

# ***Canis familiaris* allergen Can f 7: Expression, purification and analysis of B cell epitopes in Chinese children with dog allergies**

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**Abstract.** Dogs are a major source of indoor allergens. However, the prevalence of dog allergies in China remains unclear, especially in children. In the present study, Can f 7, a canine allergen belonging to the Niemann pick type C2 protein family, was selected to study its sensitization rate in Chinese children with dog allergies. The Can f 7 gene was subcloned into a pET-28a vector and expressed in *Escherichia coli* BL21 (DE3) cells. Recombinant Can f 7 was purified by nickel affinity chromatography, identified by SDS-PAGE electrophoresis, and had its allergenicity assessed by western blot, ELISA and basophil activation tests. Through a series of bioinformatical approaches, B-cell epitopes, secondary structures, and 3 dimensional (3D) homology modeling of Can f 7 were predicted. The activity of the B cell epitopes was verified by ELISA. The recombinant Can f 7 showed a distinct band with a molecular weight of 14 kDa. Six of 20 sera from dog-allergic children reacted positively to the Can f 7. Can f 7 induced an ~4.0-fold increase in cluster of differentiation 63 and C-C motif chemokine receptor R3 expression in basophils sensitized

with the serum of dog-allergic children compared with those of non-allergic controls. The secondary structure analysis showed that Can f 7 contains 6  $\beta$ -sheets. Five B cell epitopes of Can f 7 were predicted, and two of these were confirmed by ELISA. These results indicate that Can f 7 is an important canine allergen in Chinese children and provide novel data for further research concerning the use of Can f 7 in the diagnosis and treatment of Chinese children with canine allergy symptoms.

## **Introduction**

Immunoglobulin (Ig)E-associated inhalational allergies are the most common allergy diseases, affecting >25% of the world's population. Allergy symptoms include asthma, rhinitis, eczema and even life-threatening systemic allergic reaction. Allergies to furred animals, especially dogs and cats, are key factors in the development of rhinitis and asthma (1-3). Dogs are considered family pets worldwide. However, even if there is no dog in a person's house, canine allergens can also be found in schools and other public places as they can be carried on clothes, making them almost impossible to avoid. Canine allergens can cause symptoms ranging from rhinoconjunctivitis to severe asthma attacks (4,5). In one large study conducted in a European population, 27% of people with suspected allergic disease had a positive skin prick test when exposed to canine extract (6). In a population-based cross-sectional study in Germany, up to 9% of children and adolescents were canine dandruff-susceptible, consistent with data obtained from the Swedish birth cohort BAMSE (7,8).

To date, seven dog allergens have been identified (www.allergen.org). Among these allergens, Can f 1 is a lipocalin, which is secreted from canine sebaceous glands and is found in dog hair and dander as well as saliva; it is considered a major canine allergen (9). Additionally, other known allergens include albumin (Can f 3) (10), prostatic kallikrein (Can f 5) (11) and lipocalin [Can f 2 (12), Can f 4 (13) and Can f 6 (14)]; these show various molecular weights ranging from 16 to 69 kDa. Can f 7 was initially identified from canine

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allergen testing extracts by sequencing a distinct protein from Coomassie-stained 2 dimension (2D)-PAGE gels. It belongs to the Niemann pick type C2 protein family and reportedly shows 16.9% sensitivity in dog-sensitized subjects (15). With the rapid increase in the number of pet dogs, several typical allergic diseases caused by dog allergens, such as asthma, atopic dermatitis and eczema, are becoming increasingly frequent in China (16). Li *et al* (17) discovered that, among 6,304 patients aged 5-65 years in China, the prevalence of positive skin prick responses was 14.0% for dog extract. In addition, given the population's frequent contact with dogs, the incidence of allergic diseases in children is increasing. Among 153 recruited Chinese children with infant food protein allergies, 114 (74.51%) manifested skin symptoms such as atopic dermatitis or urticaria, and 28 (18.30%) were presented with early respiratory symptoms (18). However, except for Can f 6 (3), there have been few studies regarding specific dog allergens in China, particularly for Can f 7, although these may seriously impact children's health.

Identification of an allergen's 3D structure can be used to understand the body's immune response to allergenic proteins, including cross-reactivity between allergens (19). Furthermore, predicting the secondary structure of a protein from its amino acid sequence is an important step in predicting its 3D structure (20). B cell epitopes show fundamental differences depending on the location of the allergen molecule and the subsequent initiation of the immune response. B cell epitopes can be linear or conformational, and they are usually located on the surface of allergens for easy binding to antibodies. The primary purpose of epitope prediction is to design and propose hypoallergenic molecules that can replace crude allergen extracts. Therefore, identification of B cell epitopes in Can f 7 may aid the design of novel therapeutic modalities and diagnostic tests for canine allergies (21,22). Overall, these results could provide critical information regarding the distribution and characteristics of canine allergies and provide a foundation for improving the diagnosis and treatment of canine allergies in Chinese children.

## Patients and methods

**Patients and samples.** Twenty pediatric patients with positive skin prick test results (allergens were supplied by ALK-Abelló, Inc., Hørsholm, Denmark) and positive serum IgE test results to dog extracts [via ImmunoCAP assay (Pharmacia Diagnostics AB, Uppsala, Sweden) as described previously (23)], as well as six healthy children, were recruited for the present study. Clinical information for these patients, including specific IgE levels, is shown in Table I. Serum samples (4 ml) were collected from peripheral venous blood from each patient and healthy participant after centrifugation at 2,000 x g for 10 min at 4°C. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (2015-SRFA-055). According to the Declaration of Helsinki, all participants written consent was obtained from their parents or legal guardians for the use of their blood samples prior to admission.

**Expression and purification of Can f 7 in *Escherichia coli* (*E. coli*).** Can f 7 has 450 base pairs and encodes 149

Table I. Information of participants.

No.	Sex	Age	ImmunoCAP (KU/L)	Associated disease
1	Female	1	0.96	Asthma
2	Female	1	59.6	Asthma
3	Male	2	2.16	Pollinosis
4	Female	3	91.7	Asthma, rhinitis
5	Female	4	5.61	Rhinitis
6	Female	5	13.8	Asthma, rhinitis
7	Female	6	21.1	Rhinitis
8	Male	6	56.1	Asthma, rhinitis
9	Male	7	31.3	Rhinitis
10	Male	7	18.4	Asthma
11	Female	7	9.85	Asthma, rhinitis
12	Female	8	24.5	Cough
13	Female	8	>100	Asthma
14	Male	9	17.3	Rhinitis
15	Male	9	63.6	Asthma
16	Female	10	77.4	Asthma
17	Female	10	20.2	Dermatitis
18	Female	11	13.9	Asthma, rhinitis
19	Female	12	8.57	Asthma, rhinitis
20	Female	12	36.6	Rhinitis
N1	Female	9	0.37	None
N2	Male	11	0.57	None
N3	Male	11	0.48	None
N4	Male	14	0.22	None
N5	Female	16	0.32	None
N6	Male	16	0.44	None

amino acids (GenBank accession no. 945178). The Can f 7 gene was synthesized (GenScript, Nanjing, China) using the GenScript rare codon analysis tool ([www.genscript.com/tools/rare-codon-analysis/](http://www.genscript.com/tools/rare-codon-analysis/)), optimized on the basis of constant amino acid sequences, subcloned into the pET-28a vector via *Nco*I and *Xho*I sites and verified by DNA sequencing (24).

