Development and evaluation of the first immunochromatographic test that can detect specific antibodies against *Cryptosporidium parvum*

Ragab M. Fereig[a, b, c], Hanan H. Abdelbaky[a], Fumiaki Ihara[a], and Yoshifumi Nishikawa[a,*]

[a]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

[b]Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

[c]Department of Animal Medicine, Faculty of Veterinary Medicine, South Valley University, Qena City, Qena 83523, Egypt

***Correspondence**

Yoshifumi Nishikawa, PhD

Obihiro University of Agriculture and Veterinary Medicine, Inada-Cho, Obihiro, Hokkaido 080-8555, Japan

Tel: +81-155-495642, Fax: +81-155-49643, e-mail: nisikawa@obihiro.ac.jp

Abbreviations: CpGP15, *Cryptosporidium parvum* 15-kDa recombinant glycoprotein; CpP23, a 23-kDa glycoprotein of *C. parvum*; HRP, horseradish peroxidase; ICT, immunochromatographic test; iELISA, indirect enzyme-linked immunosorbent assay; IgG, immunoglobulin G; PBS, phosphate buffered saline; PBS-T, Tween 20 formulated in PBS; SK, skim milk.

Abstract

Cryptosporidium parvum is a major cause of diarrhea among human and calves, resulting in severe health hazards and drastic economic losses, respectively. Although *C. parvum* infection leads to high morbidity and mortality in immunocompromised patients and bovine calves, this infection remains a neglected disease. Currently available diagnostic tests for *C. parvum* are primarily based on detection of oocysts, DNA, or secreted antigens in fecal specimens. Demonstration of specific antibodies with a rapid immunochromatographic test (ICT) will be advantageous not only in providing a simple, rapid, accurate, and affordable tool but also in surveillance because of the ability to recognize recent and past infections. Herein, we developed two ICTs using the diagnostic antigen CpP23 and immunodominant antigen CpGP15 to detect *C. parvum*-specific antibodies in cattle sera. Because of unavailability of a reference test for antibody detection, evaluation and validation of our developed ICTs were conducted using reference cattle samples and unknown field cattle sera. Serum samples were simultaneously tested by a previously validated enzyme-linked immunosorbent assay (ELISA) using the same antigens (CpGP15 and CpP23). ICTs showed substantial ability to discriminate between positive and negative control cattle sera for both CpGP15 and CpP23. Even against field sera, high sensitivity, specificity, and agreement rates were recorded for ICTs compared with the previously validated ELISA with the same antigens (CpGP15 = 78.78%, 100%, and 85.11%; CpP23 = 80%, 100%, and 80.56%, respectively). Moreover, a high correlation was observed between the test band intensity of ICTs and optical density of ELISA, particularly in the case of CpP23-specific IgM. To our knowledge, this study represents the first development of ICTs that can detect *C. parvum*-specific antibodies. Our tests will contribute greatly to *C. parvum* infection control in cattle by providing a method for on-site diagnosis of early and latent infections.

Keywords:

Cryptosporidiosis; Cattle; Serodiagnosis; CpP23; CpGP15

1. Introduction

The genus *Cryptosporidium* consists of obligatory intracellular protozoan parasites that are globally distributed and invade intestinal cells of animals and human (Xiao *et al.,* 2004). Among the 14 identified species of genus *Cryptosporidium*, the two major species of medical importance include *C. parvum*, which infects animals and human, and *C. hominis*, which primarily infects human (Morgan-Ryan *et al.,* 2002; Rose *et al.,* 2002). The fecal-oral route is the common mode of transmission of *Cryptosporidium* disease, which occurs via ingestion of food or water contaminated with oocysts or direct contact with infected humans, animals, tools, or soil (Arrowood, 1997; Rose, 1997). Infective oocysts are shed in stools of diarrheic animals or patients and can survive for several weeks in harsh environmental conditions (Rose and Slifko, 1999; Ramirez *et al.,* 2004). Cryptosporidiosis is recognized as a ubiquitous cause of severe diarrhea in pre-weaned calves and accompanied with high mortalities because of resultant dehydration and electrolyte imbalance. Moreover, this disease causes high mortality rates in malnourished children and AIDS patients due to persistent diarrhea (Rose *et al.,* 2002).

A number of studies have focused on cell surface antigens of *C. parvum* sporozoites or merozoites as potential immunomodulators or diagnostic markers, of which CpGP15 and CpP23 are recognized as the most promising antigens (Boulter-Bitzer *et al.,* 2007; Chekley *et al.,* 2015). CpGP15 is a glycoprotein that has a crucial role in pathogenesis of *C. parvum* infection because of its contribution to parasite motility, attachment, and invasion of host epithelial cells (Tilley *et al.,* 1991; Reperant *et al.,* 1994). As an immunodominant antigen, specific antibody to CpGP15 is recognized in sera of children infected with *C. parvum* or *C. hominis* (Ajjampur *et al.,* 2011; Allison *et al.,* 2011), and in serum and colostrum of cattle infected with *C. parvum* (Mead *et al.,* 1988; Tilley *et al.,* 1990). The glycoprotein CpP23 is an immunogenic protein in human and cattle (Arrowood *et al.,* 1991), and identified during sporozoite gliding and locomotion (Enriquez and Riggs, 1998). Moreover, several studies

reported CpP23 as a potential antigen for serodiagnosis of *C. parvum*-specific antibodies in field animal samples, particularly among cattle (Wyatt and Perryman, 2000; Bannai *et al.*, 2006; Inpankaew *et al.*, 2009; Wang *et al.*, 2009; Fereig *et al.*, 2016). Accordingly, CpGP15 and CpP23 effectively contribute to pathogenesis of *C. parvum* infection and their diagnostic potentials are strongly anticipated.

