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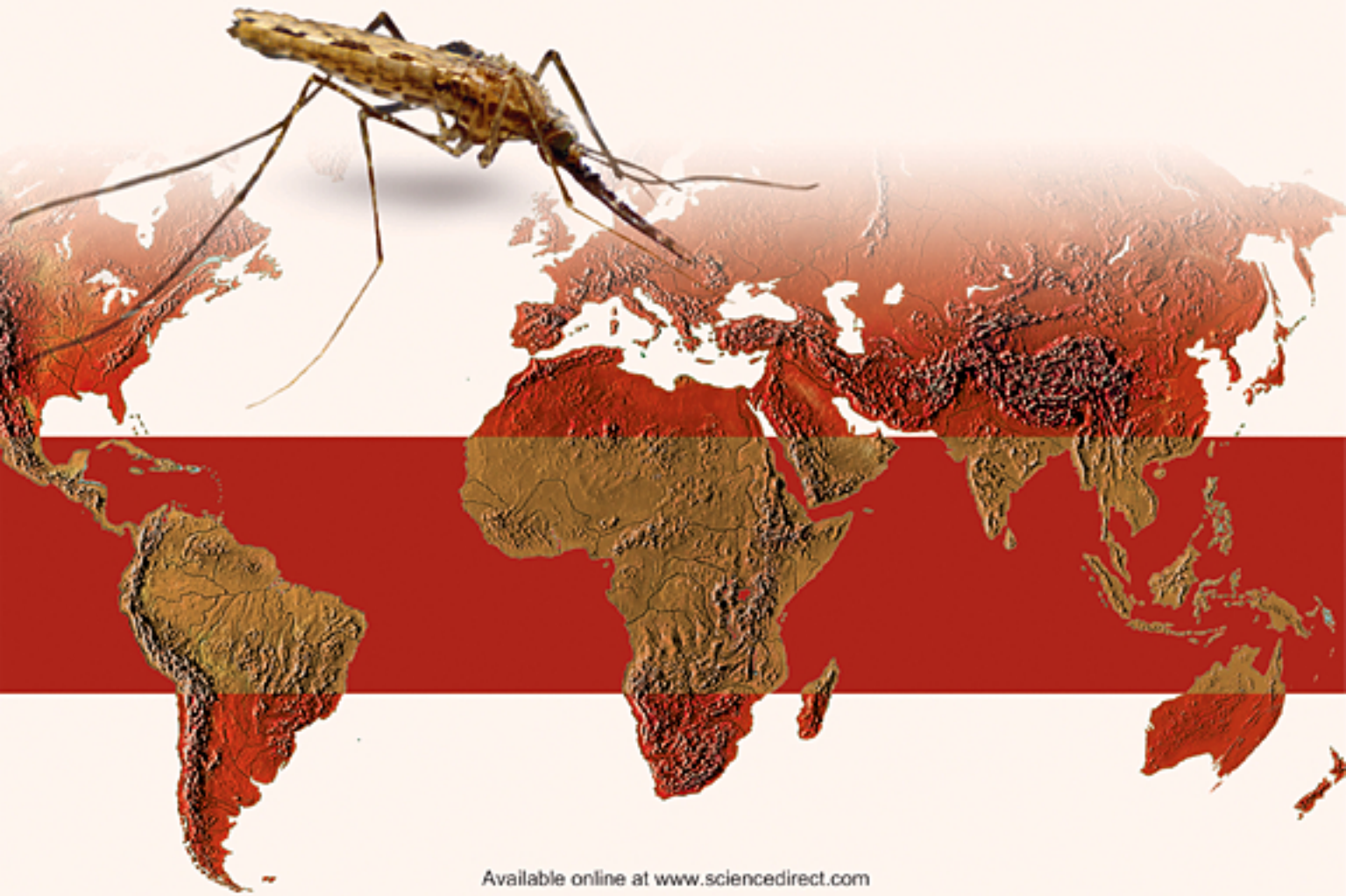

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Recombinant Expression And Indirect ELISA For COWP And HSP70 Proteins From *Cryptosporidium andersoni*