The pET28a-Can f 7 plasmid was transformed into BL21 (DE3) pLysS *E. coli* cells. Positive colonies were selected and incubated overnight at 37°C. All broths were inoculated into 200 ml of LB-Kanamycin broth and cultured with shaking at 37°C until the optical density value at 600 nm reached 0.6-0.8. Isopropyl-β-D-thiogalactopyranoside (Takara Bio, Inc., Otsu, Japan) was added to a final concentration of 0.5 mM in the broth and incubated for a further 16 h. Bacterial cells were pelleted by centrifugation at 2,000 x g for 30 min at 4°C, and the pellet was collected. The pellet was lysed in lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0) by sonication with 50 short bursts of 10 sec at 100 W and a 10 sec cooling period between each burst. The lysate was centrifuged at 13,523 x g for 30 min at 4°C. As recombinant Can f 7 is mainly found in inclusion bodies, inclusion bodies were harvested by centrifugation at 13,523 x g for 20 min at 4°C. After solubilizing the inclusion bodies with 8 M urea,

the supernatant was applied to High Affinity Ni-NTA Resin (GenScript, Nanjing, China), then the resin was washed with a buffer containing 20 mM Tris-HCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM imidazole, and 8 M urea, and eluted with a buffer containing 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM imidazole, and 8 M urea, pH 8.0 (25). Eluted fractions were collected and reduced overnight by adding a final concentration of 50 mM dithiothreitol. Refolding was performed via 1:50 dilution of the denatured protein in a redox refolding buffer (0.05 mg/ml final concentration) consisting of 0.1 M Tris, pH 8.5, 0.5 M L-arginine, 1 mM EDTA, 5 mM reduced glutathione (GSH) and 1 mM oxidized glutathione (GSSG) for 48 h at 15°C. The refolded rCan f 7 was dialyzed against PBS (pH 7.4), and the supernatant was concentrated using Amicon® Ultra 15 ml Centrifugal Filters (MWCO 3 kDa; Merck KGaA, Darmstadt, Germany), the final concentration was 0.3 mg/ml.

**Circular dichroism (CD) analysis of rCan f 7 expressed in *E. coli*.** CD analysis of rCan f 7 was carried out using a Chirascan CD spectrometer (Applied Photophysics Ltd., Surrey, UK). To record far ultra violet CD (200–250 nm) spectra, 0.1 mg/ml refolded Can f 7 in PBS was analyzed in a 10-mm path-length quartz cuvette at a 1 nm bandwidth and 0.5 sec per point. The final spectra were averaged from three consecutive scans and baseline-corrected by subtracting a control PBS spectrum. The results were expressed as the mean residue ellipticity in deg·cm<sup>2</sup> × dmol<sup>-1</sup> and analyzed using the K2D3 program (cbdm-01.zdv.uni-mainz.de/~andrade/k2d3/) (26).

**IgE binding activity of Can f 7 identified by western blot analysis.** Western blot analysis was used to detect the IgE-binding activity of Can f 7. Recombinant Can f 7 (2 µg) was separated by 13% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany). The PVDF membrane was then blocked with 5% skim milk for 2 h at room temperature and subsequently incubated with sera from 20 children with dog allergies [diluted 1:20 in Tris-buffered saline Tween-20 (TBST)] as the primary antibody and incubated overnight at 4°C. After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgE monoclonal antibody (cat. no. 074-1004; KPL, Inc., Gaithersburg, MD, USA) diluted 1:5,000 in TBST for 1 h at room temperature. Positive protein bands were visualized by incubating the membrane with Immobilon™ Western HRP Substrate Luminol Reagent (Merck KGaA). Six healthy children's sera were used as negative controls and PBST were used as a blank control.

**Immunoreactivity of human sera with recombinant Can f 7 by ELISA.** A 96-well microplate (Corning Inc., NY, USA) was coated with 100 µl 10 µg/ml recombinant Can f 7 in PBS and incubated at 4°C overnight. The wells of the plate were then blocked with bovine serum albumin (BSA; Biosharp, Hefei, China; 1 mg/ml; 200 µl/well) for 1 h at 25°C and washed once with PBS-0.05% Tween-20 (PBST). Subsequently, 100 µl of the 20 Can f 7-allergic children's sera (1:10 dilution in PBST with 0.1% BSA) was incubated for 2 h at 25°C. After incubation, the wells were washed three times with PBST and incubated with HRP-conjugated

goat anti-human IgE monoclonal antibody (1:2,500) for 1 h at 25°C. Subsequently, the wells of the plate were washed and incubated with 100 µl Tetramethylbenzidine (TMB) substrate solution (Nanjing KeyGEN Biotech Co., Ltd., Nanjing, China). After incubating for 30 min at room temperature, the reaction was stopped by adding 50 µl 2 M H<sub>2</sub>SO<sub>4</sub> in the wells. The absorbance value was then read at 450 nm on a Eon microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA; www.biotek.com/). Sera from six healthy subjects were used as control normal human serum (NHS). Absorbance values were regarded as positive if they have statistical significance compared to the mean value of the healthy controls.