Currently, the diagnosis of *Cryptosporidium* generally relies on detection of oocysts or antigens in fecal specimens from infected human or animals. The conventional method comprises detection of oocytes using a fecal smear with modified acid-fast staining and microscopical examination. This method is fast and inexpensive but has low sensitivity and specificity and requires technical expertise (Chalmers *et al.*, 2011). In addition to high sensitivity and specificity, molecular detection methods based on amplification of species-specific gene sequences provide additional advantages for genotyping, quantification, and differentiation of multiple *Cryptosporidium* species (Robinson *et al.*, 2010; Hadfield *et al.*, 2011; Ichikawa-Seki *et al.*, 2015). However, the use of such methods is limited because they require specialized equipment, expensive reagents, and high technical expertise. In the same context, although antigen detection methods using enzyme-linked immunosorbent assay (ELISA), immunofluorescence, or immunochromatographic assays have several advantages such as feasible sensitivity, specificity, and practicability, their high costs and short lifetime of antigen release render them transient methods for diagnosis of acute and symptomatic cryptosporidiosis (Garcia and Shimizu, 1997; Uga *et al.*, 2000). Although ELISA and immunoblot with various recombinant antigens are used for detection of *Cryptosporidium*-specific antibodies (Ares-Mazás *et al.*, 1999; Wyatt and Perryman, 2000; Priest *et al.*, 2001; Bannai *et al.*, 2006; Inpankaew *et al.*, 2009; Wang *et al.*, 2009; Fereig *et al.*, 2016), they are time-consuming, laborious, and require specialized instruments and reagents, which decrease their feasibility for field applications. In the same context, tremendous advances in

nanotechnology are greatly contributing to the fight against protozoan parasites including *C. parvum* (Benelli, 2018). The coupling of anti-*C. parvum* cyst antibody and alkaline phosphatase with gold nanoparticles has improved the sensitivity of the conventional immuno-dot blot assay by 500-fold (Thiruppathiraja *et al*., 2011).

IgG and IgM antibodies have been extensively investigated in immunological and seroepidemiological studies of *C. parvum* in human and different animal species (Mtambo *et al.*, 1995; De Graaf and Peeters, 1997; Wang *et al.*, 2009; Ajjampur *et al.*, 2011; Allison *et al.*, 2011). IgG is generally considered as a marker for chronic infection, while IgM is widely accepted as an indicator for acute infection. In mice, the response to IgM was specified for the early stage post-infection (5 weeks post-infection), whereas the IgG response was recorded during a later stage (25 weeks post-infection) after infection with *C. parvum* oocysts (Yu and Lee, 2007). Another study reported an earlier increase and peak of IgG than IgM (within a period of 2 weeks post-infection) in a mouse model of cryptosporidiosis (Martín-Gómez *et al.*, 2006).

In the current study, we attempted to evaluate the diagnostic performance of previously recognized potent antigens CpGP15 and CpP23 for demonstration of their use as relevant antibodies in the immunochromatographic test (ICT). Our developed ICTs were evaluated using positive and negative control cattle sera and validated against a number of field cattle sera. Additionally, the performance of ICT was compared with IgG and IgM ELISAs using the relevant antigens.

2. Materials and methods

2.1. Control and field serum samples from cattle

Blood samples were obtained from cattle after obtaining the consent of all animal owners. Samples were identified with a unique code and placed in an ice box. Sera were then separated by centrifugation and stored at −20°C until use. Sera from calves before feeding with colostrum were used as negative control samples (n = 6). Sera from cattle with cryptosporidiosis were used as *C. parvum*-positive sera (n = 8). *C. parvum* was confirmed in calves showing diarrhea by a commercial ICT kit (Bio-X Diagnostics SPRL, Jemelle, Belgium). The time interval from demonstration of infection to sample collection was approximately 2 years to detect persistence of specific antibody. Field samples (n = 47) were collected from a cattle farm at which monthly diarrheal cases were observed.

2.2. Preparation of recombinant antigens

Genomic DNA was used for amplification of the target gene sequence for CpGP15 using the following primers: forward primer including restriction enzyme *Eco*RI (underlined) 5'-GGG GAA TTC GAA ACC AGT GAA GCT GCT GCA ACC-3' and reverse primer including restriction enzyme *Bam*HI (underlined) 5'-GGG GGA TCC ATC CTT CAA AAG AAC TGT GTT GTC-3'. PCR products were digested with the respective restriction enzymes and then ligated to a similarly cut pGEX-6P1 vector containing an open reading frame encoding glutathione-*S*-transferase (GST) fused to the N-terminus of the protein (GE Healthcare, Uppsala, Sweden) using DNA Ligation Kit Mighty Mix (Takara Bio Inc., Shiga, Japan). The constructed plasmid was transformed into *Escherichia coli* (BL21) cells. Recombinant proteins were expressed as GST fusion proteins as illustrated previously for CpP23 antigen (Bannai *et al.,* 2006, Fereig *et al.,* 2016) and CpGP15, with slight modifications. Protein expression was induced by 1 mM isopropyl b-D-1-thiogalactopyranoside (Wako Inc., Osaka, Japan) at 37°C

overnight. The eluted protein lots were dialyzed in phosphate-buffered saline (PBS) overnight and filtered using a 0.45-μm low-protein binding Supor membrane (Pall Life Sciences, Ann Arbor, MI, USA). Protein purity and quantity were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue R250 staining (MP Biomedicals Inc., Illkirch-Graffenstaden, France). Protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

2.3. Production of antisera and IgG purification

Polyclonal antibodies against CpGP15-GST or CpP23-GST were generated and purified as IgG. The recombinant proteins (1 mg) were emulsified in Freund's complete adjuvant (Sigma, St Louis, MO, USA) and subcutaneously injected into female Japanese white rabbits (Kitayama Labes, Nagano, Japan) on day 0. The same protein in Freund's incomplete adjuvant (Sigma) was injected into the rabbits on days 14, 28, and 42 after the first immunization. Sera were collected from immunized rabbits at −2, 12, 26, and 40 days from the ear vein and with heart puncture after 49 days. Presacrifice serum was collected and checked for antibody titers before euthanization. IgG titers were estimated by indirect ELISA using the method described below. Two milliliters of rabbit serum were used for IgG purification using protein A chromatography columns (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Purified IgGs were checked for protein purity and quantity by SDS-PAGE and the concentration was measured with a BCA protein assay kit.

