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Development of CpGP15 recombinant antigen of *Cryptosporidium parvum* for detection of the specific antibodies in cattle

Madoka Ichikawa-Seki[a], Ragab M. Fereig[b,c], Tatsunori Masatani[d], Aiko Kinami[e], Yoko Takahashi[f], Katsuya Kida[g], Yoshifumi Nishikawa[b,*]

[a] Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka 020-8550, Japan
[b] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-13 Inada-cho, Obihiro 080-8555, Japan
[c] Department of Animal Medicine, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt
[d] Transboundary Animal Diseases Research Center, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan
[e] Apartment House Iris B101, 142-12 Shinogikurohata, Takizawa, Iwate 020-0735, Japan
[f] Tyubu Area Center Veterinary Clinic, Iwate Agricultural Mutual Aid Association, 821 Shimoneko, Hanamaki, Iwate 025-0025, Japan
[g] Field Center of Animal Science and Agriculture, Obihiro University of Agriculture and Veterinary Medicine, Japan

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ABSTRACT

The infection of neonatal calves with *Cryptosporidium parvum* can have a huge economic impact because diarrhea caused by the parasite sometimes results in death. A serodiagnostic system will be helpful in the diagnosis of *C. parvum* infection. CpP23 is commonly used as an antigen for enzyme-linked immunosorbent assay (ELISA); however, some positive sera show low reactivities, as shown in this study. Herein, we focused on three other antigens, CpGP15, CpP2 and CpGP60, in addition to CpP23, to detect *C. parvum*-specific antibodies in cattle sera. CpP23 and CpGP15 showed substantial ability to discriminate between positive (n = 10) and negative (n = 10) control cattle sera. Unlike our previous report, both the sensitivity and the specificity were 100% when the two antigens were employed for the ELISA. The newly developed ELISA was applied to a total of 344 sera obtained from 9 cattle farms. Two farms among them had suffered from *C. parvum* infections before, and were regarded as the *C. parvum*-positive farms. The positive rates of antibodies against CpP23 and CpGP15 in the *C. parvum*-positive farms were 42.7% and 49.8%, respectively, whereas the positive rate for either of the antigens was 63.0% in the farms. In contrast, 14.3% and 9.8% were positive for CpP23 and CpGP15 in the *C. parvum*-negative farms, respectively, whereas 18.8% was positive for either of the antigens. This study revealed that the ELISAs employing both of CpP23 and CpGP15 can avoid false-negative results and are useful for monitoring of the *C. parvum* infection in cattle farms.

1. Introduction

Cryptosporidium parvum is a coccidian protozoan parasite that infects a wide range of animals, including humans. The infection of neonatal calves with *C. parvum* can have a considerable economic impact because severe diarrhea caused by *C. parvum* results in inadequate development and sometimes death. Fecal examinations to identify *C. parvum* oocysts as well as the molecular detection of oocysts from feces using PCR has contributed to the diagnosis of the species [1]. Molecular characterizations of *C. parvum* from fecal samples have been reported from all over Japan [2–4]. However, the duration of oocyst shedding is reported to be within 2 weeks only [5], and oocysts are not detected anymore from feces after a host recovers from diarrhea. Therefore, obtaining accurate epidemiological data on this species is difficult because of the limitation of the optimal duration for fecal examination. In contrast, the advantage of an enzyme-linked immunosorbent assay (ELISA) is to use long duration for detection of the antibodies. Therefore, a combined examination with the ELISA and fecal examination seems to be the optimal way to determine the existence of *C. parvum*.

The ELISA using sonicated or whole oocysts of *C. parvum* were previously reported [6–9]; however, the specificity of the ELISA was insufficient, which was only 4.0% in one of the previous reports [9]. A recombinant 23-kDa glycoprotein of *C. parvum* (CpP23) was used for ELISA in previous studies [10–12]. CpP23 was identified as a surface antigen of *C. parvum,* which contains neutralization-sensitive epitopes [13]. The seroprevalence of antibody against CpP23 in cattle sera was 41.1% in China [14], 4.4% in Thailand [11] and 35.9% in Egypt [12].