**Basophil activation analysis.** Allergens induce protein expression levels of the Cluster of differentiation (CD)63 and the C-C motif chemokine receptor (CCR)3 on basophils which is considered an indicator of basophil activation (27). The ability of rCan f 7 to activate basophils in a modified basophil activation test, was examined (28–31). Briefly, a total of 4.3×10<sup>7</sup> peripheral blood mononuclear cells were obtained from 20 ml of blood collected from healthy individuals using a Ficoll-Paque density gradient (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. Then, the cells were incubated with 10 ml of lactic acid buffer (containing 1.3 M NaCl, 0.005 M KCl and 0.01 M lactic acid, pH 3.9) for 2 min on ice. After neutralization with 12% Tris (pH 10.9), nonspecific IgE on basophils was removed. The cells were washed and aliquots of a cell suspension were passively sensitized by incubation in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% patient serum for 2 h at 37°C. The six serum groups used in this test consisted of six patients' sera (No. 2, No. 4, No. 6, No. 13, No. 17 and No. 20 in Table I) and six normal sera. The cells were then incubated with Can f 7 (1.0 µg/ml) for 15 min at 37°C. HRP-conjugated goat anti-human IgE antibody (1.0 µg/ml) was used as a positive control. After incubation, the cells were washed and resuspended in 100 µl stain buffer, then 20 µl of anti-human CD63-FITC antibody (cat. no. MA1-19602; Invitrogen, Camarillo, CA, USA) and 5 µl of CCR3-PE-labeled antibody (cat. no. A18365; eBioscience, Inc., San Diego, CA, USA) were added to the cells for 15 min at 37°C in the dark. Surface-labeled flow cytometric analysis was performed on a FACS Aria flow cytometer at 488 nm and analyzed with FACSDiva software v.6.1.3 (both BD Biosciences, Franklin Lakes, NJ, USA) (32).

**Structure modeling and B cell epitopes prediction.** A structural model of Can f 7 protein was generated by aligning patterns in SWISS-MODEL (swissmodel.expasy.org/interactive) (33). Briefly, the amino acid sequences of Can f 7 were used to search the homologous template. A template for Can f 7 was selected from the SWISS-MODEL server (34). TMFMM 2.0 software (<http://www.cbs.dtu.dk/services/TMHMM/>) can predict transmembrane helices of Can f 7 (35). The secondary structure of Can f 7 ( $\alpha$ -helix,  $\beta$ -sheet, and random coil) was predicted using the PyMOL molecular graphics system (pymol.org/2/) (36).

Using the DNASTar (www.dnastar.com/) protean system, the BepiPred 2.0 server (www.cbs.dtu.dk/services/BepiPred/) and the BPAP system (imed.med.ucm.es/Tools/antigenic.

1		60
After	ATGCGTCTGCTGGTTGCTGCTTTCTGCTGCTGGCTCTGGGTGACCTGGGTCCGGGTGGT	
Before	ATGCGTCTCTTGGTCGCCGCGTTCTGCTCCTGGCGCTCGGCACCTCGGCCCTGGCGGA	
	M R L L V A A F L L L A L G D L G P G G	
61		120
After	GCTGTCACTTCAAAGACTGCGGTTCTGCTGTTGGTGTATCAAAGAACTGAACGTTAAC	
Before	GCGGTGCATTTCAGGACTGTGGTTCTGCAGTTGGAGTTATAAAAGAACTGAATGTGAAC	
	A V H F K D C G S A V G V I K E L N V N	
121		180
After	CCGTGCCCCGCTCAGCCGTGCAAAGTGCACAAAGGTGAGTCTTACTCTGTAACTGTTACC	
Before	CCATGCCCTGCCAGCCTTGCAAAGTGCACAAAGGCCAATCTTACAGTGTCAATGTCACC	
	P C P A Q P C K L H K G Q S Y S V N V T	
181		240
After	TTCACCTCTAACATCCCGTCTCAGTCTTCTAAAGCTGTTGTTACGGTATCGTTCTGGGT	
Before	TTCACCAGTAATATTCCATCTCAAAGTAGCAAAGCCGTGGTGCATGGCATCGTGTGGGC	
	F T S N I P S Q S S K A V V H G I V L G	
241		300
After	GTTGCTGTTCCGTTCCCGATCCCGGAAGCTGACGGTTGCAAATCTGGTATCAACTGCCCG	
Before	GTCGCAGTTCCTTTCCATTCTGAGGCTGATGGTTGTAAGAGTGAATCAACTGCCCC	
	V A V P F P I P E A D G C K S G I N C P	
301		360
After	ATCCAGAAAGATAAGACCTACAGCTACCTGAACAAACTGCCAGTGAAGAACGAATACCCC	
Before	ATCCAGAAAGATAAGACCTACAGCTACCTGAACAAACTGCCAGTGAAGAACGAATACCCC	
	I Q K D K T Y S Y L N K L P V K N E Y P	
361		420
After	TCTATCAAAGTGGTTGTTGAGTGGATGCTGCTGGGTGACAACAACCAGCACCTGTTCTGC	
Before	TCTATAAAAGTGGTGGTGCAGTGGATGCTTCTGGGCGACAACAATCAGCATCTCTTCTGC	
	S I K L V V Q W M L L G D N N Q H L F C	
421		450
After	TGGGAAATCCCGTTTCAGATCGAAGGTTAA	
Before	TGGGAAATCCAGTTTCAGATTGAAGGCTAG	
	W E I P V Q I E G *	

Figure 1. Codon-optimized Can f 7 nucleotide sequences prior to and following codon optimization. Can f 7, *Canis familiaris*.

pl) predicted B cell epitopes of Can f 7 (37,38). The final consensus epitope results were obtained by combining the results of these three tools with previous methods (39-41). In the DNASTar Protean system, four properties (hydrophilicity, flexibility, accessibility and antigenicity) of the amino acid sequence were chosen as parameters for epitope prediction. Peptide regions with good hydrophilicity, high sensitivity, surface accessibility, and high antigen index were selected as candidate epitopes for further study. The BPAP system and the BepiPred 2.0 server require only amino acid sequences, which provide more direct results, together with the physico-chemical properties of amino acids, such as hydrophilicity, flexibility, accessibility, corners and exposed surfaces. Five predicted B cell epitopes were synthesized by GenScript with a purity >90% and named as P1, P2, P3, P4 and P5 respectively.

**Verification of B cell epitopes by ELISA.** The IgE binding of the predicted B cell peptides was detected using ELISA (42). A 96-well microplate was coated with each epitope in PBS at 2 µg/100 µl/well (0.02 µg/µl). The plate was then blocked with 1 mg/ml BSA (200 µl/well) for 1 h at 25°C and washed once with PBST. Subsequently, 100 µl of four Can f 7 allergic children's sera (No. 2, No. 4, No. 6 and No. 13 in Table I; diluted 1:10 in

PBST with 0.1% BSA) was incubated for 2 h at 25°C. After incubation, the plates were washed three times with PBST. The plates were incubated with a 1:2,500-diluted HRP-conjugated goat anti-human IgE monoclonal antibody for 1 h at 25°C and washed with PBST three times. Subsequently, 100 µl TMB color liquid was added to each well. After staining for 30 min at room temperature, the reaction was stopped by the addition of 50 µl of 2 M H<sub>2</sub>SO<sub>4</sub>, and the serum of four healthy participants without a history of allergic symptoms was used as control, NHS. After incubation, plates were processed as described above, and absorbances were read at 450 nm.

