* oocysts can be disinfected by treatment at 70°C for 5 sec (Fujino *et al.* 2002), and coating porous surfaces with milk of lime. Milk of lime is a form of hydrated lime, $Ca(OH)_2$ with a pH of about 12, and it has been used to sterilize solid or waste water based on its antiviral, antimicrobial, and antipatasitic effects (Zintl *et al.* 2010). The effectiveness of hydrated lime or quicklime on reducing the viability of *Cryptosporidium* spp. has been reported (Robertson *et al.* 1992; Graczyk *et al.* 2008), although the utility of these treatments remains controversial (Rimhanen-Finne *et al.* 2004). This is the first report to describe mixed infection of both parasites on a farm in Japan and to demonstrate the treatment of lethal cryptosporidiosis using milk of lime and boiling water.

Materials and Methods

History of the study farm

The dairy farm is located in Chiba Prefecture, Japan. On this farm, Japanese black and crossbreed cattle were kept in housing as follows: stall barn for 48 cows, pasture for 10 breeding stock, pasture for dry cows, and calf pens for up to seven calves. For almost 10 years before May 2014, watery diarrhea was often reported among the calves at this farm. In recent years, 37 of 42 newborn calves between May 2014 and April 2015 showed diarrhea, and neurological symptoms and debility in addition to diarrhea were observed, with the most severe cases (n = 7) resulting in death. In most cases, diarrhea appeared in the calves at the age of approximately 7 days old, followed by the onset of intense symptoms. The calves were administered toltrazuril at 3 to 5 days after birth, and symptomatic calves were given antibiotic treatments, such as oxytetracycline and sulfadimethoxine, transfusions to prevent metabolic acidosis, and management of the housing conditions, including thermal management were conducted. However, there were no changes in incidence or outcome.

To date, some of the calves that died due to diarrhea were examined histopathologically, bacteriologically, and virologically at a public livestock hygiene service center in Chiba Prefecture, but the cause of death could not be determined based on the results of the following examinations. Intestinal contents and samples from some organs including the lungs, heart, kidneys, spleen, or intestinal lymph nodes were cultured for *Escherichia coli* on various agars such as trypto-soya agar, triple sugar iron agar, and LIM agar (Eiken Chemical, Tokyo, Japan). Colonies detected in these cultures were determined to be in the O serogroups based on agglutination using commercially available antisera (Statens Serum Institut, Copenhagen, Denmark) according to the manufacturer instructions. Genes encoding virulence factors of toxins (LT, STa, STb, eas, Stx1, and Stx2) were examined by PCR as described by Vu-Khac *et al.* (2007), and in these findings, none were detected. Samples were analyzed for *Campylobacter* spp. *Clostridium perfringens*, and *Clostridium difficile* by Sanritsu Co. Ltd. (Chiba, Japan), and no isolates were detected. The intestinal contents were inspected using species-specific reverse transcription (RT)-PCR for coronavirus, rotavirus, bovine torovirus, and bovine viral diarrhea virus (Vilcek S 1994; Fukuda, M. 2012), but no viruses were detected.

On April 2014, following the deaths of two calves (Nos. A and B) (No. C recovered), feces of four calves (Nos. D-G) (Fig. 1) were collected for detailed examination at the National Institute of Animal Health at Tsukuba, Japan as described below.
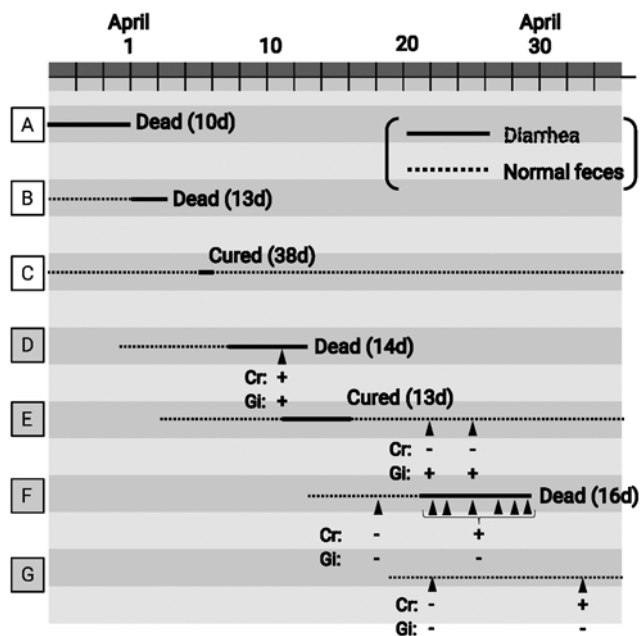
Fig. 1. Summary of occurrence of diarrhea in seven calves (Nos. A-G) over approximately one month from March to May 2014 at the farm and detection of parasites. Parentheses indicate the age of the calves (d; days old) and cattle (Nos. D-G) was moved to the farm at the age of 0 days old. Arrowheads indicate the timing of the microscopy and PCR results shown for *Cryptosporidium* spp. (Cr) and *G. intestinalis* (Gi)