Although CpP23 has commonly been used as an antigen for

* Corresponding author.
E-mail address: nisikawa@obihiro.ac.jp (Y. Nishikawa).

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serodiagnostic ELISA in previous studies [10–12], the sensitivity of an ELISA calculated by using the general cut-off point (average plus 3 times standard deviation of negative controls) was 20.0% in the previous study [10]. The low sensitivity may cause false-negative results. Herein, in addition to CpP23, we focused on three other antigens, CpP2 (reported as a vaccine candidate) [15,16], and 15- and 60-kDa recombinant *C. parvum* glycoproteins (CpGP15 and CpGP60, respectively) [16] to detect *C. parvum*-specific antibodies in cattle sera. CpP2 is a ribosomal protein of *C. parvum*, and recombinant CpP2 was reported as a vaccine candidate with sufficient antigenicity in a host [15,16]. CpGP60 is a mucin-like glycoprotein antigen synthesized as a single precursor protein and proteolytically cleaved into the mature glycoproteins, CpGP40 and CpGP15 [17]. CpGP15 is anchored in the sporozoite membrane by a glycosylphosphatidyl inositol (GPI) moiety, while the CpGP40 does not contain any predicted transmembrane domains or GPI anchors and is predicted to be soluble [18]. Subtyping of the glycoproteins has suggested a relationship with species-specific infection. Some *C. parvum* subtypes including IIaA15G2R1 are responsible for zoonotic cryptosporidiosis, whereas other subtypes infect certain animal species [19]. However, there is no comparative study using CpP23, CpP2, CpGP15, and CpGP60 for serodiagnosis system against *C. parvum* infection. Therefore, the objective of this study was to perform a comparative evaluation of various antigens of *C. parvum*, which is able to select the best combination of the antigens for ELISA detecting antibodies against the *C. parvum* antigens in cattle sera.

2. Materials and methods

2.1. Sample population

Blood samples were obtained from cattle, and sera were separated by centrifugation and stored at −20 °C until use. Sera from cattle that had experienced cryptosporidiosis were used as positive control sera ($n = 10$). The infection was proven using a commercial immunochromatographic test kit (Bio-X Diagnostics SPRL, Jemelle, Belgium) in neonatal calves exhibiting diarrhea. The existence of *C. parvum* oocysts was also confirmed by fecal examination. At the point of serum collection, positive control cattle were approximately two years old. Sera from calves before being given colostrum were used as negative control sera ($n = 10$).

A total of 344 sera from 9 cattle farms were obtained to determine the seroprevalence of *C. parvum* (Table 1). Farms #1 to #3, and #7 were located in Hanamaki, Iwate prefecture. Farms #4 to # 6 were located in Kitakami, Iwate prefecture while farms #8 and #9 were located in Shizukuishi, Iwate and Obihiro, Hokkaido prefectures, respectively.

Actually, based on the information from veterinarians in charge of each farm, little or none diarrhea cases were observed in farms #1 to #7, while farms #8 and #9 were reported to have many cases of *C. parvum*-related neonatal calf diarrhea, which diagnosed by the fecal examination to detect the oocysts. Accordingly, farms #1 to #7 and farms #8 and #9 were regarded as the "*C. parvum*-negative farms" and the "*C. parvum*-positive farms" respectively (Table 1). The age of animals from the farms at the point of blood collection was listed in Table 1.

