

Vaccination with a cocktail vaccine elicits significant protection against *Sarcoptes scabiei* in rabbits, whereas the multi-epitope vaccine offers limited protection

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ABSTRACT

Sarcoptes scabiei cause scabies in humans or sarcoptic mange in animals. Currently, information regarding vaccines against *S. scabiei* is limited and no commercial vaccine is available. In present study, we expressed and mixed recombinant *S. scabiei* serpin (rSs-serpin), recombinant *S. scabiei* chitinase-like protein-5 [rSs-CLP5] and -12 [rSs-CLP12] as a cocktail vaccine (three proteins mixed), and also a multi-epitope protein derived from these three *S. scabiei* genes was expressed as a vaccine candidate to evaluate the effects of two vaccine strategies. Four test groups (n = 12 per group) and a control group (n = 12 per group) were involved in this vaccination trial. The results showed that 91.67% (11/12) and 83.33% (10/12) of rabbits exhibited no detectable skin lesions from *S. scabiei* infestation in cocktail vaccine groups, whereas two multi-epitope groups produced only a few rabbits (5/12, 6/12) having no detectable skin lesions. Four test groups displayed significant increases in specific IgG antibodies (Abs) and total IgE Abs after immunized with recombinant proteins. Taken together, our data demonstrated a mixture of rSs-serpin, rSs-CLP5 and rSs-CLP12 was a promising vaccine candidate that induced robust immune protection and could significantly decrease mite populations to reduce the direct transmission between rabbits. However, vaccination with the multi-epitope protein showed limited protection in rabbits.

1. Introduction

Sarcoptes scabiei is a burrowing mite that can cause itching, allergy, and inflammation after infestation. Severely infested individuals or animals can suffer hair loss, epidermal crusts, and chronic diseases, leading to weight loss. The disease caused by *S. scabiei* is called scabies in humans and has a long history (NA, 1949; Roncalli, 1987). Increased attention has been paid to this public disease, with scabies being included in the list of neglected tropical diseases in 2017 (Engelman et al., 2018). In developing countries (Heukelbach and Feldmeier, 2006) or indigenous peoples (McCarthy, 2004), the incidence of scabies in children is higher than that in adults. Scabies mites can infest more than 100 mammals (Pence and Ueckermann, 2002), such as pigs, cattle, goats, sheep, cats, and dogs, and other wild animals. New cases of wild species infestation are frequently reported (Francisca et al., 2018). This disease seriously threatens the health of humans and animals (Hengge

et al., 2006), and causes economic losses to modern animal husbandry and wildlife protection.

Currently, acaricides are the only way to control scabies mites; however, the use of acaricides in mass infestations is inconvenient, and scabies is prone to relapse with rapid onset after recurrence. Importantly, the continued use of acaricides will eventually lead to the development of drug resistance in *S. scabiei* and environmental pollution (Pasay et al., 2006; Currie et al., 2004; Terada et al., 2010). In addition, some acaricides have side effects (Wagner and Wendlberger, 2000). Thus, the development of vaccines against scabies is significant for the prevention and control of scabies mites.

In the past few years, research into the -omics, pathogenesis, immune regulation, disease diagnosis, and vaccines of *S. scabiei* has accelerated, providing new references for the development of a scabies mite vaccine. Infestation of scabies mites can induce the host's innate and adaptive immune responses (Cote et al., 2013; Arlian et al., 1994). Studies have

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found that immunization with a total extract of scabies mites could induce antibodies to more antigens than those produced by rabbits infested with mites (Morgan and Arlian, 1994). However, currently, scabies mites cannot be cultured in vitro, making it very difficult to obtain a large amount of scabies mites and their total extract.

Recombinant protein-based vaccines represent easily available potential vaccine candidates against scabies mite. Studies have found that after immunization with recombinant *S. scabiei* chitinase-like protein 5 (rSs-CLP5), high levels of specific antibodies could be produced and the number of infesting mites could be reduced (Shen et al., 2018). In our previous investigation, recombinant *S. scabiei* serpin (rSs-serpin) showed good immunogenicity and vaccination with rSs-serpin produced immune protection in rabbits (unpublished). Likewise, we found that rSs-CLP5 and recombinant *S. scabiei* chitinase-like protein 12 (rSs-CLP12) have potential as vaccine candidate molecules, and the rabbit's immune protection rate and the rate of mite reduction improved after immunization with a mixture of the two recombinant proteins (Shen et al., 2020). These findings suggested that a combination of different candidate vaccine molecules might increase the level of immune protection offered by the vaccine.

In the present study, we aimed to use a mixture of three vaccine candidate molecules, rSs-serpin, rSs-CLP5, and rSs-CLP12, to immunize rabbits. In addition, we aimed to produce a multi-epitope vaccine comprising a recombinant fusion protein containing epitopes of the three proteins, which was used to immunize rabbits. Evaluation of the immune protection effect of the cocktail vaccine and the multi-epitope vaccine will provide more information and new methods for research into vaccines against *S. scabiei*.

2. Material and methods

2.1. Mites and animals

In this experiment, all the scabies-free New Zealand white rabbits (*Oryctolagus cuniculus*, 3-month-old) weighing $2.3 \text{ kg} \pm 0.2 \text{ kg}$ were purchased from Chengdu Tatsuo Biological Technology Co., Ltd. (Chengdu, China). The live *S. scabiei* var. *cuniculi* mites are provided by Department of Parasitology, Sichuan Agricultural University (Chengdu, China). In these rabbits, ten (five females and five males) were infested with *S. scabiei* var. *cuniculi* for 1 month to obtain large number of live mites (adults, nymphs, and larvae) for challenge test of vaccination trial. Remaining sixty rabbits (30 females and 30 males) were prepared to complete the vaccination trial, which were housed individually in 0.3 m^2 wire cages, with ivermectin spraying, in rooms having no associated mite-animals breeding history. All the rabbits were provided with food and water *ad libitum*, and all of the rooms had good air conditioning and ventilation.

