Recombinant cold shock domain containing protein is a potential antigen to detect specific antibody during early and late infections of Haemonchus contortus in goat

Muhammad Ali-ul-Husnain Naqvi
Nanjing Agricultural University
KaLiBiXiaTi Aimulajiang
Nanjing Agricultural University
Muhammad Ali Memon
Nanjing Agricultural University
Muhammad Waqqas Hasan
Nanjing Agricultural University
Sana Zahra Naqvi
Nanjing Agricultural University
Shakeel Ahmed Lakho
Nanjing Agricultural University
Wen Chu
Nanjing Agricultural University
Lixin Xu
Nanjing Agricultural University
Xiaokai Song
Nanjing Agricultural University
XiangRui Li
Nanjing Agricultural University
RuoFeng Yan (yanruofeng@njau.edu.cn)
Nanjing Agricultural University https://orcid.org/0000-0002-6637-0886

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Abstract

Background: Haemonchus contortus (H. contortus) is the most abundant nematode causing haemonchosis with major economic losses to the small ruminant industry farming worldwide. Effective prognosis and treatment depend upon the early diagnosis of H. contortus infection. To date, no widely-approved methods are available to identify prepatent H. contortus infection. The aim of this study was to evaluate the diagnostic potential of recombinant cold shock H. contortus protein (rHc-CS) during early and late infections of H .contortus in goat.

Results: Purified rHc-CS exhibited a clear band, with a molecular weight about 38 kDa. Fecal egg count technique was unable to detect H. contortus eggs in feces collected between 0 and 14 days post infection. Eggs were detected at 21, 28 and 35 days post infection. However, Specific anti rHc-CS antibodies were detectable in sera of all infected goats during early stage (2nd week of infection) and late stage (3rd to 14th week of infection) using immunoblotting assay. Furthermore, no cross reactivity was observed against most commonly found helminths (Trichinella spiralis, Fasciola hepatica, and Toxoplasma gondii) and uninfected goats. The format variables for rHc-CS indirect-ELISA were optimized. The optimum antigen coating concentration was found 0.28μg/well at 37℃ 1h and overnight at 4°C. Optimum dilution ratio of serum and rabbit anti-goat IgG was recorded 1:100 and 1:4000 respectively. The best blocking buffer was 5% bovine serum albumin (BSA) and the best time for blocking, serum incubation and TMB reaction was recorded as 60, 120 and 10 minutes respectively. The cut-off value for positive and negative interpretation was determined as 0.352 (OD450). The diagnostic specificity and sensitivity of the rHc-CS, both were recorded 100%.

Conclusion: These results demonstrated that rHc-CS is a potential immunodiagnostic antigen to detect specific antibodies at early and late H. contortus infections in goat.

Background

H. contortus is an important haematophagous parasite found in abomasum that may remove about 0.05ml of blood per day [1, 2]. Heavy infestation of this helminth may result acute anemia, diarrhea, edema, weight loss, severe frailty and ultimately death [3, 4]. Haemonchosis causes significant economic losses to small ruminant farming industry, particularly in humid, tropical and subtropical regions of the world as a consequence of high mortality and morbidity [5, 6]. China contributes 17.3% of the total world goat population [7] with capricious prevalence rate of *H. contortus* between different provinces [8].

The accurate diagnosis of this parasitic infection is crucial to control. The clinical diagnosis of haemonchosis mainly relies on the use of conventional fecal egg counts technique and it is difficult to detect *H. contortus* eggs in feces before 3rd week of infection (21–25 days) [4]. Last larval stages of this parasite feed on blood [9] and loss up to one fifth of the total circulating erythrocyte volume from young animals [10]. *H. contortus* starts blood feeding on 11 days post infection (D. P.I) [11] and clinical signs usually become apparent when the infection becomes severerious [12]. Another way to diagnose the

infection of *H. contortus* is the degree of anemia using FAMACHA system in which an ocular conjunctiva color chart is used for assessment of anemia to decide which animal requires treatment for haemonchosisnematodal infestation [13]. However, these methods are often time-consuming, inaccurate, unreliable, laborious to perform [14] and lacking the efficacy to detect the infection at early stage. Moreover, these methods are also challenging for the intensive farming. Hence, early (2–3 week of infection) detection of this parasite is crucial to control haemonchosis effectively [15].

Several diagnostic assays such as immunoblotting and ELISA based on somatic antigens, crude protein and ES antigen have been described previously to detect *H. contortus* specific antibodies [4, 15–17]. Although, shared antigenic composition which leads to cross-reactivity in diagnosis of *H. contortus* infection is major disadvantage of these tools [18]. Currently, there is lack of potential immunogenic antigen based assay which can accurately detect the specific infectious stage of this parasitic helminth in goat. Excretory and secretory products (ESPs) are produced and released by the parasites during infection [19]. ESPs have important immunological role during early stage of infection and have widely used as diagnostic antigen because of good sensitivity and specificity [20]. ESPs contain numerous proteins which depress the immunity of host and modulate immune system from the early stages of infection [21]. In recent research, suppressive effects of *Heaemonchus* ESPs (HcESPs) were evaluated. Cold shock domain containing protein (CS), one of these HcESPs, that binds to goat peripheral blood mononuclear cells (PBMCs) at L4 and L5 development stages of *H. contortus* and it can also be specifically recognized from infected goat sera [22]. CS domains are constituent part of nearly all organisms (prokaryotes and eukaryotes) and present in every cellular compartment. In animals, CS proteins exhibit broad functions related to growth and development. These proteins have unique ability to bind with nucleic acid and they regulate not only their own expression but also involve in the regulation of virulent genes [23]. The presence of cold shock protein may serve for diagnostic purposes as biomarker [24]. Thus, these proteins can perfectly act as immunodiagnostic tool in early stage of infection [20]. To overcome the challenges in immunodiagnosis of *H. contortus* and to improve control strategies, a potential antigen based immunodiagnostic assay is needed.

This study was aimed to evaluate the diagnostic capacity of rHc-CS to detect antibodies during early and late *H. contortus* infections in goat using immunodiagnostic assays.

