International Journal of
Molecular Sciences

MDPI

Article

Engineering Epitope-Specific IgY Antibodies to Neutralize the Major Fel d 1 Allergen in Cats

Xinya Tian [1,2,†], Chunxiao Li [1,2,†], Wei An [1,2], Yifei Wang [3], Zhenlong Wang [1,2,*], Jiaxue Wang [1,2], Xiaoxin Fu [1,2], Bing Han [1,2], Hui Tao [1,2], Andrews Jon Christopher [4], Jinquan Wang [1,2] and Xiumin Wang [1,2,*]

[1] Key Laboratory of Feed Biotechnology, Ministry of Agriculture and Rural Affairs, Beijing 100081, China; 82101232041@caas.cn (X.T.); 82101212039@caas.cn (C.L.)
[2] Institute of Feed Research, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[3] College of Animal Science & Technology, Nanjing Agricultural University, Nanjing 210095, China
[4] School of Agriculture and Environment, Massey University, Palmerston North 4410, New Zealand; c.j.andrews@massey.ac.nz
* Correspondence: wangzhenlong02@caas.cn (Z.W.); wangxiumin@caas.cn (X.W.)
† These authors contributed equally to this work.

Abstract

Approximately 10–24% of people suffer from a cat allergy. Fel d 1, the major allergen, triggers reactions in approximately 94% of sensitized individuals. Current therapeutic strategies for allergic diseases primarily involve medication or immunotherapy to alleviate symptoms, which are often burdened by low efficacy, high cost, and extended duration, posing significant challenges for patients. In contrast, IgY antibodies offer a promising alternative by reducing the level of allergens produced by cats. In this study, T-cell epitopes of Fel d 1 were predicted using ProPred/CTLpred, connected via a suitable linker (GGGGS), and expressed in *E. coli*. Immunization of mice and hens with recombinant cFel d 1 yielded high-titer specific antibodies (IgG: 1:301,500; IgY: 1:4,194,304). Cats administered anti-cFel d 1 IgY-enriched yolk powder (1–3% of diet) for four weeks exhibited a 30–71% reduction in salivary Fel d 1. These findings indicate that the allergen epitope-targeted IgY strategy effectively reduces allergen levels in cats, providing a promising basis for preventing and treating allergic conditions.

Keywords: allergy; feline allergen; cFel d 1; IgY antibody; recombinant expression

1. Introduction

Cat allergens represent the third most prevalent category of inhalation allergens affecting humans [1]. The global prevalence of allergy to cats is approximately 24% in children and 15% in adults, with recent studies suggesting an increased trend [2,3]. The World Health Organization (WHO) and the International Union of Immunological Societies (IUIS) have identified eight cat allergens responsible for respiratory allergies in humans. Among these, Fel d 1 is the immunodominant allergen, responsible for triggering allergic reactions in 93% of sensitized individuals [2]. Serological analyses reveal that 60–90% of immunoglobulin E (IgE) antibodies in allergic patients specifically target Fel d 1 [4]. This secretory protein, which belongs to the globin family, is produced principally in the sublingual and sebaceous glands, with additional production occurring in the anal and lacrimal glands [4]. Fel d 1 binds to microscopic dander particles, which facilitates their environmental dispersion and subsequent human exposure, presenting a significant public health concern [5,6]. However, current therapeutic interventions for human allergies to cats (such

Academic Editor: Paolo Colombo

Received: 18 January 2026
Revised: 3 March 2026
Accepted: 3 March 2026
Published: 9 March 2026

as pharmacotherapy, immunotherapy, etc.) continue to be limited by insufficient efficacy and safety concerns [7–10]. In contrast, interventions targeting the animal source—such as reducing the active allergen secreted in cats—provide a direct and complementary strategy.