Examination of the calves

The fecal samples (Nos. D-G) collected in April 2014 were examined by the sugar floatation method based on methods previously reported (Uga *et al.* 2000). Briefly, 1 g of feces was suspended in distilled water. A sucrose solution with a specific gravity of 1.200 was added, the upper layers were recovered onto slide glasses after the centrifugation, and oocysts or cysts of intestinal protozoan parasites or eggs of helminthes were examined under a microscope. For samples that were positive for *Cryptosporidium* spp. or *G. intestinalis*, oocysts or cysts were purified from the feces by the sugar floatation method. Additionally, samples were tested with *Cryptosporidium*/ *Giardia* detection kits using immunofluorescence (Easy Stain™, BTF, Australia).

For molecular analyses, genomic DNA of the parasites was extracted using a QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer instructions after freeze-thawing three times. The purified DNA was stored at -20°C until further analysis. For identification of *Cryptosporidium* spp., nested PCR with primer pairs F1 and R1 and F2 and R2 targeting the *Cryptosporidium* 18S ribosomal RNA (18S rRNA) gene (approximately 770 bp) were performed as previously described (Xiao *et al.* 1999; Nagano *et al.* 2007). Additionally, the *GP60* gene (approximately 450 bp) of *C. parvum* was amplified by nested PCR to determine subtype, as previously reported (Peng *et al.* 2001). For identification of *G. in-*

testinalis, diagnostic fragments were amplified using primer pairs G7 and G759 and G376 and G759, which are specific for the *Giardia β-giardin* gene (approximately 380 bp) (Cacciò *et al.* 2002). These PCR products were sequenced in both directions using the primers on an ABI 3130 automated sequencer (Applied Biosystems Inc., CA, USA). The obtained sequences were aligned with the representative nucleotide sequences of *Cryptosporidium* or *Giardia* species or genotypes using ClustalW with initial fixed parameter values (DNA Data Bank of Japan (DDBJ), http://clustalw.ddbj.njg.ac. jp/top-j.html). Homology searches using the obtained partial gene sequences were performed using the FASTA program (http://www.ddbj. nig.ac.jp/search/fasta-j.html) (Yui *et al.* 2014).

Autopsy of dead calf

The calf (No. F) that died at the age of 16 days was necropsied, and rumen, reticulum, omasum, abomasum, small and large intestines, kidneys, liver, spleen, and lungs were removed. The removed tissues were fixed in 10% buffered formalin at room temperature for 7 days and then embedded in paraffin. Each tissue section was cut to a thickness of 3 μm, stained with hematoxylin and eosin (H&E) stain, and examined using light microscopy. To confirm the infection, immunohistochemistry was performed with the universal immune-enzyme polymer method using a Histofine Simple Stain MAX-PO Kit (Nichirei, Tokyo, Japan). Briefly, serial intestinal sections were pretreated with 0.1% actinase for 20 min at 37°C, and endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 30 min at room temperature. The rabbit polyclonal antibody against *C. parvum* (Matsubayashi *et al.* 2013) was applied as the primary antibody at a dilution of 1:128 for 30 min at room temperature. The sections were lightly counterstained with hematoxylin and assessed using light microscopy. Simultaneously, sections of porcine intestine known to be infected with *Cryptosporidium* spp. were immunolabeled as positive controls. Negative controls were prepared by replacing the primary antibody with a commercial Tris–HCl buffer (antibody diluent with background reducing components) (Dako, Tokyo, Japan).

Parasitic surveys of all cattle on the farm

In June 2014, all the cattle on the farm (Fig. 2), namely 12 breeding stock in calf hatches and barn for breeding cattle, and 48 adult dairy cattle, were parasitologically investigated in order to survey the source of the infections and understand the prevalence of *Cryptosporidium* spp. and *G. intestinalis* in non-symptomatic individuals. Fecal samples were collected and examined by the sugar floatation method, as described above.

