

# Molecular characterization and serodiagnostic potential of two serpin proteins in *Psoroptes cuniculi*

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## Research

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# Abstract

**Background:** *Psoroptes cuniculi* is a global common ectoparasite of wild and domestic rabbits and causes an economically devastating loss and serious welfare issues of commercial rabbit husbandry. Serine proteinase inhibitor (Serpins) is present in almost all organisms that are involved in host-pathogen interactions, inflammatory responses, and reproductive development, etc. However, the research on *P. cuniculi* serpins is still limited.

**Methods:** In this study, two serpin genes of *P. cuniculi* (Pso c 27 and PsoSP2 cDNAs) were cloned, and the molecular characterization was analyzed. The transcriptional profiles and tissue localization of these two serpins in *P. cuniculi* were investigated by quantitative real-time PCR and immunohistochemistry, respectively. The potential function of recombinant Pso c 27 and PsoSP2 (rPso c 27 and rPsoSP2) in the serodiagnosis of *P. cuniculi* infestation in rabbits were evaluated by indirect enzyme-linked immunosorbent assay (iELISA).

**Results:** Both of the 523 residue Pso c 27 and the 240 residue PsoSP2 proteins contained typical serpin domains and signatures. Both Pso c 27 and PsoSP2 cDNAs expressed throughout the life cycle, more specifically, significantly higher expression in female mites than the larva, nymph, and male mites (Pso c 27,  $F(3, 8) = 1935.953, p < 0.0001$ ; PsoSP2,  $F(3, 8) = 660.669, p < 0.0001$ ). The native Pso c 27 and PsoSP2 localized in ovary and mouthpart of adult female mites, respectively. Compared to rPsoSP2, the rPso c 27 displayed better diagnostic efficiency with higher values of sensitivity, specificity and the area under the receiver operating characteristic curve (AUC) (rPso c 27 - rPsoSP2: 96.0% - 90.0%; 90.91% - 78.18%; 0.988 - 0.964, respectively). Moreover, the rPso c 27 showed seropositive in 80% rabbits as early as the 2<sup>nd</sup> week post-infestation (p.i.), prior to visible clinical signs and microscopy-positive of skin scrapings.

**Conclusions:** These results suggested that these two serpins may play essential roles in reproductive development, serum-feeding, and pathogenicity of *P. cuniculi*. Compared to PsoSP2, Pso c 27 appeared as a potential antigen for serodiagnosis of *P. cuniculi* infestation in rabbits, especially at the early stage of infestation.

## Background

*Psoroptes cuniculi* is a common ectoparasite of wild and domestic rabbits worldwide [1, 2]. This mite causes psoroptic mange of rabbits, mainly characterized as intense cutaneous inflammation, extreme pruritus and crusted skin lesions [1, 2]. Additionally, it causes severe economic losses and welfare issues in rabbits

feeds on serous fluids, lymph, and red blood cells [5]. Consequently, mite produces essential proteins to resist the host complement system for successful feeding and self-proliferation. Meanwhile, it excretes allergens to promote the subsequent cutaneous inflammatory response [6, 7]. expressed in almost all organisms, has shown a variety of fundamental physiological functions in arthropods including

anticoagulation, regulation inflammation response and reproductive development etc. [8]. It also played an essential role in host-pathogen interaction [9]. Besides, serpin may serve as a promising diagnostic antigen or vaccine candidate [10, 11].

Recently, transcriptome analyses revealed that serpins existed in *P. cuniculi* [12], but beyond that, no research has been reported on *P. cuniculi* serpins. Therefore, we are highly interested in the function of two serpin genes of *P. cuniculi* (Pso c 27 and PsoSP2 cDNAs) which were identified based on our transcriptomic data [12]. In this study, we cloned and expressed the two recombinant Pso c 27 and PsoSP2 in prokaryotic expression vectors, performed the sequence analysis. Additionally, we also investigated the transcriptional profiles as well as tissue localization in mites, and their potential efficiencies in the diagnosis of *P. cuniculi* infestation in rabbits were accessed by indirect enzyme-linked immunosorbent assay (iELISA). This is a preliminary study in relevance to the roles of these two proteins in *P. cuniculi*, which laying the foundation for further understanding of their functions.

## Methods

### Mite collection and RNA extraction

*P. cuniculi* were harvested from an infested New Zealand White rabbit maintained at the Department of Parasitology, Sichuan Agricultural University (Sichuan, China). About 300 mites, a pool of larvae, nymphs and adults, were collected and processed for the total RNA extraction.

### Expression and Purification of two recombinant serpin proteins

Total RNA was converted into cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). The two serpin genes were amplified from cDNA using the following primers: 5'-CGGGATCCGCTCATGTTGGTCAACATC-3' (forward) and 5' CCAAGCTTTTAAAATCATGAATTTACC-3' (reverse) for Pso c 27 with underlined restriction enzymes of *Bam*HI and *Hind*III, and 5'-CGGGATCCCTGAATGCGAATTCATTGCTG-3' (forward) and 5'-CCCTCGAG TCAAATCCATGCATTTACC-3' (reverse) for PsoSP2 with underlined restriction enzymes of *Bam*HI and *Xho*I. The cDNA fragments were sub-cloned into pET32a (+) (Invitrogen, Beijing, China). The recombinant proteins were expressed in *E. coli* BL21 (DE3) and purified as the previous method described by Gu et al. [2]. The eluted fractions were concentrated by Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). Two purified serpin proteins were detected by 12% SDS-PAGE..

### Sequence analysis

DNAMAN version 7.0 was applied to compare the similarity between paralogous genes, and SignalP 5.0 (<http://www.cbs.dtu.dk/Services/SignalP/>) was used to predict signal peptides. Transmembrane regions were analyzed using the Transmembrane Prediction Server (<http://www.cbs.dtu.dk/services/TMHMM-2.0>). B-cell epitopes were predicted by BaCelLo (<http://gpcr.biocomp.unibo.it/bacello/pred.htm>). The serine protease inhibition domains were analyzed by Inter-ProScan EMBL-EBI Software

(<http://pfam.xfam.org/>). Amino acid sequences were aligned using MEGA5.0. Secondary structure predictions were performed by JPred 4.0 (<http://www.compbio.dundee.ac.uk/jpred/>). The neighbor-joining (NJ) tree, including values of 1000 replications resampled tests, was constructed by MEGA 5.0 software [13].

## **Rabbit sera**

Fifty *P. cuniculi*-positive rabbit sera were collected from a farm located in Chengdu, Sichuan, China. All rabbits were confirmed *P. cuniculi*-positive by observation of ear scab and skin scrapings by microscopy [14]. Twenty-five negative sera from *P. cuniculi*-free rabbits were obtained from a farm without a history of psoroptic mange. For cross-reaction testing, another 30 sera included *Sarcoptes scabiei*-positive sera, *Eimeria* spp.-positive sera, and *Cysticercus pisiformis*-positive sera (ten/group) were provided by the Department of Parasitology, Sichuan Agricultural University.