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ABSTRACT

Cryptosporidium spp. infect cattle at a high rates, and reduce milk production. Cryptosporidiosis has caused economic losses for the dairy industry. Studies in Western countries have shown that *Cryptosporidium* can also infect humans. Therefore, the development of methods for the early detection of *Cryptosporidium* is an important public health objective. Total RNA isolated from *C. andersoni* was used as template for generating cDNA encoding the COWP and HSP70 proteins. The recombinant plasmid, pET-32a(+)-COWP-HSP70, was constructed by double digestion and subcloning. The expression of the three recombinant proteins was induced in *Escherichia coli* BL21 using isopropyl-β-D-thiogalactopyranoside. The antigenicity of the recombinant proteins was examined using western blotting and indirect ELISA. The identities of the COWP fusion protein (CFP), HSP70 fusion protein (HFP), and COWP-HSP70 fusion protein (CHFP) were confirmed by BLAST searches of known sequences in GenBank respectively. The ELISA and western blot analyses indicated that all three of the proteins were highly immunogenic and antigenic. An indirect ELISA method was developed using the three recombinant proteins as coating antigens for the analysis of 40 clinical samples. The results showed that CHFP was the best candidate antigen for clinical testing, with a detection rate of 100%, compared with general parasitological screening. Above of all, the recombinant CHFP protein represents the best candidate antigen among three ones for detecting anti-*Cryptosporidium* antibodies in clinical samples. The development of the indirect ELISA lays the foundation for further research in immunodiagnosis and disease prevention of cryptosporidiosis.

1. Intrduction

Cryptosporidium species are protozoan parasites that infect a broad range of hosts including livestock and humans worldwide (Ryan et al., 2014; O'Donoghue, 1995). The major clinical sign of cryptosporidiosis is self-limiting diarrhea, which can lead to life-threatening chronic diarrhea in immunodeficient hosts (Fayer et al., 2008). *Cryptosporidium* spp. have been detected in humans with diarrhea in England (Leoni et al., 2006), and in pediatric patients in Malawi (Morse et al., 2007). Chronic infection with *C. andersoni* induces gastritis, reduced milk production, and poor weight gain in cattle (Matsubayashi et al., 2008).

Four species of *Cryptosporidium*, namely *C. parvum, C. andersoni, C. bovis*, and *C. ryanae*, commonly infect cattle, and cause bovine cryptosporidiosis (Zhao et al., 2013; Rzezutka and Kaupke, 2013). Among these, *C. andersoni* is the predominant species in infected cows in China which associated with gastritis, reduced milk yield and poor weight gain in adult cattle, as well as high morbidity rates (Wang et al., 2011). Meanwhile, *C. andersoni* oocysts detected in humans were reported (Checkley et al., 2015; Wang et al., 2017). The transmission of cryptosporidiosis is dependent upon the ingestion of oocysts excreted in the feces. Feces-contaminated surface water (Almeida et al., 2010; Mac Kenzie et al., 1994) is the main route of infection in humans (Nagano et al., 2007;). The threat to public health posed by cryptosporidiosis has become the focus of current research, and the lack of specific chemotherapeutics for the treating the disease increases the importance of early detection and the introduction of efficient control measures to

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minimize the spread of oocysts in the environment.

Numerous techniques have been used to detect the proteins and DNA of *Cryptosporidium* for the diagnosis of infections in humans and animals. A veterinary diagnosis of cryptosporidiosis relies primarily on the use of immunoassays or microscopic methods for the detection of oocysts in fecal samples. Traditional microscopic methods may not identify low-grade *Cryptosporidium* infections because of the low numbers oocysts in the feces. The identification of conserved gene sequences using polymerase chain reaction (PCR) is more sensitive than microscopic methods, and can be used to identify asymptomatic *Cryptosporidium* infections (Rzezutka and Kaupke, 2013).

Immunodetection methods represent an alternative to genetic and microscopic methods of detection. Serological assays for the detection of anti-*Cryptosporidium* antibodies can be useful prior to general parasitological screening and PCR-based testing. Current immunodetection methods rely primarily on *Cryptosporidium* antigens from ruptured oocysts, and variability in the range and abundance of these antigens occurs according to the oocyst purification technique used, which leads to variability in the testing results (Kjos et al., 2005). Kjobs *et al.* (Kjos et al., 2005) used recombinant rCP41 protein to develop an enzyme-linked immunosorbent (ELISA) assay that detected anti--*Cryptosporidium* antibodies in 192 human serum samples, which represented a notable improvement in the uniformity of serological methods available for the detection of cryptosporidia.

In our current study, we cloned the *COWP* and *hsp70* genes from *C. andersoni*, and expressed the recombinant COWP and HSP70 proteins using the prokaryotic expression plasmid, pET-32a(+). According to the western blotting and ELISA analyses, these two recombinant proteins exhibited good immunogenicity. To the best of our knowledge, there are no previous reports of the expression of these two recombinant proteins from *C. andersoni*. The aim of the current study was to use the recombinant COWP and HSP70 proteins to develop a highly sensitive indirect ELISA that could be used to identify *Cryptosporidium* infections, especially in animals with asymptomatic infections.