Immunoglobulin Y (IgY) represents the predominant antibody class produced in avian, reptilian, and amphibian species. It is transferred in large quantities to the egg yolk, conferring passive immunity to the developing embryo. IgY antibodies can be easily harvested from the yolk and serve as an ideal alternative to mammalian IgG antibodies [11]. Notably, IgY demonstrates remarkable biochemical stability, maintaining functionality across a broad pH range (3.5–11.0) and temperature spectrum (30–70 °C). Therapeutic applications of IgY have been well-documented against diverse pathogens and toxins [12,13], including snake venom, norovirus, *Taenia solium*, and pathogenic *Escherichia coli* strains [14–17]. For cat allergen management, rabbit polyclonal antibodies and chicken IgY antibodies specific to Fel d 1 have been demonstrated to effectively bind to the allergen in cat saliva. This interaction may reduce the concentration of native Fel d 1 at its source, thereby lowering exposure levels for allergic individuals. Subsequent research confirmed that specific IgY against natural Fel d 1 protein significantly decreased allergen concentrations by 47% in cats after a 10-week period [18]. Importantly, comprehensive safety evaluations revealed no mutagenic effects (7 to 66 ppm concentrations) in *Salmonella typhimurium* and *E. coli* tests, nor any observable chromosomal aberration or toxicological consequences following six-month dietary supplementation in cats [19,20]. These findings collectively establish IgY as both a safe and effective treatment for managing allergies caused by Fel d 1. However, the use of natural allergens for the systematic production of specific, high-affinity IgY remains a significant challenge.

In this study, we used bioinformatics approaches to predict and characterize T-cell epitopes of the major feline allergen Fel d 1. To optimize immunogenicity and structural stability, we engineered a chimeric protein (cFel d 1) by conjugating dominant epitopes with a flexible linker (GGGGS). The recombinant cFel d 1 was successfully expressed in *E. coli* expression systems. Subsequent immunization protocols in mice and layer hens generated high-affinity IgG (mice) and IgY (layer hens) antibodies specific to cFel d 1. The effect of IgY antibodies was then evaluated through a feeding trial, where cats received the different contents of anti-cFel d 1 IgY-enriched egg yolk powder.

2. Results

2.1. High Sequence Similarity of Fel d 1 Protein Among Felidae Species

There was a high sequence conservation for Fel d 1 among *Felidae* species. Specifically, Fel d 1 chain v exhibited the highest similarity (91%) with major allergen I polypeptide chain 1 (XP_058563703.1) from *Puma yagouaroundi* and *Lynx rufus* (XP_046934163.1). Additionally, it showed approximately 90% identity with homologs from *Neofelis nebulosa* (XP_058563703.1), *Prionailurus bengalensis* (XP_043454992.1), and *Panthera onca* (XP_060508755.1) (Figure S1). For Fel d 1 chain 2, the highest homology (90%) was observed with *Prionailurus bengalensis* (XP_043454990.1), followed by *Leopardus geoffroyi* (89%; XP_045298454.1). Lower, but still significant, similarity was detected with *Panthera uncia* (88%; XP_049477030.1) and *Panthera tigris* (87%; XP_007096807.1) (Figure S2).

2.2. Structural Characterization of Fel d 1 Protein

Secondary structure analysis revealed distinct conformational differences between Fel d 1 chain 1 and chain 2. Chain 1 predominantly comprises an α-helix conformation (74%), with minor contributions from β-sheet (6%), β-turn (1%), and random coil (19%) (Figure S3). In contrast, chain 2 exhibits a more balanced distribution, with 58% α-helices, 14% β-sheets, 4% β-turns, and 24% random coils (Figure S4). Tertiary structure model

demonstrated that the N-terminal region of Fel d 1 is dominated by α-helices, while the C-terminus adopts a random coil configuration (Figure S5).

Homology modeling via Swiss-Model confirmed that Fel d 1 forms a tetrameric glycoprotein composed of two heterodimers (Figure S5). The high global model quality estimation (GMQE) score of 0.81 indicates excellent reliability in both overall folding and local structural accuracy. Structural alignment with experimentally resolved PDB entries revealed strong similarity, particularly in the α-helix and random coil regions (Figure 1A). Local quality assessment further supported model robustness, with most regions scoring above 0.6—a threshold for structural reliability (Figure 1B). Ramachandran plot analysis provided additional validation, with 98% of residues occupying energetically favorable (φ, ψ) angles (Figure 1C). Notably, β-sheets clustered in the upper left quadrant, while α-helices populated both upper right and lower left regions. The dense distribution of points within the dark green (optimal) zone—representing >90% of residues—confirms the structural stability of Fel d 1 [21,22].