Results

Purification, and Immunoblotting and Early Diagnostic Potential

The rHc-Cs was purified as Histidine- tagged fusion protein and resolved on 12% SDS-PAGE and had a molecular weight (MW) of about 38 kDa (Fig. 1a). Immunoblotting analysis revealed that HcESPs could be recognized by antibodies generated against rHc-CS protein in SD rats. The molecular mass of the native CS protein was 20 kDa, which was the same molecular weight as that of the rHc-CS after reduction

from pET–32a protein (Fig. 1b, Lane 1). While, no protein was detected with normal rat sera (Fig.1b, Lane 2)

Initial antibodies were detected by immunoblotting in sera of all artificially infected goats (n = 5) of group 1, collected at prepatent stage (14 D. P.I). The sera of all *H. contortus*-infected goats had detectable antigenicity at 21, 35, 49, 63, 85, and 103 days of infection, while, no IgG antibody against rHc-CS was detected in sera collected before infection and 7th day of infection (Fig. 2). On the other hand, FECs showed that strongylid eggs were detected in all samples collected at 21 D. P.I (mean±SEM = 7.4±0.6), 28 D. P.I (mean±SEM = 9.8±0.78) and 35 D. P.I (mean±SEM = 10.8±1.08) and no eggs were detected in feces collected at 0, 7 and 14 D. P. I. Moreover, presence of *H. contortus* worms was confirmed at 30 D. P.I by necropsy in all tested goats. Furthermore, the rHc-CS antigen did not show any cross-reaction with goat anti-sera of commonly found helminths (*T. spiralis, F. hepatica* and *T. gondii)* spp., and no false positive results were observed (Fig. 3). These findings suggest that protein rHc-CS had good immunorectivity and antigenic characteristics which detected the specific antibody during early and late stages of *H. contortus* infection. Conversely, the microscopic examination couldn't detect *H. contortus* infection at early stage (7 and 14 D. P.I).

Optimization of rHc-CS Indirect-ELISA

The rHc-CS indirect-ELISA was optimized by evaluation of several format variables utilizing a small set of four samples that included two known positive and two known negative sera (group 3). Through checkerboard titration methods, the optimum coating concentration of rHc-CS was to be found 0.28µg/well and the optimum dilution ratio of serum was 1:100 with the highest P/N (OD_{450} = 5.085) value (Table 21). Subsequently, the best working concentrations of the rabbit anti-goat IgG was determined 1:4000 (the antibody titer was 5.706). Then, optimization of other format variables was carried out. The results showed that the best incubating condition was 37℃ 1h and overnight at 4°C with 5.117 P/N value (Fig. 4A). Among different percentages of blocking buffers (milk and BSA), 5% BSA showed best performance with P/N = 5.222 (Fig. 4B). The best dilution of rabbit anti-goat IgG was recorded 1:4000 that showed highest titration (5.706) results (Fig. 4C). The optimum incubation time of serum and blocking buffer was 60 and 120 minutes respectively (Fig. 4D; Fig. 4E), while best TMB reactive time was 10 minutes (Fig. 4F). Using this optimized rHc-CS indirect-ELISA format, P/N ratios were determined for all sera that were already categorized as either *H. contortus* positive or negative for optimization of format variables.

Determination of the cut-off value

The cut-off value in indirect-ELISA for negative sera was 0.352 as determined by mean value (0.244 ± 0.004) plus three multiplied by standard deviations (3 × 0.036) of the OD values obtained from 35 known negative goat sera. The sera isolated from positive group showed significantly higher OD_{450} (> 0.352) as

compared to negative group (OD < 0.352). While, significant differences (P < 0.05) in mean OD values between *H. contortus* infected goats' sera (0.645 ± 0.012) and uninfected sera (0.244 ± 0.004) were observed by t-test.

ROC curve analysis

The influence of different cut-off values on the sensitivity and specificity of this antigen was investigated by using the ROC curve analysis. This analysis resulted in a maximum value for the combined sensitivity and specificity at a cut-off value of 0.352 (100% sensitivity, 100% specificity). The least difference between sensitivity and specificity occurred at a cut-off value of 0.387 (96.8% sensitivity, 100% specificity). Furthermore, at a cut-off value of 0.316 100% sensitivity and 96.8% specificity were recorded. The area under the ROC curve (AUC) with this cut-off was 1 (P < 0.001), which is the perfect classification value indicating high accuracy of the cut-off for classifying serum samples into positive or negative. Thus, rHc-CS indirect-ELISA cut-off value of ≥ 0.352 was selected for further use to provide maximum sensitivity with very good specificity (Fig. 5).

Sero-diagnostic potential of rHc-CS indirect-ELISA

The rHc-CS has definite potential for serological diagnosis through the established indirect ELISA. The rHc-CS indirect-ELISA showed 100% sensitivity against well-characterized positive sera and 100% specificity against helminth free sera while no false positive/negative results were observed. For Analytical sensitivity, 33 known positive sera were analyzed. rHc-CS indirect-ELISA had a sensitivity of 100% as all (33/33) positive sera were above the cut-off line (0.352). The analytical specificity was evaluated for sera positive to *T. spiralis* (n = 4), *F. hepatica (*n = 4) and *T. gondii* (n = 4), which are other commonly co-infecting parasites with *H. contortus* in goats from endemic areas. All of these other parasites infected sera and known non-infected sera were below the cut-off line in the rHc-CS indirect-ELISA, demonstrating suitable analytical specificity against closely related co-infecting parasites. Scatter plot analysis showed significant differences (P < 0.05) between *H. contortus* infected sera and other parasites positive sera, while no significant difference (P > 0.05) was observed between *H. contortus* free negative sera and other parasites' positive sera (Fig. 65). In order to diagnose haemonchosis in goats, 100% sensitivity of this antigen was measured while; no negative control was higher than the cut-off point.

Discussion

H. contortus is the most predominant nematode causing haemonchosis with major economic losses to the small ruminant's industry worldwide. It may cause high mortality and morbidity rates in domestic animals [5, 6]. Early diagnosis of the infection is crucial for effective control of *H. contortus* infection because last two larval stages of this parasite feed on blood and loss up to one fifth of the total circulating erythrocyte volume from young animals and may cause fatal anemia [10]. The serological

methods become important for the diagnosis of parasite as they are more accurate in comparison to EPG test and they are able to diagnose infection in a short time [25].

Recently different antigens such as somatic antigens, larval antigen and crude antigen were evaluated for immunodiagnosis against *H. contortus* infection [17, 26, 27]. However the cross reactivity among different helminthes and low sensitivity are the limiting factors of these antigens. ESPs are released by parasites within the body of host and these products played vital role in pathogenesis and modulation of the immune response at the early stage of infection. *H. contortus* interaction with host starts after the transition of L_3 into L_4 stage [28]. Recombinant protein, having high immunoreactive concentration, may act as best immunodiagnostic antigen by achieving high sensitivity and specificity [29–36]. Recombinant Hc-CS is a major constituent of HcES proteins [22] which may have potential to diagnose *H. contortus* at early stage of infection. This study was designed to evaluate the specific antibodies detection during early and late infections of *H. contortus* using immunoblotting and indirect-ELISA based on rHc-CS in experimentally infected goat.