2.2. Protein expression

Recombinant CpP23, CpP2, CpGP15, and CpGP60 were expressed as glutathione-S-transferase (GST) fusion proteins. Briefly, the DNA fragments encoding these proteins were amplified from genomic DNA using the primers listed in Table 2. The PCR products were digested with the respective restriction enzymes and then ligated to a similarly cut pGEX-6P1vector containing an open reading frame encoding GST fused to the N-terminus of the protein (GE Healthcare, Uppsala, Sweden) using a DNA Ligation Kit Mighty Mix (Takara Bio Inc., Shiga, Japan). After verification of the proper in-frame position of the sequences, the recombinant plasmids were used to transform *Escherichia coli* (BL21) cells. The expression was performed at 37 °C for 6 h, after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (Wako Inc., Osaka, Japan). Supernatant of disrupted *E. coli* was purified with Glutathione–Sepharose 4 B beads according to the manufacturer's instructions (GE Healthcare). The GST-fused proteins were eluted with elution buffer (pH 8, 100 mM Tris-Hcl, 100 mM NaCl, 5 mM EDTA and 20 mM reduced glutathione (Wako)). The proteins were filtered using a 0.45-μm low-protein binding Supor membrane (Pall Life Sciences, Ann Arbor, MI, USA). The purity and quantity of the proteins were detected as a single band by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue R250 staining (MP Biomedicals Inc., Santa Ana, CA, USA). The concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Serological test

The presence of *C. parvum* antibodies was evaluated by ELISA as described previously [12]. Fifty microliters of purified recombinant antigens, at a final concentration of 0.1 μM, were coated onto ELISA plates (Nunc, Denmark) overnight at 4 °C, with a carbonate–bicarbonate buffer (pH 9.6). Plates were washed once with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), and blocking was performed for 1 h at 37 °C with PBS containing 3% skim milk (PBS-SM).

Table 1
Seroprevalences of antibodies against CpP23 and CpGP15 for 9 cattle farms located in Iwate or Hokkaido Prefectures.

Farm ID	*C. parvum*	Location	No. tested	Age[b]	No. positive (%)				95% CI[a]			
					CpP23	CpGP15	Both	Either	CpP23	CpP15	both	either
#1	Negative	Hanamaki, Iwate	17	1 to 9 years old	2 (11.8)	3 (17.6)	1 (5.9)	4 (23.5)	2.1–37.7	4.7–44.2	0.3–30.8	7.8–50.2
#2	Negative	Hanamaki, Iwate	4	1 to 3 years old	1 (25.0)	1 (25.0)	0	2 (50.0)	1.3–78.1	1.3–78.1	0–60.4	9.2–90.8
#3	Negative	Hanamaki, Iwate	16	1 to 7 years old	2 (12.5)	1 (6.3)	1 (6.3)	2 (12.5)	2.2–39.6	0.3–32.3	0.3–32.3	0.3–32.3
#4	Negative	Kitakami, Iwate	50	1 to 9 years old	11 (22.0)	2 (4.0)	0	13 (26.0)	12.0–36.3	0.7–14.9	0–9.0	15.1–40.6
#5	Negative	Kitakami, Iwate	18	0 to 1 year old	3 (16.7)	3 (16.7)	1 (5.6)	5 (27.8)	4.4–42.3	4.4–42.3	0.3–29.4	10.7–53.6
#6	Negative	Kitakami, Iwate	19	0 to 1 year old	0	3 (15.8)	0	3 (15.8)	0–20.9	4.2–40.5	0–20.9	0–20.9
#7	Negative	Hanamaki, Iwate	9	1 to 2 years old	0	0	0	0	0–37.1	0–37.1	0–37.1	0–37.1
Subtotal			133		19* (14.3)	13* (9.8)	3* (2.3)	29* (21.8)	9.0–21.7	5.5–16.5	0.6–7.0	12.8–26.7
#8	Positive	Shizukuishi, Iwate	66	2 to 15 years old	15 (22.7)	11 (16.7)	5 (7.6)	21 (31.8)	13.7–35.0	9.0–28.3	2.8–17.5	21.2–44.6
#9	Positive	Obihiro, Hokkaido	145	No data	75 (51.7)	94 (64.8)	57 (39.3)	112 (77.2)	43.3–60.0	56.4–72.5	31.4–47.8	69.4–83.6
Subtotal			211		90* (42.7)	105* (49.8)	62* (29.4)	133* (63.0)	35.9–49.6	42.9–56.7	23.4–36.1	56.1–69.5

[a] 95% CI = confidence interval.
[b] The age of animals at the point of blood collection.
* Chi-square test, $p < .01$.

Table 2
Primers used for amplification of the target proteins.