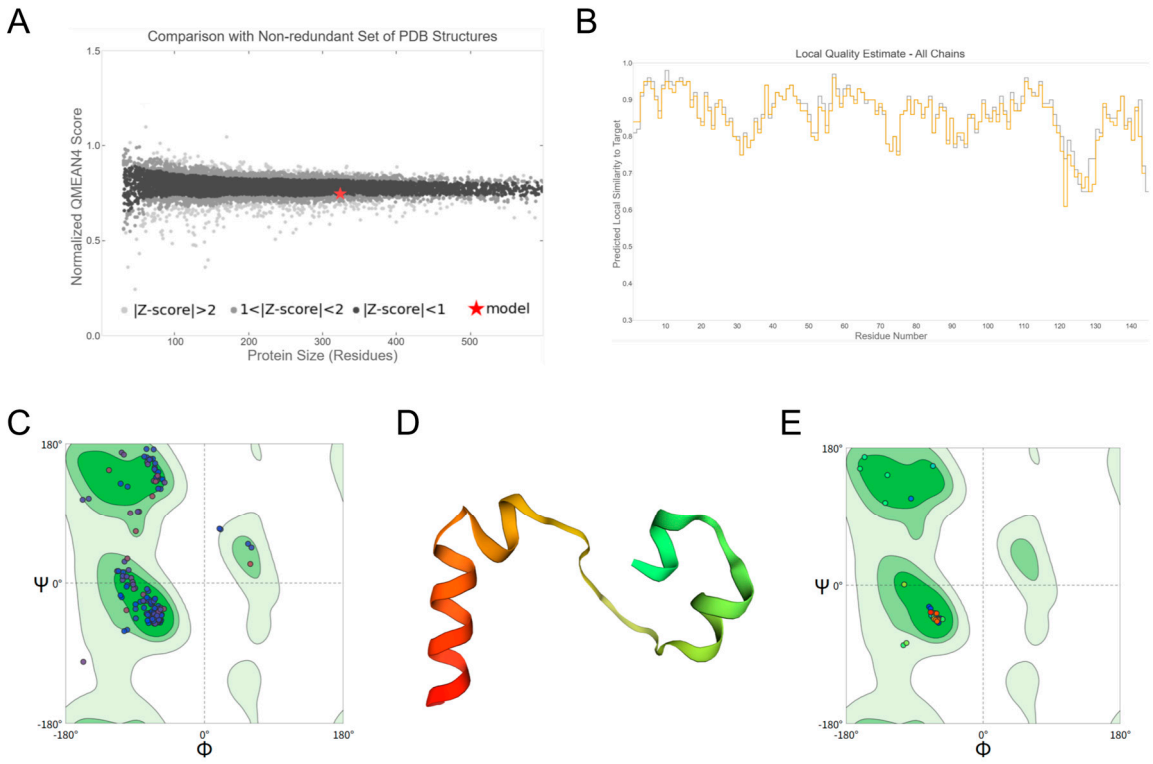

Figure 1. Structural analysis of Fel d 1 and its chimeric cFel d 1 protein based on epitopes. (**A**) Comparison of PDB database structure models of Fel d 1 protein. The *x*-axis represents the length of the protein structure and the *y*-axis represents th protein feature scores. Each point in the diagram represents a structure in the PDB database. The red star represents the prediction model, and the dark region represents the structure in which the absolute value of the Z-score is less than 1, which is equivalent to the data within the standard deviation of statistical normal distribution. (**B**) The local quality estimation of the prediction model of Fel d 1 protein with the yellow and blue lines representing chains 1 and 2, respectively. An area of >0.6 indicates high reliability. (**C**) Raman diagram of Fel d 1 protein. The dark green region indicates the optimal region of amino acids (φ, ψ). (**D**) 3D structure of chimeric cFel d 1 protein based on epitopes. Swiss-Model homologous modeling of cFel d 1 was constructed with the p30440.1.a major allergen I polypeptide chain 2 as the template. The score of GMQE is 0.41. (**E**) Raman diagram of chimeric cFel d 1 protein based on epitopes.

