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

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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.

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; Checkley 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,

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

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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 immunodot 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 ($n = 8$). Sera from cattle with cryptosporidiosis were used as *C. parvum*-positive sera ($n = 6$). *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.