## **Preparation of polyclonal antibodies and Western blotting**

Polyclonal antibodies were obtained by experimentally immunization with purified rPso c 27 and rPsoSP2, respectively. The produces were raised following slightly modified procedures described by Gu et al. [2]. Briefly, rabbits were immunized with about 1 mg purified recombinant protein four times by subcutaneous injection. Sera were collected via the marginal ear vein before immunization and 7 days after the fourth infection, and then purified by HiTrap Protein A affinity chromatography (Bio-scale™ Mini UNOsphere SUPRA™ Cartridge) to obtain the IgG of anti-rPso c 27 and anti-rPsoSP2.

Two purified recombinant proteins were separated by 12% SDS-PAGE and transferred to the nitrocellulose membranes using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The membranes were blocked using 5% skim milk powder for 2 h. After three times of 5-min washes with TBST (0.02 M Tris-HCl, pH7.6, 0.15 M NaCl, 0.05% Tween-20), membranes were incubated with rabbit anti-*P. cuniculi* antibody or anti-rPso c 27 IgG or anti-rPsoSP2 IgG (1:150 v/v) overnight at 4°C. Non-infested rabbit serum was used as a negative control. After washing three times with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:1000 dilution; Boster Bio-project Co. Dalian, China) for 1 h at room temperature. Following three washes with TBST, the signal was detected using an Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen, Beijing, China).

## **Immunolocalisation of two serpin proteins in adult female *P. cuniculi***

The immunolocalisation of two serpin proteins were performed as previously described [15]. Briefly, adult female mites were collected and sliced into 5 µm histological section, then were treated with 0.01 M citrate buffer and incubated with purified rabbit anti-rPso c 27 IgG or anti-rPsoSP2 IgG or pre-immune IgG (1:200 v/v). After three times washing with PBS, sections were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200 v/v) and finally visualized by a fluorescent microscope (BX53, Olympus, Japan).

## Transcriptional profiles of serpin at different life stages of *P. cuniculi*

The total RNA was extracted from larvae, nymph, and adult (male/female) mites using a MiniBest universal RNA extraction kit (TaKaRa), respectively. Relative gene expression was evaluated by two-step qRT-PCR with the following primers: Pso c 27, 5'-TGGCAGCAGTGGATCAGAATCATC-3' (forward) and 5'-AATGCAACAGCAACACTGTATGGC-3' (reverse); PsoSP2, 5'-TCCTACATACACGTCCATCAACA-3' (forward) and 5'-TGGTACAATAGCGACGGCTG-3' (reverse). The  $\beta$ -actin gene was used as a housekeeping control to correct the relative fluorescence signal of the target genes using primers 5'-TGAATTGCCTGATGGTCAAG-3' (forward) and 5'-TGGCGAACAAGTCTTTACGG-3' (reverse). Gene transcription was assessed according to the manufacturers' recommendations of the real-time PCR System (LightCycler® 96 System, Roche, Switzerland) and the SYBR Premix Ex Taq II Kit (TaKaRa, Dalin, China). Each sample was performed in triplicate. An equal volume of ddH<sub>2</sub>O replaced the template cDNA as blank control. Thermal cycling was performed as follows: 95 °C for 30 s, 95 °C for 5 s, 58 °C for 30 s, then followed by 40 cycles at 95 °C for 0 s, 59 °C for 15 s, and 95 °C for 0 s. Melting curves were plotted, and relative expression levels of the target genes were calculated by the  $2^{-DDCt}$  method.

## Establishment of an Indirect ELISA (iELISA)

The establishment of iELISA was performed as described by Crowther [16]. The concentrations of antigen and primary serum samples were determined by the checkerboard titration tests. Briefly, the purified proteins were diluted two-fold in 0.1 M carbonate buffer (pH 9.6) to the different concentrations and coated in 96-well plates with 100  $\mu$ L/well overnight at 4 °C. The dishes were washed three times with PBS containing Tween-20 (PBST, pH 7.4) (5 min per wash), then incubated with 5 % (w/v) skim milk powder at 37 °C for 90 min, then with 100  $\mu$ L of two-fold gradient dilution of *P. cuniculi*-positive and negative serum samples (ranging from 1:20 to 1:320) were added and incubated at 37 °C for 1 h. The plates were washed 3 times and incubated 1 h at 37 °C with 100  $\mu$ L HRP-labeled goat anti-rabbit IgG (1: 3000 dilution with 0.01 M PBS) (Boster Bio-project Co., Wuhan, China). After 4 times washing, 100  $\mu$ L of TMB chromogenic solution (TIANGEN, Beijing, China) was added at 37 °C for 20 min, then the reaction was stopped with 100  $\mu$ L / well of 2 M H<sub>2</sub>SO<sub>4</sub>. Optical densities (OD) were read at 450 nm by a microplate reader (Thermo Scientific, Pittsburgh, PA, USA). The optimal working conditions were determined with the highest P/N (positive/negative serum) value. The cut-off value of iELISA was determined as the mean OD<sub>450</sub> value plus three standard deviations (SD) using 25 negative serum samples from naïve rabbits [2].

To further evaluate the feasibility of the iELISA, 50 *P. cuniculi*-positive serum samples were assessed by the iELISA, and the sensitivity was calculated as (ELISA positive  $\times$  100) / true *P. cuniculi*-positive [2]. Thirty serum samples from rabbit infected with *S. scabiei*, *Eimeria spp* and *C. pisiformis* (10 samples for each species) were used to evaluate the cross-activity of the iELISA. Twenty-five negative serum samples from naïve rabbits and 30 serum samples in the cross-activity assay were used to determine the specificity of the iELISA, which was calculated as (ELISA negative  $\times$  100) / real *P. cuniculi*-negative [2]. After that, the area under the receiver operating characteristic curve (AUC), a graph of the sensitivity (true positive rate) versus 1-specificity (false positive rate), was calculated by MedCalc 19.0.7 [17].

The repeatability (intra-assay variability) and reproducibility (inter-assay variability) of the iELISA were evaluated using three *P. cuniculi*-positive serum samples, substantially as previously described [18].

## **The experimental infestation of rabbits with *P. cuniculi* and serological testing using the established iELISA**

Rabbits infected with *P. cuniculi* were performed strictly as previously described [2]. Briefly, ten 3-month-old naive New Zealand rabbits (5 females and 5 males) were infested with *P. cuniculi*, and three non-infested rabbits were applied as controls. Serum samples from 13 rabbits were collected at weeks 0, 1, 2, 3, and 4. Afterward, in a total of 65 serum samples (50 from the *P. cuniculi* infestation rabbits and 15 from the non-infestation rabbits) were examined by the establishment optimal iELISA method. Each serum sample in triplicate was analyzed in one ELISA plate, and positive and negative controls were contained in the dishes.

## **Statistical analysis**

All data are represented as mean  $\pm$  standard deviation (SD), and statistical differences between groups were evaluated using Mann-Whitney U tests in SPSS software v.17.0. *P*-values  $< 0.05$  were considered as statistically significant.

# **Results**

## **Sequence analyses of two serpins**

The 1572-bp open reading frame (ORF) in Pso c 27 cDNA (GenBank: MT707535) encodes 523 amino acids (aa), while the 723-bp ORF in PsoSP2 cDNA (GenBank: MT707536) encodes 240 aa. The Pso c 27 protein contains a signal peptide but no transmembrane region, whereas PsoSP2 appears to contain no signal peptide and a transmembrane region.