2. Materials and Methods

2.1. Sample collection and examination

Fecal samples were collected from the rectum of cattle at farms in Nanning City in Guangxi, China. The average age of the cattle was 11 months. The fecal samples were stored at 4°C until laboratory examination. *Cryptosporidium* oocysts were purified by the sugar flotation method reported by Matsubayashi *et al.* (Matsubayashi et al., 2008), with some modification. The purified *Cryptosporidium* oocysts were suspended in phosphate-buffered saline (PBS), and were microscopically examined at × 400 magnification. *Cryptosporidium* DNA was extracted from 0.1 g (100 μL) of animal feces using the methods previously described by Millar *et al.* (Millar et al., 2001) and Nichols and Smith (Nichols and Smith, 2004). The DNA sequence of the *Cryptosporidium* oocyst wall protein (*COWP*) gene was amplified by PCR to differentiate between different *Cryptosporidium* species. All of the isolates were identified as *C. andersoni* based on their COWP gene sequence (Matsubayashi et al., 2008) and the results of the microscopic examination.

Our study was performed in strict accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals* of the Ministry of Health, China. Our study protocol was approved by the Animal Care and Use Committee of the Guangxi Veterinary Research Institute. Permission was obtained from the farm owners before the fecal samples were collected.

2.2. RNA extraction and complementary DNA (cDNA) production

Total RNA was isolated from purified *C. andersoni* oocysts using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. The RNA were suspended in diethyl pyrocarbonate-treated water, and stored at -80°C. The cDNA of the target genes was generated by reverse transcription and polymerase chain reaction (RT-PCR) using the TransScript One-Step RT-PCR Kit (Transgen Biotech, Beijing, China), according to the manufacturer's instruction. For the amplification of the *COWP* gene sequence (GenBank accession no. DQ060431), a 549-bp PCR product was amplified using the forward primer, 5′-CCG*GAATTC*GTAGATAATGGAAGAGATTGTG-3′, and the reverse primer, 5′-CCC*AAGCTT*TGAAATACAGGCATTATATTG-3′, which contained *Eco*RI and *Hin*dIII restriction enzyme sites (italicized), respectively. Thermal cycling was performed using an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 40 s, 50°C for 40 s, and 72°C for 40 s, with a final elongation at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel. A 342-bp fragment of the *hsp70* gene (GenBank accession no. AB610481) was PCR amplified using the forward primer, 5′-CCC*AAGCTT*GACTTT-GATAATAGATTAGTTGA-3', and the reverse primer, 5′-CCG*CTCGA-G*ATCATGTACTGACCTCTTATCC-3′, which contained *Xho*I and *Hin*dIII restriction enzyme sites (italicized), respectively. The thermal cycling conditions used for the *hsp70* gene were identical to those used for the *COWP* gene, except that an annealing temperature of 49°C was used.

2.3. DNA sequencing and sequence analysis

The RT-PCR products were purified, and cloned into the TA cloning plasmid, pMD-18T (Takara Bio, Shiga, Japan), following the manufacturer's protocol. The DNA sequences of the recombinant clones were compared to known sequences in GenBank using the BLAST computational tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Recombinant protein expression and purification

Based on the DNA sequencing results, the amplicons were cloned into the bacterial expression vector pET-32a(+) (Invitrogen) to produce the pET-32a(+)-COWP and pET-32a(+)-HSP70 expression plasmids, respectively. The plasmids were selected based on ampicillin-resistance, and the sequence of the open reading frame of each plasmid was verified by DNA sequencing. Chemically competent BL21 *E. coli* (Invitrogen) were separately transformed with the two expression plasmids.

The expression conditions, including expression time, temperature, and concentration of isopropyl-β-D-thiogalactopyranoside (IPTG; Promega, Madison, WI, USA), were varied to optimize the expression of soluble COWP fusion protein (CFP) and HSP70 fusion protein (HFP). The bacterial lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% acrylamide gel, and the resolved bands were visualized by staining with Coomassie brilliant blue. The recombinant His_6-tagged proteins were purified from the soluble fraction of the lysates using a Ni-NTA chromatographic column (GenScript, Piscataway, NJ, USA), and the purified protein was eluted with 300 mM imidazole (Sigma-Aldrich, St. Louis, MO, USA). The purified recombinant proteins were analyzed using a UV spectrophotometer (Beckman, Fullerton, CA, USA), and the concentration was determined at 593 nm using a standard curve.