Immunoblotting is a useful technique to detect specific pathogen and for antigen potency immunodiagnostic studies [37]. A confirmatory test for immunoblotting-positive sera was required to overcome the possible false positive results that may affect the certain diagnosis of infection [20]. In this study immunoblotting results indicated that rHc-CS showed high immunoreactivity against polyclonal antibodies generated in sera of SD rats. The ELISA is a powerful tool as it provides a less time consuming, easy and safe way to perform serological detection. The association between ELISA and immunoblotting techniques can be seen as a potent way to increase sensitivity for immunodiagnosis purposes [38]. Previously one study was performed to detect *H. contortus*-specific antibody in prepatency, early and late patency of *H. contortus* infections using Hc26-based ELISA and immunoblotting but in sheep [37]. Another study was performed using recombinant protein reclgE1–2 to detect anti-*H. contortus* antibody in infected sheep's serum collected at 2–4 weeks post infection but immunoblotting assay showed cross-reactivity against sheep sera [39]. In contrast, the immunoblotting results of this study revealed that rHc-CS specifically reacted with antibodies present in all *H. contortus* infected-goats' sera collected from not only in early stage (14 D. P.I) but also collected sera up to 103 D. P. I. Hence, antibody detection has been proved to be more sensitive than traditional parasitological microscopic techniques during early stage of infection. It is needed in areas characterized by a low level of transmission, low prevalence and particular low intensity [40]. Furthermore, rHc-CS based immunoblotting did not show any cross-reaction with the sera of goats infected with *T. spiralis, F. hepatica* and *T. gondii.* Antigenicity, sensitivity and specificity may be influenced by the secretion time of antigen. It has been reported that rHc-CS releases at 14 and 60 D. P.I [22] and antibodies against this molecule are produced.

In this study, the diagnostic sensitivity and specificity of the rHc-CS indirect-ELISA were much higher than the performance reported in a previous study [17] in which a somatic antigen design in a conventional indirect-ELISA format was performed. This contrast could be related to higher binding efficiency of rHc-CS antigen, as type of antigens may influence sensitivity, specificity and cross reactivity of assay [41]. In a research, crude Excretory-Secretory (ES) protein of *H. contortus* was used in Dot-ELISA in which solid dot

formation took place with 4th day and 1–3 weeks post infection sera but cross reactivity of this antigen with other nematode and trematode was not checked [15]. In previous study, indirect ELISA using somatic and adult larval antigen results in relatively lower diagnostic specificity because of cross-reactivity with closely-related *H. contortus* species and possibly other genera [9]. In present study, both sensitivity and specificity of the rHc-CS indirect-ELISA were measured as 100%, that is similar to the findings of previous study in which His-ES24-based ELISA was performed to detect specific antibodies in *H. contortus* infected serum samples of sheep [20].

Various types of false negative and false positive reactions may influence, irrespective of antigens in indirect-ELISA [42]. To determine the background noise reactions, optimization of best antigen and antibodies dilution are crucial to improve the diagnostic potential of assay. After standardization of rHc-CS based indirect-ELISA, checkerboard titration results showed best performance with optimal antigen coating concentration (0.28µg/well), optimum serum 1:100 dilution ratio and the best working concentrations of the rabbit anti-goat IgG (1:4000). Another factor which may influence in the assay and encourage the false positive/negative reaction is blocking buffer as well as its incubation time [43]. To minimize the false positive/negative reaction, different blocking buffer's dilutions were optimized with different incubating times. Incubation time of buffer has significant effects on the performance of the assay. The optimized results showed that the best blocking buffer was 5% bovine serum albumin (BSA) which is consistent with the findings of previous study. [44]. The optimum reactive time of blocking and TMB was recorded as 60 and 10 minutes respectively. The best P/N value (6.44) was recorded when serum was incubated for 120 minutes. To reduce time consumption, serum can be incubated for 60 minutes because P/N value was observed positive (P/N>2.1). This standardized rHc-CS indirect-ELISA will be more authenticated tool for identifying goats in the prepatent stage of *H. contortus* infection than other antigen-based assays. The rHc-CS antigen based indirect-ELISA is a pivotal tool for identifying *H. contortus* -infected goats in endemic areas and preventing their movement to non-endemic and free areas. This assay has high sensitivity and specificity for downstream application in field studies.

The results of this study will facilitate the low cost early serological diagnosis of large number of animals and possibly vaccine development against the infection of *H. contortus.* However, further study is needed to evaluate the cross reactivity of rHc-CS against other gastrointestinal nematodes belonging to Trichostrongylidae family. Additionally, epidemiological surveillance studies with large number of samples are required to fine tune this assay.

Conclusion

To the best of our knowledge, it is the first report on the use of rHc-CS in indirect-ELISA format for prepatent immunodiagnosis of *H. contortus* infection in goats. Combine use of immunoblotting and indirect ELISA showed that rHc-CS is a specific, sensitive, and potential immunodiagnostic antigen which can consistently detect *H. contortus* antibodies in goats during early as well as late infection of *H. contortus.*

Methods

Ethical statement

All experimental protocols were approved by the Science and Technology Agency of Jiangsu Province (Approval ID: SYXK (SU) 2010-0005).

Animals

Local crossbred female goats (n=35), aged approximately 6 months, were bought from a farm in Xuyu city of Jiangsu province and kept in animal house of Nanjing Agricultural University (NAU) under controlled conditions. All goats were orally administered Levamisole (8 mg/kg body weight) twice at two week intervals to remove natural parasitic infections. Microscopic analysis of fecal samples was performed twice per week for helminth eggs. Helminthes free goats were used in further experiments. After 25 days of first deworming, goats were divided randomly into group 1 (n=5), group 2 (n=28) and group 3 (n=2).

Female Sprague Dawley (SD) rats of 150 g body weight (n=6) were bought from the Experimental Animal Center of Jiangsu, PR China (Certified: SCXK 2008-0004). Rats were randomly divided into two groups, group 1 (n=3) and group 2 (n=3) to collect polyclonal antibodies. Rats were kept in sterilized room and supplied with food and water.

H.contortus infective larva (L_3)

The *H. contortus* strain was maintained by serial passages in helminth-free goats, at MOE laboratory NAU. To collect *H. contortus* infective L_3, two local male goats (02 years old) were raised under nematode free condition and dewormed twice at 15 days interval by anthelmintic drug. Both goats were orally infected with 10,000 L_3 of *H. contortus* and to confirm the infection, fecal samples were collected and checked twice in week for the presence of *H. contortus* eggs. After confirmation of infection, infected larvae of *H. contortus* (L_3) were obtained through conventional method [22]. Briefly, feces from *H. contortus* infected-goat were collected, crushed, mixed with water and combined with vermiculite to keep mixture moist. The pan was covered with aluminum foil having several holes to allow air flow and put at room temperature for ten days. To recover the larvae, mixture was filtered through cheesecloth and larvae were examined microscopically. Then preserved at 4°C in penicillin G added water until use.