Pso c 27 and PsoSP2 shared 31.33 % ~ 50.85 % and 28.99 % ~ 69.92 % amino acid sequence identity with orthologs in other mites (Fig. 1). Interestingly, Pso c 27 and PsoSP2 shared 100% amino acid sequence identity with the reported serpin-like proteins of *P. ovis* PSOVI22g04610 and PSOVI22g04560, respectively [19] (Fig. 1). Pso c 27 was comprised of 12 helix and 3-sheets, while PsoSP2 contained 5 helices and 3-sheets (Fig. 1a). A serpin domain was identified in the amino acid sequence from Arg78 to His493 for Pso c 27 and Asn4 to Met237 for PsoSP2 [20] Moreover, both proteins appeared to possess the specific serpin signature at the deduced amino acid sequences from 496 to 506 (LRFDHPFLYFV) for Pso c 27 and from 213 to 223 (LSFDHPFLYFL) for PsoSP2, respectively (Fig. 1a and 1b). The NJ tree revealed that Pso c 27 had the closest relationship with *P. ovis*-leukocyte elastase inhibitor-like protein (PSOVI22g04610), then clustered with *D. farinae*-Der f 27 allergen, *D. pteroyssinus*-Der f 27-like allergen, and *E. maynei*-serpin (Bf=100%), whereas PsoSP2 had the closest relationship with *P. ovis* serpin B5 (PSOVI22g04560) (Bf=100%), then grouped with *E. maynei*-Serpine-like and *D. pteroyssinus*-Serpine B10-like (Bf=98%, Fig. 2).

## Expression and Identification of two recombinant serpins

The rPso c 27 were mainly present in the supernatant with an expected size of ~75 kDa, whereas rPsoSP2 principally present in insoluble inclusion bodies with an expected size of ~ 46 kDa (including ~18 kDa His-tag fusion peptide from pET-32a) (Fig. 3). Western blotting showed that rPso c 27 and rPsoSP2 were reacted with *P. cuniculi*-positive sera and the correspondent anti-serum IgG from rabbits, but not negative sera, revealing the favorable reactivity and antigenicity (Fig. 3).

## Tissue localization of two serpins in adult female *P. cuniculi*

Native Pso c 27 and PsoSP2 were located in ovary and mouthpart of female mites, respectively (Fig. 4b and 4c). No fluorescence signal was observed in adult female mites using pre-immunized rabbit IgG antibodies (Fig. 4a).

## Transcriptional profiles of two serpins in *P. cuniculi*

The qRT-PCR data revealed that Pso c 27 and PsoSP2 cDNAs were expressed throughout the life cycle of mites, more specifically, significantly higher expression in female mites than larva, nymph, and male mites, respectively. (Pso c 27,  $F(3, 8) = 1935.953$ ,  $p < 0.0001$ ; PsoSP2,  $F(3, 8) = 660.669$ ,  $p < 0.0001$ ) (Fig. 5).

## Serodiagnosis potential of two recombinant serpin proteins

By checkerboard titration, the optimal working conditions of iELISA were 46.0 µg/mL of rPso c 27, 64.5 µg/mL of rPsoSP2 for coated antigens and a 1:100 dilution for rabbit sera. The cut-off values of OD450 were 0.633 of rPso c 27 and 0.490 of rPsoSP2, respectively.

The sensitivities were determined as the results of positive sera with 96.0% of rPso c 27 (48/50) (Fig. 6a) and 90.0% of rPsoSP2 (45/50) (Fig. 6b). The specificities showed 90.91% of rPso c 27 (50/55) and 78.18% of rPsoSP2 (43/55). Consequently, the AUC was 0.988 of rPso c 27-iELISA (95% confidence interval (CI), 0.944~0.999) and 0.964 of rPsoSP2-iELISA (95% CI, 0.908~0.991), indicating that the rPso c 27-iELISA showed a better accuracy to detect specific antibodies against *P. cuniculi* than rPsoSP2-iELISA (Fig. 7). The intra- and inter-assay variabilities of rPso c 27-iELISA were < 5%, indicating the established rPso c 27-iELISA was stable and reproducible.

## Serodiagnostic test of rabbits experimentally infested with *P. cuniculi*

After 4 weeks of post-infestation (p.i.), all infested rabbits were observed with the visible ear scabs. Meanwhile, *P. cuniculi* showed positive in skin scrapings. By rPso c 27-iELISA, the mean value of the anti-rPso c 27 level from the infestation group revealed an increase from 1 to 4 weeks p.i (Fig. 8). The positive anti-rPso c 27 above cut-off value was firstly detected with 2/10 serum samples at 1-week p.i. in the infestation group. Afterward, the rate of positive serum gradually increased to 80 % (8/10) at 2 and 3

weeks p.i., then up to 100 % (10/10) at 4 weeks p.i. (Fig. 8). In the non-infestation group, the anti-rPso c 27 antibody appeared below the cut-off value throughout the experiments.

## Discussion

In the present study, two *P. cuniculi* serpins were characterized, and the potential of the recombinant proteins was evaluated for serodiagnosis of *P. cuniculi* infestation in rabbits. The predicted amino acid sequence showed the low overall identity of serpins compared to other mites, however, these two target proteins were identified as typical serpins due to the presence of the features such as serpin domain and serpin signature in C-terminal end [20]. Pso c 27 shared 50.85% amino acid sequence identity with the newly characterized *D. farinae* Der f 27 allergens, which has been proven to orchestrate the pulmonary inflammatory response and mediate Th2 type response in mouse [21]. Besides, NJ analysis revealed that Pso c 27 yielded a close relationship with Der f 27. In combination with the homology and the genetic relationship between Pso c 27 and Der f 27, Pso c 27 may be considered as an allergen of *P. cuniculi*, which was possibly associated with the instigation of the host cutaneous pro-inflammatory response [22]. Additionally, this cutaneous inflammation resulted in serum extravasation to provide sufficient food for mite population growth and cause aggravation of scabby lesions [5, 14]. The expression of Pso c 27 and PsoSP2 in all stages of mites indicated that Pso c 27 and PsoSP2 possibly play an essential role in the development of *P. cuniculi*. However, significant differences were seen for the transcription of Pso c 27 in female mites being the highest level of expression with a 347-fold change. In addition, the native protein was located in the ovary of female mites, indicating that Pso c 27 possibly was essential in vitellogenesis [19, 23]. This role of serpin being involved in vitellogenesis has been proven in a recent research, which indicated RNAi of serpin gene resulted in a reduction of yolk granule accumulation in *Rhipicephalus haemaphysaloides* [24]. *Psoroptes* mites are serum-feeding ectoparasites [5] and possess ability to counter host's complement attack. In this study, PsoSP2 showed homologous to the *S. scabiei* serpin family genes (20.98 % ~ 54.13 % amino acid sequence identity), some of which have been confirmed to inhibit the activation of complement pathways [25, 26]. Moreover, the native PsoSP2 localized in mouthpart of female mites and its cDNA expression throughout the life stages of mites suggested that PsoSP2 may appear to be vital in mites for anti-complement activity to successful serum-feeding [5, 9], and PsoSP2 could be a potential vaccine candidate.