2.5. Production of immune sera

The anti-CFP and anti-HFP immune sera were prepared in mice, as described previously (Liu et al., 2014), with some modification. The recombinant proteins were diluted, and emulsified in an equal volume of complete or incomplete Freund's adjuvant (Sigma-Aldrich). Six-week-old BALB/c mice were immunized with 100 μg of protein in complete Freund's adjuvant, and the mice received a booster immunization consisting 50 μg of protein in incomplete Freund's adjuvant at 2 and 4 weeks following the initial immunization. Blood was collected 7 days after the second booster immunization. The mouse anti-CFP and anti-HFP polyclonal antibodies were purified from the blood of the mice, and stored at -80°C.

2.6. Immunogenicity analysis of the recombinant proteins

Each of the CFP and HFP proteins was subjected to SDS-PAGE in a 12% acrylamide gel, and transferred to a polyvinylidene fluoride (PVDF) membrane for 45 to 60 min at 20 V. After washing with Tris-buffered saline (TBS) for 10 min, the membranes were blocked with 5% nonfat dried milk for 1 h at 37°C. After blocking, the membranes were incubated with anti-His_6 monoclonal antibody, mouse anti-CFP polyclonal antibody, or mouse anti-HFP polyclonal antibody at 37°C for 1 h. After washing five times for 10 min with TBS containing 0.03% Tween-20, the membrane-bound primary antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and the reactive bands were visualized using the HRP substrate, diaminobenzidine (Sigma-Aldrich). Cultures of BL21 *E. coli* transformed with pET-32a(+) plasmid were used as negative controls.

Antibody levels in the mouse serum samples were determined using an indirect ELISA. The purified CFP and HFP proteins were diluted in carbonate buffer (pH 9.6), and 96-well microtiter plates were coated with 2 μg of purified protein per well. The plates were incubated at 37°C for 2 h. The plates were washed with PBS (pH 8.0) three times for 15 min, and blocked with 1% nonfat dried milk at 37°C for 1 h. Diluted mouse serum was added to each well, and the plates were incubated at 37°C for 1 h. After washing the plates three times for 15 min with PBS containing 0.03% Tween-20 (PBST), HRP-conjugated goat anti-mouse IgG was added to each well. The HRP substrate, tetramethylbenzidine (TMB), was added to detect the immune complexes, and the reaction was stopped by adding 2 M H_2SO_4. The absorbance of the contents of each well was measured at 450 nm. All of the samples were analyzed in triplicate. Naive mouse serum was used as a negative control.

2.7. Expression of CHFP fusion protein

The pMD-18T-HSP70 and pET-32a(+)-COWP plasmids were digested using *Xho*I and *Hin*dIII restriction enzymes. The DNA fragments were purified, and ligated at 16°C overnight to produce the pET-32a (+)-COWP-HSP70 expression plasmid. Chemically competent BL21 *E. coli* were transformed with the ligation product. Recombinant protein expression was performed under the optimized conditions described above. The COWP-HSP70 fusion protein (CHFP) was purified using a Ni-NTA chromatographic column. A mouse anti-CHFP polyclonal antibody was also generated using the methods described above. The CHFP protein was analyzed by western blotting and ELISA as described above.

2.8. Development of indirect ELISA for detecting Cryptosporidium

The immune serum was in mice immunized with *C. andersoni* oocysts. The purified oocysts were sonicated, and filtered through a 0.22-μm filter membrane. The sonicated proteins were analyzed using a UV spectrophotometer (Beckman), and the concentration was determined based on absorbance at 593 nm using a standard curve. BLAB/c were immunized with 100 μg of oocyst proteins in complete Freund's adjuvant. The mice received a booster immunization consisting of 50 μg of oocyst proteins in incomplete Freund's adjuvant at 2 and 4 weeks following the initial immunization. Seven days after the third immunization, the anti-oocyst protein polyclonal antibody was purified from the mouse blood, and stored at -80°C.

The conditions for the indirect ELISA were optimized (data not shown). Ninety-six-well microtiter plates were coated with 0.078 μg/mL CFP, 0.081 μg/mL HFP, or 0.053 μg/mL CHFP in carbonate buffer (pH 9.6), and incubated at 37°C for 2 h. The plates were washed with PBS (pH 8.0) three times for 15 min, and blocked with 1% nonfat dried milk at 37°C for 1 h. A 1: 800 dilution of the anti-oocyst protein mouse serum was added to each well, and the plates were incubated at 37°C for 1 h. The plates were washed three times with PBST for 15 min. Diluted HRP-conjugated goat anti-mouse IgG (1:5000 for CFP and HFP; 1:4000 for CHFP) was added to each well, and the plates were incubated at 37°C for 2 h for CFP, 1 h for HFP, or 0.5 h for CHFP. The immune complexes were detected by adding TMB to each well, and the plates were incubated for 30 min for CFP, 20 min for HFP, or 5 min for CHFP. The reaction was stopped by adding 2 M H_2SO_4, and the absorbance of the contents of each well was measured at 450 nm. All of the samples were analyzed in triplicate. An optical density (OD) > 0.177 for CFP, > 0.179 for HFP, and > 0.215 for CHFP was considered as a positive result.