**ELISA inhibition of B cell epitopes.** The five B cell epitopes, BSA and the rCan f 7 were tested via inhibition ELISAs of Can f 7 with the sera of two Can f 7-allergic children (No. 4 and No. 13 in Table I). In this experiment, 96-well microplates were coated with 2 µg recombinant Can f 7 (100 µl/well) in PBS overnight 4°C. The plate was washed three times with PBST. The plate was then blocked with BSA (1 mg/ml; 200 µl/well) for 1 h at 25°C and washed once with PBST. The plates were then incubated with 100 µl of 2 allergic children's sera (1:10 diluted in PBST, which were previously preincubated with synthesized epitopes for 1 h at 37°C with 8 gradient concentrations (0, 10<sup>-4</sup>, 10<sup>-3</sup>, 10<sup>-2</sup>, 10<sup>-1</sup>,

1, 10, and  $10^2$   $\mu\text{g/ml}$ ) at  $25^\circ\text{C}$  for 2 h. After incubation, the plates were washed three times with PBST. The plates were incubated with a 1:2,500-diluted HRP-labeled anti-human IgE monoclonal antibody for 1 h at  $25^\circ\text{C}$  and washed with PBST three times. Subsequently, 100  $\mu\text{l}$  TMB color liquid was added to each well. After staining for 30 min at room temperature, the reaction was stopped by the addition of 50  $\mu\text{l}$  of 2 M  $\text{H}_2\text{SO}_4$ . After incubation, plates were processed as described above, and the absorbance was read at 450 nm (43).

**Statistical analysis.** Statistical significance between mean values of three experiments was analyzed by Dunnett's t-test following one-way analysis of variance or the Student's t-test utilizing the SPSS 13.0 version (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression and purification of Can f 7 in *E. coli*.** The optimized codon sequence of Can f 7 used for expression in *E. coli* is shown in Fig. 1. The codon-optimized Can f 7 gene was then subcloned into the pET-28a (+) vector and transformed into BL21 (DE3) pLysS *E. coli* cells. Fig. 1 shows a comparison of the sequences prior and following optimization. By SDS-PAGE, Can f 7 was shown to be primarily expressed in inclusion bodies (Fig. 2A). Recombinant Can f 7 was then purified on a Ni-NTA resin under denaturing conditions. Purified Can f 7 is shown as a single band in the subsequent SDS-PAGE analysis. The apparent molecular weight of purified recombinant Can f 7 was  $\sim 14$  kDa (Fig. 2B). The denatured proteins were refolded in a glutathione redox system. The far UV spectra of refolded Can f 7 showed a trend towards  $\beta$ -sheet structures (Fig. 3). Estimation of the secondary structure using the K2D3 program resulted in a determination of  $\sim 1.43\%$   $\alpha$ -helices and  $44.13\%$   $\beta$ -sheets.

**Immunoreactivity of Can f 7 to IgE by western blot and ELISA.** To determine the ability of Can f 7 to bind specific IgE in sera from dog-allergic children, western blot analysis and ELISA were performed. A total of 20 pediatric patients with dog allergies were included. As shown in Fig. 4, six of 20 dog-allergic sera from pediatric patients showed positive IgE binding to Can f 7 (numbers of the positive children are No. 2, 4, 6, 13, 17 and 20 in Table I), whereas healthy control sera failed to do so.

**Basophil activation analysis.** Can f 7 induced an  $\sim 4.0$ -fold increase in CD63 and CCR3 expression in basophils sensitized by serum from dog-allergic children compared with healthy controls (Fig. 5).

**Structure modeling and B cell epitope prediction.** Looking for a protein with a similar amino acid sequences to Can f 7 in SWISS-MODEL, an epididymal secretory protein E1 (PDB ID: 2hka.1.A; [www.rcsb.org/](http://www.rcsb.org/)) demonstrated the highest sequence identity with Can f 7 (76.15%). Therefore, the template 2hka.1. A was selected as a template for homology

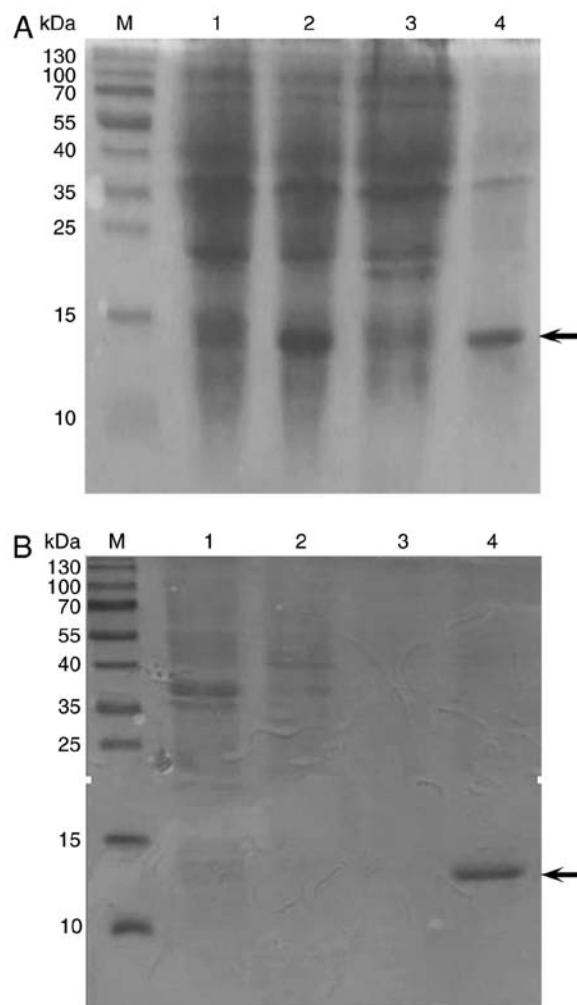


Figure 2. Expression and purification of Can f 7 in *E. coli*. (A) Lane M, protein molecular weight standard; Lane 1, noninduced recombinant Can f 7 whole cell lysate; Lane 2, isopropyl- $\beta$ -D-thiogalactopyranoside-induced recombinant Can f 7 whole cell lysate; Lane 3, supernatant fraction after ultrasonication; Lane 4, precipitated fraction (inclusion bodies) after ultrasonication. (B) SDS-PAGE after affinity chromatography of Can f 7. Lane M, protein molecular weight standard; Lane 1, unbound fractions of the supernatant of Can f 7; Lane 2, first elution fraction using 500 mM imidazole; Lane 3, second elution fraction using 500 mM imidazole; Lane 4, third elution fraction using 500 mM imidazole. The arrow indicates Can f 7 and its purified version. Can f 7, *Canis familiaris*.