2.2. Expression and purification of recombinant proteins

For the recombinant proteins, the soluble recombinant *S. scabiei* chitinase-like protein 5 (rSs-CLP5) protein and the inclusion-body retained recombinant *S. scabiei* chitinase-like protein 12 (rSs-CLP12) protein were prepared as described previously (Shen et al., 2018; He et al., 2017). Primers (Invitrogen, Beijing, China) for *S. scabiei* serpin (*Ss-serpin*) were designed using Primer Premier 5.0 software (PREMIER Biosoft International San Francisco, CA, USA): forward 5'-CGG GAT CCT GGC CTG AAC CAT TGG-3', with a *Bam*HI restriction site (underlined), and reverse 5'-CCC TCG AGT CAA GGC TTA TTG ATC TCA-3', with a *Xho*I restriction site (underlined). *Ss-serpin* was amplified using PCR cycling conditions of 94 °C for 5 min; followed by 30 cycles of amplification at 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 45 s; with a final extension at 72 °C for 10 min. The PCR fragment was cloned into the pET-32a (+) expression vector (Invitrogen), which was transformed into *Escherichia coli* BL21 (DE3), and induced using 0.6 mM IPTG (isopropyl- β -D-thiogalactoside) at 37 °C for 8 h for protein expression.

The epitopes of Ss-serpin, Ss-CLP5, and Ss-CLP12 were predicted using the online website (<http://tools.immuneepitope.org/bcell/>) (Antibody Epitope Prediction, 2020), and the epitope-intensive areas of each encoding gene was selected for amplification. Then, the multi-fragment homologous recombination method was used to fuse the three fragments encoding the epitope-intensive areas, followed by expression of the fusion protein. The primers (Invitrogen, Beijing, China) were as follows: *Ss-serpin*, forward 5'-gcc atg gct gat atc GGA TCC ATG GGA AAA ATC AAT CAA TTT CTT C-3', with a *Bam*HI restriction site (underlined), and reverse 5'-cag aat atG ATG CAG CTG CCT CGG T-3'; *Ss-CLP5*, forward 5'-ggc agc tgc atc aTA TTC TGA TGC ATC ACG ACC CG-3', and reverse 5'-gaa CCG TCC GGA TGA TTC TTT GTA-3'; and *Ss-CLP12*, forward 5'-aag aat cat ccg gac ggT TCG ACG ACC CTA AGA AAG CTT-3'; and reverse 5'-gtg gtg gtg gtg gtg CTC GAG CTA TCA TTA ATC TTT TGA AGA GGG ATT C-3', with a *Xho*I restriction site (underlined). The amplifications were completed using PCR cycling conditions comprising 98 °C for 3 min; followed by 35 cycles of amplification at 98 °C for 10 s, 52 °C–63 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. According to the instructions of the pEA-SY®-Basic Seamless Cloning and Assembly Kit (TRANS, Beijing, China), the target fragment, the vector, and the reagent were mixed to form a connection system at a suitable concentration, and then the recombinant product was transferred into Trans1-T1 phage cells for culture. The successfully constructed recombinant plasmid was transformed into *E. coli* BL21 (DE3) and expressed. The recombinant proteins were purified using chromatography with a Ni-NTA His-tag affinity kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, as described previously (Shen et al., 2020). After the expression and purification of recombinant proteins, they were separated by 12% SDS-PAGE and checked. All proteins were stored at $-80 \text{ }^{\circ}\text{C}$ until further use.

2.3. Vaccination trial and mite challenge

For the vaccination trial, rabbits (30 females and 30 males) were immunized twice at a 14-day interval by subcutaneous injection into the neck. Rabbits were randomly divided into five groups of 12 rabbits each. The first group was injected with 1 ml 0.01 M phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4) as unvaccinated controls; the second group was immunized with 300 μg of purified mixed proteins (100 μg rSs-CLP5, 100 μg rSs-CLP12, and 100 μg rSs-serpin) with 1 ml of Quil-A saponin at a concentration of 1 mg/ml (dissolved in PBS) for the first and second immunizations, respectively; the third group was immunized using the same methods as group two as a repeat experiment; the fourth group was immunized with 300 μg of multi-epitope fusion protein with 1 ml Quil-A saponin at a concentration of 1 mg/ml (dissolved in PBS); and the fifth group was immunized using the same methods as group four as a repeat experiment.

Two weeks after the second vaccination, the 10 previously infested rabbits were euthanized by intravenous injection of 100 mg/kg barbiturate to collect the mites from the scraping skin. After *S. scabiei* mites were collected and counted, the challenge test was carried out immediately, as described previously (Shen et al., 2018, 2020). Briefly, each rabbit was challenged with approximately 2000 live mites onto the two hind limbs. The challenge area was selected because mange lesions in naturally infested rabbits are most frequently initially observed in limbs. After fixing rabbit, first cut the hair around the toes of rabbits' hind limbs, then evenly distribute mites on the skin around toes, and finally wrap the rabbit's hind limbs with gauze. Serum samples were separated from blood samples obtained with marginal ear veins (MEV) from experimental rabbits before vaccination and every week during the experiment until 4 weeks post-infestation (wpi). All sera samples were stored at $-20 \text{ }^{\circ}\text{C}$ until use.

2.4. Lesion score and mite burden

During the vaccination trial, areas that were mite-free before infestation and skin lesions of the hind legs were photographed after challenge. At the same time, the rabbits were observed for itching after the challenge test. Mange lesion areas and body weights were measured during the vaccination trial on a weekly basis. After challenge, skin lesions caused by the mites were assessed at weekly intervals from 1 to 4 wpi. The lesion areas measured using calipers. The inflammatory reaction and skin lesions were graded as follows: 0 – no inflammatory reaction; 1 – mild inflammatory reaction; 2 – severe inflammatory reaction; 3 – lesions first observed on the limbs (lesions ≤ 7.75 cm²); 4 – lesions between 7.75 and 15.5 cm² (including 15.5 cm²), and 5 – lesions between 15.5 and 31 cm² (Shen et al., 2020; Casais et al., 2014).

