Development and evaluation of an enzyme-linked immunosorbent assay using recombinant p23 for the detection of antibodies to Cryptosporidium parvum in cattle

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Development and evaluation of an enzyme-linked immunosorbent assay using recombinant p23 for the detection of antibodies to *Cryptosporidium parvum* in cattle

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) based on the recombinant p23 of *Cryptosporidium parvum* was established for the detection of antibodies against *C. parvum* in cattle. The sensitivity and specificity of the ELISA were evaluated with the standard indirect fluorescent antibody test (IFAT) using sporozoites as antigens. Of 77 bovine sera collected from China, 20 (26.0%) were identified as positive by the IFAT. The same samples were tested with the ELISA. The optical densities at 415 nm were compared to the IFAT results and statistically analyzed to designate a provisional cut-off point. As a result, the cut-off point was concluded to be 0.08, which was considered to be the best condition in the light of its sensitivity (80%) and specificity (73.7%). Thus, it was demonstrated that the ELISA could be a useful tool for the diagnosis of *C. parvum* infection in cattle under field conditions to estimate the magnitude of asymptomatic infection in a cattle population and subsequent risk to humans.

Key words: *Cryptosporidium parvum*, p23, ELISA, IFAT

INTRODUCTION

Cryptosporidium parvum is a coccidian parasite that infects the intestinal epithelium in humans and animals (Fayer et al., 1997). Exposure of immunocompetent individuals to *C. parvum* results in a transient infection that may be asymptomatic or can result in self-limited diarrhea. In contrast, this parasite causes a life-threatening disease characterized by profuse watery diarrhea and dehydration in immunocompromised patients, especially, individuals with the human immunodeficiency virus-acquired immune deficiency syndrome (Brasseur, 1997). Feces of infected cattle present a risk as the source of infection to farm workers. The contamination of watersheds by cattle feces has been indicated as a primary source of outbreaks in human populations (Smith and Rose, 1990). Thus, *C. parvum* epidemics in cattle must be properly monitored to prevent any risk to humans.

The diagnosis of *C. parvum* infection has relied almost exclusively on the microscopic detection of oocysts in feces. However, this method is relatively slow, subject to the expertise of the microscopist, and, thus, often not cost-effective. Furthermore, this method may overlook past infections because of the short and sporadic duration of shedding oocysts (Fayer et al., 1998; O'Handley et al., 1999), and the potential for the number of oocysts shed was below the limit of detection of the test (Weber et al., 1991; Webster et al.,

1996). To be able to screen large populations in epidemiological studies, including those that may not be at risk for shedding high concentrations of oocysts, a diagnostic test that is at once rapid, accurate, partially automated, and cost-effective is needed. An indirect fluorescent antibody test (IFAT) could detect antibodies against *C. parvum* in infected cattle sera with relatively high sensitivity (87.5%) (Lorenzo-Lorenzo et al., 1993). This serological test could partially fulfill the requirements because antibodies against *C. parvum* persist longer than shedding oocysts in feces (Whitmore and Harp, 1991; Quilez et al., 1996). However, immuno-cross-reactivity between *C. parvum* and *Eimeria spp*. has often been observed (Lorenzo et al., 1998) in the IFAT. Since a more specific serological test was needed, we established an enzyme-linked immunosorbent assay (ELISA) using a parasite-specific antigen.

P23, a 23-kDa glycoprotein of *C. parvum*, was identified as a surface antigen of the parasite. Since it contains neutralization-sensitive epitopes, it was considered to be a hopeful candidate for the development of an effective vaccine against cryptosporidiosis (Perryman et al., 1996). It was reported that immunization with the truncated recombinant p23 induced immune bovine colostrums that protected calves against cryptosporidiosis (Perryman et al., 1999). Recently, Takashima et al. (2003) reported that a recombinant bovine helpesvirus-1 expressing *C. parvum* p23 induced neutralizing antibodies in rabbits.