2.4. Indirect ELISA

Recombinant CpGP15 and CpP23 antigens at a final concentration of 0.1 μM were coated onto ELISA plates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C with carbonate-bicarbonate buffer (pH 9.6). After blocking with PBS containing 3% skim milk (PBS-SM), plates were washed once with washing buffer (0.05% Tween 20 in PBS), and 50 μl of serum samples (diluted with PBS-SM at 1:100) were added to each well. Plates were then incubated at 37°C for 1 h with horseradish-peroxidase-conjugated anti-bovine IgG or IgM (Bethyl Laboratories, Montgomery, TX, USA), diluted with PBS-SM at 1:4,000 and 1:6,000 for IgG and IgM, respectively. After six washes, 100 μl of 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-sulfonic acid) (Sigma)] was added to each well and incubated for 1 h at room temperature. The absorbance was measured using an ELISA reader at a wavelength of 415 nm. ELISA results for specific antibody to CpGP15 or CpP23 were determined by measuring the mean optical density (OD) of GST protein subtracted from that of the relevant antigen. Sera from calves before feeding with colostrum were used as *C. parvum*-negative sera ($n = 8$). The ELISA cutoff value was calculated from the OD value obtained from each antigen against negative controls and after subtraction of the OD value of GST (cutoff values for CpGP15: IgG = 0.1, IgM = 0.05; cutoff values for CpP23: IgG = 0.03, IgM = 0.035).

2.5. Design and preparation of ICTs

Recombinant CpGP15-GST or CpP23-GST antigen at a concentration of 0.5 mg/ml was conjugated with a gold colloid (British Biocell International, Cardiff, UK) (1:10, vol/vol) at pH 6.3 by incubation at room temperature for 10 min. To stabilize and block conjugate particles, 0.05% polyethylene glycol 20000 (PEG) and 1% bovine serum albumin (BSA) were added to the solution. After centrifugation at 18,000 × *g* for 30 min, around 90% of the

supernatant was discarded, the pellet was resuspended by sonication, washed with PBS containing 0.05% PEG and 0.5% BSA, and re-centrifuged. The pellet was then resuspended in PBS with 0.5% BSA and 0.05% PEG. The concentration of the conjugate was adjusted until the absorbance at 520 nm reached 5. The conjugate was diluted in 5% sucrose prepared in 10 mM Tris-HCl (pH 8.2), sprayed onto glass fiber (Schleicher & Schuell BioScience, Inc., Keene, NH, USA), and then dried overnight. Rabbit anti-CpGP15-GST or anti-CpP23-GST IgG was purified with an Econo-Pac protein A kit (Bio-Rad Laboratories). Purified antigens (CpGP15-GST, CpP23-GST, or GST alone) and IgG (rabbit anti-CpGP15-GST or anti-CpP23-GST) were diluted in PBS at an optimal concentration of 0.5 mg/ml and linearly jetted onto nitrocellulose (Schleicher & Schuell) using a BioJet Quanti 3050 quanti-dispenser (BioDot Inc., Irvine, CA, USA). After desiccation of the membrane at 50°C for 30 min, blocking solution (0.5% casein in 50 mM boric acid buffer, pH 8.5) was applied for 30 min. The membrane was directly incubated in a solution of 50 mM Tris-HCl (pH 7.4) containing 0.5% sucrose and 0.05% sodium cholate for washing, followed by overnight incubation in air. The nitrocellulose, absorbent pad, conjugate pad, and sample pad were assembled onto an adhesive card (Schleicher & Schuell) and cut into 3-mm-wide strips by using a BioDot cutter (Fig. 1). For sample testing, 40 µl of diluted serum in PBS (1:2, vol/vol) was placed on the sample pad by pipetting. The development of band coloration was judged within 20 min for test or control lines. To optimize ICT condition and reactions, various antigen concentrations, serum dilutions, and gold colloidal pHs were tested.

2.6. Statistical analyses

Significant differences in ELISA values were calculated using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical analyses were performed

with Student's *t* test for group comparisons. Specificity, sensitivity, agreement proportion, kappa values, and 95% confidence intervals were calculated using an online statistical tool (www.vassarstats.net). The strength of agreement was graded with kappa values of fair (0.21–0.40), moderate (0.41–0.60), and substantial (> 0.61). The correlation coefficients between relative intensity in the ICT band and absorbance values in the ELISA were calculated using Pearson's correlation coefficient. To calculate the relative intensity in the ICT, ICT pictures were converted into 8-bit JPEG images, then the intensity of the gray scale images was analyzed using ImageJ software v. 1.49 (Mac version of NIH Image, http//rsb.info.nih.gov/nih-image/).

3. Results and discussion

Cryptosporidium is implicated as the potential causative agent in most recorded waterborne outbreaks of parasitic origin worldwide, in which diarrhea or abdominal distress was revealed as a major clinical sign (Xiao, 2010; Efstratiou *et al.*, 2017). To perform this study, we had to overcome several challenges such as: 1) unavailability of a reference test for detection of *C. parvum*-specific antibodies, 2) lack of previous studies or information on the technology of developing an ICT for detection of anti-*Cryptosporidium* antibodies, and 3) validation of our ICTs against a number of authentic *C. parvum*-negative and -positive samples obtained from the field.

Because our goal is to develop an ICT that can detect *Cryptosporidium*-specific antibodies from infected cattle, positive and negative control sera obtained from field cattle were used to evaluate and validate our test. Strikingly, all negative samples (n = 8) were also negative for CpGP15 and CpP23 ICTs, and all positive samples (n = 6) were positive in the ICTs (Fig. 2). These results were identical to IgG or IgM ELISA results (Fig. 3), indicating the high performance of the ICT with CpGP15 and CpP23 for discrimination between *C. parvum*-negative and -positive cattle sera. Testing of both IgG and IgM was proven as a necessary step for accurate diagnosis of *C. parvum* infection (Wang *et al.*, 2003; Yu and Lee, 2007; Campbell and Mutharasan, 2008) and for comprehensive evaluation and analyses of ICT results because of the difference in the dynamicity and reactivity of both antibodies. CpGP15 and CpP23 have previously been demonstrated as highly conserved antigens among diverse *Cryptosporidium* species and *C. parvum* isolates, respectively, which render them as potential diagnostic antigens for *C. parvum* infection with no or low possibility for cross-reactions with other microbial infections (Boulter-Bitzer *et al.*, 2007; Checkley *et al.*, 2015).