Measures to control infection

After the parasitic survey, we determined that only the calf pens were contaminated with *Cryptosporidium* spp. and
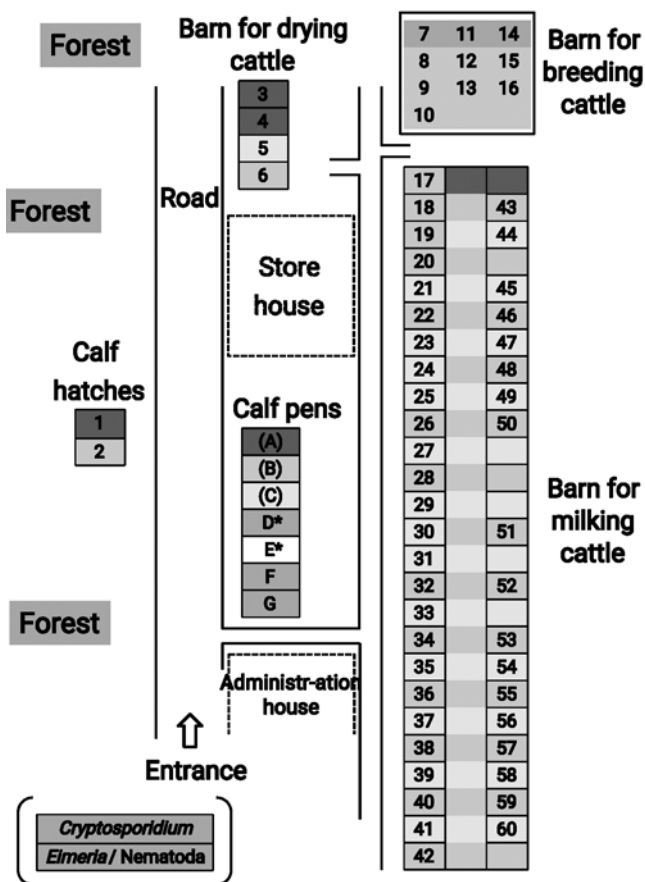
Fig. 2. Diagram of pens for calves (Nos. A-G) and barns for dairy cattle (Nos. 1–60) at the study farm. The numbers of gray or shaded boxes indicate positive for *C. parvum* and *Eimeria* spp., respectively. Calves Nos. A-C were not examined because of death (A, B) or transfer to another farm (C). Additionally, calves Nos. D* and E* were infected with *G. intestinalis*

G. intestinalis. Thus, we took measures to control the protozoan infectious agents around the calf pens in June to July 2014. After relocating the remaining calves to new locations, we removed bedding and fecal matter to the extent possible. All of the wooden board panels dividing the pens were removed and exchanged with new ones, and the pens were dried for several days. Water was boiled in pans using household gas stoves on the market (heated to over approximately 95°C) and poured by dipper on the boards of the partitions between the calf pens and on the floors, and then again on the floors being careful to make contact with all areas at the edges of the pens. This was repeated several times until the temperature on the surfaces reached about over 90°C, and then the pens were allowed to dry. After the wood was dried, we coated all the boards with milk of lime (Ca[OH]$_2$) (Tagen Sekkai Kogy, Tochigi, Japan) and followed up with spreading it on/around the pens to achieve full coverage on gaps and crevices around the floors and walls in order to coat any oocysts that might survived the boiling water treatment with lime.

Ethics statement

All experiments were carried out without using live animals. Thus, no approval from the ethical review board for animal experimentation was necessary. Veterinarians of NOSAI at Chiba Prefecture were asked by the owners to conduct examinations of the dead animals and to conduct field surveys on the farm. Permission was granted by the farm owners to conduct all examinations in this study. No animals were indisposed for the purpose of this study. No human participants were involved in this study.

Results

The onset of diarrhea, the prognosis, and parasitic infection findings are summarized in Fig. 1. Microscopic examination of feces in this survey identified oocysts of *Cryptosporidium* (4 to 5 μm in diameter) in three calves (Nos. D, F, and G) and cysts of *G. intestinalis* in two calves (Nos. D and E). In one calf (No. D), both oocysts and cysts were identified. Results by immunofluorescence staining agreed with those of the floatation method. In the survey month, four out of seven calves, including two individuals (Nos. D and F) with confirmed *Cryptosporidium* infection, died. Based on PCR analyses, three calves (Nos. D, F, and G) were positive for *Cryptosporidium* and two calves (Nos. D, and E) were positive for *G. intestinalis*. Detection results by PCR were completely identical to results of the floatation method.

Sequences of PCR products targeting the *Cryptosporidium* 18S rRNA gene showed that all *Cryptosporidium* isolates in the present study were identical to those of *C. parvum* (100% homology over 765 bp), which was also isolated from many other animals such as a calf (Accession No. AF308600) and horse (Accession No. KT948751). Based on the partial sequences of the *GP60* gene, all of the isolates were identified as zoonotic *C. parvum* subtype IIaA15G2R1 (Accession No. LC012018 from cattle) (100% homology over 436 bp). By sequencing the *Giardia β-giardin* genes, *Giardia* isolates (Nos. D and E) were identified as *G. intestinalis* assemblage E (Accession No. KT731978 from cattle) (100% homology over 359 bp).