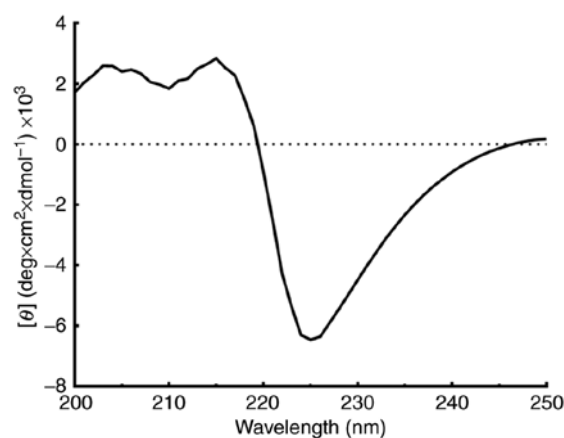


Figure 3. Circular dichroism analysis of recombinant Can f 7 expressed in *E. coli*. The molecular ellipticities (y-axis) at different wavelengths (200–250 nm, x-axis) are displayed. Can f 7, *Canis familiaris*.

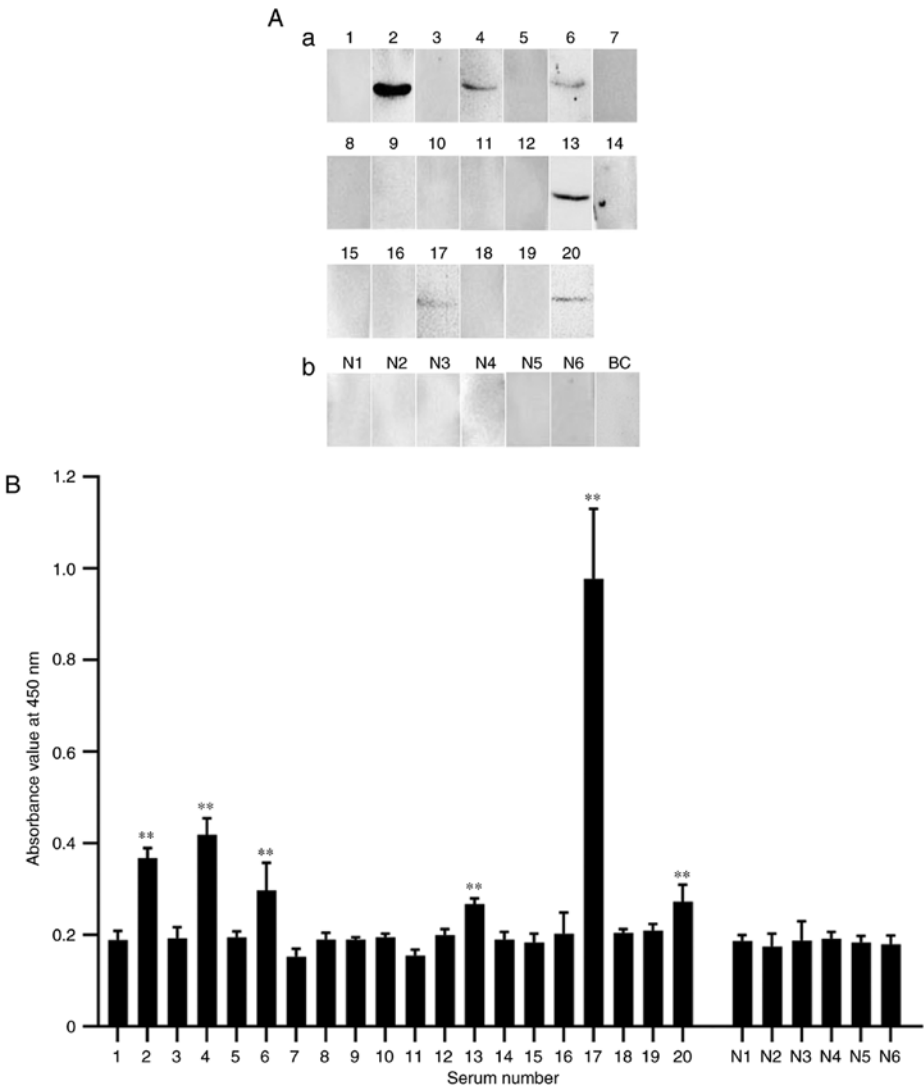


Figure 4. IgE reactivity of human sera against rCan f 7. (A) IgE binding of Can f 7 identified by western blot analysis. (a) Can f 7 was incubated with sera from 20 dog-allergic patients (line 1-20). (b) Can f 7 was incubated with sera from 6 healthy individuals as negative serum controls (N1-N6) and PBST as BC. (B) Immuno-reactivity of Can f 7 to IgE was assessed by ELISA. The values are shown as the mean  $\pm$  standard error from three experiments. \*\* $P < 0.01$  vs. the healthy controls. BC, blank control; Can f7, *Canis familiaris*; Ig, immunoglobulin; N, negative; PBST, PBS with Tween-20.

modeling. Fig. 6A shows the overall 3D structure of Can f 7 determined via homology modeling.

Can f 7 was predicted to contain no transmembrane helix by TMHMM 2.0. Secondary structure prediction showed that Can f 7 contains 6  $\beta$ -sheets and no  $\alpha$ -helices (Fig. 6B).

Based on four main properties (hydrophilicity, flexibility, reachability and antigenicity), the final predicted B-cell epitopes in Can f 7 allergen by DNASTar were amino acid regions 64-87, 89-106 and 117-127. In contrast, the BPAP system predicted regions 18-29, 65-87, 89-101, 106-117, 120-128 and 136-145 as B cell epitopes in Can f 7. The BepiPred 2.0 server predicted regions 34-47, 88-103, 105-118, and 136-148 as B cell epitopes in Can f 7. The final potential B cell epitopes of Can f 7 were determined based on the results from all three tools. The final results of the three immunological informatics tools ultimately predicted five B cell epitopes in Can f 7: P1: IPSQSSKAVVHGIVLGVAVPFPI; P2: EADGCKSGINCPI; P3: TYSYLNKLPVKN; P4: PSIKLVVQW; and P5: QHLFCWEIPV (Table II; Fig. 6C).