Psoroptic mange spreads rapidly under crowded conditions and causes major morbidity in the rabbit breeding industry in China [27]. Thus, timely diagnosis and treatment of *P. cuniculi* infestation in rabbits are paramountly important to reduce the risk of disease transmission and improve profitability. In China, the current microscopic diagnosis for this disease is extremely time-consuming and inefficient in the low mite carriers and sub-clinical infestations in rabbits. Thus, it is imperative to seek for the effectively immunoreactive antigens for rapid and accurate diagnosis of the *P. cuniculi* infestation in rabbits. Furthermore, animals infested *P. ovis* could evoke sero-specific antibody [1, 28], and this sero-specific antibody was induced at the early phase of parasite infestation when animals appeared asymptomatic [1, 2, 29]. Thus, enzyme-linked immunosorbent assay (ELISA) can be considered as an accurate method compared to the microscopy of skin scrapings at the low mite carriers and sub-clinical infestations

because of its speedy, high sensitivity, and handling convenience. In a previous study, serpin of *Schistosoma mansoni* was considered as a promising species-specific diagnostic antigen in human schistosomiasis [10]. Therefore, in this study, we evaluated the serodiagnostic potential of rPso c 27 and rPsoSP2 by the establishment of the iELISA. Compared to rPsoSP2-iELISA, the rPso c 27-iELISA displayed better diagnostic efficiency with higher values of sensitivity, specificity and AUC (rPso c 27 - rPsoSP2: 96.0% - 90.0%; 90.91% - 78.18%; 0.988 - 0.964, respectively). Although rPso c 27 showed cross-reaction with sera from 3/10 *S. scabiei*-infestation, the cross-reaction between these two ectoparasites have been proved commonly in other studies [2, 29, 30]. Fortunately, these two mite species were effectively treated with the same acaricide [3, 31]. Besides, 1/10 rabbits infested with *S. scabiei*, *C. pisiformis* and *Eimeria* spp. showed sero-reaction with rPso c 27, however, their OD values were close to the cut-off value and appeared markedly lower than those rabbits infested with *P. cuniculi* ( $F(1, 78) = 115.444, p < 0.0001$ ). Moreover, rPso c 27-iELISA can detect seropositive in 80% (8/10) rabbits as early as the 2nd-week p.i., prior to visible clinical signs and microscopy-positive of skin scrapings. Regarding the high sensitivity and specificity, Pso c 27 was more suitable as a candidate antigen for serodiagnosis of *P. cuniculi* infestation in rabbits, especially at the early stage of infestation.

## Conclusions

In conclusion, Pso c 27 and PsoSP2 cDNAs displayed the typical characterization of serpin superfamily with the regular serpin domain and signature. The gene expressions of Pso c 27 and PsoSP2 were found in all life stages of mites, with significantly high expressions in adult female mites. Compared to rPsoSP2, rPso c 27 seemed to display a better diagnostic efficiency than PsoSP2 by iELISA, suggesting that Pso c 27 could be developed as a potential antigen for serological diagnosis of *P. cuniculi* infestation in rabbits, especially at the early stage of infestation.

## Declarations

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### Ethics approval and consent to participate

In this study, the animal procedure was reviewed and approved by the Animal Care and Use Committee of Sichuan Agricultural University (SYXK 2019-187). All the rabbits were strictly managed under the Guide for the Care and Use of Laboratory Animals (National Research Council, Bethesda, MD, USA) and the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>).

### Consent for publication

Not applicable.

## Availability of data and materials

The nucleotide sequences of serpin genes from *P. cuniculi* in this article are available in the GenBank databases under the accession no. MT707535 for Pso c 27 and MT707536 for PsoSP2. The other data supporting our findings and conclusions are available in the article.

## Competing interests

The authors declare that they have no competing interests.

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## Authors' contributions

XBG conceived and designed the experiments. YHC and CYZ performed the experiments including RT-PCR, qRT-PCR, recombinant protein expression, indirect ELISA. NXS and CW performed the tissue localization. XBG, YHC, YX, RH and XZ achieved the data analysis. GYY, XRP, DYY and ZH contributed reagents/materials/analysis tools. ZH and ZJZ provide experimental technical assistance. XBG and YHC wrote the initial manuscript. All authors critically revised and approved the final version of the manuscript.

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## Abbreviations

*P. cuniculi*: *Psoroptes cuniculi*; *P. ovis*: *Psoroptes ovis*; *S. scabiei*: *Sarcoptes scabiei*; *C. pisiformis*: *Cysticercus pisiformis*; *E. coli*: *Escherichia coli*; Serpin: Serine protease inhibitor; PCR: Polymerase Chain Reaction; qRT-PCR: quantitative real-time polymerase chain reaction; rPso c 27/rPsoSP2: recombinant Pso c 27/ recombinant PsoSP2; p.i: post-infestation; PAGE: polyacrylamide gel electrophoresis; ELISA: enzyme-linked immunosorbent assay; iELISA: indirect enzyme-linked immunosorbent assay; NJ tree: neighbor-joining tree; HRP: horseradish peroxidase; OD: Optical densities; P/N value: positive/negative serum value; SD: standard deviation; ROC: receiver operating characteristic curve ; AUC: area under the

receiver operating characteristic curve; ORF: open reading frame; aa: amino acids; Bf: bootstrapping frequency; CI: confidence interval; RNAi: RNA interference; PBS: phosphate-buffered saline; PBST: phosphate-buffered saline containing tween-20; TBST: Tris-buffered saline containing Tween-20

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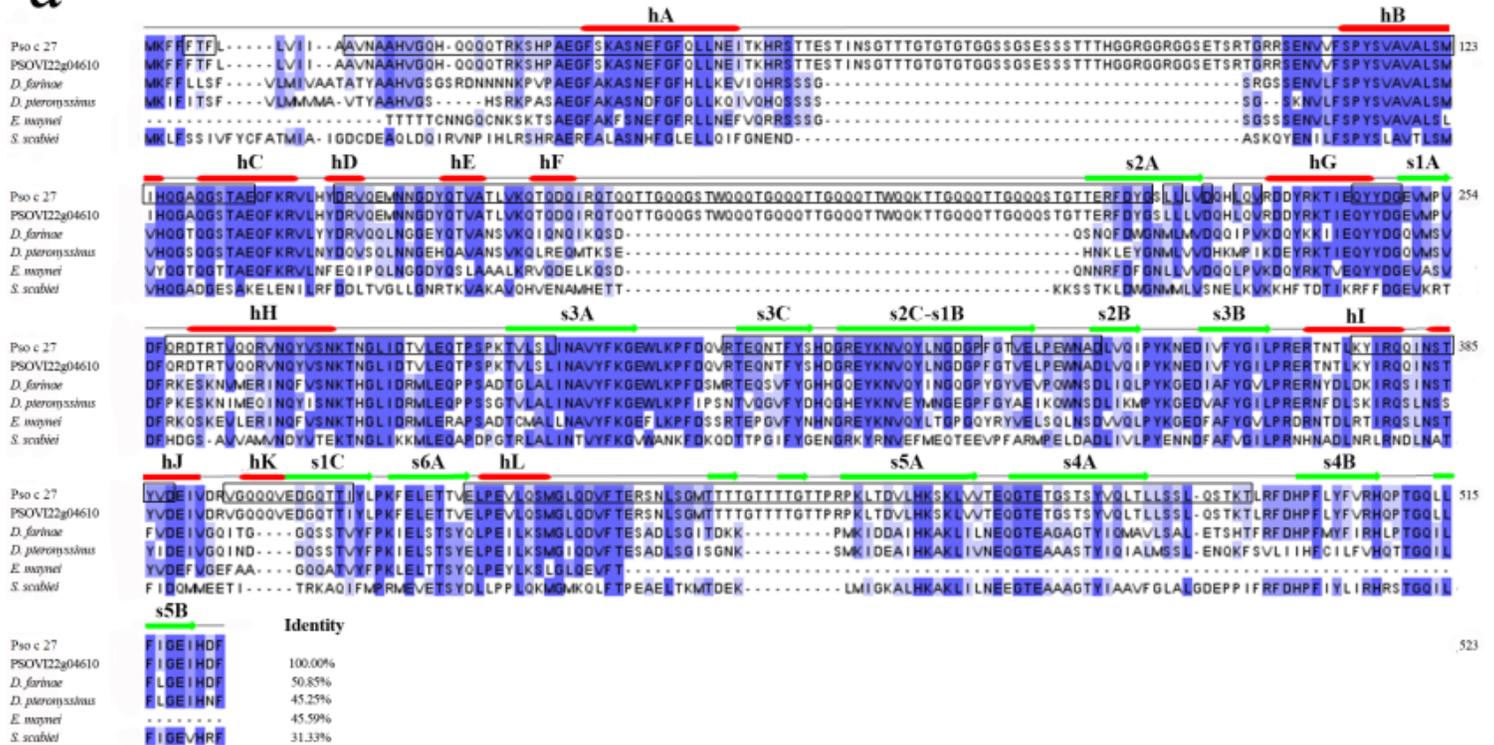
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## Figures