The autopsy of calf No. F showed the cause of death to be hyperemia. Some hemorrhages were observed in the intestinal mucosa, and clots of blood were detected in lumen of the colon. A large number of yellowish-white granular nodules were observed at the cortex of the kidney. Yellow and slight muddy ascites were increased, and liver showed swelling and was surrounded with precipitated fibrin. Mesenteric lymph nodes showed swelling. Histopathological examination of the calf showed *Cryptosporidium* zoites at the surface of the intestinal crypt, and these reacted positively to anti-*Cryptosporidium* antibody (Fig. 3). Additionally, abscesses including some masses of bacteria in the serous membrane and at the cortex of the kidney, and infiltration of neutrophil in the renal tubules were also observed (images not shown).
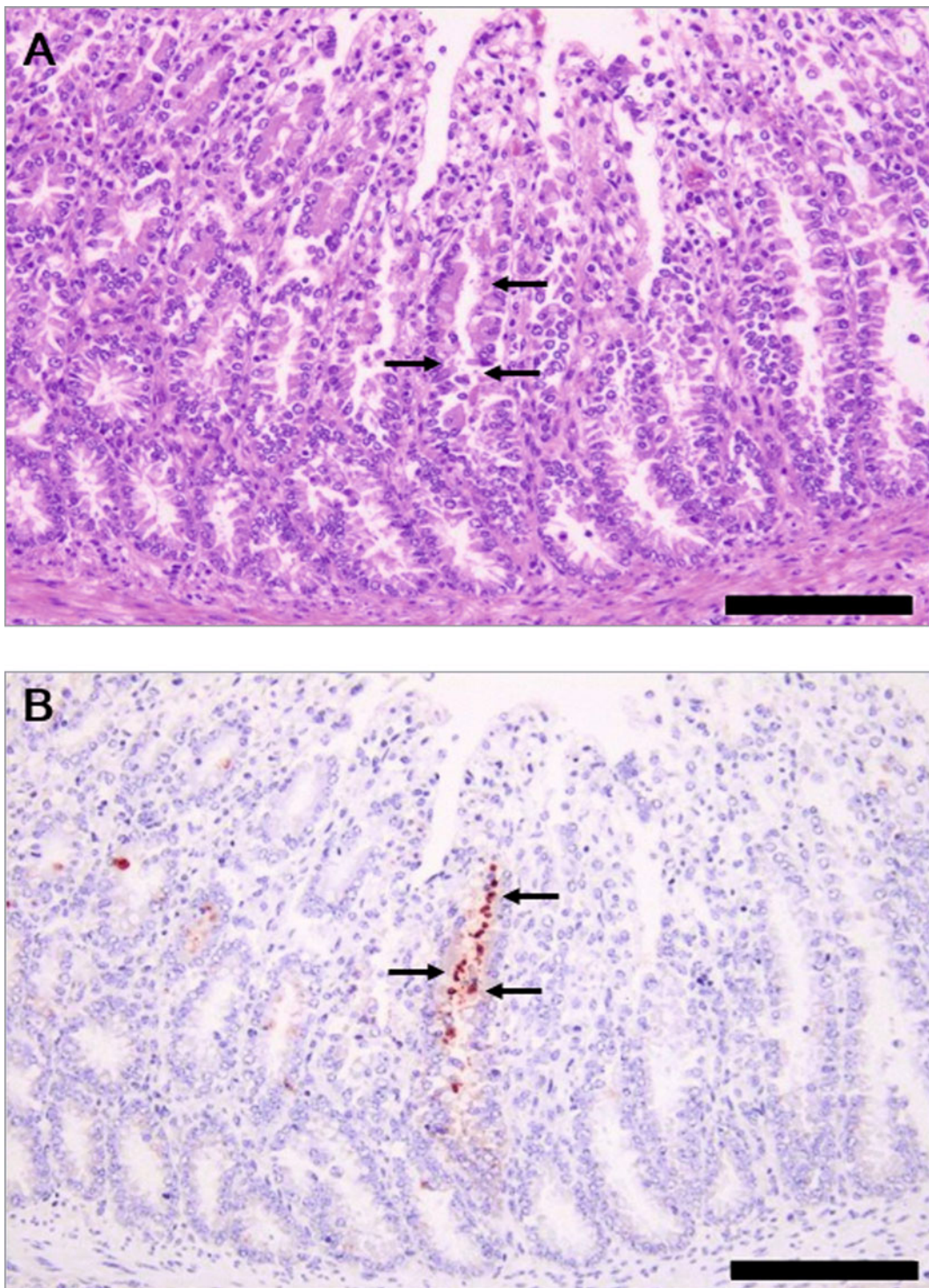
Fig. 3. Photographs of histopathological sections of ileum stained by H&E (A) and anti-*C. parvum* serum (B). Arrows indicate the zoites of *C. parvum* parasitizing at the surface of epithelium cells. Scale bars are 500 μm