Protein	Direction	Sequence[a]	Restriction enzyme
CpP23	Forward	ggg GGA TCC ggt tgt tca tca tca aag cc	*Bam*HI
	Reverse	ggg GAA TTC tta ggc atc agc tgg ctt gtc	*Eco*RI
CpP2	Forward	ggg GAA TTC ggt atg aaa tac gtt gca gct tac	*Eco*RI
	Reverse	ggg CTC GAG tta gtc aaa caa tga gaa acc tag g	*Xho*I
CpGP60	Forward	ggg GAA TTC ctc aga gga act tta aag gat gtt cct g	*Eco*RI
	Reverse	ggg CTC G AG tta caa cac gaa taa ggc tgc a	*Xho*I
CpGP15	Forward	ggg GAA TTC gaa acc agt gaa gct gct gca acc	*Eco*RI
	Reverse	ggg GGA TCC atc ctt caa aag aac tgt gtt gtc	*Bam*HI

[a] Capitals indicate recognition site of the restriction enzyme.

Cattle sera were diluted with PBS-SM at 1:100. After the plates were washed once with PBS-T, 50 µl of serum sample was added to the wells. Plates were incubated at 37 °C for 1 h. After washing six times with PBS-T, plates were incubated with horseradish peroxidase (HRP)-conjugated anti-bovine IgG (Bethyl Laboratories, Montgomery, TX, USA) diluted with PBS-SM at 1:4000 at 37 °C for 1 h. Plates were washed again six times before the substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, and 0.3 mg/ml 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) (Sigma-Aldrich, St. Louis, MO) was added to each well in 100-µl aliquots. The absorbance at 415 nm was read after 1 h of incubation at room temperature. Absorbance values were determined as the difference in the mean optical density of the duplicate wells at a value of 415 nm ($OD_{415\ nm}$). The readings for the recombinant antigens were subtracted from those of the GST protein. The cut-off point was determined as the mean $OD_{415\ nm}$ value for standard *C. parvum*-negative sera ($n = 10$) plus three standard deviations.

2.4. Statistical analyses

Student's *t*-test was employed for the comparison of $OD_{415\ nm}$ values among *C. parvum*-negative ($n = 10$) and positive ($n = 10$) sera for the recombinant antigens, CpP23, CpP2, CpGP15, and CpGP60. For the seroprevalence results, the 95% confidence intervals of a proportion including continuity correction were calculated using the website for statistical computation (VassarStats: http://www.vassarstats.net). The Chi-square test was used to detect differences in the seroprevalence between farms with *C. parvum*-related diarrhea cases and non-diagnosed cases. A P value of < 0.05 was considered statistically significant. The correlation coefficient (r) was calculated using the Microsoft Excel software (Microsoft Corporation, Redmond, WA) to examine the correlation between the $OD_{415\ nm}$ values of CpP23 and CpGP15 in this study.

2.5. Sensitivity and specificity

By using positive ($n = 10$) and negative ($n = 10$) control sera, the sensitivity and specificity of the ELISA were determined. The sensitivity was the probability that the ELISA will indicate positive among the positive controls, whereas the specificity was the fraction of negative controls who will have a negative result in the ELISA.

3. Results

Among the recombinant antigens, the $OD_{415\ nm}$ values between positive and negative control sera were significantly different for CpP23, CpGP15, and CpGP60 recombinant antigens, whereas there was no significant difference in the $OD_{415\ nm}$ values for CpP2 (Fig. 1). Furthermore, no difference was observed in the reaction of positive control sera to CpGP15 and CpGP60 ($p = .61$). Because CpGP60 is proteolytically cleaved into CpGP40 and CpGP15 [17], CpGP15 and CpP23 were selected for further analyses. Each of the sensitivities