a



b

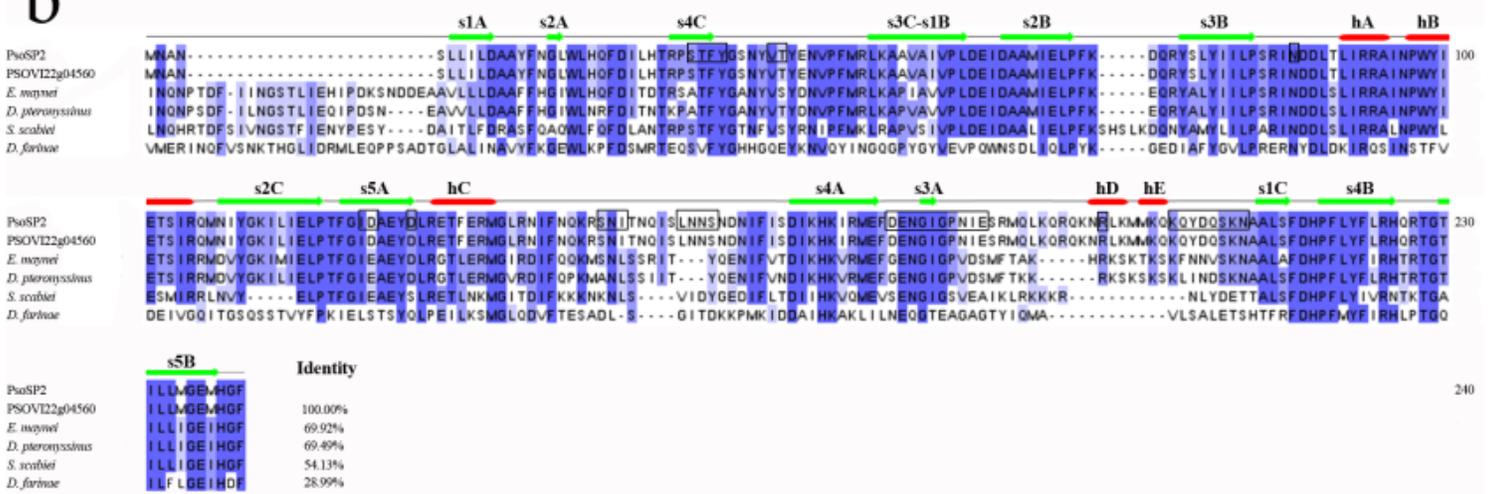
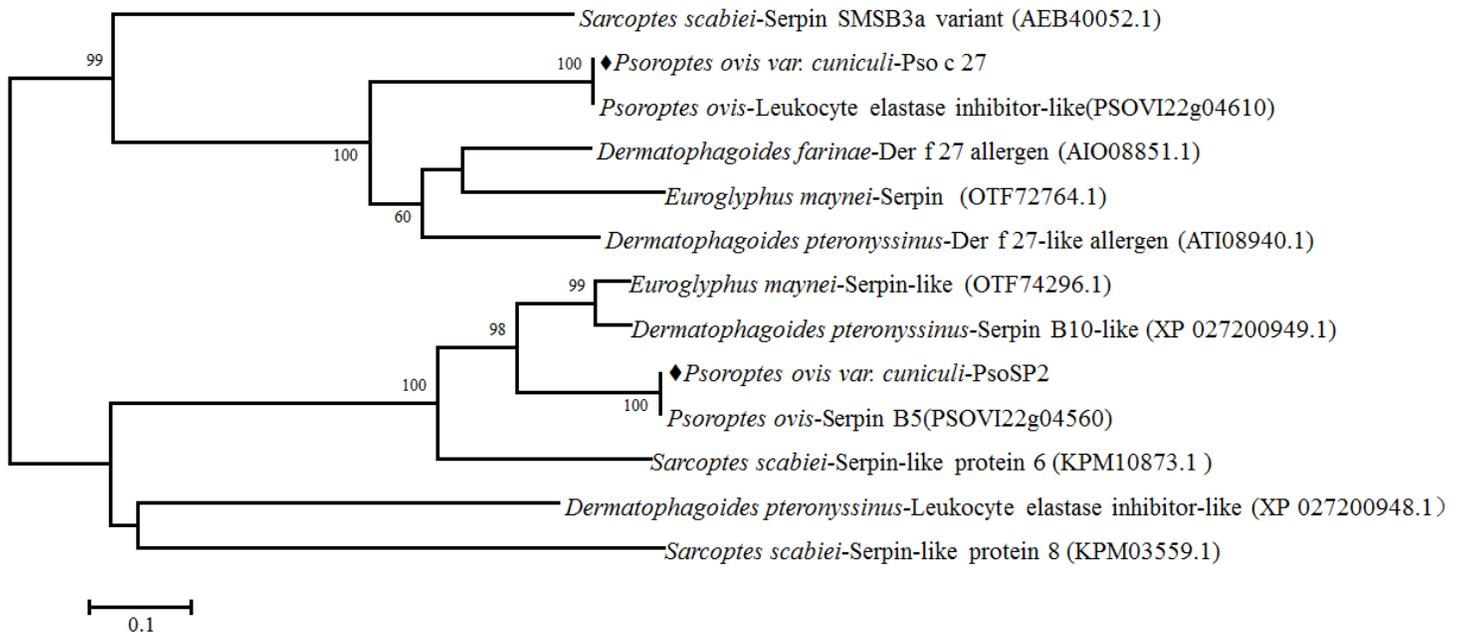