253 Despite the long time interval (approximately 2 years) from diagnosis of infection to
254 sample collection of positive control sera, specific IgM antibodies were detectable by ELISA
255 using both antigens (Fig. 3). IgM is generally involved in the early response to microbial
256 infections (Geisberger *et al.*, 2006); thus, it is difficult to explain the exact reason for this result.
257 However, available information suggests reinfection of these animals because these samples
258 were collected from a farm with many *C. parvum*-related diarrheal cases. Based on information
259 obtained from veterinarians in charge of this farm, diarrheal cases occurred monthly. For this
260 reason, negative control samples were collected from newly born calves before being fed with
261 colostrum of dams because colostrum is a rich source of anti-*C. parvum* antibodies (Tilley *et*
262 *al.*, 1990). In addition to IgG antibody, the persistence of IgM antibody may be the triggering
263 factor for the induction of long-lasting protective immunity and this may explain the high rate
264 of self-recovery in immunocompetent individuals (Racine *et al.*, 2011). IgM is crucial for the
265 activation of complement system for pathogen removal (Cooper *et al.*, 1983), B cell
266 development (Baker and Ehrenstein, 2002), and regulation of inflammatory responses (Zhang
267 *et al.*, 2004). Consistently, numerous reports on *C. parvum* and other pathogens indicate the
268 crucial role of IgM in chronic infection (Shehab *et al.*, 2002; Couper *et al.*, 2005; Martín-
269 Gómez *et al.*, 2006; Secundino *et al.*, 2006; Ortarik *et al.*, 2011; Racine *et al.*, 2011; Matteucci
270 *et al.*, 2014). Additional studies are required to understand the contribution of IgM in immunity
271 against *C. parvum* infection and its usefulness for diagnostic purposes.

272 Furthermore, a number of field serum samples obtained from cattle (n = 47) were also
273 checked using the two ICTs and compared with the previously validated ELISA. Similar results
274 were obtained from ICTs and ELISAs when CpGP15 and CpP23 were used as the coated
275 antigen (Fig. 4). Next, we applied objective evaluation to estimate the correlation between ICT
276 band intensity and ELISA OD values using ImageJ software v. 1.49 (Fig. 5). A weak to
277 moderate correlation for CpGP15 was observed between ICT and IgG and IgM ELISAs

(Pearson's r = 0.1193 and 0.3287, respectively). In the case of CpP23, a weak to moderate (Pearson's r = 0.3960) and a moderately strong correlation (Pearson's r = 6880) between the color gradient of the ICT test band and IgG and IgM ELISAs were found, respectively.

Consistently high, positive rates were obtained for field cattle sera against both antigens using ICTs (CpGP15 = 55.32%, CpP23 = 68.01%) and combined ELISA (either IgG or IgM: CpGP15 = 70.21%, CpP23 = 97.87%) (Table 1). These results may be attributed to the high number of infected animals; particularly, these samples were collected from a cattle farm close to a *C. parvum*-endemic area. Another factor for the higher IgM prevalence may be related to the higher binding affinity of IgM than IgG because of topological structure (Roux *et al.*, 1999).

As shown in Table 2, sensitivity, specificity, kappa value, and agreement proportion of our developed ICTs were evaluated and compared with previously validated ELISAs for both antigens. CpGP15-ICT demonstrated moderate and substantial concordance with IgM ELISA and IgM and/or IgG ELISA results using CpGP15 antigen, as evidenced by kappa values of 0.566 and 0.689 to 1, respectively (Table 2). Moreover, CpP23-ICT showed moderate concordance with IgG ELISA results using CpP23 (kappa value = 0.431) (Table 2). The divergence in responses of antigens (CpP23 and CpGP15) and antibodies (IgM and IgG) may be related to the different role of both antigens in the pathogenesis of *C. parvum* (Tilley *et al.*, 1991; Reperant *et al.*, 1994; Enriquez and Riggs, 1998). Previous reports also indicated the correlation of CpP23-specific IgG antibody to chronic or latent infection, whereas the CpGP15-specific IgM response is primarily concerned with recent infection (Boulter-Bitzer *et al.*, 2007; Checkley *et al.*, 2015). Thus, our data suggest that CpGP15-ICT and CpP23-ICT may be practical and reliable tests for the serodiagnosis of *C. parvum* based on IgM- and IgG-specific antibodies, respectively.

Herein, we provide additional evidence about the usefulness of CpGP15 and CpP23 in detection of specific antibodies in sera from *C. parvum*-infected cattle using ICT as the first serodiagnostic system and previously validated ELISAs. The potential use of CpP23 and, to a lesser extent, CpGP15 for detection of relevant antibodies was previously demonstrated by numerous reports using ELISA and immunoblotting (Wyatt and Perryman, 2000; Bannai *et al.*, 2006; Shayan *et al.*, 2008; Inpankaew *et al.*, 2009; Wang *et al.*, 2009; Ajjampur *et al.*, 2011; Allison *et al.*, 2011; Fereig *et al.*, 2016). This potential may be attributed to the high proportion of epitopes sensitive for antibody neutralization in the case of CpP23 (Perryman *et al.*, 1996). We anticipate our developed ICTs will be able to supplement or replace currently available diagnostic tests because ICT is a rapid, simple, and cost-effective method that can be applied for research purposes or survey in remote endemic areas that lack research facilities. Since our ICTs are efficient in detecting IgG and IgM-specific antibodies, they will be helpful in the diagnosis of early or latent *C. parvum* infection. Hence, more accurate seroprevalence and epidemiological investigations will be conducted. In the future, we could expect the ability of ICT for direct demonstration of specific antibodies without requiring secondary or detection antibodies, facilitating the diagnosis of infection caused by *C. parvum* or other *Cryptosporidium* species in animals including human.