Since p23 is one of the major targets of antibody production in *C. parvum*-infected animals, the capability of recombinant p23 as a diagnostic antigen for ELISA is expected. In this study, we established an ELISA using recombinant p23 expressed in *Escherichia coli* and demonstrated that the ELISA could provide a useful tool for the diagnosis of cryptosporidiosis.

MATERIALS AND METHODS

Parasites

Cryptosporidium parvum isolate (HNJ-1 strain) was used in all experiments (Abe et al., 2002). The parasite stock was maintained by successive passage in SCID mice, and oocysts from feces were isolated and purified as described previously (Arrowood et al., 1987; Kilani et al., 1987). The oocysts were incubated on ice for 10 min with phosphate-buffered saline (PBS) containing 0.5% sodium hypochloride. The treated oocysts were centrifuged at 8,300x*g* for 1 min and washed with PBS. After being washed four times, oocysts were used for the following experiments.

Indirect fluorescent antibody test

C. parvum oocysts were incubated in 0.01N HCl for 1 h at 37^{o}C. After washing three times in PBS, oocysts were incubated in 1% Gall/DMEM (SIGMA, USA) for 1 h at 37^{o}C for the excystation of sporozoites. After washing three times in PBS, the shed sporozoites were suspended in PBS and placed on glass slides. The slides were dried and then fixed with acetone before use. Serum samples were diluted at 1:100 in 3% FBS/0.1% NaN_3/PBS and placed on the slides. After incubation for 30 min at 37^{o}C, the slides were washed once quickly in PBS, followed by two washes in PBS within 10 min. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-bovine IgG antibodies (SIGMA, USA) diluted at 1:400 in 3% FBS/0.1% NaN_3/PBS were added to the slides, incubated for 30 min at 37^{o}C, and then washed as described above. The slides were examined using a fluorescence microscope (Nikon, Japan).

ELISA

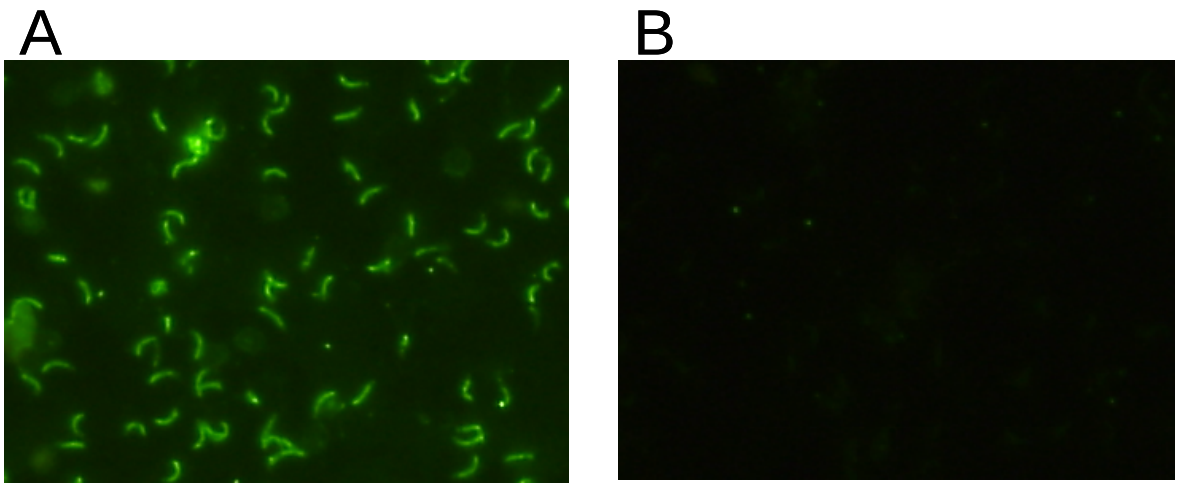

Fig. 1. Detection of antibodies in cattle serum against *C. parvum* sporozoite with an indirect fluorescent antibody test (IFAT).