Four weeks after challenge, rabbits were euthanized and the hind limbs were collected and treated (hair removal and bandaging incision). Then, hind limbs were put in a 38 °C incubator, and the live mites (larvae, nymphs and adults) would crawl out from the skin and were counted total number within 12 h. After that, the per square centimeter crust from each rabbit's hind limb was collected to count the number of dead *S. scabiei* (larvae, nymphs and adults) under the microscope after 10% KOH digestion of the crusts and concentration of the mites by floating with saturated sucrose solution (Shen et al., 2018; Rodríguez-cadenas et al., 2010).

2.5. Measurement of antibody responses

In the vaccination trial, serum samples were analyzed for IgG antibodies using an rSsCLP5-based indirect enzyme-linked immunosorbent assay (ELISA), an rSsCLP12-based indirect ELISA, an rSs-serpin-based indirect ELISA, a multi-epitope protein-based indirect ELISA, and an IgE antibody ELISA kit (BioVision, Milpitas, CA, USA). The indirect ELISA procedures were performed as previously described (Shen et al., 2017, 2018; He et al., 2017) and the IgE ELISA procedures were performed according to the manufacturer's instructions.

2.6. Statistical analyses

All statistical tests were performed using SPSS 16.0 (IBM Corp., Armonk, NY, USA) and all data were analyzed using the immunized group and time as fixed factors, and the rabbit as a random factor to account for repeated measures variability. The one-sample Kolmogorov–Smirnov test was performed to determine whether the paired-difference variables of the OD 450 nm of the IgG and IgE values were normally distributed at same time point within each group. The mean $\pm$ SD of the data were presented in the appropriate sections. Duncan's Multiple range test, with the alpha value set as 0.05, was applied to compare IgG levels, IgE levels, and skin lesions scores at different time points within the same infestation group, and at the same time point between different infestation groups.

3. Results

3.1. Antigenic epitope prediction and expression of a multi-epitope fusion protein

The epitope prediction of *Ss-serpin*, *SsCLP5* and *SsCLP12* genes was performed using an online prediction algorithm. The results showed that the proteins encoded by all three genes have dense epitope regions, among which the epitope region of *SsCLP12* is longer than that of *Ss-serpin* and *SsCLP5* (Fig. 1). The regions encoding the epitope-intensive areas of each protein were amplified (*Ss-serpin*: 184–376 aa, *SsCLP5*: 129–217 aa, and *SsCLP12*: 76–312 aa) (Fig. 1). Then, the multi-fragment homologous recombination method was used to fuse the fragments encoding these three epitope-intensive areas and express the fusion protein. The regions encoding the epitope dense regions of the three

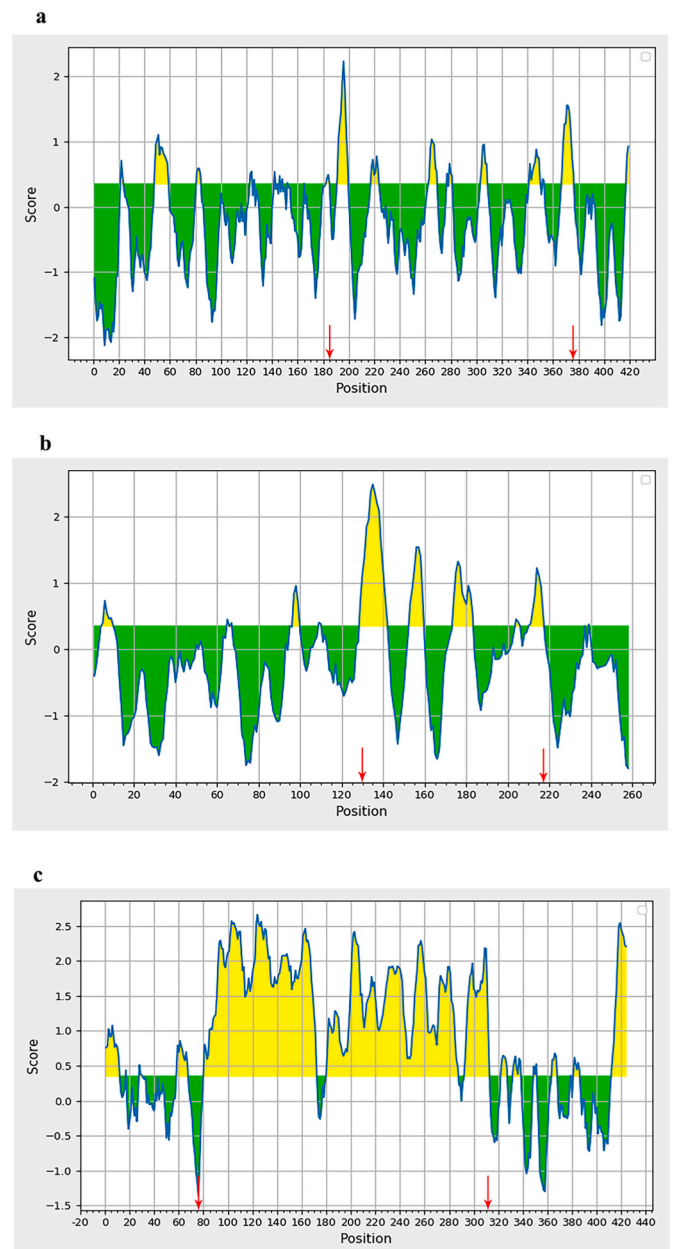


Fig. 1. The B cell epitope prediction of the proteins encoded by the *Ss-serpin* (a), *SsCLP5* (b) and *SsCLP12* (c) genes. The 'position' on horizontal coordinate represents the position of the amino acid residue, and the 'score' on vertical coordinate represents the hydrophilicity parameter score of amino acid residue. Peaks above the threshold (yellow) represent hydrophilic residues, and peaks below the threshold (green) represent hydrophobic residues. The fragments between the two red arrows represents peptide encoded by the amplified fragment of the genes (*Ss-serpin*: 184–376 aa, *SsCLP5*: 129–217 aa and *SsCLP12*: 76–312 aa).