2.3. Prediction and Assembly of Dominant Epitopes in Fel d 1 Protein

Immunoinformatic analysis was performed to identify key immunogenic epitopes of Fel d 1. Using the ProPred program, we predicted three prominent Th epitopes: VVLE-

NARIL (positions 33–41), VKMAETCPI (positions 71–79), and FAVANGNEL (positions 85–93), all located in irregular coil and turn regions (Table 1 and Figures S6–S10). CTL epitope prediction using IEDB (https://nextgen-tools.iedb.org/ (accessed on 2 March 2023)) or CTLpred workplace (http://crdd.osdd.net/raghava/ctlpred/ (accessed on 2 March 2023)) with an ANN threshold of 0.51 identified three dominant epitopes: AETCPIFYD (positions 74–82), YTSPLCVKM (positions 65–73), and GEAVQNTVE (positions 145–153), which were similarly localized to the coil and turn regions (Table 1 and Figures S6–S10).

Table 1. Th and CTL epitope prediction of Fel d 1 protein.

Type	Segment Position	Sequence	Score
Th epitopes			
DRB1-0101	33–41	VVLENARIL	2.29
	71–79	VKMAETCPI	1.00
	85–93	FAVANGNEL	0.80
DRB1–0102	33–41	VVLENARIL	3.29
	71–79	VKMAETCPI	2.00
	85–93	FAVANGNEL	0.80
DRB1–0301	71–78	VKMAETCP	0.50
	79–87	IFYDVFFAV	4.37
	93–101	LLLDLSLTK	4.20
	132–140	LVMTTISSS	3.90
CTL epitopes			
	74–82	AETCPIFYD	1.00
	65–73	YTSPLCVKM	0.98
	145–153	GEAVQNTVE	0.98

Structural analysis revealed that while several regions (16–24, 26–30, 48–58, 64–67, 103–117, 138–140, and 151–158) exhibited high surface accessibility, these areas contained relatively few predicted epitopes (Figure S8). Notably, the CTL epitope GEAVQNTVE (145–153) was located in a hydrophilic region, while other epitopes showed poor hydrophilicity (Figure S9). Importantly, epitopes YTSPLCVKM (65–73) and GEAVQNTVE (145–153) were situated in highly flexible regions (Figure S10).

Based on these findings, we engineered a chimeric cFel d 1 protein by linking key epitope-containing regions (25–41, 46–59, 85–98, 132–140, and 145–153) with flexible GGGGS linkers. Structural characterization revealed the chimeric cFel d 1 protein contained 32% α-helices, 29% β-sheets, 18% β-turns, and 21% random coils (Figure S11). The tertiary structure maintained these secondary structure elements (Figure 1D), and Ramachandran plot analysis confirmed structural stability with 98% of residues in favored regions (Figure 1E).

2.4. Recombinant Expression and Purification of Chimeric cFel d 1 Protein

The chimeric cFel d 1 gene (254 bp) was chemically synthesized with flanking *Xho* I/*Nco* I restriction sites and an N-terminal 6×His tag sequence (Figure 2A). The gene fragment was subsequently cloned into the pET-28a expression vector, and recombinant plasmid construction was verified by both PCR amplification and DNA sequencing (Figure S12).