Experiment 1

First experiment was performed to evaluate the early diagnostic potential of rHc-CS protein during different stages of *H. contortus* infection using immunoblotting assay. For this purpose, group 1 (n=5)

was artificially infected with 8000 infected larva of *H. contortus* (L_3) orally and group 3 was kept un-infected as control. Serum samples were collected from group 1 (infected) and group 3 (uninfected) at specified days; one day prior challenging infection and 7 to 103 days post challenging infection with week interval for antibody detection.

Moreover, McMaster egg count technique was performed to examine the fecal samples collected at 7, 14, 21, 28 and 35 D.P.I by dispersing 2g of feces in saturated NaCl (58 mL) providing a diagnostic sensitivity of 50 epg (eggs per gram). Eggs of *H. contortus* were characterized based on body dimensions; shape and dark brown blastomeres [45].

Experiment 2

Experiment 2 was performed to develop and optimize indirect ELISA based on rHc-CS. Group 2 (n=28) was infected with 8000 *H. contortus* L_3 orally and serum samples were collected after the confirmation of *H. contortus* infection by fecal examination. Moreover, at 30 D.P.I the goats of group 2 were euthanized by injecting sodium pentobarbital (>150 mg/kg) intravenously [46], immediately opened the abomasum and the luminal content was removed. After washing the abomasum, presence of adult worms embedded in the mucosa was confirmed.

Purification of recombinant protein of *H. contortus*

The recombinant plasmid of CS (pET-32a+rHc-CS) was provided by MOE joint international Research Laboratory, Preventive Veterinary Medicine, NAU (GenBank: CDJ84294.1) and purified by standard protocol described previously [47]. Briefly, transformation of recombinant plasmid into *E.coli* BL21 (DE3) was performed and cultured in ampicillin (100μg/ml) containing LB medium (Luria Bertini). After that, induction of protein expression was done at 37°C using 1mM IPTG (Isopropyl β-D-thiogalactopyranoside; Sigma-Aldrich) to make 0.6 (OD_{600}). Culture was centrifuged at 4500rpm for 15 min and supernatant was discarded. Pellet was lysed by using lysozyme (10μg/ml Sigma-Aldrich) followed by sonication. 12% (w/v) SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was used to examine the sonication products. Manufacturer's instructions were practiced to purify recombinant protein using Ni2+ nitrilotriacetic acid column (GE Healthcare, USA). The rHc-CS protein purity and quantification were performed by 12% SDS-PAGE with Coomassie blue stain and Bradford method respectively [48]. Protein toxins were removed utilizing Toxin Eraser^{TM} Endotoxin Removal kit (GeneScript, USA).

Immunoblotting assay

Immunoblotting plays preliminary role in selection of target protein to diagnose the infection of *H. contortus* and to evaluate the immunogenicity and immunoreactivity of antigen [49]. Complete Freund's adjuvant mixed equally with rHc-CS protein (300μg) was injected subcutaneously in SD rats of

experimental group to obtain polyclonal antibodies. After 2 weeks, three more doses of this protein mixed with Freund's incomplete adjuvant were injected at 1-week interval. After one week of the last dose, SD rats were anesthetized with 25% isoflurane (inhaling anesthesia) by open drop method [46] and blood samples were collected from eye. Finally, SD rats were euthanized by head dislocation. The serum samples from experimental group and control group were prepared and used in immunoblotting to evaluate the immunoreactivity of *rHc-CS*. The sera from untreated rats were also used as negative control.

Immunoblotting analysis was also performed to evaluate the antigenic characteristics of recombinant *H. contortus* CS (rHc-CS) at early stage as well as late stage of *H. contortus* infection in sera of experimentally infected goats (Group 1). The purified rHc-CS was first separated on 12% SDS-PAGE and transferred onto PVDF (polyvinyl difluoride membrane, Millipore, Billerica, USA) with the help of semi dry system (Novablot Hoefer, USA) and transfer solution (Tris 48 mM, glycine 39 mM, SDS 0.0375%, methanol 20%). The membrane was blocked with 5% skimmed milk diluted in TBS-T (Tris-Buffered Saline containing 0.05% Tween 20) for 2h at 37℃. The PVDF membrane was cut into five strips, washed (3 times) and incubated with anti-*H. cortortus* 1:100 diluted goat serum (primary antibody) for 2h at 37℃. Following 3 washes with TBS-T the strips were incubated with 1:4,000 diluted Horseradish Peroxidase (HRP) conjugated rabbit anti-goat IgG (Sigma, Hilden, Germany). Subsequently, the strips were washed five times and immunoreactions were observed by virtue of substrate (Tanon™ High-sig ECL Western Blotting). Upon each blood sampling day, the whole immunoblotting practice was repeated whereas the primary antibody differed. All anti -*H. contortus* sera separated from infected goats (Group 1) and non-infected goats (Group 3), on those sampling days were taken as the primary antibody. Immunoblotting also helped to check the specificity of rHc-CS against infected sera of *Trichinella spiralis* (*T. spiralis*), *Fasciola hepatica* (*F. hepatica*) and *Toxoplasma gondii* (*T. gondii*) as primary antibody.

Optimization of indirect-ELISA

Indirect-ELISA was performed to assess the immunodiagnostic potential of rHc-CS [50]. The rHc-CS indirect-ELISA was optimized by evaluation of numerous format variables including working dilution of rHc-CS antigen (4.5 to 0.07 µg/well), serum samples (1:25 to 1:200 dilutions), secondary antibody HRP conjugated rabbit anti-goat IgG (1:2000 to 1:16000) and working time of antigen coating (37℃ 2h-4℃ overnight, 37℃ 1h- 4℃ overnight), blocking (30-120 min), serum incubation (30-120 min) and tetramethylbenzidine (TMB) reaction (5-15 min). Furthermore, best blocking buffer (2, 3, 4, 5% BSA and 2, 3, 4, 5% Milk) was also determined using same method. The optical density (OD) values derived from the checkerboard were calculated and showed as the positive to negative (P/N) ratio [51]. The highest P/N (OD_{450} of infected serum / OD_{450} of non-infected serum) value (>2.1) was used to optimize the experimental conditions.