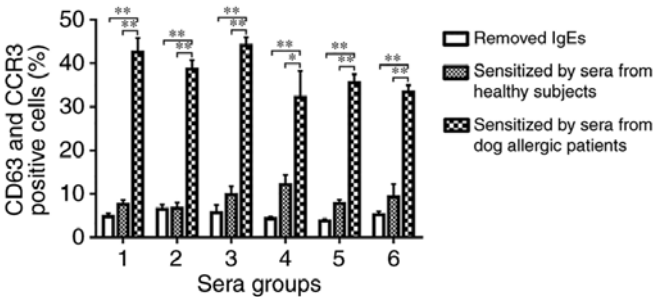


Figure 5. Induction of basophil activation by purified recombinant Can f 7. After removal of nonspecific IgE on basophils, cells from each donor were sensitized with sera from six healthy subjects or six dog-allergic patients and treated with Can f 7 at 1.0  $\mu\text{g}/\text{ml}$ . The values shown are the mean  $\pm$  standard error from six different participants. \* $P < 0.05$  and \*\* $P < 0.01$  vs. the sera derived from the dog allergic patient. Can f7, *Canis familiaris*; CCR, C-C motif chemokine receptor; CD, cluster of differentiation; Ig, immunoglobulin.

*B cell epitope verification.* The specific IgE binding of predicted B cell peptides was detected by ELISA using sera

Table II. Location of the B cell epitopes of the Can f 7 in *Canis familiaris* predicted by immunoinformatics tools.

Peptide	Position	Sequence
P1	65-87	IPSQSSKAVVHGIVLGVAVPFPI
P2	89-101	EADGCKSGINCPI
P3	106-117	TYSYLNKLPVKN
P4	120-128	PSIKLVVQW
P5	136-145	QHLFCWEIPV

from children with dog allergies (patient No. 2, No. 4, No. 6 and No. 13 in Table I). The results showed that the mean absorbance values in P3 and P4 groups were approximately twice that of the control NHS. However, there was no prominent increase in the absorbance values in P1, P2 and P5 groups. Therefore, among the five predicted B cell peptides, P3 (TYSYLNKLPVKN) and P4 (PSIKLVVQW) reacted with four dog-allergic children's sera and showed significantly different IgE binding affinity compared with NHS ( $P < 0.05$ ; Fig. 7).

**Inhibition ELISA experiments.** Inhibition ELISA experiments were performed with the five B cell epitopes, BSA and rCan f 7. P3 and P4 could inhibit the binding of specific IgE antibodies to the recombinant fusion protein Can f 7. In Patient 4, the maximum inhibition rates of P3 and P4 were 13% and 17% at a concentration of 100  $\mu\text{g/ml}$ , respectively, whereas 96% inhibition could be achieved by rCan f 7 (the inhibition rates of P1, P2, P5 and BSA were  $< 5\%$ ). In Patient 13, the maximal inhibition of P3 and P4 were 22 and 26%, respectively. Similarly, the maximal inhibition of rCan f 7 was 93%, and the others were all  $< 10\%$  (Fig. 8).

## Discussion

To better understand the prevalence and mechanism of Can f 7-associated dog allergy in children in China, recombinant Can f 7 was isolated from *E. coli*. Can f 7 has three potential glycosylation sites and can reportedly be glycosylated in yeast expression systems (15). However, it has also been reported that glycosylation of Can f 7 does not affect IgE binding to Can f 7 (15). The present study primarily sought to examine the sensitization rate of Can f 7 in Chinese children with allergies to dogs; therefore, glycosylation of the protein was not taken into consideration, which led to the use of the more convenient *E. coli* expression system.

In the present study, Can f 7 was expressed in inclusion bodies, while the same protein was reported to be expressed in a soluble form in *E. coli* (15). This may be due to different factors between the two studies, including the cloning vectors, the optimized codons, the host cells, and the expression conditions, among others. The inclusion bodies were purified on a Ni-NTA column under denaturing conditions. To allow correct folding and disulfide bond formation, a GSH/GSSG redox system was used (44). This system has been widely used for refolding proteins with disulfide bonds (45-47). CD analysis of the refolded Can f 7 found that the secondary structure of

this protein was estimated to be  $\sim 1.43\%$   $\alpha$ -helix and  $44.13\%$   $\beta$ -sheets, which was consistent with the predicted result that Can f 7 contains six  $\beta$ -sheets.

Can f 7 showed immunological activity, binding with IgE in dog-allergic children's sera with a sensitization rate of 30% (6/20) in Chinese children. In Fig. 4, not all patients' sera show high reactivity to rCan f 7 since the children were all reacted with dog extracts and then they would be diagnosed with dog allergies. However, dog extracts are complex substances which contain not only Can f 7 but also other allergens, such as Can f 1-6 and even unreported allergens. Thus, not all children were allergic to rCan f 7. This is a preliminary assessment of the IgE-binding activity of dog-allergic children in China and should be confirmed in studies with larger populations in the future. In addition, basophil activation testing, which is well-established as a functional assay for allergenicity was performed. In this test, the expression of CD63 and CCR3 on basophil surfaces is considered an indicator of basophil activation; it was found that Can f 7 could induce up to a 4.0-fold increase in their expression. Therefore, it was confirmed that Can f 7 is an active canine allergen. Compared with a previous study of Can f 7, a major innovation of this experiment is that allergic reactions in Chinese children was analyzed, identifying a higher sensitization rate (30%) compared with that (16.9%) in a previous report (15). These findings indicate that the sensitization rate of Can f 7 is higher in Chinese children than adults and show that Can f 7 is also an important allergen in Chinese children. Taking into account the increase in the number of pet dogs in China, the present analysis of dog allergens is significant.

$\alpha$ -Helices and  $\beta$ -sheets are the two major secondary structures of proteins whose structure is maintained by hydrogen bonding, making it impossible for the epitope sequence to be located therein (3). In contrast, random coils always contain epitope sequences as they are located in areas of surface exposure (48). The secondary structure of Can f 7 is predicted to contain six  $\beta$ -sheets. In addition to a sequence consisting of  $\beta$ -sheets, many other residues consist of random coils and may also be associated with certain immunological features of Can f 7. Greater analysis of the structure and function of Can f 7 will likely contribute to improved peptide-based vaccine design for dog allergies.