genes were successfully connected, and the sequence is shown in Fig. 2. The recombinant protein was successfully expressed in *E. coli* BL21 (DE3) cells. Expression of the recombinant multi-fragment fusion protein was maximal after 8 h of induction with 1 mM IPTG, and the protein was expressed in inclusion bodies. The purified recombinant protein could be identified using the sera of rabbits infested with *S. scabiei*, and using anti-rSs-serpin, anti-rSsCLP5, and anti-rSsCLP12 antibodies, respectively (data not shown).

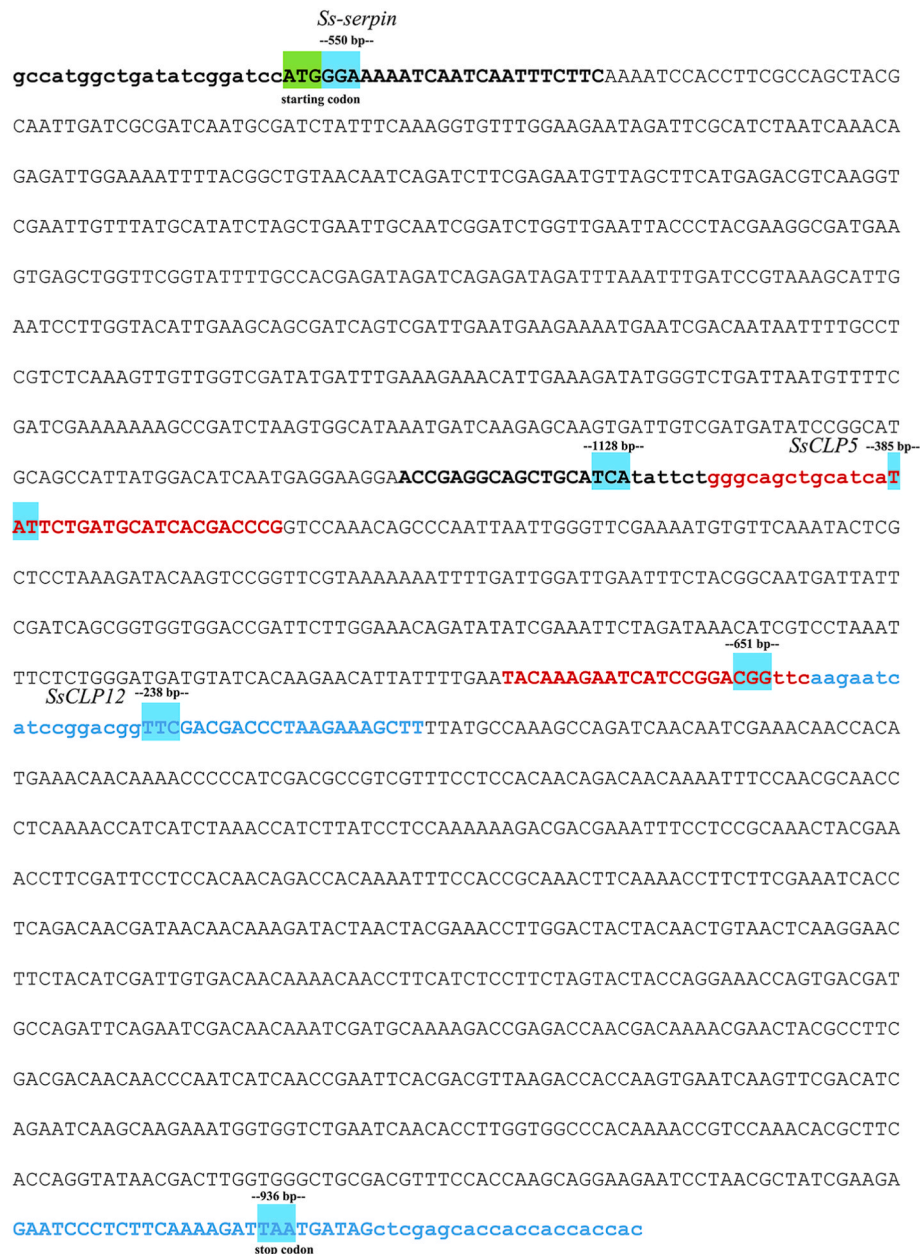


Fig. 2. Successfully linked multiple-epitope sequence of three genes. The bold uppercase letters represent the upstream and downstream primers of each gene, and the lowercase letters before and after the primers represent the flexible fragments when connected. The position of each amplified fragment of a gene refers to the length of the ORF of that gene (*Ss-serpin*: 550–1128 bp, *SsCLP5*: 385–651 bp and *SsCLP12*: 23–936 bp).

3.2. Vaccination trial and clinical monitoring

During the vaccination trial, the severities of the inflammatory reaction were recorded every week. There were significant differences between the four test groups and the control group from 1 wpi until the end of the experiment at 4 wpi ($P < 0.01$; Table 1). The itching and inflammation signs appeared at 5 day-post-infestation (dpi) in the experimental rabbit population. At 10 dpi, clinical signs of most of rabbits in the two mixed proteins (cocktail vaccine) groups become weaker or disappeared, showing no significant differences compared with the mite free rabbits. However, in the multi-epitope groups, some rabbits had obvious clinical signs, and a low percentage of rabbits were relieved of their signs at 10 dpi. As the infestation progressed, the clinical signs of scabies in the two multi-epitope groups and the control group became serious. Most of the rabbits in cocktail vaccine groups showed a healthy state, free from scabies, with 91.67% (11/12) and

83.33% (10/12) of rabbits exhibiting no detectable skin lesions of *S. scabiei* infestation, respectively. There were significantly fewer rabbits with mild scabies in the cocktail vaccine groups compared with those in the multi-epitope groups and the controls (Fig. 3).