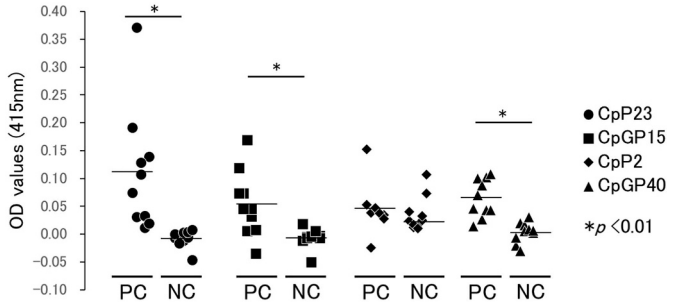

Fig. 1. ELISA with recombinant antigens using sera from a farm with confirmed *Cryptosporidium parvum*-associated diarrhea. Positive controls (PC, $n = 10$) and negative controls (NC, $n = 10$) were from the sera of cattle previously infected with *C. parvum* and those of neonatal cattle before being given colostrum, respectively. Solid lines indicate average values of the samples. * Indicates significant differences between positive and negative sera in CpP23, CpGP15, and CpGP60 as determined by Student's *t*-test ($p < .01$).

employing CpP23 or CpGP15 independently were calculated as 60.0% respectively by using the cut-off points determined in this study; however, results showed no significant correlation between the $OD_{415\ nm}$ values of positive control sera against CpP23 and CpGP15 ($r = 0.28$, $p = .23$) (Fig. 2). This means that some positive control sera were positive for one antigen, but negative for the other. Both of the sensitivity and specificity were 100% when the two antigens were employed for the ELISA (Fig. 2). The age of the positive control cattle at the point of blood collection as well as the oocysts number of the individuals at the point of exhibiting cryptosporidiosis were not related with the $OD_{415\ nm}$ values (detailed data not shown).

The $OD_{415\ nm}$ values of CpP23 and CpGP15 among the 9 farms were summarized in Fig. 3. Out of 211 sera from *C. parvum*-positive farms (farms #8 and #9), antibodies against CpP23 and CpGP15 were present

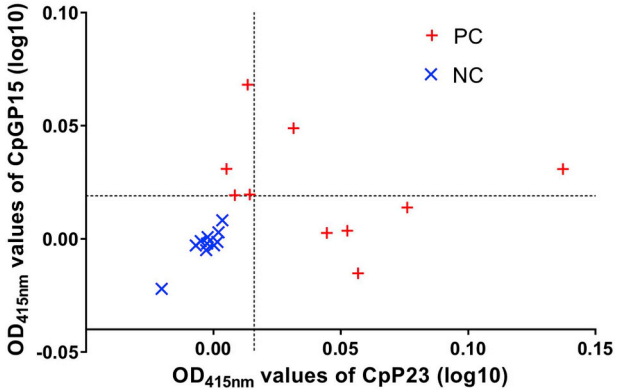

Fig. 2. Relation between $OD_{415\ nm}$ values (log10 scale) of CpP23 and CpP15 for positive (+, $n = 10$) and negative controls (×, $n = 10$). Dotted lines indicate cut-off points. No significant correlation was observed between the $OD_{415\ nm}$ values of both of the antigens ($r = 0.28$, $p = .23$).