2.9. Clinical sample detection

The specificity of the indirect ELISA was evaluated to ensure a minimal false positive rate. Serum samples from animals infected with *Coccidia* spp., *Trypanosoma evansi*, or *Toxoplasma gondii* were analyzed to determine specificity. Thirty serum samples were collected from cows with microscopically and PCR-confirmed *C. andersoni* infections, and the samples were numbered from 1 to 30. Ten serum samples were collected from cows at a farm where with no cryptosporidiosis cases, and numbered from 31 to 40. These 40 samples were analyzed in triplicate using the indirect ELISA above.

2.10. Statistical analysis

The statistical analysis was performed using the SPSS, version 16.0, software (IBM, Armonk, NY, USA). The data were analyzed using a one-way analysis of variance. The mean values for the results were compared using Duncan's multiple range test.

3. Results

3.1. Molecular cloning and sequence analysis

The primers used to amplify the target genes in *C. andersoni* were designed based on the *COWP* and *hsp70* genes from *Cryptosporidium* in GenBank. The *COWP* and *hsp70* cDNAs were 549 and 342 bp in size, respectively, and shared 100% identity with the sequences of *COWP* and *hsp70* from *Cryptosporidium* in GenBank.

3.2. Recombinant protein expression and purification

Under the optimized expression conditions, the bacteria transformed with pET-32a(+)-COWP and induced using 0.5 mM IPTG for 5 h at 30°C expressed a soluble recombinant protein approximately 40 kDa in size. The bacteria transformed with pET-32a(+)-HSP70 and induced using 1.2 mM IPTG for 7 h at 30°C expressed a soluble recombinant protein approximately 30 kDa in size. The COWP and HSP70 proteins were purified using Ni-NTA chromatography, and the purified proteins had concentrations of 0.78 and 0.65 mg/mL, respectively.

3.3. Immunogenicity of the recombinant proteins

Western blotting was used to analyze the immunogenicity of the recombinant proteins. One band was detected for each protein using the His_6-tagged monoclonal antibody (Fig. 1A and 1B) and the mouse anti-COWP and anti-HSP70 polyclonal antibodies (Fig. 1C and 1D). The proteins were not detected in the bacteria transformed with pET-32a(+). The evaluation of the antibody levels in the mouse serum samples generated against the two recombinant proteins indicated levels of immunogenicity that were acceptable for the development of the indirect ELISA (data not shown).

3.4. Expression of the CHFP fusion protein

The bacteria transformed with pET-32a(+)-COWP-HSP70 (Fig. 2A) and induced using 1.5 mM IPTG for 6 h at 30°C expressed soluble CHFP that was approximately 50 kDa in size (Fig. 2B). The CHFP protein was purified using Ni-NTA chromatography, and had a concentration of