Establishment of Indirect ELISA

In order to evaluate the diagnostic potential and to ratify the immunoblotting results of the rHc-CS, an indirect-ELISA with optimal conditions was performed as discussed previously [52]. 96-well flat bottom without lid high binding plates (Costar, Bodenheim, USA) were used to perform the experiment in triplicate. The wells were coated with 100µl of diluted rHc-CS in 0.05M carbonate buffer solution (CBS; pH 9.6) followed by overnight incubation at 4°C. All wells were blocked with 100µl of 5% BSA (bovine serum albumin) for 2h at 37℃. 100µl of positive and negative goat sera, diluted in blocking buffer, were added to known well after washing with TBS-T and incubated at 37 °C. After 2h incubation, wells were washed again (3 times) and incubated with 100µl of diluted secondary antibody HRP conjugated rabbit anti-goat IgG at 37℃ for 1 h. Then the last three washings were performed to add 100µl TMB in each well and incubated for 10 min in the dark at room temperature. The color reaction was stopped by adding 100µl/well of 2M H_2SO_4. The OD of wells was read at a wavelength of 450 nm using micro plate reader (Thermo Fischer Scientific, Waltham, MA, USA). Moreover, to investigate the further feasibility of indirect ELISA, serum samples (n=33) from goats infected with *H. contortus* were used to calculate diagnostic sensitivity and serum samples of all goats before artificial infection (n=35) were evaluated to calculate diagnostic specificity. The sensitivity and specificity were defined using following formula described previously [53].

$$Sensitivity = \frac{True\ Positive}{True\ Positive + False\ Negative}$$

$$Specificity = \frac{True\ Negative}{True\ Negative + False\ Positive}$$

Additionally, a total of 12 anti- *T. spiralis*, -*F. hepatica* and -*T. gondii* goat serum samples (4 samples for each parasite) provided by Prof. Yan, were used to confirm the cross-reactivity against commonly found helminths. All the experiments were performed in duplicate.

Determination of cut-off value

Sera (n=35), collected from all goats before the induction of *H. contortus* infection were used to determine the cut-off value. The cut-off value was calculated by taking “mean absorbance values of known negative sera + (3× standard deviation)” [54]. The experiment was repeated twice and for the interpretation, any goat sera that had an OD_{450} value greater than the cut off value was considered as seropositive while OD_{450} value lower than cut-off value was considered as sero-negative [34].

Statistical analysis

The Receiver operating characteristic (ROC) analysis was used to simulate the influence of different cut-off values on the sensitivity and specificity of the test [38]. ROC curves were obtained using statistical

software MedCalc (version 15; http://www.medcalc.be). Statistical analysis of the data was evaluated by using the Graph Pad Prism™ v6 07 software.

Abbreviations

rHc-CS: Recombinant Cold Shock domain containing protein; D. P.I: Days post infection; SD: Sprague Dawley; ROC: Receiver-operating characteristics; HcESPs: Excretory and secretory products of *Haemonchus contortus;* PBMCs: Peripheral blood mononuclear cells; NAU: Nanjing Agricultural University; AEC: Animal Ethics Committee; IPTG: Isopropyl-b-D-thiogalactopyranoside; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF: Polyvinyl difluoride membrane; HRP: Horseradish peroxidase; TMB: Tetramethylbenzidine; OD: Optical density; P/N: Positive to negative; BSA: Bovine serum albumin; TBS-T: Tris-Buffered Saline containing 0.05% Tween 20; MW: Molecular weight; kDa: Kilodalton.

Declarations

Ethics approval and consent to participate

The study was conducted following the guidelines of the Animal Ethics Committee, Nanjing Agricultural University (NAU), China. All experimental protocols were approved by the Science and Technology Agency of Jiangsu Province (Approval ID: SYXK (SU) 2010–0005).

Consent to publish

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Development of Jiangsu Higher Education Institutions (PAPD). The funding bodies played no role in the design of the study, the collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions

YRF directed the project and participated in the coordination and management of the study. MAHN performed the laboratory tests and the data analysis and wrote the manuscript. KA and SZN provided some ideas for the experimental design. MAM and CW helped in blood sampling. MWH and SAL helped in parasite collection. LXR, SXK and XLX provided new analytical reagents and tools. All authors read and approved the final manuscript.

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Authors' Information

Not applicable

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Tables

Table 1 Determination of the optimal rHc-CS coating concentration and serum dilution for indirect-ELISA

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Serum Dilution		OD_{450} values of rHc-CS at increasing coating concentrations						
		0.07	0.14	0.28	0.56	1.12	2.25	4.5
1:25	(P)	0.46	0.79	1.21	1.20	1.36	1.56	1.85
	(N)	0.19	0.30	0.35	0.53	0.64	0.94	1.61
	P/N	2.45	2.67	3.41	2.27	2.13	1.66	1.15
1:50	(P)	0.40	0.68	1.19	1.18	1.21	1.49	1.63
	(N)	0.18	0.21	0.29	0.34	0.55	0.77	1.41
	P/N	2.20	3.21	4.05	3.45	2.21	1.94	1.15
1:100	(P)	0.35	0.51	1.01	1.20	1.28	1.38	1.40
	(N)	0.17	0.17	0.20	0.26	0.44	0.68	1.29
	P/N	2.07	2.88	**5.08**	4.57	2.91	2.01	1.08
1:200	(P)	0.23	0.41	0.78	0.86	0.92	0.97	1.22
	(N)	0.17	0.13	0.16	0.22	0.30	0.50	1.00
	P/N	1.36	3.08	4.69	3.82	3.02	1.92	1.22

Abbreviations: P: positive serum; N: negative serum.
Note: Bold represent the optimum conditions for this indirect-ELISA method, the highest P/N value is 5.085.