3.3. Lesion size and mite burden

The protective effects of the vaccine candidates were also assessed by measuring the lesion area after challenge (Fig. 4). As the grades of the lesion area show, there were significant differences between the two cocktail vaccine groups, the two multi-epitope groups, and the PBS control after 4 wpi ($P < 0.01$; Fig. 4), respectively, and the differences became more apparent over the course of the infestation. The mite burden was detected as an important indicator to evaluate the effects of the candidate vaccines. Large differences in the average number of mites were shown, with more than 4000 mites in the PBS control group and

Table 1

Mean mange lesion scores at different times after rabbits were challenged with *S. scabiei*.

	Before challenge	Week 1 after challenge	Week 2 after challenge	Week 3 after challenge	Week 4 after challenge
group 1 (PBS) (n = 12)	0	1.92 ± 0.90 ^a	1.92 ± 0.79 ^a	2.83 ± 1.03 ^a	4.33 ± 0.65 ^a
group 2 (Cocktail vaccine) (n = 12)	0	0.42 ± 0.67 ^b	0.42 ± 0.67 ^b	0.42 ± 0.79 ^b	0.50 ± 0.80 ^b
group 3 (Cocktail vaccine') (n = 12)	0	0.58 ± 0.90 ^b	0.50 ± 0.67 ^b	0.58 ± 1.00 ^b	0.83 ± 1.03 ^b
group 4 (Multi-epitope group) (n = 12)	0	0.58 ± 0.67 ^b	0.75 ± 0.87 ^a	2.00 ± 1.28 ^b	2.50 ± 0.67 ^b
group 5 (Multi-epitope group') (n = 12)	0	0.33 ± 0.49 ^b	0.83 ± 0.83 ^a	2.17 ± 1.11 ^b	2.67 ± 0.49 ^b

Note: Values within a row for the same group followed by different subscript lowercase letters were significantly different compared with different weeks-post-infestation (wpi) ($p < 0.05$), while the same subscript lowercase letters were not significantly different ($p > 0.05$). Values within a column for different groups followed by different superscript lowercase letters were significantly different compared with the same wpi ($p < 0.05$), while the same superscript lowercase letters were not significantly different ($p > 0.05$). The results of the mange lesion scores are expressed as the mean ± standard deviation (SD). PBS, phosphate-buffered saline.

more than 2000 mites in the multi-epitope groups. However, 669 mites and 829 mites were recorded in cocktail vaccine groups, respectively (Table 2). In particular, the mite reduction rates were 84.84% and 81.21% in the cocktail vaccine groups, whereas in the two multi-epitope protein groups, the rate was less than 50% (Table 2).

3.4. Antibody responses

The results of specific IgG antibody and total IgE antibody responses are shown in Figs. 5 and 6, respectively. Before immunization, the antibodies levels were low (<0.5 for IgG OD and <300 pg/ml for IgE) in all rabbits. After the vaccination, most of rabbits in test groups developed specific serum IgG and total IgE antibodies, and the levels were significantly higher than those of the PBS control group ($P < 0.01$; Figs. 5 and

6). A significant increase in IgG levels was first detected at one week after immunization, which increased strongly and peaked at two weeks after the last vaccination, after which the OD values levelled to a plateau. A significant increase in the IgE level was also first detected at one week after second immunization and then increased progressively. The total IgE level showed a rapid and strong increase in the first two weeks and reached its highest value at 2 weeks after immunization. Specific IgG levels were not observed in the PBS control group during the trial. The IgE antibodies levels observed in the PBS group increased after infestation; however, the levels were lower than in the four test groups, respectively ($P < 0.01$; Fig. 6).

4. Discussion

Scabies is a serious threat to the health of animals and humans (Hengge et al., 2006; Alasaad et al., 2013) and is a neglected tropical disease. An effective vaccine will greatly reduce the problems and losses caused by this disease. However, although a vaccine against *S. scabiei* mites is highly necessary, the development of an anti-mite vaccine is difficult.

Chitinase-like proteins (CLPs) are homologous to chitinases, but lack the ability to degrade chitin (Nair et al., 2005), and are mediators related to infection, T cell-mediated inflammation, wound healing, allergy, and asthma (Sutherland et al., 2009). In previous studies, we found that two CLPs from scabies mites could induce immune protection in rabbits (Shen et al., 2018, 2020). Serine protease inhibitors (serpins) play important roles in various of fundamental physiological functions in arthropods (Meekins et al., 2017) and are involved in the host-pathogen interaction (Jialing et al., 2018). Vaccination of cattle with a combination of rRAS-1 and -2 (serpins) conferred significant protective immunity against ticks, resulting in a 61.4% reduction in the nymph engorgement rate (Imamura et al., 2006). In addition, rabbits immunized with another serpin, rHLS2, acquired immune protection against ticks, with 44.6% and 43.0% mortality against nymphal and adult ticks, respectively (Imamura et al., 2005). We expressed the multi-epitopes of three proteins (Ss-serpin, SsCLP5, and SsCLP12) based on our previous studies, and used a mixture of rSs-serpin, rSsCLP5, and rSsCLP12 to immunize rabbits, and constructed a multi-epitope fusion protein to immunize rabbits to evaluate the immune protective effects of the two different immune components.