In this study, B-cell epitopes of Can f 7 were analyzed and projected them on the 3D structure of Can f 7. The synthesis of allergen-specific IgE is a key step in the development of allergic symptoms (3,49). IgE production requires B cells to be in close contact with allergen-specific helper T cells for translational recombination (50). Therefore, identification of relevant epitopes is important to understand the development of allergy symptoms. Computer prediction has become a useful method of identifying epitopes from immune-related proteins (51). Hydrophilicity, antigenicity, segmental motility, flexibility and accessibility have all been used to predict linear B cell epitopes on protein sequences based on the propensity values of amino acid properties in many algorithms (43). Actually, conformational epitopes are likely the most significant ones since the results between Fig. 4A and B are not fully consistent. For instance, in Fig. 4A, serum sIgE from patient 17 shows the lowest reactivity



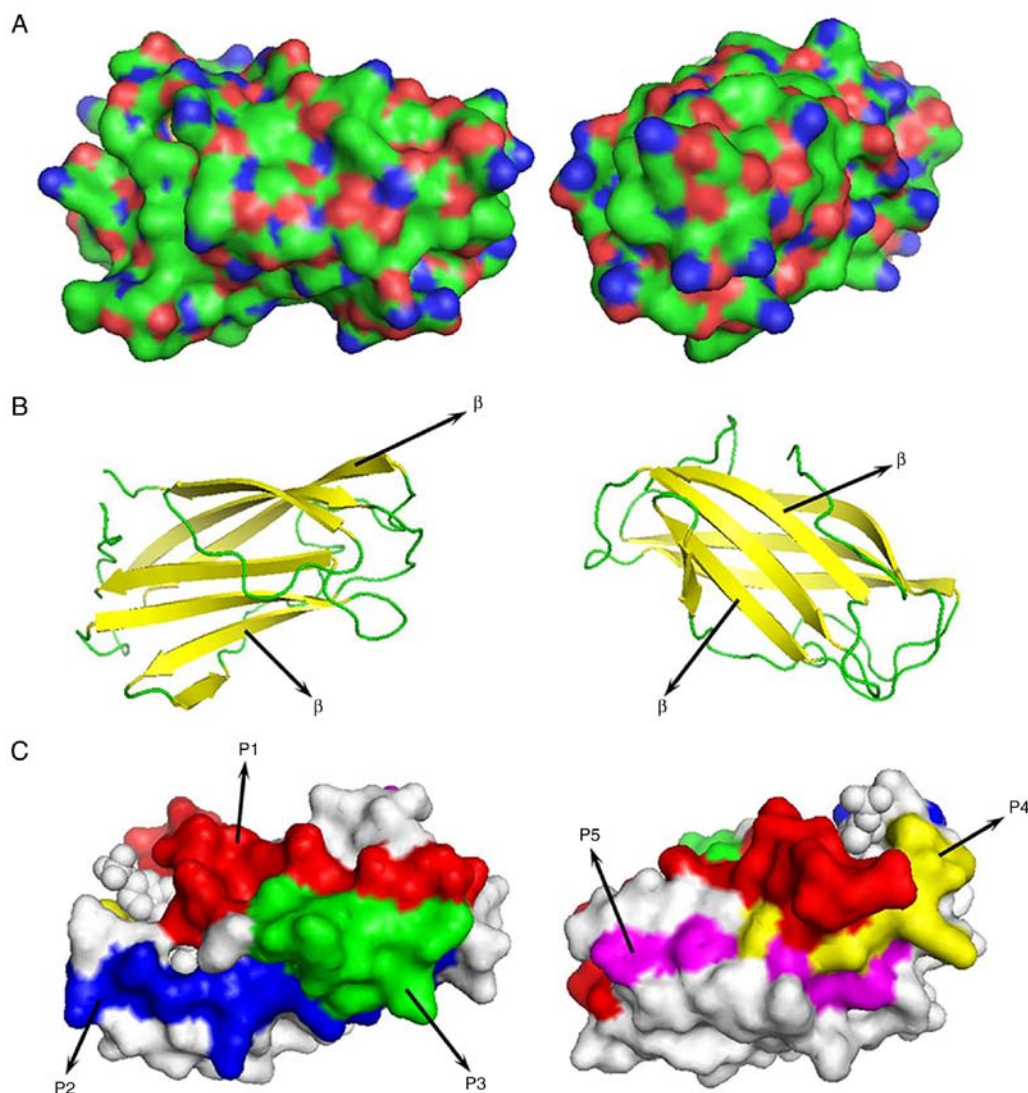


Figure 6. Three-dimensional structure of Can f 7. (A) Protein structure of a Can f 7 homology model. (B) The predicted secondary structure is shown on the surface of the Can f 7 structure. The green symbolizes  $\beta$ -sheets (marked with arrows). (C) Predicted B cell epitopes are shown on the surface of the Can f 7 structure (marked with arrows): P1 (red), IPSQSSKAVVHGIVLGVAVPFPI; P2 (blue), EADGCKSGINCPI, P3 (green), TYSYLNKLPVKN; P4 (yellow): PSIKLVVQW, P5 (pink): QHLFCWEIPV. Can f7, *Canis familiaris*.

to rCan f 7, but in Fig. 4B it shows the highest reactivity. This may due to the conformational epitopes of rCan f 7 were fully destroyed during the sample preparation step in WB and the sIgE in the serum from patient 17 was mainly target to the conformational epitopes of rCan f 7. Although the conformational epitopes in allergen play an important role in allergy, their prediction algorithms are still far less than satisfactory (52). Thus, in the present study, the focus was on the prediction of five linear peptides (P1-P5) as potential B cell epitopes of Can f 7. In addition, it was attempted to confirm the predicted allergenicity of these B cell epitopes by ELISA. The present results identified that only P3 and P4 showed a positive association with sera from children with dog allergies. Moreover, these B cell epitopes are composed of consecutive amino acids, meaning that they belong to sequential B cell epitopes. However, although the two predicted epitopes could be detected by IgE raised against dog allergens, the positive signals in ELISA were relatively weak compared with that of the negative control. This is most likely an artifact of the testing methodology, as a single

epitope coated on an ELISA plate has limited ability to bind with Can f 7-specific IgE, which may have led to a weaker ELISA value. The inhibition ELSIA demonstrated that both P3 and P4 could inhibit the binding of specific IgE antibodies to rCan f 7. In this experiment, the sera from patient 4 and 13 were selected as they had significant reactions to P3 and P4. Moreover, the inhibition rate calculated from the low OD value would lead to large deviation. Therefore, other patient's sera were not included in this experiment. Finally, the present results suggest that sequential epitopes may serve an important role in Can f 7-related dog allergies.

In summary, Can f 7 recombinant protein was expressed and purified, and its sensitization rate was determined by Western blot, ELISA, and basophil activation analysis. The homology of the Can f 7 protein was modeled, and two B cell epitopes were predicted and confirmed by ELISA. Currently, there is no commercially available method to detect sensitization to Can f 7. The present study provides a potential epitope prediction strategy that may be used for the future diagnosis and treatment of canine allergy in children.