4. Conclusions

In the current study, we showed the successful development of ICTs for detection of *C. parvum*-specific antibodies using two immunodominant *C. parvum* antigens, CpGP15 and CpP23. Both ICTs exhibited excellent performance with control cattle sera. Additionally, high agreement was observed between the result of our developed ICTs and previously validated ELISAs in unknown field cattle sera. Our developed ICTs are regarded as specific diagnostic tools because of the use of conserved antigens CpP15 (among *Cryptosporidium* species) and

CpP23 (among *C. parvum* isolates). On-site diagnosis using ICTs will contribute to *Cryptosporidium* surveillance because antibody detection can provide an inclusive record of the endemic status of cryptosporidiosis in cattle, including acute, sub-acute, or chronic infections. Our developed ICTs will be valuable for diagnosis of *Cryptosporidium* infection and are expected to reduce economic losses caused by cryptosporidiosis in cattle if accompanied with proper control strategies, such as quarantine measures, symptomatic treatment of infected animals, hygienic disposal of infected excreta, and disinfection of contaminated utensils with chlorine.

Conflict of interest

The authors declare that they have no conflicts of interest.

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509

Figure legends

Fig. 1. Schematic diagram for ICT using CpGP15 or CpP23 to detect *C. parvum*-specific antibodies in sera. Three lines were sprayed onto the nitrocellulose membrane including two control lines containing recombinant GST-tagged protein or anti-rabbit IgG and one test line containing recombinant CpGP15 or CpP23 antigen. The concentration of all antigens or antibody used in this study was optimized at 500 μg/ml.

Fig. 2. Reactivity of ICT against control sera. Strip tests using CpGP15 (A) and CpP23 (B) are shown as before testing (untreated) and after testing against control cattle sera. No. 1: pretest, No. 2: PBS, Nos. 3–10: *C. parvum*-negative control sera, Nos. 11–16: *C. parvum*-positive control sera.

Fig. 3. Reactivity of ELISA against control sera. (A) Responses of both recombinant antigens (CpGP15 and CpP23) against cattle sera using IgG ELISA. (B) Reactivity of both antigens using IgM ELISA. Sera from *C. parvum*-infected ($n = 8$) and non-infected ($n = 6$) cattle were tested against each antigen. *** $P < 0.0001$, Student's *t* test.

Fig. 4. Comparison of ICT and ELISA for detection of *C. parvum*-specific antibodies in cattle. Serum samples ($n = 47$) collected from a cattle farm were tested using ICTs for CpGP15 and CpP23 and compared against IgG and IgM ELISAs of both antigens. Dotted horizontal lines represent ELISA cutoff values. ELISA results were considered positive at an OD_{415} of (A) > 0.1 for CpGP15 IgG, (B) > 0.05 for CpGP15 IgM, (C) > 0.03 for CpP23 IgG, and (D) > 0.035 for CpP23 IgG and IgM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, Student's *t* test.

Fig. 5. Analyses of ICT results with different antibodies and ELISA OD values. Scatter graphs show the correlation between relative intensity in the ICT and absorbance values in the ELISA using serum samples. The equation represents the approximation formula. The break line represents the calculated line of best fit. Correlation coefficients were calculated using Pearson's correlation coefficient: $|r| = 0.70$, strong correlation; $0.5 < |r| < 0.7$, moderately strong correlation; and $|r| = 0.3$ - 0.5 weak to moderate correlation. Correlation coefficient (r): (A) $r = 0.1193$; (B) $r = 0.3287$; (C) $r = 0.3960$; (D) $r = 0.6880$.

Fig. 1. Fereig et al.