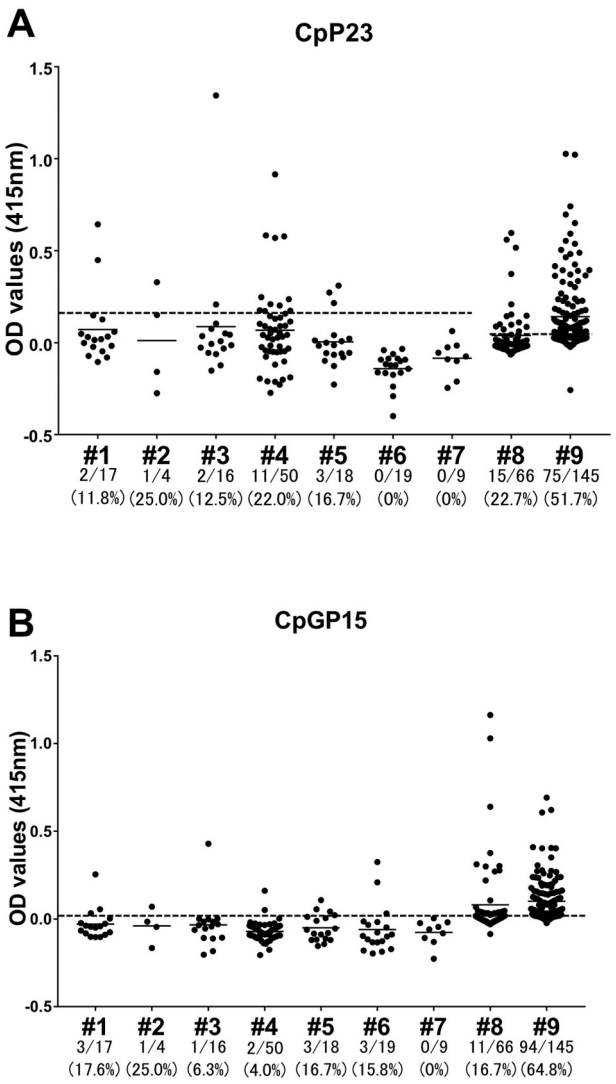

Fig. 3. ELISA with (A) CpP23 and (B) CpGP15 using sera from 9 farms located in Iwate (#1–8) or Hokkaido (#9) prefectures. The sera from farms #1 to #7 were *C. parvum*-negative, whereas those from farms #8 and #9 were *C. parvum*-positive. The seroprevalence for each farm is shown below the ID number. Dotted and solid lines indicate the cut-off and average values, respectively.

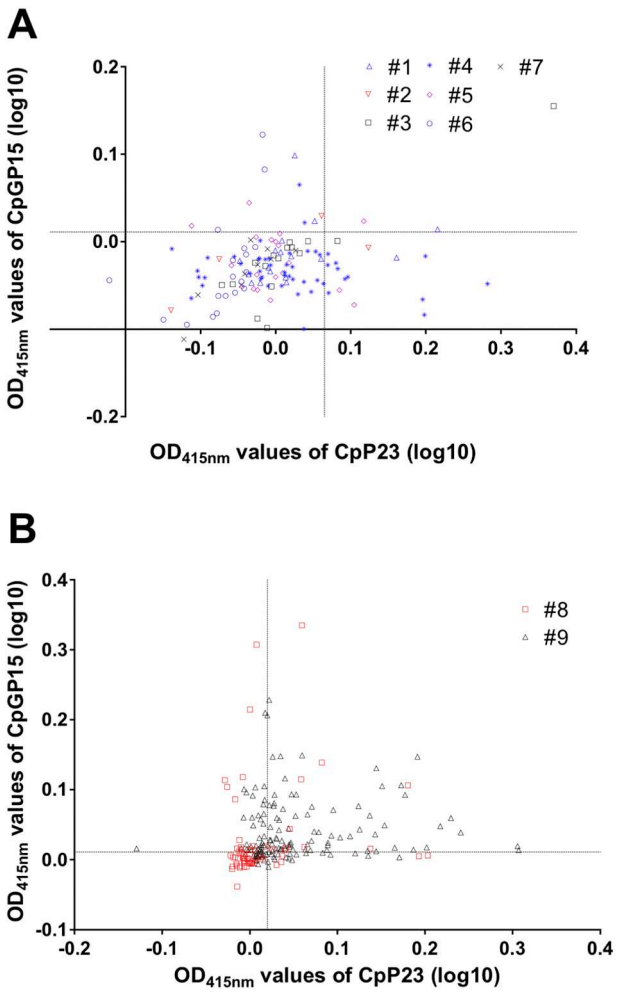

Fig. 4. Relation between $OD_{415\ nm}$ values (log10 scale) of CpP23 and CpP15 for the (A) *C. parvum*-negative sera (farms #1 to #7) and (B) *C. parvum*-positive sera (farms #8 and #9). Dotted lines indicate cut-off points. A farm ID was shown by a symbol. (A) A weak correlation between CpP23 and CpGP15 ($r = 0.30, p < .001$); however, there was no correlation when the samples with negative values are excluded ($r = -0.16$, $p = .40$). (B) No significant correlation was observed between the two antigens ($r = 0.12$, $p = .07$).

in 90 (42.7%) and 105 (49.8%), respectively, whereas 133 (63.0%) were positive for either of the antigens. In contrast, out of 133 sera from *C. parvum*-negative farms (farms #1 to #7), 19 (14.3%) and 13 (9.8%) were positive for CpP23 and CpGP15, respectively, whereas 25 (18.8%) were positive for either of the antigens (Table 1).