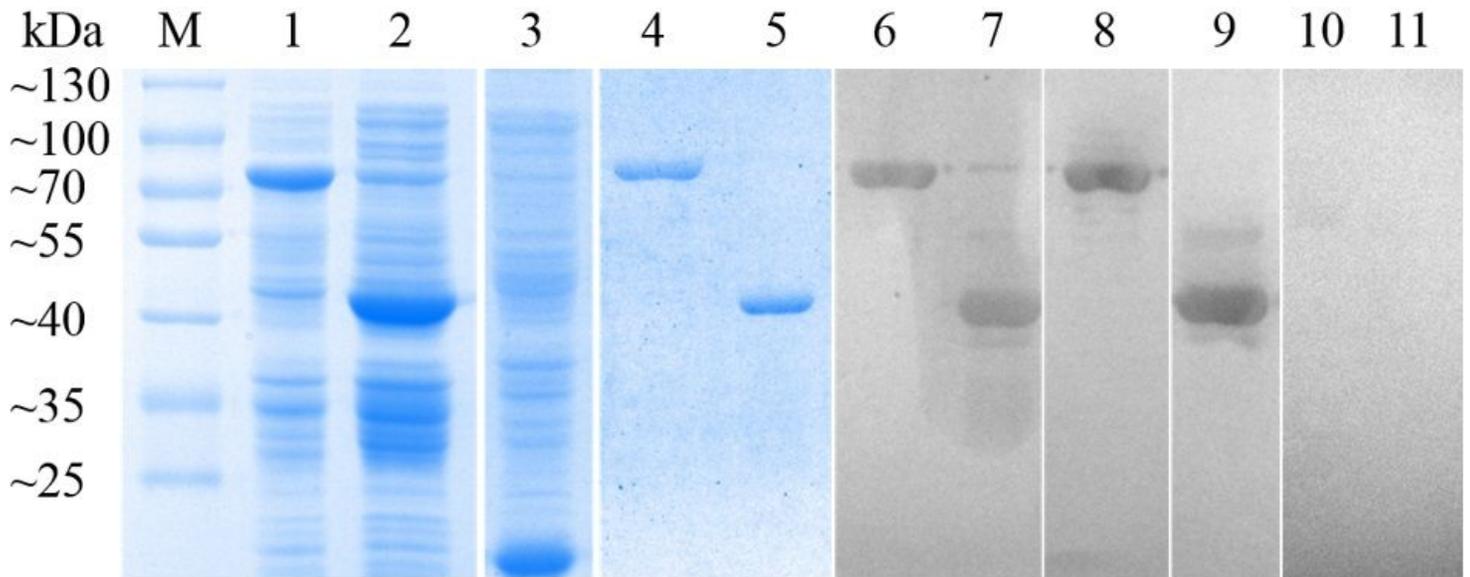
Figure 1

Multiple sequence alignment of Pso c 27 (a) and PsoSP2 (b). a: Pso c 27: Multiple sequence alignment of the deduced amino acid sequence of Pso c 27 with homologous sequences of related proteins of other parasites: *P. ovis* (PSOVI22g04610), *Dermatophagoides farinae* (GenBank: AIO08851.1), *D. pteronyssinus* (GenBank: ATI08940.1), *Euroglyphus maynei* (GenBank: OTF72764.1) and *Sarcoptes scabiei* (GenBank: AEB40052.1). b: PsoSP2: Multiple sequence alignment of the deduced amino acid sequence of PsoSP2 with homologous sequences of related proteins of other parasites: *P. ovis* (PSOVI22g04560), *Euroglyphus maynei* (GenBank: OTF74296.1), *Dermatophagoides pteronyssinus* (GenBank: XP\_027200949.1), *Sarcoptes scabiei* (GenBank: KPM10873.1) and *Dermatophagoides farinae* (GenBank: AAP35082.1). *P. ovis* (PSOVI22g04610) and *P. ovis* (PSOVI22g04560) are obtained from the Online Resource for Community Annotation of Eukaryotes (OrcAE) (<https://bioinformatics.psb.ugent.be/orcae/overview/Psovi>). Helices are marked as red tubes, and sheets as dark green arrows on the sequence. Elements of secondary structure are labeled as follows: (hA, hB, etc.) A-helix, B-helix, etc.; (s1A, s2A, etc.) strand 1 of the A  $\beta$ -sheet, strand 2 of the A  $\beta$ -sheet, etc. Consistent residues are highlighted with a dark blue background, and consistent partial residues are highlighted with a light blue background. B-cell epitopes are marked with a black box.



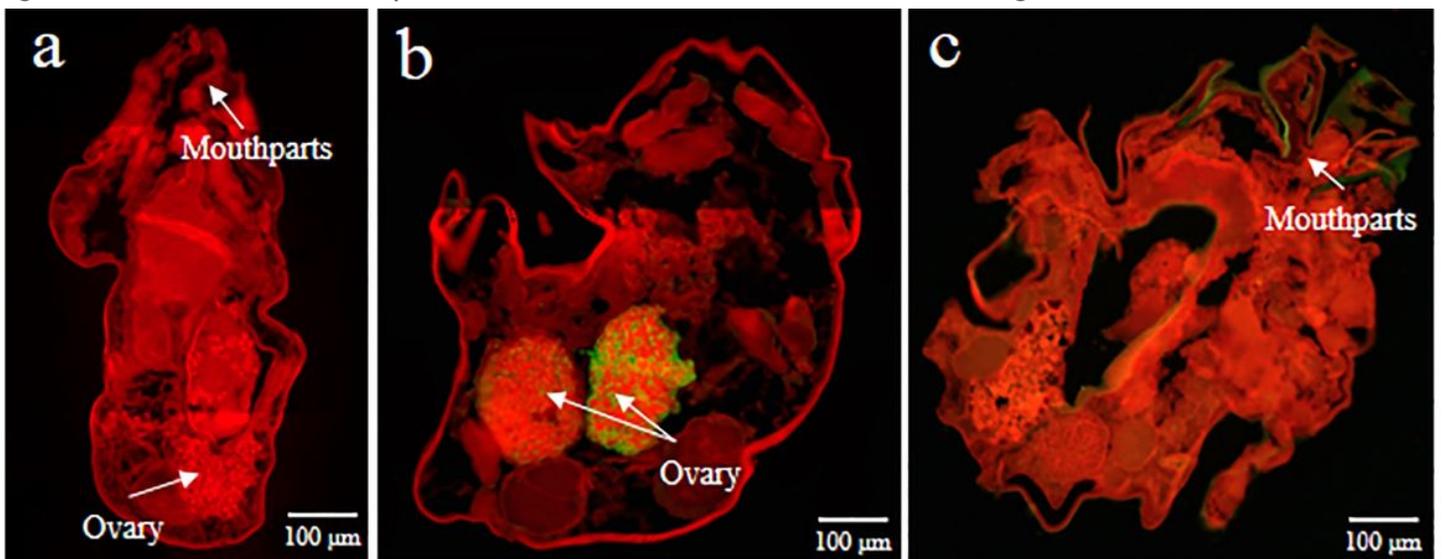
**Figure 2**

The neighbor-joining (NJ) tree was constructed based on the deduced amino acid sequence of serpin. The numbers at nodes are the bootstrapping frequency (Bf) values of 1000 replications.