Throughout the development of vaccines against scabies mites, a variety of studies have been published, including those reporting whole body extract vaccines (Morgan and Arlian, 1994; Tarigan and Huntley, 2005), a DNA vaccine (Gu et al., 2014), and recombinant protein vaccines (Casais et al., 2016; Harumal et al., 2003). Recombinant subunit vaccines are promising candidates and have been used successfully in some fields, such as the EG95 vaccine against hydatid disease (Lightowlers et al., 1999; Larrieu et al., 2013), a cap vaccine for porcine

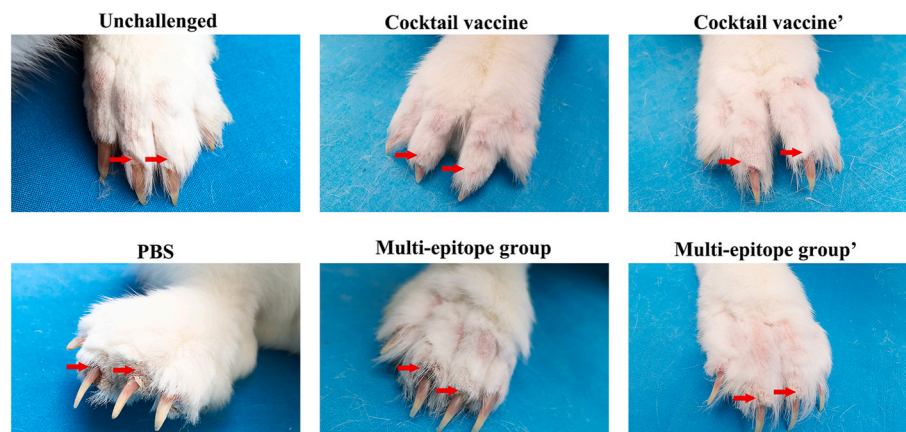


Fig. 3. Clinical signs in rabbits at 4 wpi. Unchallenged, the rabbits were free of mite infestation; PBS, vaccinated with 1 mL 0.01 M PBS each time; cocktail vaccine and cocktail vaccine' refer to the test groups immunized with 300 µg of purified mixed proteins (100 µg rSsCLP5, 100 µg rSsCLP12, and 100 µg rSs-serpin) with 1 mL Quil-A saponin at a concentration of 1 mg/mL (dissolved in PBS) for the first and second immunizations, respectively; multi-epitope group and multi-epitope group' refer to the test groups immunized with 300 µg of multi-epitope fusion protein with 1 mL Quil-A saponin at a concentration of 1 mg/mL (dissolved in PBS) for the first and second immunizations, respectively.

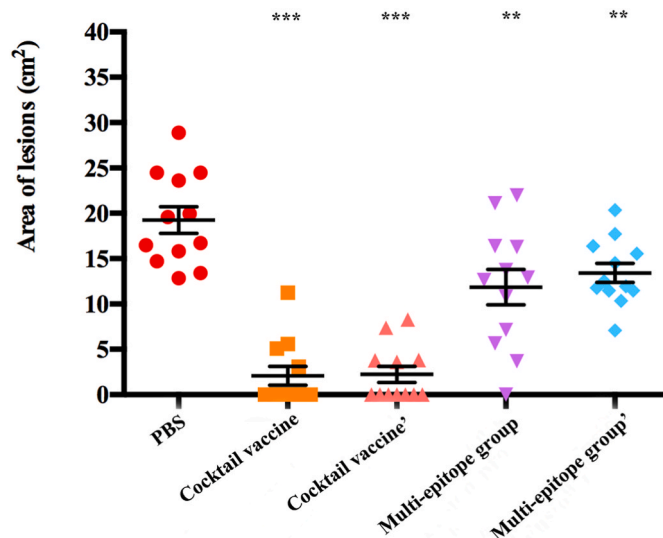


Fig. 4. Lesion areas of the rabbit hind limbs at 4 wpi. PBS, vaccinated with 1 mL 0.01 M PBS each time; cocktail vaccine and cocktail vaccine' refer to the test groups immunized with 300 μ g of purified mixed proteins (100 μ g rSsCLP5, 100 μ g rSsCLP12, and 100 μ g rSs-serpin) with 1 mL Quil-A saponin at a concentration of 1 mg/mL (dissolved in PBS) for the first and second immunizations, respectively; multi-epitope group and multi-epitope group' refer to the test groups immunized with 300 μ g of multi-epitope fusion protein with 1 mL Quil-A saponin at a concentration of 1 mg/mL (dissolved in PBS) for the first and second immunizations, respectively. The lesion areas in the two cocktail vaccine groups were significantly lower than those in the PBS control and two multi-epitope vaccine groups ($***P < 0.001$), respectively. The lesion areas in the two multi-epitope vaccine groups were significantly lower than that in the PBS control ($**P < 0.01$).

Table 2

Mean mange mite burden at 4 weeks after challenge and the mite reduction rate of the rabbits.

	Number of mites	Reduction rate of mites
group 1 (PBS) (n = 12)	4413 $\pm$ 1529.79a	–
group 2 (Cocktail vaccine) (n = 12)	669 $\pm$ 831.74b	84.84%
group 3 (Cocktail vaccine') (n = 12)	829 $\pm$ 572.67b	81.21%
group 4 (Multi-epitope group) (n = 12)	2224 $\pm$ 1541.86b	49.60%
group 5 (Multi-epitope group') (n = 12)	2496 $\pm$ 1331.64b	43.44%

Note: Values within the column of number of mites for the different groups followed by different lowercase letters were significantly different ($p < 0.05$), while the same lowercase letters were not significantly different ($p > 0.05$). The results of the number of mites are expressed as the mean $\pm$ standard deviation (SD). PBS, phosphate-buffered saline.