As an additional survey to understand the prevalence of intestinal protozoan parasites, including *C. parvum* and *G. intestinalis*, we examined feces from all of the cattle on the farm. No oocysts of *Cryptosporidium* spp. or cysts of *G. intestinalis* were identified from the examined cattle. *Eimeria* spp. was detected in most of the breeding stock and a few of the dairy cattle (15 of 60 examined cattle, 25%), and the eggs of Nematoda spp. were found in two breeding stock and three dairy cattle (6/60, 10%) (summarized in Fig. 2).

Finally, we took measures on the calf pens to prevent further protozoan infections, especially *C. parvum*. After the treatments, the incidence of death in calves (92 calves) was zero as of October 2016, and no oocysts of *C. parvum* or cysts of *G. intestinalis* were found in subsequent fecal examinations of some calves.

Discussion

To date, there are many reports on the detection of *Cryptosporidium* spp. in cattle throughout the world, including Japan (Bajer 2008; Ryan *et al.* 2014; Ichikawa-Seki *et al.* 2015). However, sequential occurrences of frequent diarrhea or fatal cases on individual farms have rarely been reported, although only cases of calf deaths due to cryptosporidiosis were pathologically examined (De Cesaro *et al.* 2014). At the farm examined in the present study, cases of diarrhea in calves had been occurring continuously for approximately ten years and resulted in some deaths; however, the causative agent had unfortunately not been determined by a livestock hygiene service center. As a simple method for detection, oocysts can be observed by showing a bright pink color in the sucrose floatation method as described in previous reports (Abe *et al.* 2002; Koompapong *et al.* 2009). Experts can easily detect oocysts by this method, and the results in this study were identical to those of immunofluorescence staining and PCR. However, because oocysts of *Cryptosporidium* spp. are extremely small compared to eimerian oocysts, the diagnosis of cryptosporidiosis is often difficult, especially when parasitic specialists or commercial simple detection kits are not available. No bacterial or viral pathogens were detected on this farm and, therefore, in the present study, it is thought that *C. parvum* is the likely causative agent of the cases of diarrhea in calves in this farm over an extended time, although parasitological examination was not performed on previous cases of calves that died following diarrhea.

Genetically, the isolates of *Cryptosporidium* spp. and *G. intestinalis* were identified as *C. parvum* (subtype IIaA15G2R1) and *G. intestinalis* assemblage E. To date, many subtypes of *C. parvum* have been recognized in the *GP60* gene region. Interestingly, only subtype IIaA15G2R1 was detected in cattle in Japan (Ichikawa-Seki *et al.* 2015) and, therefore, our findings corroborate the report that this subtype is widespread in Japan. This subtype of *C. parvum* was found in humans, identifying it as a zoonotic one (Xiao 2010). While infected cattle can be a source of infection to humans as well as other cattle, none of the farmers on this particular farm showed any signs of diarrhea. Assemblage E of *G. intestinalis* has been implicated in causing diarrhea in calves (Barigye *et al.* 2008; Gillhuber *et al.* 2014), but other researchers have described this assemblage as being mostly asymptomatic (Liu *et al.* 2012). Therefore, the pathogenicity of the assemblage remains unknown. In Japan, there are only two known reports of *G. intestinalis* infections in cattle (Itagaki *et al.* 2005; Matsubayashi *et al.* 2005), and thus, this is the third report of *G. intestinalis* infection in cattle and the first occurrence of the mixed infection of *Cryptosporidium* spp. and *G. intestinalis* in Japan.

As an additional survey to understand the distribution of parasites, we examined the feces from all cattle on the farm. We could not detect any *Cryptosporidium* oocysts and *Giardia* cysts from the cattle, except for among the calves reared in calf pens. Thus, we focused treatments against cryptosporidiosis on only the calf pens. At the time of writing, no new infections have been reported, suggesting that these control measures are appropriate for repressing infections. However, more detailed evaluations of the effectiveness of these control methods is warranted.

Compliance with ethical standards

None of the authors have any conflicts of interest.

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