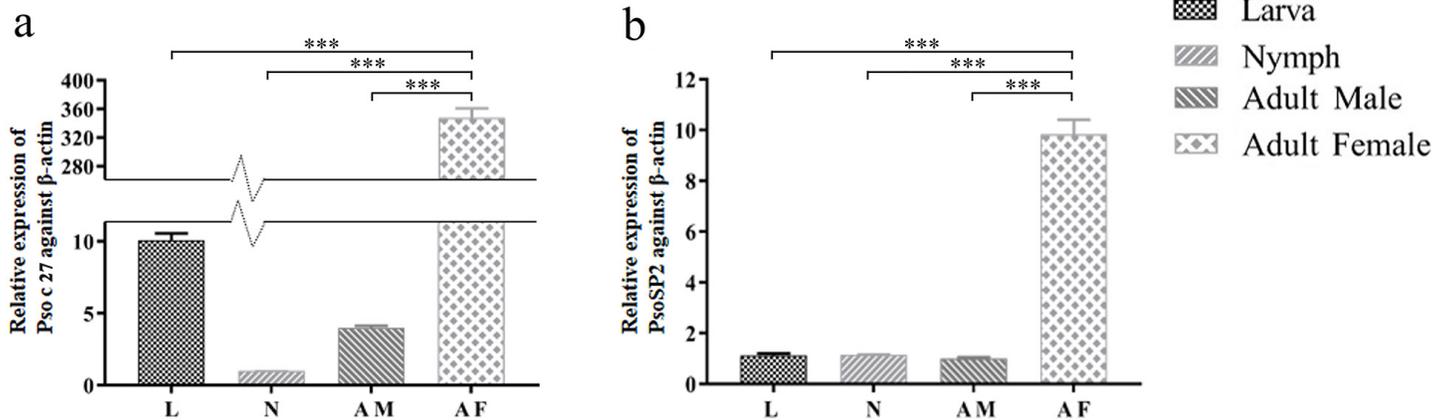
**Figure 3**

Immunoblotting with the serum binding recombinant Pso c 27 and PsoSP2. Lane M: protein molecular weight marker; Lane 1: recombinant proteins of *E. coli* expressing pET32a (+)-Pso c 27 induced by IPTG; Lane 2: recombinant proteins of *E. coli* expressing pET32a (+)-PsoSP2 produced by IPTG; Lane 3: the proteins of *E. coli* expressing pET32a (+); Lane 4: the purified rPso c 27; Lane 5: the purified rPsoSP2; Lane 6: the purified rPso c 27 immunoblotted with the positive serum of the rabbits with psoroptic mange; Lane 7: the purified rPsoSP2 immunoblotted with the positive serum of the rabbits with psoroptic mange; Lane 8: the purified rPso c 27 immunoblotted with the anti-rPso c 27 IgG; Lane 9: the purified rPsoSP2 immunoblotted with the anti-rPsoSP2 IgG; Lane 10: the purified rPso c 27 immunoblotted with the negative serum; Lane 11: the purified rPsoSP2 immunoblotted with the negative serum.



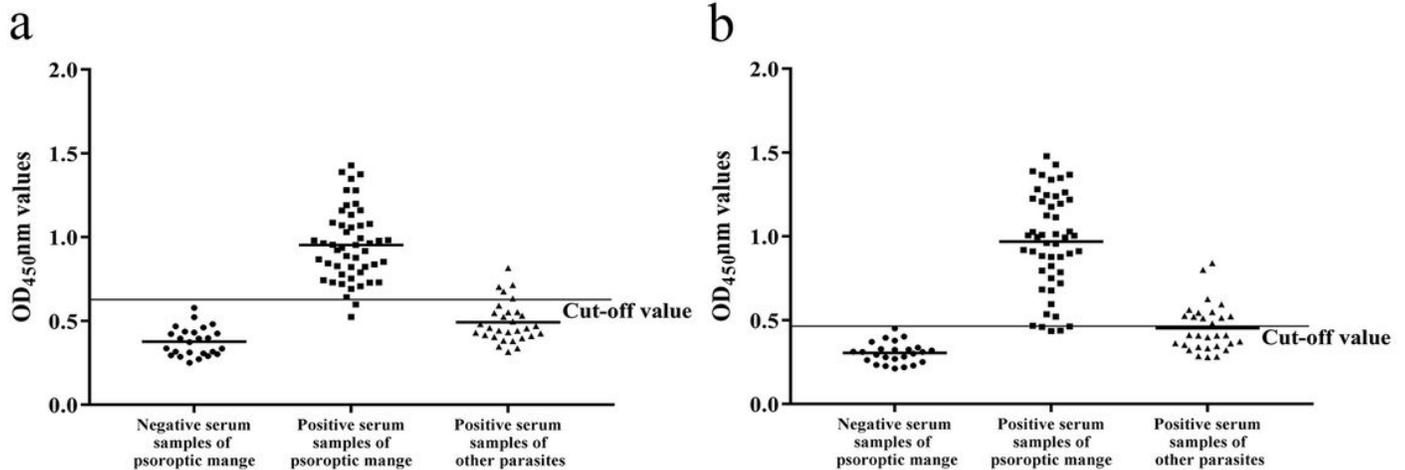
**Figure 4**

Immunolocalization of Pso c 27 and PsoSP2 in the adult female of *Psoroptes cuniculi*. a: Incubated with the negative IgG of the rabbit before immunization; b: Incubated with the specific IgG of anti-rPso c 27; c: Incubated with the specific IgG of anti-rPsoSP2. All images were taken under a fluorescent microscope at the magnification of 100 ×.