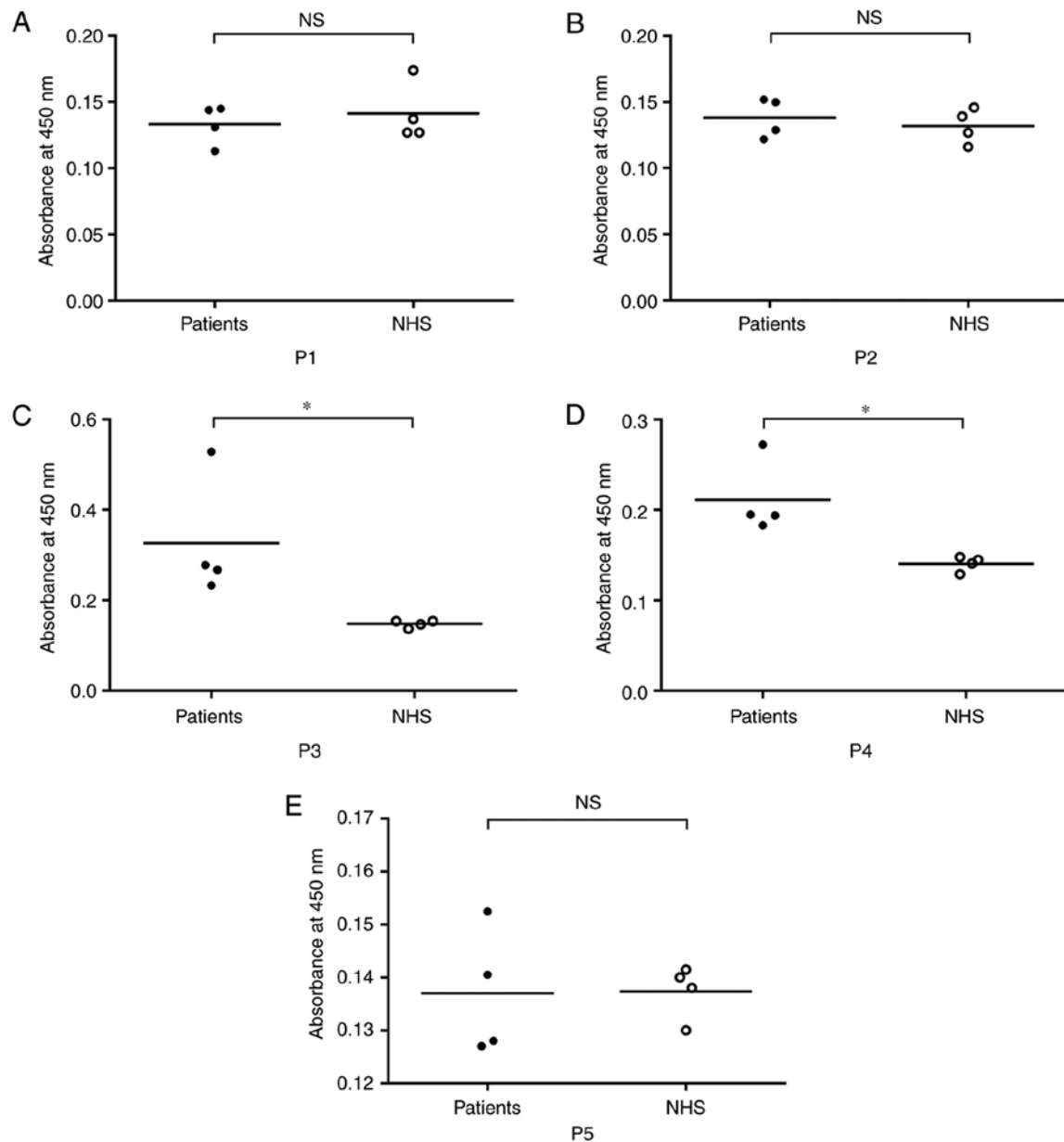


Figure 7. IgE binding of Can f 7 B Cell peptides to sera from dog-allergic children and NHS as detected by ELISA. (A) Dog-allergic children's sera results compared with NHS for P1. (B) Results of dog-allergic children's sera compared with NHS for P2. (C) Results of dog-allergic children's sera compared with NHS for P3. (D) Results of dog-allergic children's sera compared with NHS for P4. (E) Results of dog-allergic children's sera compared with NHS for P5. \*P<0.05 vs. NHS. Can f7, *Canis familiaris*; Ig, immunoglobulin; NHS, normal human serum; ns, non-significant.

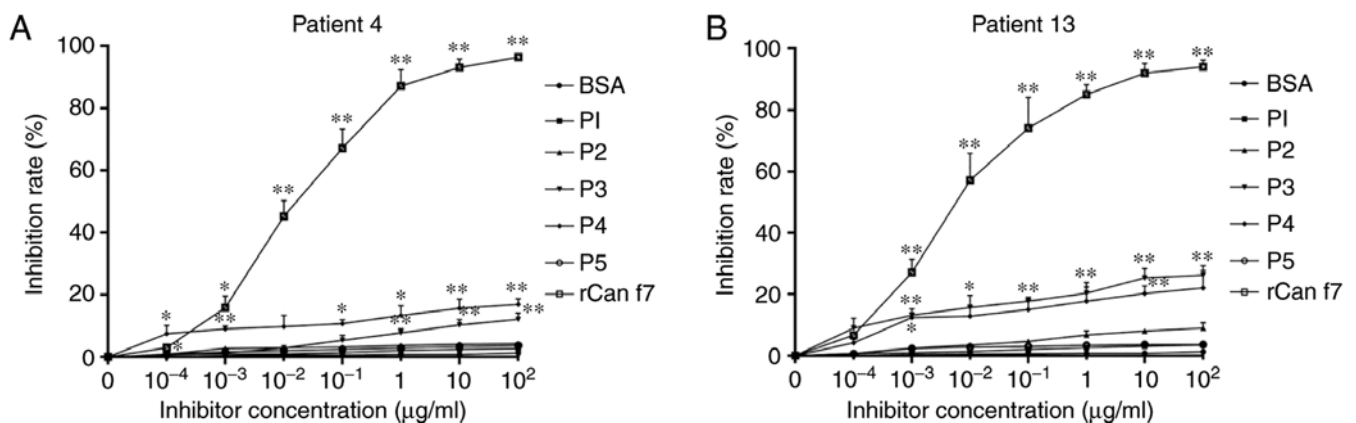


Figure 8. Inhibition ELISA for Can f 7 with two positive B cell epitopes. (A) Results of inhibition experiments in patient 4 and (B) in patient 13. \*P<0.05 and \*\*P<0.01 in comparison with the uninhibited control. BSA, bovine serum albumin; Can f7, *Canis familiaris*; r, recombinant.

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## Availability of data and materials

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

## Authors' contributions

JLS and JFW contributed to the conception and design of the study. RQW, YJW and ZQX performed all experiments and verified the analytical data. YJZ, MDC and WZ contributed to the statistical analysis and helped to interpret the results. YJW, ZQX and WZ wrote the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (2015-SRFA-055).

## Patient consent for publication

All participants written consent was obtained from their parents or legal guardians for the use of their blood samples prior to admission.

## Competing interests

The authors declare that they have no competing interests.

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