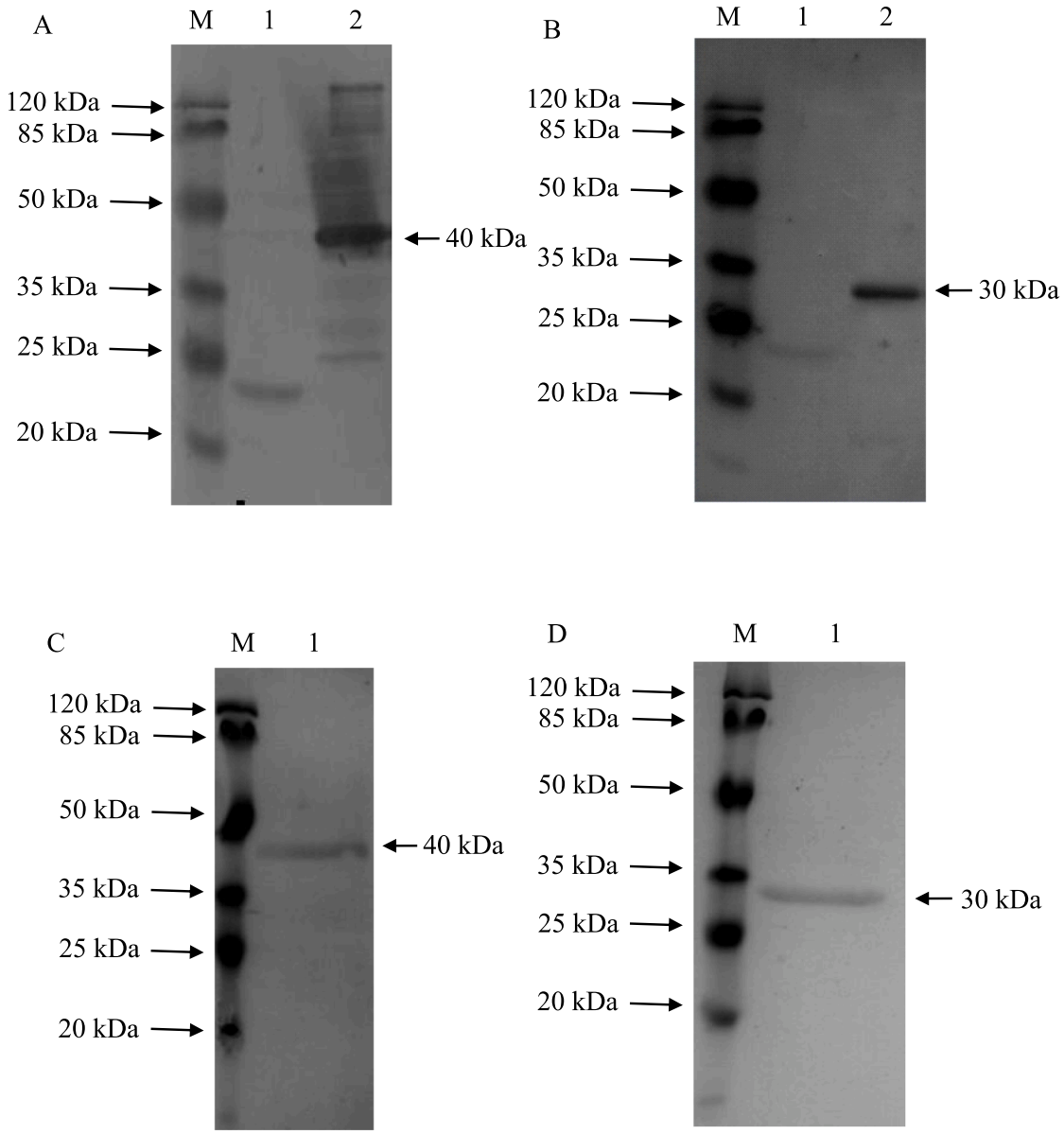

Fig. 1. Western blotting analysis of CFP and HFP. The expressed protein was separated by SDS-PAGE and transferred to PVDF membranes, at the same time the wild type vector transferred bacterial was used as control. In (A) and (B), A was CFP and B was HFP, anti-His_6 tag monoclonal antibody were used as first antibody. Protein marker (lane M), the His_6 tag control (lane 1) and the recombinant protein (lane 2). In (C) and (D) mouse anti-CFP and mouse anti-HFP polyclonal antibody were used as first antibody, respectively. Protein marker (lane M), the recombinant protein (lane 1).

1.055 mg/mL. The western blotting analysis showed that the His_6-tagged CHFP protein was expressed as a single polypeptide (Fig. 2C). The indirect ELISA was performed using the mouse anti-CHFP polyclonal antibody. The results indicated an acceptable level of immunogenicity (data not shown), and that CHFP can induce an immune response (Fig. 2D).

3.5. Clinical detection

The indirect ELISA was used to analyze serum collected from cows infected with *Coccidia* spp., *Trypanosoma evansi*, or *Toxoplasma gondii*, and the OD values were all below the critical value, indicating that no cross-reactivity occurred between the recombinant proteins and the antigens of the other species. In samples 1 to 30 (*Cryptosporidium*-positive), the detection rate was 93.3%, with only 2 samples producing an OD below the critical value using CFP as the antigen. Identical results were obtained using HFP as the antigen. However, when CHFP was used as the antigen, the detection rate was 100%. In samples 31 to 40 (*Cryptosporidium*-negative.), the detection rate was 100%. All of the OD values for all three of the recombinant proteins were less than the critical value, which was consistent with the results of general parasitological screening (data not shown). Our results collectively show that the three recombinant proteins can be used to detect the *Cryptosporidium* infections with a high level of specificity, and that the CHFP antigen was the best candidate for clinical detection.