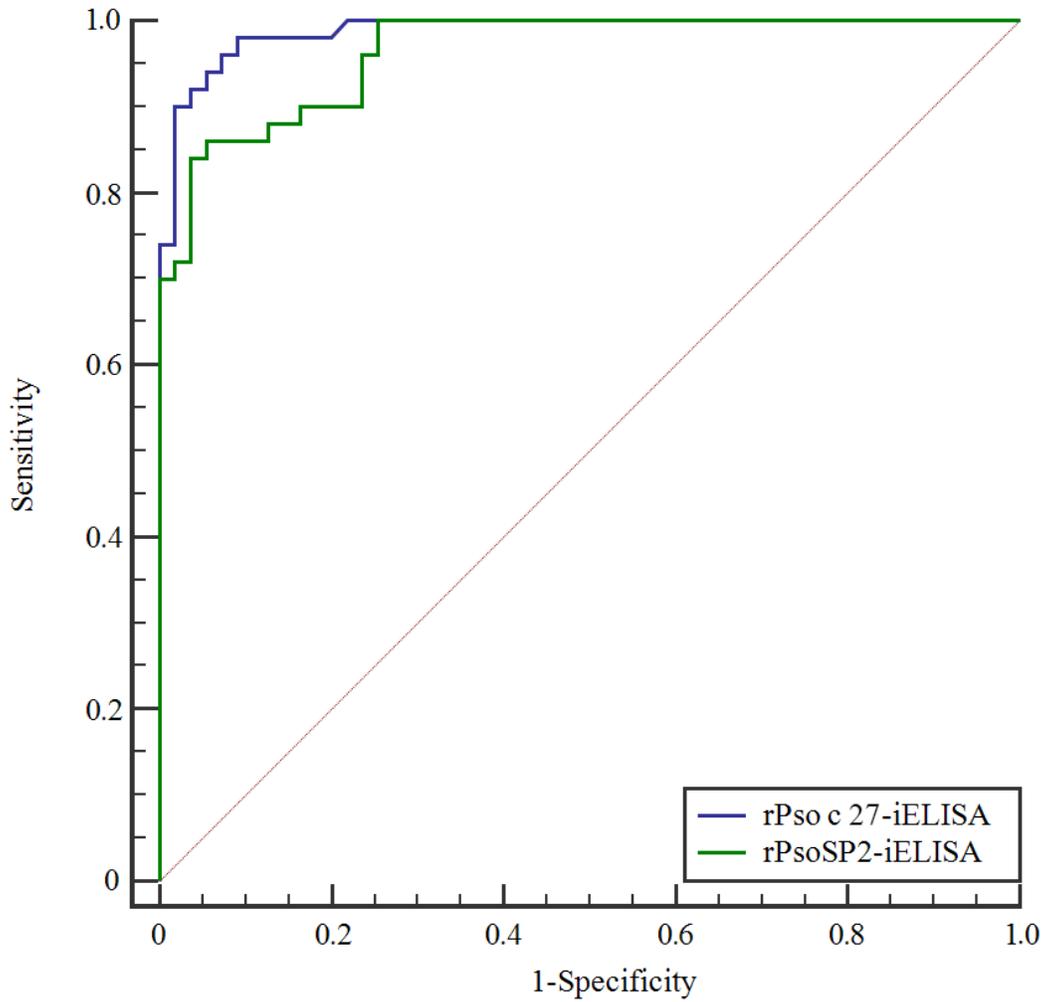
**Figure 5**

Relative transcriptional profiles of Pso c 27 (a) and PsoSP2 (b). The internal reference gene was  $\beta$ -actin in the study. Data are represented as the mean with standard deviation (SD) in triplicate (\*\*\*) indicates the statistical significance of  $p < 0.0001$ ).



**Figure 6**

Specificity, sensitivity and cross-reactivity of rPso c 27 (a) and rPsoSP2 (b) by indirect ELISA. The thin horizontal line represents the cut-off value (rPso c 27-iELISA: 0.633; rPsoSP2-iELISA: 0.490). Statistically significant differences were compared between *P. cuniculi*-positive serum and the other serum samples, including *Eimeria* spp, *C. pisiformis*, *S. scabiei*-positive, and *P. cuniculi*-negative serum samples (\*\*\*) indicates the statistical significance of  $p < 0.0001$ ).



**Figure 7**

The Receiver operating characteristic (ROC) curves of the rPso c 27-iELISA and rPsoSP2-iELISA for the detection of antibodies against *P. cuniculi*. The ordinate represents the sensitivity of the iELISA. The abscissa represents the 1-specificity of the iELISA. The purple line shows the mean area under the curve (AUC) plot of rPso c 27-iELISA, and the green line shows the mean area under the curve (AUC) plot of rPsoSP2-iELISA.

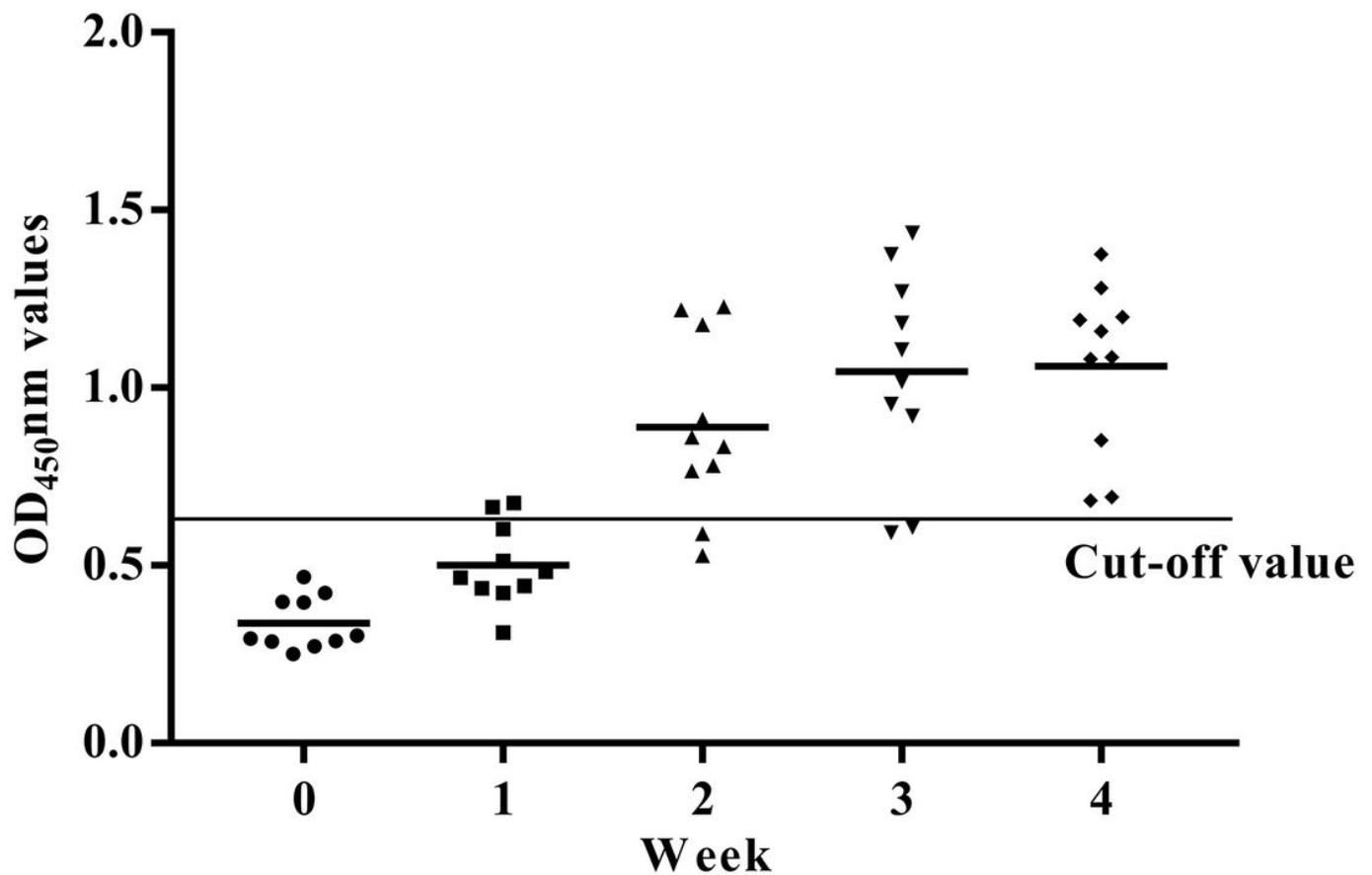


Figure 8

Serum antibody profiles detected by rPso c 27-iELISA in rabbits experimentally infected with *P. cuniculi*. The ordinate represents the OD<sub>450</sub> value of serum. The abscissa represents the serum of different infection period. The thin horizontal line represents the cut-off value (rPso c 27-iELISA: 0.633).

## Supplementary Files

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