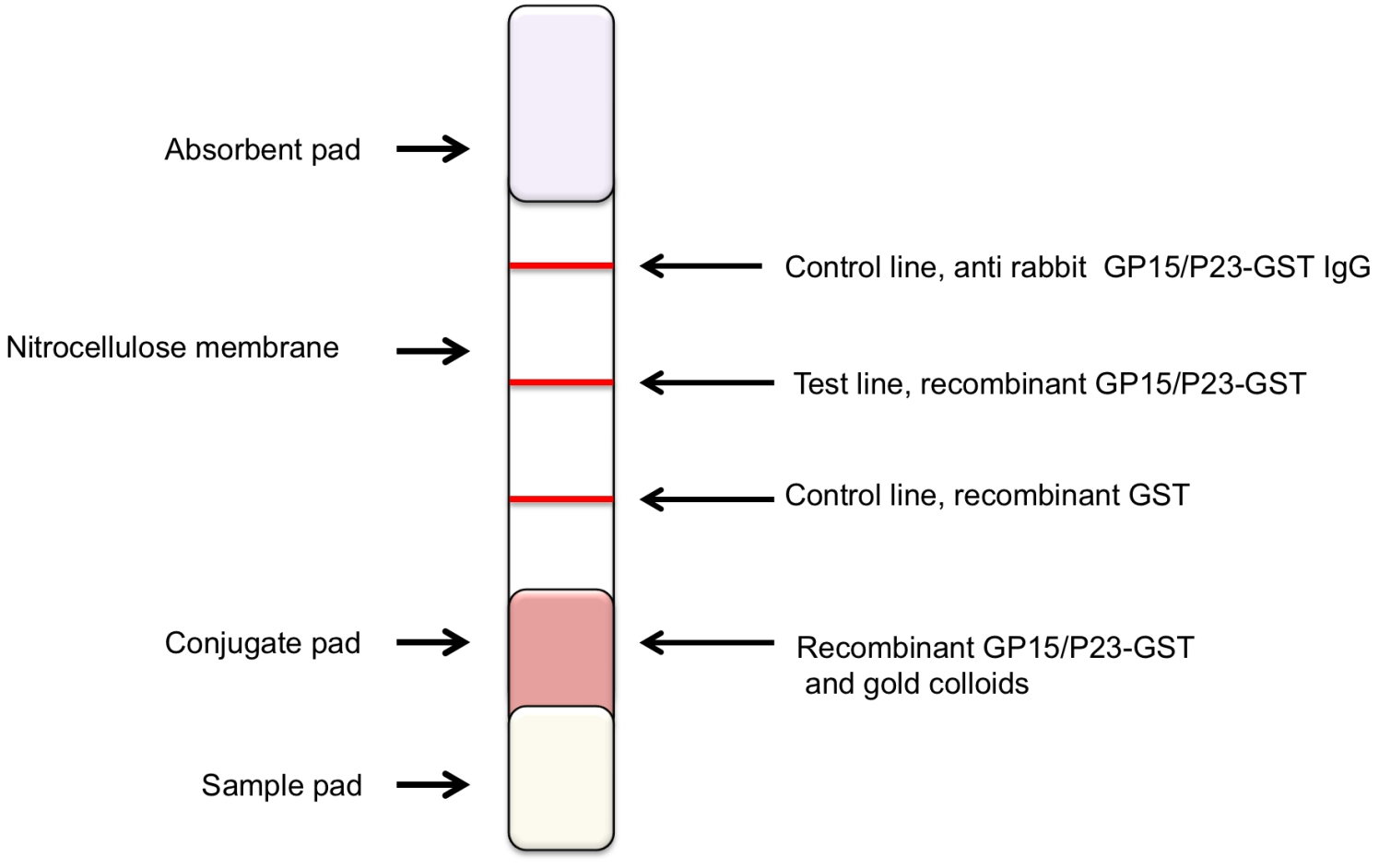
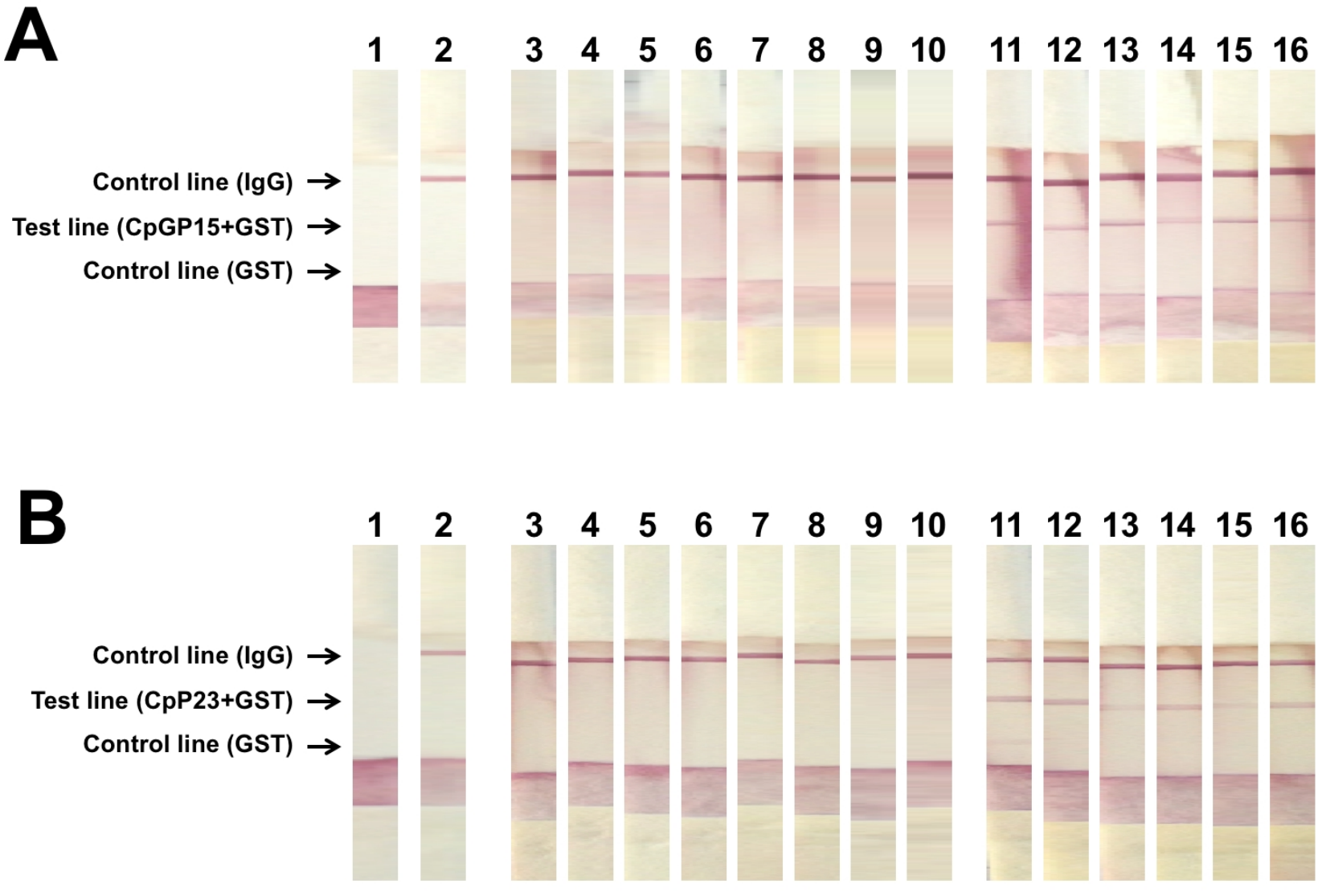


Fig. 2. Fereig et al.