The glutathione S-transferase (GST)-fused recombinant p23 (GST-p23) of *C. parvum* was expressed in JM109 *E. coli* (Promega, USA) as previously described (Takashima et al., 2003). The purified GST-p23 was diluted with a carbonate-bicarbonate buffer (pH 9.6) to 5 μg/ml and then dispensed into the wells of flat-bottom 96-well microplates in 50 μl aliquots. After incubation for 24 h at 4°C, the unabsorbed GST-p23 was discarded, and 100 μl of the blocking solution (PBS containing 3% skim milk) was added. After incubation for 1 h at 37°C, the blocking solution was discarded. The plates were washed six times with a washing solution (PBS containing 0.05% Tween 20), and 50 μl of serum diluted in PBS was added to each well. After incubation for 1 h at 37°C, the plates were washed six times with the washing solution, and 100 μl of horseradish peroxidase-conjugated rabbit anti-bovine IgG antibodies (Bethyl Lab. Inc., USA) diluted in PBS was added to each well. After incubation for 1 h at 37°C, the plates were washed six times with a washing solution. Then, a substrate solution [0.1 M citric acid, 0.2 M sodium phosphate, 0.0003% H_2O_2, 0.5 mg of 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) per ml] was added to each well in 100 μl aliquots. After incubation for 1 h, the absorbance at 415 nm was measured. The optical density values tested in the GST control were deducted from the values tested in GST-p23 in order to eliminate the effects of non-specific antibodies. The statistic analysis method of sensitivity and specificity developed by Frössling et al. (2006) and Nishikawa et al. (2001) was used.

Western blot analysis

Protein samples were electrophoresed in each lane of a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. The membrane was washed twice with the washing solution (Tris-buffered saline (TBS) containing 0.05% Tween 20 and 1% skim milk) and then incubated with serum diluted at 1:100 in TBS containing 1% skim milk for 1 h at room temperature. After washing three times with the washing solution, the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-bovine IgG antibodies (Bethyl Lab. Inc., USA) diluted at 1:100 in TBS containing 1% skim milk for 1 h at room temperature. After washing three times, the results were visualized using 3,3-diaminobenzidine and H_2O_2.