Figures

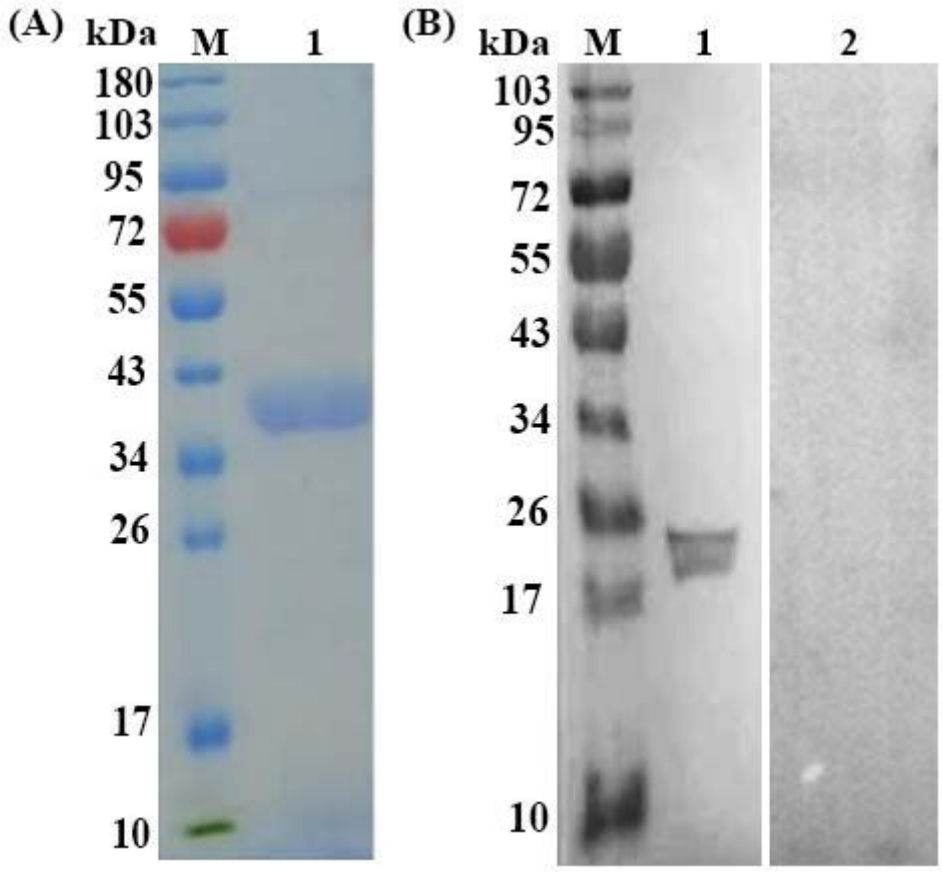

Figure 1

Purification and immunoblotting of rHc-CS protein. Lane M: standard protein pre stain molecular weight Marker. A Lane 1: Purified rHc-CS protein. B Lane 1: HcESPs were detected by rat anti-rHC-CS protein antibodies; Lane 2: membrane incubated with normal rat sera (as control).

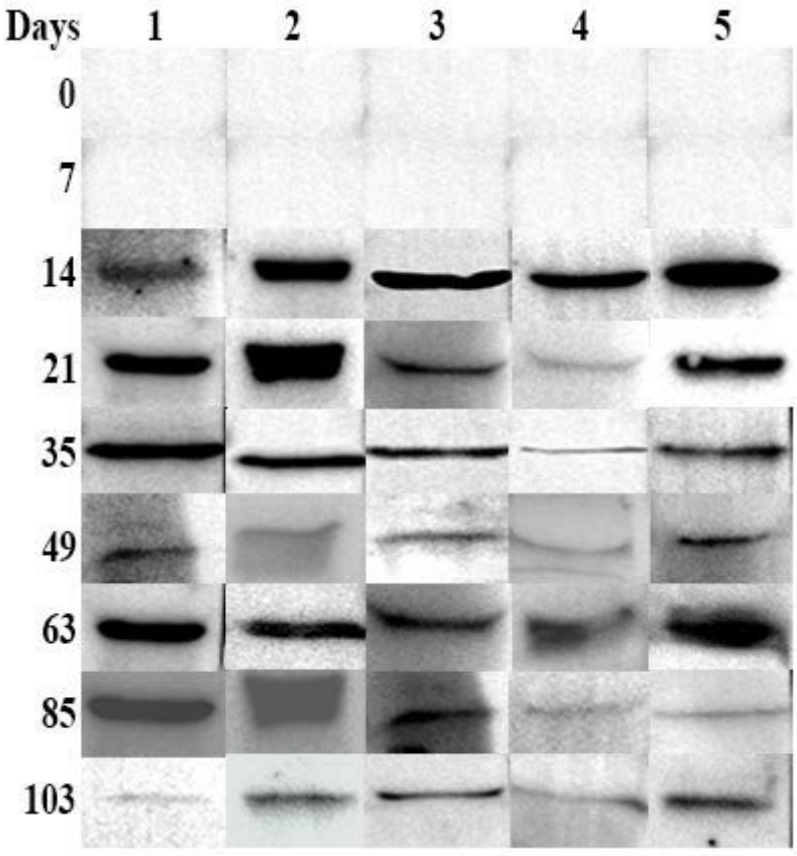

Figure 2

Immunoblotting of rHc-CS for specific antibody detection during different stages of H. contortus infections. Vertical axis: Lanes 1-5: Different Goat Labels; Horizontal axis: Different days of serum collection (0, 7, 14, 21, 35, 49, 63, 85, and 103). Antibodies against rHc-CS were detectable at early stage (14 D.P.I) and late stages (21 -103 D.P.I). No antibody detection was observed at 7 D.P.I and before challenging infection day.

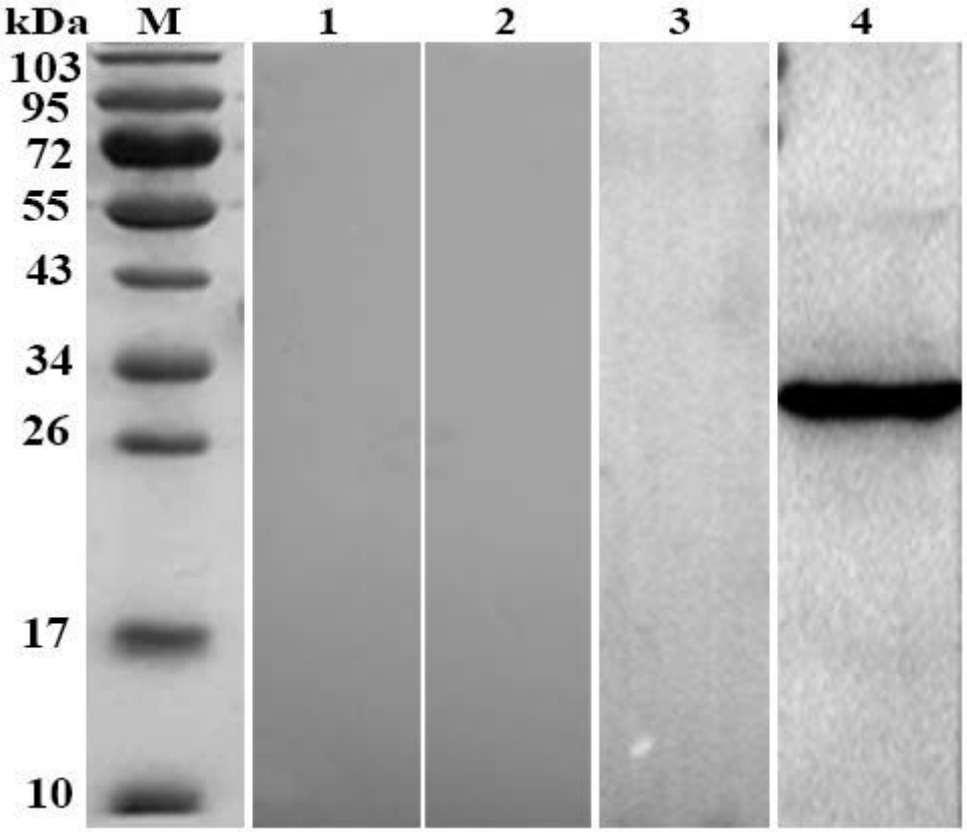

Figure 3

Immunoblotting of rHc-CS antigen to determine crossreactivity. Lanes shown are as follows: M = standard protein pre stain molecular weight Marker; Lane 1: membrane incubated with positive T. spiralis serum; Lane 2: membrane incubated with positive F. hepatica serum; Lane 3: membrane incubated with positive T. gondii serum; Lane 4: rHc-CS was recognized by goat anti-H. contortus sera (positive control).