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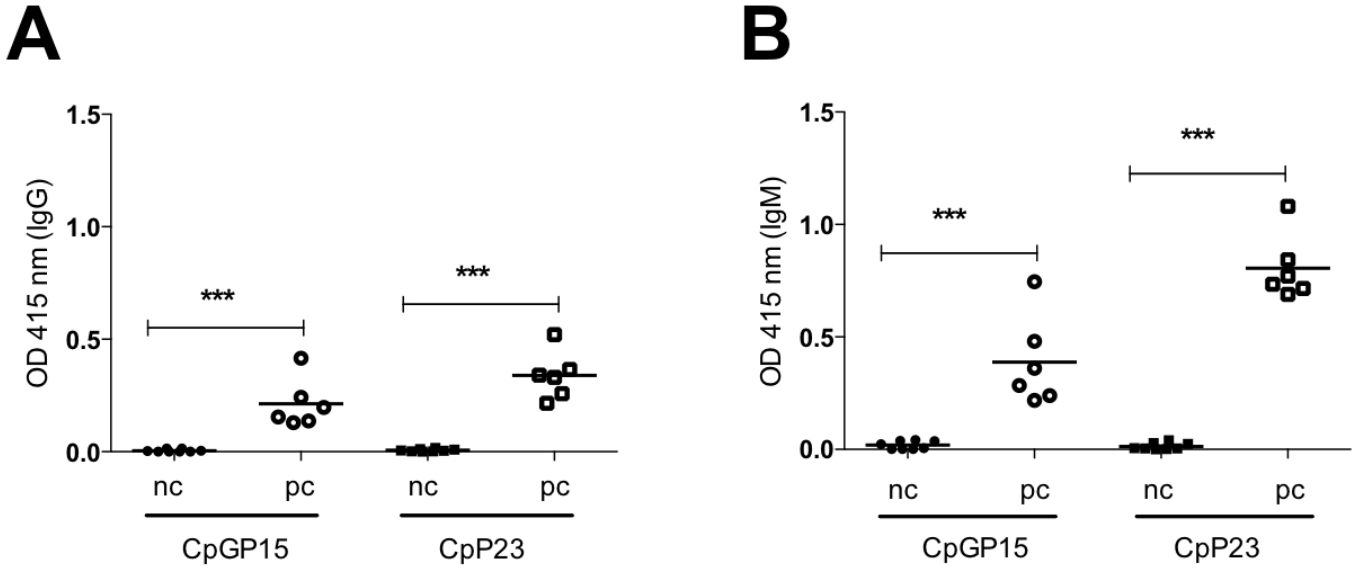

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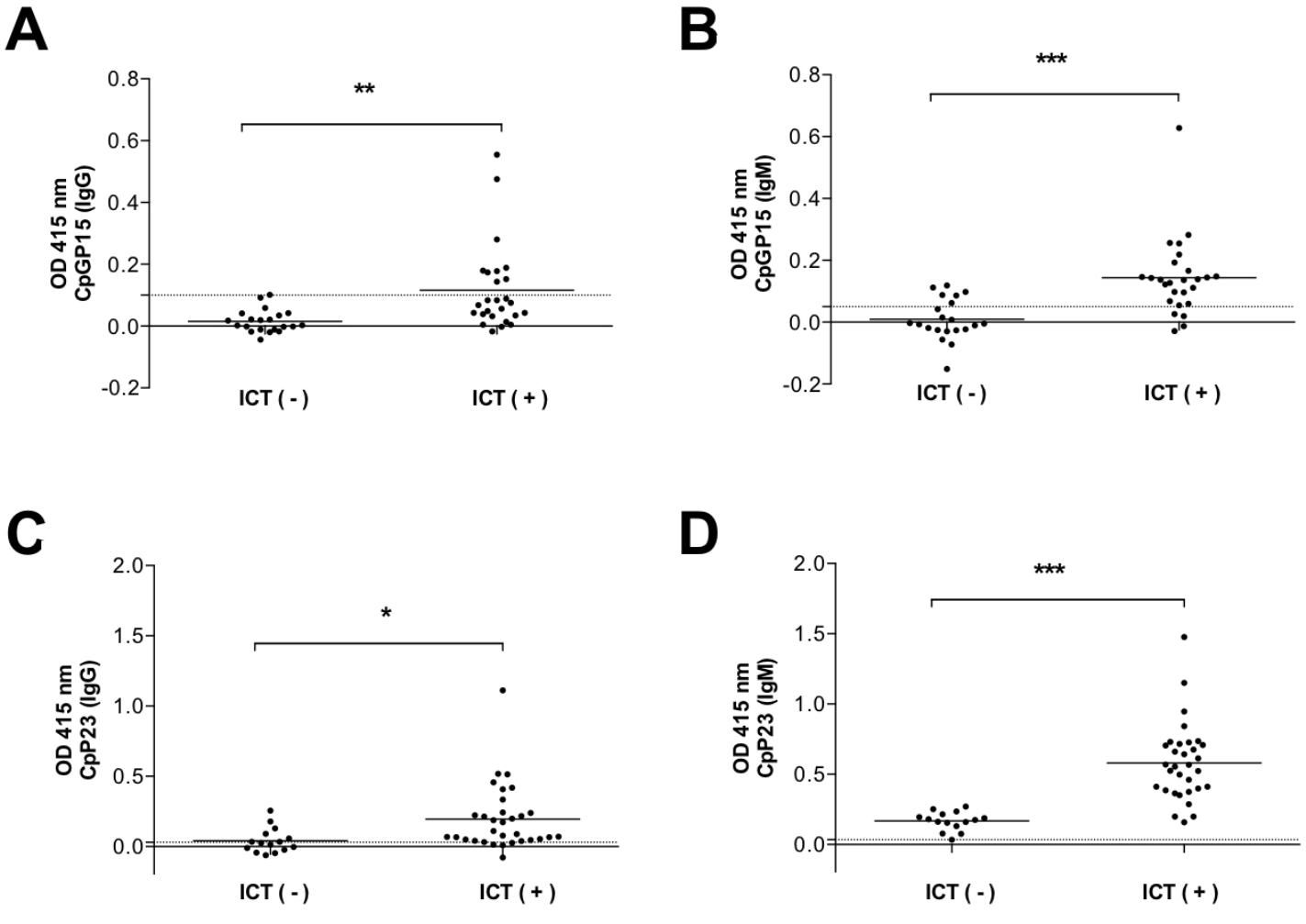