Sensitivity and specificity of the ELISA

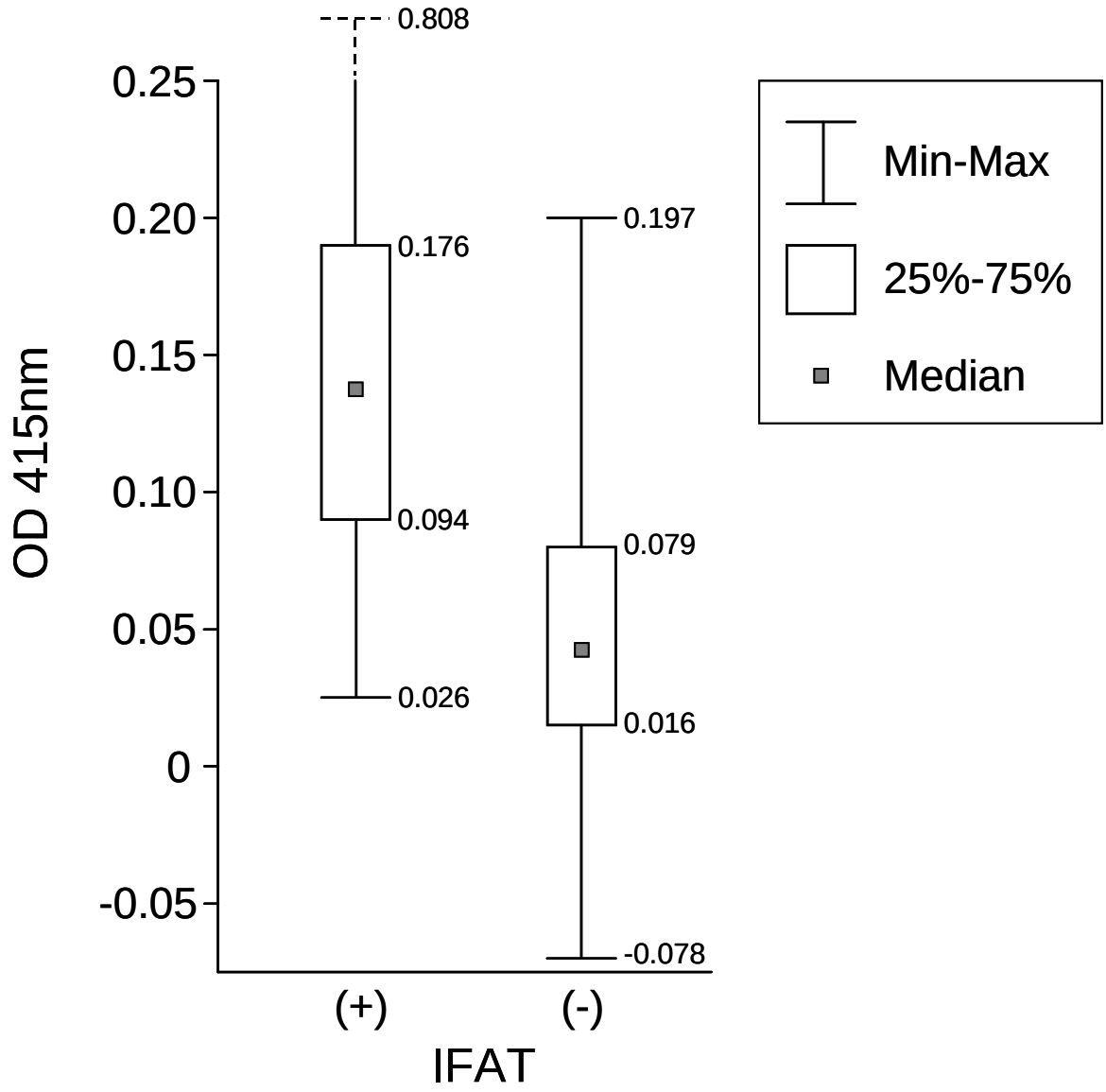

Fig. 2. An enzyme-linked immunosorbent assay (ELISA) of bovine sera using GST-p23. The optical densities at 415 nm of *C. parvum* IFAT positive (+) (n=20) and negative (-) (n=57) sera were read and are plotted on the chart. Minimum and maximum values (Min-Max), 25 and 75% percentiles (25%-75%), and median values were figured as noted.

Estimates of the sensitivity and specificity at different cut-off points in the ELISA were calculated by the standard cross-tabulation technique (Rothman and Greenland, 1998).

RESULTS AND DISCUSSION

Seventy-seven bovine serum samples collected from the Yanbian region, China, were tested with IFAT. Twenty samples were found to be positive, as shown in Fig. 1A. To confirm the specificity of positive sera to GST-p23, Western blot analyses were performed. A serum sample that was positive in the IFAT specifically reacted to the recombinant GST-p23 expressed in *E. coli* with a single sharp band with a molecular mass of approximately 40 kDa, which was the expected size, while the control GST protein was not recognized by the positive serum (data not shown). Thus, the GST-p23 could be specifically recognized by serum from cattle infected with the parasite in the field.

The ELISA with GST-p23 was performed, and the resultant optical densities at 415 nm (OD415 nm) were statistically analyzed (Fig. 2). A scattered plot indicated that the minimum and maximum values of