circovirus (Seo et al., 2014), and a VP1 vaccine used in porcine FMDV. Scabies mite infestation induces protective immune responses (Casais et al., 2014; Bhat et al., 2017), suggesting that it is possible to develop a recombinant vaccine against scabies mites. In addition, rabbit vaccination with rSsCLP5 or rSsCLP12 produced immune protection effects, resulting in 75% and 70% rabbits having no detectable crusts, respectively (Shen et al., 2020). Moreover, the results showed that rSs-serpin had good immunogenicity and vaccination with rSs-serpin produced immune protection in our previous investigation, and it demonstrated that rSs-serpin was a promising candidate for a recombinant protein-based vaccine against *S. scabiei* (Supplementary information). By contrast, studies showed that goats immunized with a scabies mite extract experienced no protective effect (Tarigan and Huntley, 2005). Although rabbits produced high levels of antibodies after vaccination

with Ssag1 and Ssag2, no immune protection from *S. scabiei* challenge and no mite burden reduction were observed (Harumal et al., 2003). Vaccination providing no protection might be related to the specific antibodies induced by the recombinant protein. Specific antibodies induced by different recombinant proteins will show different protective effects, thus some could induce host immune protection but some could not (Casais et al., 2016; Zhang et al., 2012).

We previously found that the chitin binding domain containing protein (a kind of mite gut lining protein) could bound to the antibody IgE and the biological activity of the protein would be affected in vitro (unpublished). Moreover, the level of IgE antibodies plays an important role in the host's resistance against pathogen infection (Roberts et al., 2005; Arlian et al., 2015), such as bacteria, viruses, and parasites (Hagan et al., 1991; Nyindo et al., 1999). Immunization with each fraction of *S. scabiei* proteins invoked high levels of specific IgG, but failed to induce specific IgE, and the immunized goats failed to resist challenge with *S. scabiei* (Tarigan and Huntley, 2005). By contrast, *S. scabiei* re-infestation in the goats induced a strong IgE response and resistance to mite re-challenge, which suggested that IgE is related to, and is involved in, the development of resistance against scabies mites (Rodríguezcadenas et al., 2010; Tarigan and Huntley, 2005). In the present study, the induced IgG and IgE produced immune protection against mites, suggesting that the immune-protective may be related to the coordination of IgG and IgE, rather than a separate or minor effect of a single antibody subtype. Likewise, vaccination with rSsCLP5 and rSsCLP12 produced high levels of IgG and IgE antibodies in rabbits, resulting in immune protection effects and a reduced mite burden (Shen et al., 2020). Overall, these data indicate that IgE is not only involved in hosts' defence to *S. scabiei*, but is very important to host against mites.

Cocktail vaccines have been developed in recent years as a new recombinant subunit vaccine. For instance, some types of cocktail vaccines induced immune protection responses in cattle against ticks (Imamura et al., 2006, 2008), in dogs against hydatid disease (Petavy et al., 2008; Zhang et al., 2018), and in mice against *Mycoplasma hyopneumoniae* infection (Galli et al., 2013). Sheep were immunized with a cocktail vaccine of seven recombinant proteins from the sheep scab mite, *Psoroptes ovis*, resulting in highly significant reductions in both lesion size (up to 63%) and mite burden (up to 56%) (Burgess et al., 2016). In our previous study, the combined use of rSsCLP5 and rSsCLP12 could improve the immune protection in experimental rabbits (Shen et al., 2020). These results indicated that the combined use of multiple cocktail vaccine candidates has the potential to induce strong immune protection after vaccination. In the present study, the cocktail vaccine containing three vaccine candidates and the multi-epitope fusion vaccine were used to immunize rabbits. At 1 wpi, there were no significant differences in scores among the four test groups; however, from 2 wpi, the cocktail vaccine group and the multi-epitope fusion vaccine group showed significant differences ($P < 0.01$). As the challenge progressed, the results showed that 91.67% (11/12) and 83.33% (10/12) of the experimental rabbits in two cocktail vaccine groups had no clinical signs of scabies after challenge, and the immune protection rate was higher than that of the three recombinant proteins when used separately. There was no significant difference between one of the cocktail vaccine (three proteins) groups and the reported combined use of rSsCLP5 and rSsCLP12 (Shen et al., 2020); however, the protection rate of the other cocktail vaccine (three proteins) group was 91.67%, which was higher than the effects of combined use of rSsCLP5 and rSsCLP12 (Shen et al., 2020).

The immunogenicity of a protein is closely related to its epitopes. The multi-epitope recombinant plasmid pET30a-EgA31-Eg95 was successfully constructed and recombinant protein EgA31-Eg95 was expressed, which provided the basis for research on its immunogenicity and as a candidate vaccine (Li et al., 2014). In view of this, we fused the gene fragments of the epitope-intensive regions of three proteins and expressed it as multi-epitope fusion vaccine. The results showed most rabbits had clinical signs of scabies and the immune protection effect was lower than that of combined use of three proteins, although the

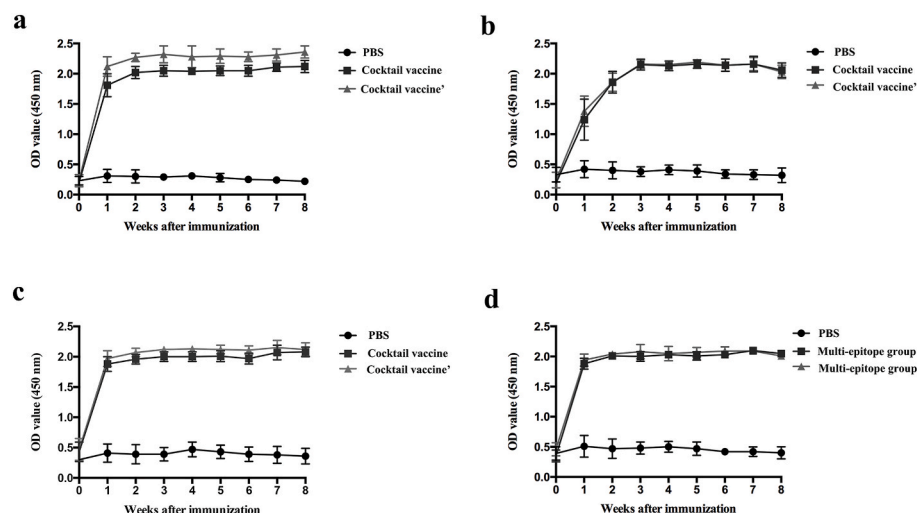


Fig. 5. Variation in specific IgG levels in the sera of immunized rabbits. a: The OD 450 nm value of the specific IgG antibodies was detected using rSs-serpin-based indirect ELISA; b: The OD 450 nm value of the specific IgG antibodies was detected using rSsCLP5-based indirect ELISA; c: The OD 450 nm value of specific IgG antibodies was detected using rSsCLP12-based indirect ELISA; and d: The OD 450 nm value of specific IgG antibodies was detected using multi-epitope-based indirect ELISA.