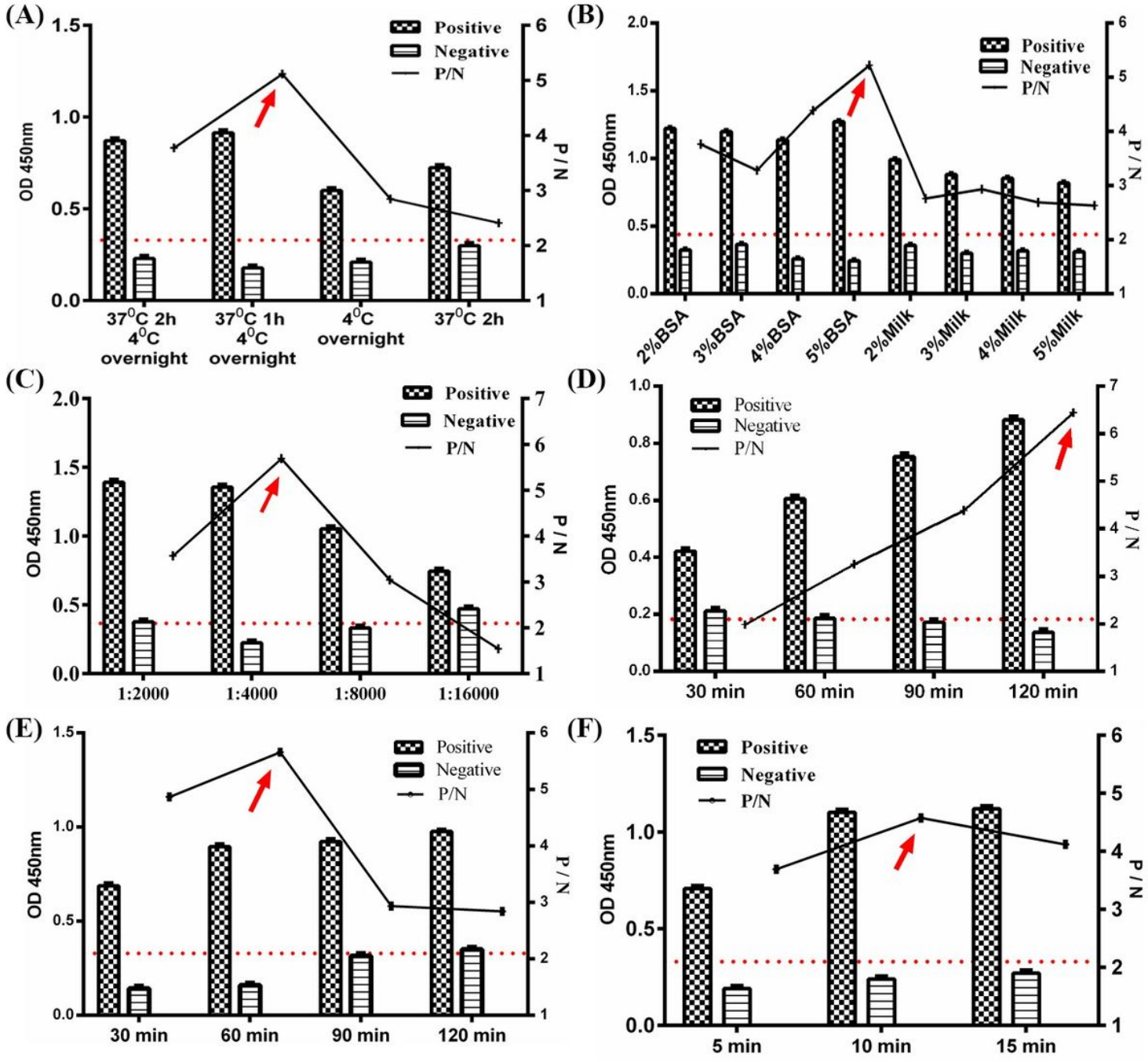

Figure 4

Optimization of indirect-ELISA by checkerboard titration. Positive bar: OD450 value of H. contortus infected goat sera; Negative bar: OD450 value of helminths frees goat sera; P/N: OD450 value of positive/ OD450 value of negative that showed on each bar and horizontal red dotted line is the cutoff line at 2.1 (right Y axis). P/N value more than 2.1 was determined to be positive while P/N value less than 2.1 was determined to be negative. The best condition (the highest P/N value) of each other was pointed by red arrows (↑). Graph A: Selection of antigen incubating condition; Graph B: Selection of blocking buffer; Graph C: Selection of optimal dilution of rabbit anti-goat IgG; Graph D: Selection of reactive time of serum; Graph E: Selection of blocking time; Graph F: Selection of reactive time of TMB. The optimum antigen incubating condition (37°C 1h and overnight at 4°C), best blocking buffer (5% BSA), optimal

dilution of rabbit anti-goat IgG (1:4000), best reactive time of serum (120 min), blocking (60 min) and reactive time of TMB (10 min) were observed.