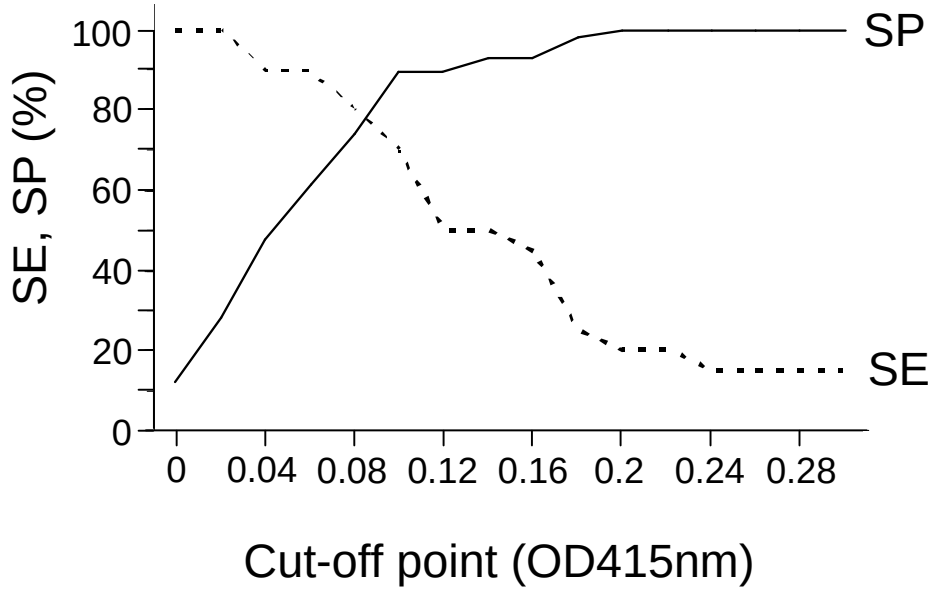

Fig. 3. Sensitivity (SE) and specificity (SP) curves of the ELISA at various cut-off points when used to detect antibodies to *C. parvum* in cattle sera from China (n=77). Dashed line, SE; solid line, SP.

the OD 415 nm of the IFAT-positive samples were 0.026 and 0.808, respectively. However, the samples between 25 and 75% percentiles were within the range from 0.094 to 0.176. In IFAT-negative samples, the minimum and maximum values were -0.078 and 0.197, respectively. The 25 and 75% percentiles were from 0.016 to 0.079. From the result of the distribution of positive/negative samples, OD values between 0.079 and 0.094, where the 25 and 75% percentiles of positives and negatives did not overlap, were thought to be a good candidates for a cut-off point (Fig. 2). However, the generally used calculation for a cut-off point (average plus 3 times the standard deviation) was 0.206. When this cut-off point was employed, only 4 samples were detected (sensitivity=20.0%) within 20 positives in the IFAT. This lesser degree of sensitivity was due to the widely scattered OD values because serum samples tested in this study were obtained from the field and not experimentally confirmed for negative sera. Thus, a more optimal and accurate cut-off point should have been set up for the detection of all sera from the infected cattle in field.

To identify the optimal point, we calculated and evaluated the sensitivities and specificities at various cut-off points (Fig. 3). Given that the objective of this diagnostic test was to detect exposure in populations rather than to identify individual clinical cases, the sensitivity should be high enough to detect primary, past, and latent infections in which the shed oocysts in feces are not detected and the antibody levels are relatively low. For this reason, the optimal cut-off point was concluded to be 0.08 (sensitivity=80.0%, specificity=73.7%).

This study was carried out to investigate whether the ELISA with recombinant p23 could be used as a test for evidence of exposure to *C. parvum* in cattle at different levels of risk of endemics in humans. The intent was to develop a test that is accurate, easy to apply, and economically efficient to be used in surveillance studies. The IgG antibodies against *C. parvum* in serum, whether they are passively derived or actively developed, could be a useful diagnostic marker. Within samples tested, although no clinical

symptoms of cryptosporidiosis were observed in cattle, the IFAT found 20 seropositives, and 16 of them were also detected as positive with the ELISA. Such data could provide valuable information of risk to human populations, especially to farmers and veterinarians. In addition, it is possible to estimate the possibility of water pollution by *C. parvum* in certain areas and to follow epidemics in a large population. In the light of its time and cost effectiveness, the ELISA with recombinant p23 could be a useful diagnostic tool with high sensitivity and specificity.

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