The comparison of $OD_{415\ nm}$ values between the two antigens in farms #1 to #9 was shown in Fig. 4. A weak correlation was detected in $OD_{415\ nm}$ values of *C. parvum*-negative farms (farms #1 to #7) ($r = 0.30$, $p < .001$) (Fig. 4A); however, no correlation was found ($r = -0.16$, $p = .40$) when the samples with negative values are excluded. Moreover, no significant correlation was observed in the *C. parvum*-positive farms (farms #8 and #9) ($r = 0.12$, $p = .07$) (Fig. 4B).

Chi-square test analysis demonstrated that the seroprevalence in the *C. parvum*-positive farms was significantly increased compared to that of *C. parvum*-negative farms ($p < .01$). A significant increase of the seroprevalence to both of the antigens as well as to either antigen was also observed in the *C. parvum*-positive farms (Table 1).

4. Discussion

By ELISA, the $OD_{415\ nm}$ values of the recombinant antigens CpP23, CpGP15, and CpGP60 were significantly higher in positive control sera than in negative control sera (Fig. 1). However, the $OD_{415\ nm}$ values of CpP23 and CpGP15 using positive control sera ($n = 10$) were not in agreement with each other (Fig. 2). This result indicates that employing the two antigens in the ELISA improves the reliability of determining the presence of antibodies against the *C. parvum* antigens in a cattle farm, because using both of the antigens can cover the positive samples that were not detected when only one of the antigens is used. It is noteworthy that 100% sensitivity was achieved when employing the two antigens (Fig. 2).

The native proteins of the two antigens are known as surface proteins of sporozoites and merozoites of *C. parvum*. The native CpGP15 is shed in trails during gliding motility [20,21]. Meanwhile, the timing of production for the native CpP23 is unknown, but it contains at least two neutralization epitopes [13]. The expression pattern of CpP23 and CpGP15, and the individual difference on the antibody production may affect the levels of the antibodies against *C. parvum*. If the duration of antibody persistence against these two antigens in the course of the cattle's life can be analyzed in the future, then diagnosis may be more precise.

In the *C. parvum*-positive and negative sera (farms #1 to #9), the number of detection of the antibodies against *C. parvum* was increased

when both of the antigens were employed for the ELISAs (Fig. 4). In the case of single use of CpP23, 43 sera were diagnosed as negative even though they were actually positive for CpGP15 (Table 1). Moreover, the positivity rates of antibodies to CpP23 and CpGP15 correlated to the existence of *C. parvum*-related diarrhea cases (Table 1). These observations support the reliability of ELISA for this study.

This ELISA can be useful for the screening of cattle farms to determine the presence of antibodies against CpP23 or CpGP15. If a farm with high prevalence of *C. parvum* antibodies is found, fecal examination should subsequently be performed to find the oocysts from neonatal calf diarrhea cases. This combination will contribute to precise diagnosis of *C. parvum* infection in farms.

5. Conclusion

The use of both of the recombinant CpP23 and CpGP15 antigens in ELISA allowed the determination of cryptosporidiosis-positive farms. The detection of the existence of *C. parvum* became more reliable when both of the antigens were employed for the ELISA because the combination of the two antigens can avoid false-negative results. Since the high seroprevalences were realized in the cattle farms used in the present study, further study based on ELISA with CpP23 and CpGP15 is required to reveal the situation of the cryptosporidiosis in cattle farms throughout Japan.

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Conflict of interest statement

None of the authors has any financial or personal relationship with other people or organizations that could inappropriately influence or bias this paper in any way.

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