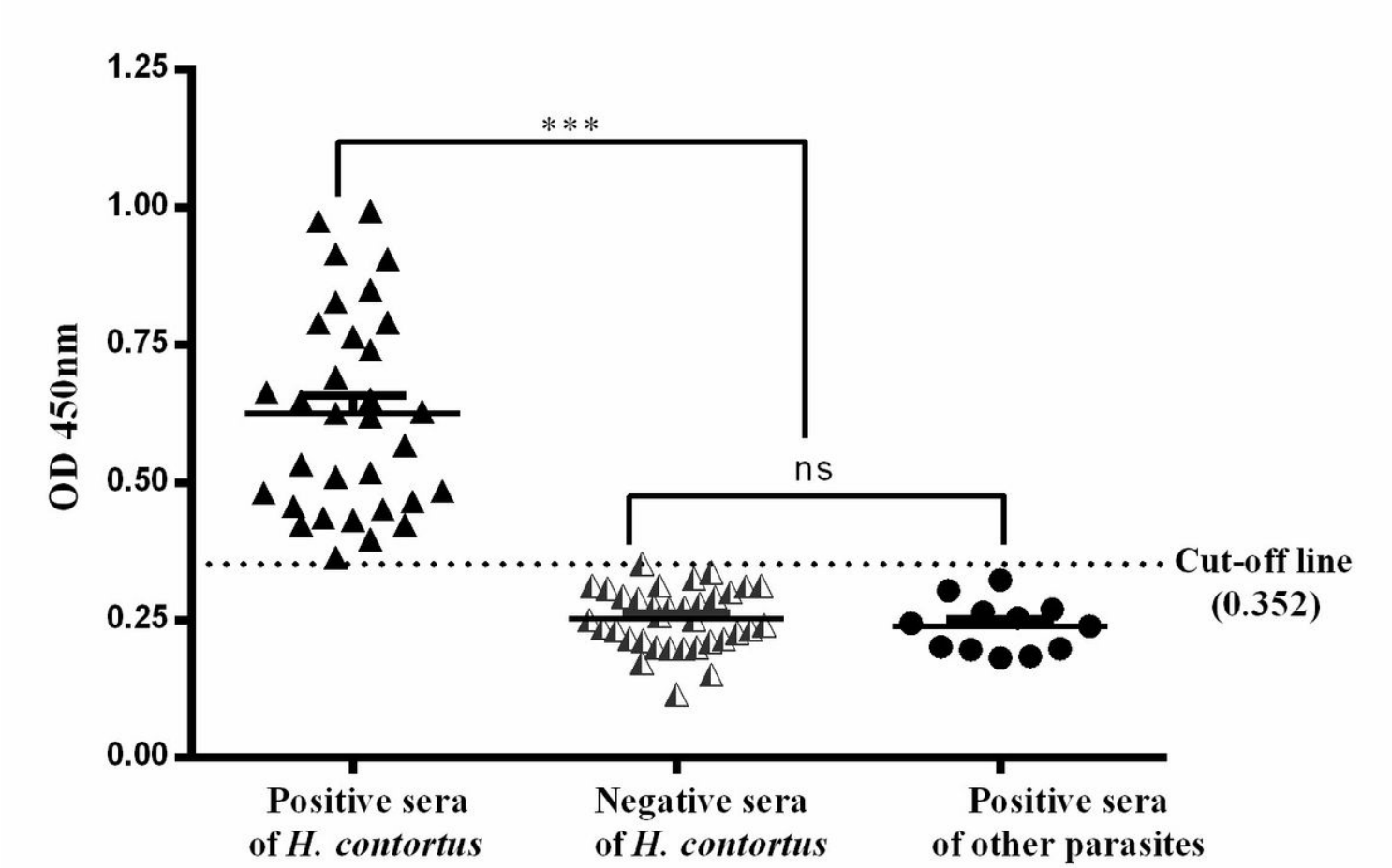

Figure 5

Sensitivity, specificity and cross-reactivity of indirect-ELISA based on rHc-CS. The dotted horizontal line represents the cut-off value (OD450=0.352) of indirect ELISA based on recombinant Hc-CS. Statistically significant differences ($P < 0.001$) were observed between H. contortus- positive sera and the other organisms sera (T. spiralis, F. hepatica and T. gondii-positive and H. contortus-negative sera). No significant difference ($P > 0.05$) was noted between the H. contortus-negative and other parasites-positive serum samples.

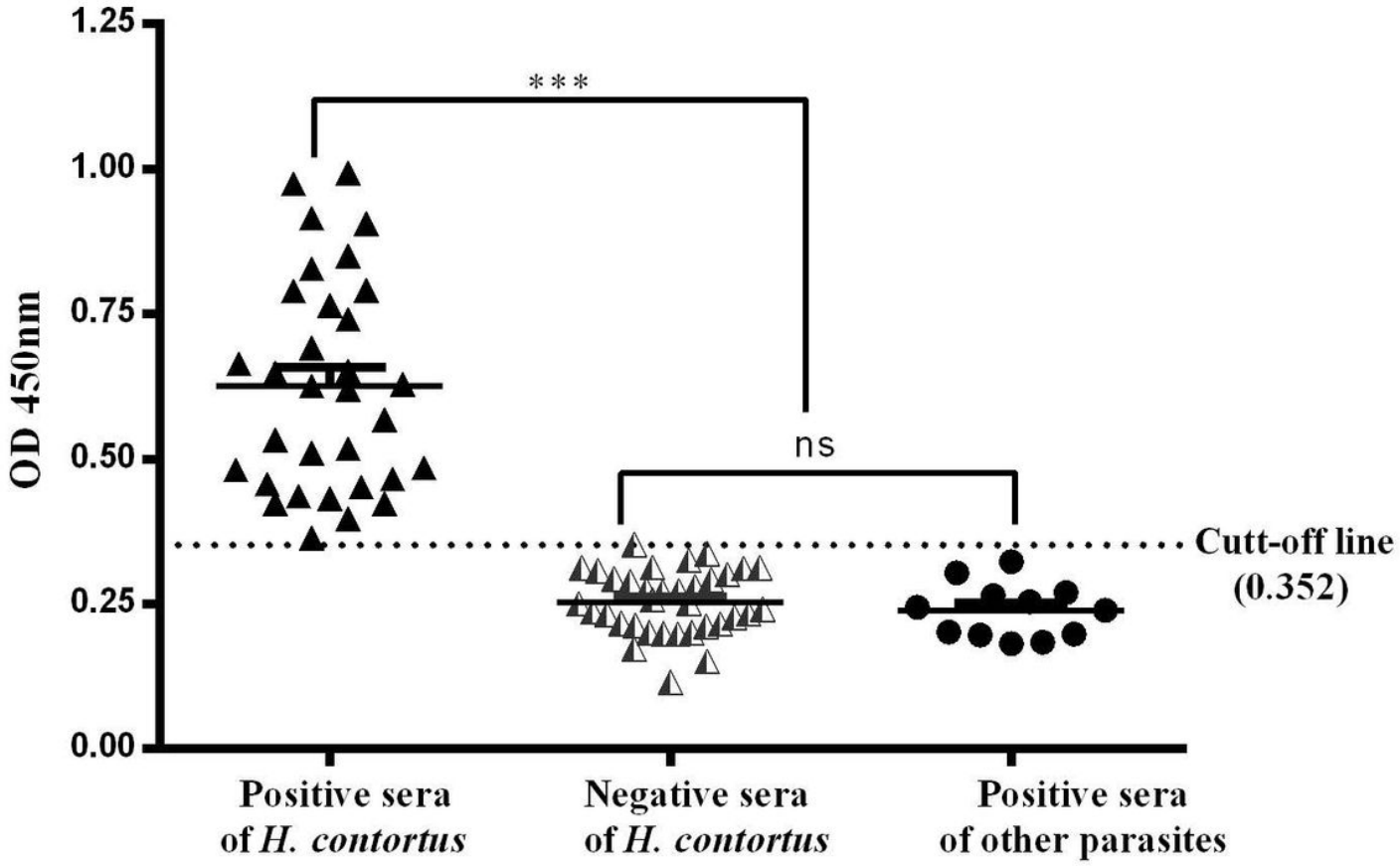

Figure 6

Sensitivity, specificity and cross-reactivity of indirect-ELISA based on rHc-CS. The dotted horizontal line represents the cut-off value (OD450=0.352). Statistically significant differences were observed between H. contortus- positive sera and the other organisms sera (T. spiralis, F. hepatica and T. gondii-positive and H. contortus-negative sera). No significant difference was noted between the H. contortus-negative and other parasites-positive serum samples.

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