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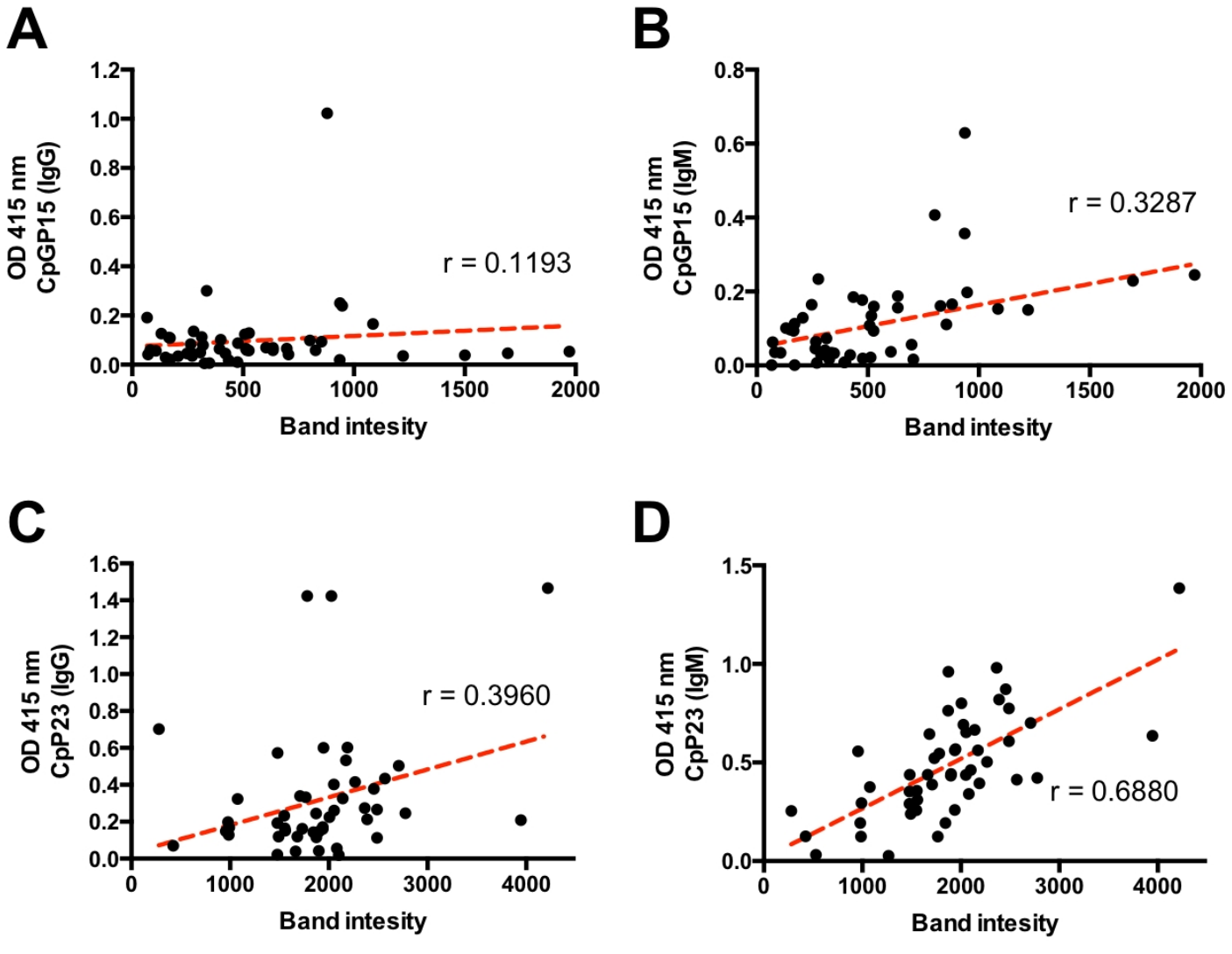

Table 1. Comparison of the reactivity of unknown field cattle sera against ELISA and ICT using the same antigen.

Item	CpGP15 (n=47)			CpP23 (n=47)		
	Positive (%)	Negative (%)	CI (95%)	Positive (%)	Negative (%)	CI (95%)
IgG-ELISA	10 (21.28)	37 (78.72)	11.2-36.07	35 (74.47)	12 (25.53)	59.36-85.58
IgM-ELISA	28 (59.57)	19 (40.43)	44.3-73.28	46 (97.87)	1 (2.13)	87.28-99.89
IgG or IgM ELISA*	33 (70.21)	14 (29.79)	54.92-82.21	46 (97.87)	1 (2.13)	87.28-99.89
IgG and IgM ELISA#	5 (10.64)	14 (29.79)	3.98-23.9	35 (74.47)	1 (2.13)	59.36-85.58
ICT	26 (55.32)	21 (44.68)	40.24-69.54	32 (68.01)	15 (31.91)	52.75-80.48

95% CI, confidence interval.

* Samples positive for one or both antibodies (IgG or IgM) ELISA.

Samples positive for both antibodies (IgG and IgM) ELISAs.

Table 2: Evaluation of ICTs against IgG and IgM ELISAs with relevant antigens.

Diagnostic parameter*	CpGP15-ICT				CpP23-ICT			
	IgG ELISA	IgM ELISA	IgG or IgM ELISA	IgG and IgM ELISA	IgG ELISA	IgM ELISA	IgG or IgM ELISA	IgG and IgM ELISA
Sensitivity	90	78.57	78.78	100	80	69.57	69.565	80
Specificity	52.77	78.95	100	100	66.66	100	100	100
Positive predictive value (PPV)	34.62	84.92	100	100	87.5	100	100	100
Negative predictive value (PPV)	95	71.43	66.66	100	53.33	66.66	66.66	12.5
Estimated prevalence (%)	21.74	59.57	70.21	26.32	74.47	97.87	97.87	97.22
Kappa value	0.271	0.566	0.689	1	0.431	0.089	0.089	0.182
Agreement proportion	60.87	78.72	85.11	100	76.6	70.21	70.21	80.56

*Parameters were calculated using an online statistical tool (www.vassarstats.net). The strength of agreement (kappa value) between each test and ELISA were graded as fair (0.21–0.40), moderate (0.41–0.60), and substantial (over 0.61).