4. Discussion

Cryptosporidiosis is a common cause of diarrhea in cattle, which has a significant clinical and economical impact (Zheng et al., 2011). Cryptosporidium in clinical detection and diagnosis is still based mainly on microscopic detection methods (Ryan et al., 2014). An effective method of serological identification is needed due to the shortcomings of conventional parasitological screening and PCR-based testing for *Cryptosporidium* spp. Recombinant antigens maybe used to develop standardized ELISAs for seroprevalence surveys (Kjos et al., 2005). The present standard for the *Cryptosporidium* antigen is a crude preparation,

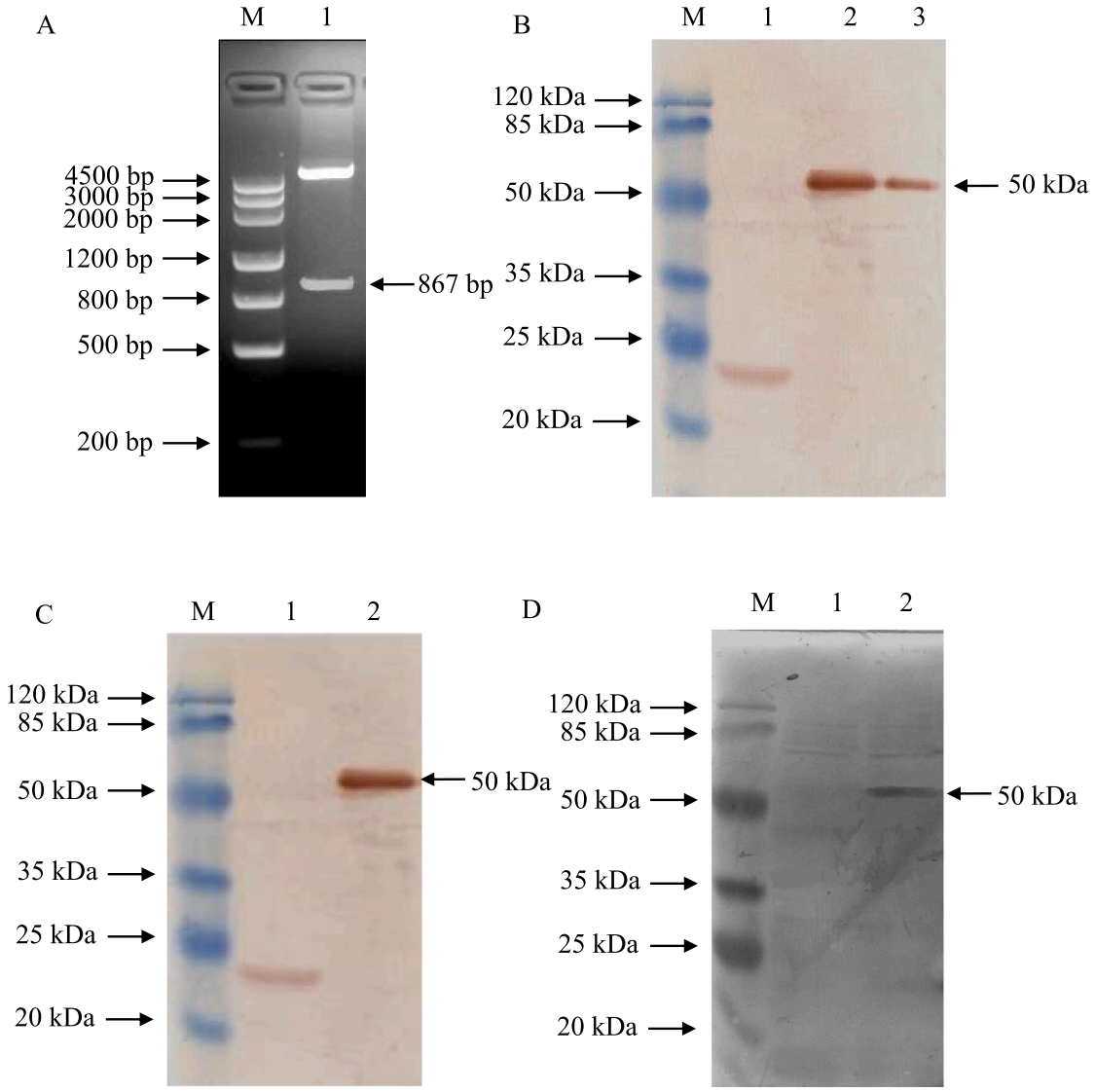

Fig. 2. Fusion expression of COWP and HSP70 proteins, the wild type vector transferred bacterial was used as control. (A) was dentification result of plasmid pET-32a(+)-COWP-HSP70 by double restriction enzyme digestion. DNA marker (lane M), digestion products (lane 1). (B) was the expression detection of CHFP, protein marker (lane M), the His_6 tag control (lane 1), the supernatant of induced product (lane 2) and precipitation of induced product (lane 3). (C) and (D) were immunogenicity analysis of CHFP. Anti-His_6 tag monoclonal antibody (C) and mouse anti-CHFP polyclonal antibody (D) were used as first antibody, individually. Protein marker (lane M), the His_6 tag control (lane 1) and the recombinant protein (lane 2).

consisting of a whole-parasite extract. However, bacterial contamination and other impurities in purified *Cryptosporidium* oocysts can reduced the sensitivity and specificity of immunodetection methods (Crowther, 1995). To overcome these defects, the use of immunodominant antigens, rather than disrupted oocysts, in the development of serological assays can improve sensitivity and specificity (Miller, 1990).