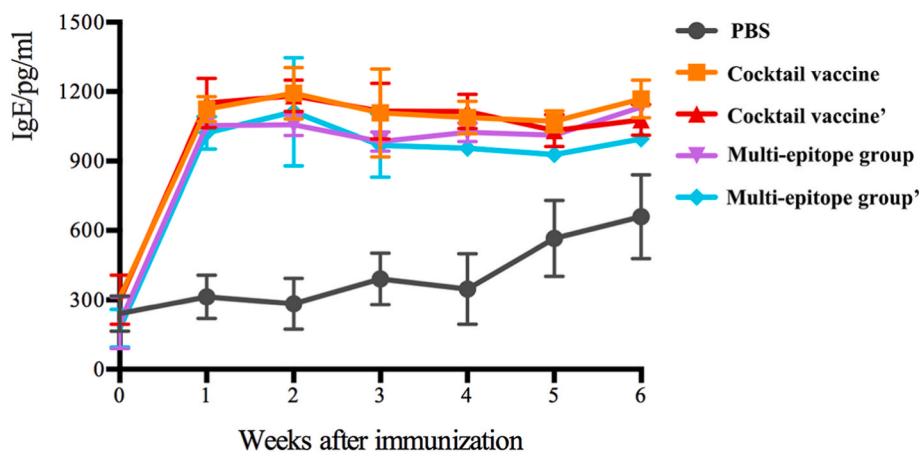


Fig. 6. Variation in total IgE levels in the sera of immunized rabbits. Testing time points: before immunization, one week and two weeks after the secondary immunization, and every week after the challenge until the fourth week. The concentration of IgE (pg/mL) was detected using an ELISA kit.

course of the disease was slower and clinical signs were milder than those in the PBS control group. The multi-epitope recombinant protein induced antibody, but did not produce good protective effect. The results might be related to the multi-epitope fusion protein was expressed by inclusion body, and the inactive protein could not induce protective antibodies. In the further investigation, these works such as optimizing the sequence of fusion protein and selecting different expression systems to express soluble active protein both need to be improved. A multi-epitope vaccine was identified as a promising vaccine candidate that could induce an immune protection effect in mice against *Helicobacter pylori* (Zhou et al., 2009). Similarly, a multi-epitope vaccine showed high immunogenicity that induced high titers of specific antibodies in mice and induce immune protection against group A streptococcus (Ma et al., 2014). Unfortunately, the multi-epitope vaccine in this study did not induce a strong immune protection; however, vaccination with the cocktail vaccine (three proteins) could produce high levels of specific IgG and total IgE antibodies, which induced strong immune protection against *S. scabiei* in the rabbits. For further vaccine research against scabies, the choice of a suitable adjuvant is very important because the antigen epitope of the recombinant protein is single and the combined use of recombinant protein and adjuvant could induce stronger immune protection. In addition, screening of vaccine candidates, molecular assembly, vaccine optimization and immune dose of fusion protein will be very important to improve immune protection effect of scabies vaccines.

5. Conclusion

Together, our data demonstrated that the mixture comprising rSsCLP5, rSsCLP12, and rSs-serpin is a promising candidate for a recombinant protein-based vaccine against *S. scabiei*. The cocktail vaccine candidate induced robust immune protection and could significantly decrease mite populations to reduce the direct transmission between rabbits. However, vaccination with the multi-epitope protein showed limited protection in rabbits.

Ethics approval and consent to participate

The animal study was reviewed and approved by the Animal Care and Use Committee of Sichuan Agricultural University (SYXK, 2014-187). All animal procedures used in this study were carried out in accordance with Guide for the Care and Use of Laboratory Animals (National Research Council, Bethesda, MD, USA) and the recommendations of the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (<http://www.nc3rs.org.uk/arrive-guidelines>). All applicable institutional and/or national guidelines for the care and use of animals were followed.

Consent for publication

Not applicable.

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Contributions

SNX participated in the design of the study, feeding the experimental animals, the experiments, statistical analysis, and manuscript writing. WWR and CYH fed the experimental animals and performed the experiments. LS, XL and XJ contributed to sample collection and performed the experiments. YGY participated in the design of the study. GXB, XY, XJ, JB and PXR helped with the study design. All authors have read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest. All authors have approved the submission of this manuscript. The results have not been previously published and are not being considered for publication in another journal.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exppara.2022.108442>.

Abbreviations

SsCLP5	<i>S. scabiei</i> chitinase-like protein 5
rSsCLP5	recombinant <i>S. scabiei</i> chitinase-like protein 5
SsCLP12	<i>S. scabiei</i> chitinase-like protein 12
rSsCLP12	recombinant <i>S. scabiei</i> chitinase-like protein 12
CLPs	chitinase-like proteins
Ss-serpin	<i>S. scabiei</i> serpin
rSs-serpin	recombinant <i>S. scabiei</i> serpin
IPTG	isopropyl-beta-D-thiogalactopyranoside;
ELISA	enzyme-linked immunosorbent assay
PBS	phosphate-buffered saline;
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
dpi	day-post-infestation; wpi: week-post-infestation

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