The oocyst wall of protozoan parasites consists mainly of proteins and lipids (Mai et al., 2009). The *Cryptosporidium* oocyst wall protein (COWP) is the main component of the oocyst wall, which protects against various potentially damaging environmental factors. Zheng *et al.* (Zheng et al., 2011) immunized BALB/c mice with a DNA vaccine consisting of the *COWP* gene in the pVAX1 plasmid. The results showed that the DNA vaccine induced an effective immunological response, compared with the control mice, which was characterized by both antibody and cellular immune responses. Heat shock proteins (HSP) are produced by cells to maintain homeostasis in response to certain stressful conditions (Burel et al., 1992), and are indispensable for maintaining normal cell function (Schlesinger, 1990) and directing the immune response (Gaston et al., 1989; Jacquier-Sarlin et al., 1994; Martinez et al., 2001). Liu *et al.* (Liu et al., 2007) cloned the 70-kDa heat shock protein (*hsp*70) gene of *C. andersoni*, and subcloned it into the pET-28a(+) plasmid for recombinant expression in BL21(DE3) *E. coli*. Their western blotting and ELISA analyses showed that the purified recombinant HSP70 protein exhibited high antigenicity, and represented a potential candidate antigen for the immunodiagnosis of cryptosporidiosis.

Because the *COWP* and *hsp70* genes are highly conserved among *Cryptosporidium* spp., we selected these genes for recombinant protein expression using the pET-32a(+) bacterial plasmid to evaluate their antigenicity for the detection anti-*Cryptosporidium* antibodies. The COWP, HSP70, and CHFP proteins were expressed in soluble form, with molecular weights of 40 kDa, 30 kDa, and 50 kDa, respectively. These recombinant proteins were purified, and used to immunize BALB/c mice. The serum antibody titers were greater than those of the controls, according to the ELISA results. Sera from *C. andersoni*-positive mice were used to analyze the antigenicity of the recombinant proteins using western blotting, and the result showed that the sera reacted with the CFP, HFP, and CHFP proteins, indicating that all three of these

recombinant proteins were highly antigenic.

The three recombinant proteins were used as antigen to develop an indirect ELISA for the detection of *Cryptosporidium* infections using serum from BALB/c mice immunized with disrupted oocysts, and the indirect ELISA detection method was optimized. We selected 40 clinical samples, 30 samples from cattle with *Cryptosporidium* infections and 10 samples from noninfected cattle, and analyzed them using the indirect ELISA. Using the CHFP protein as the antigen, the results showed that the ELISA method was highly effective for detecting anti-*Cryptosporidium* antibodies. All of the 30 samples from the *Cryptosporidium*-infected cattle had an OD value > 0.215, and all of the 10 samples from noninfected cattle had an OD value < 0.215. The test results were 100% comparison with parasitological screening. In serodiagnostic ELISA in previous studies(Fereig et al., 2016), the positive rate calculated from the OD415 nm values of the recombinant antigens in positive control sera or in negative control sera to discriminate the sensitivity and the specificity of the ELISA assay is common. Therefore, we concluded that the recombinant CHFP protein was the superior antigen for identifying *Cryptosporidium*-infected cattle, compared with the use of CFP or HFP alone, because using both of the antigens can cover the positive samples that were not detected and avoid false-negative results when only one of the antigens is used.

Although many prophylactic medications are available for cryptosporidiosis, the emergence of drug-resistant parasites in commercial production settings is increasing. Infections can be difficult to treat. The development of an effective vaccine against Cryptosporidium is still a challenge and has been hampered by incomplete understanding of the host immune response to Cryptosporidium (Ludington and Ward, 2015). Therefore, prevention and early detection are important public health measures and research on immunodominant protein like *COWP* and *hsp70* improved our understanding of both the detection of and vaccine study in cryptosporidium infections. Our results suggest that the use of CHFP protein in an indirect ELISA is highly effective for the detection of antibodies against *Cryptosporidium* and developed a novel candidate target for Cryptosporidium vaccine development, This study can provide a new direction for research of cryptosporidiosis. and will benefit efforts to control cryptosporidiosis outbreaks.

Declaration of Competing Interest

This manuscript has not been simultaneously submitted for publication in another journal and been approved by all co-authors. The authors declare that they do not have any conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105767.

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