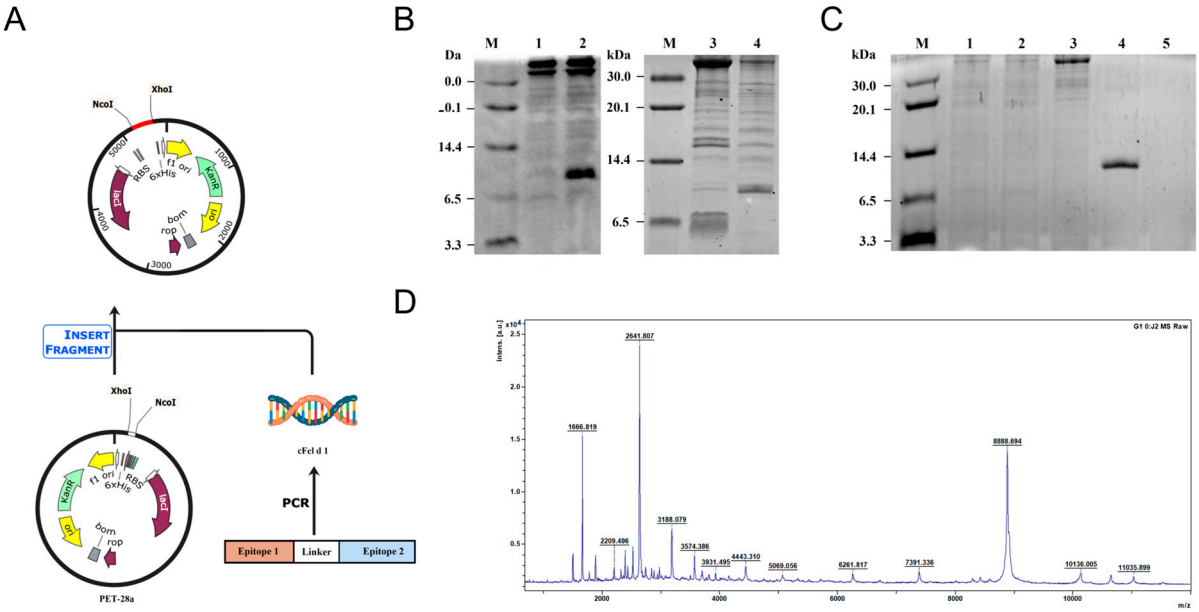

Figure 2. Recombinant chimeric cFel d 1 protein. (**A**) Construction of recombinant pET-28a-cFel d 1 plasmid. (**B**) Tris-tricine sodium dodecyl-sulfate polyacrylamide gel electrophoresis (Tris-Tricine-SDS-PAGE) electrophoresis of recombinant cFel d 1 protein. Lane M: protein marker; lane 1: protein expression without IPTG induction; lane 2: protein expression induced by IPTG; lane 3: cell precipitation after sonication; lane 4: supernatant after sonication. (**C**) Purification of cFel d 1 protein. Lane M: protein marker; lane 1: flow through the liquid; lane 2, 3: 50 mM imidazole; lane 4: 100 mM imidazole; lane 5, 6: 500 mM imidazole. (**D**) MALDI-TOF MS analysis of the purified cFel d 1 protein.

For protein expression, recombinant *E. coli* BL21 (DE3) harboring the pET-28a-cFel d 1 plasmid was induced with 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) at 30 °C for 4 h. Following large-scale culture (5 L), bacterial cells were harvested by centrifugation (10,000 rpm, 15 min, 4 °C) and lysed via sonication. The soluble fraction of the cell lysate was then subjected to immobilized metal affinity chromatography (IMAC) using Ni^{2+}-NTA resin. The purification protocol involved stepwise elution with increasing imidazole concentrations (20–250 mM). The cFel d 1 protein was predominantly expressed in the soluble fraction and efficiently eluted at 100 mM imidazole (Figure 2B). SDS-PAGE analysis and MALDI-TOF MS confirmed the molecular mass of the purified cFel d 1 protein to be approximately 9 kDa, consistent with the predicted size including the 6×His tag, with a purity of 95% (Figure 2C,D).

2.5. Specific Anti-cFel d 1 IgG and IgY Antibody Responses

Both mice and laying hens were immunized to evaluate the immunogenicity of the recombinant cFel d 1 protein (Figure 3A,B). Serum samples collected after three immunizations with recombinant cFel d 1 showed robust IgG responses in mice, with titer ranging from 1:34,500 to 1:301,500 (Figure 3C). In laying hens, the anti-cFel d 1 IgY response exhibited a classic immunization profile, reaching a peak titer of 1:4,194,304 at week 9 (Figure 3D). Subsequently, antibody levels stabilized at 1:524,288 between weeks 11 and 14, and then gradually declined.

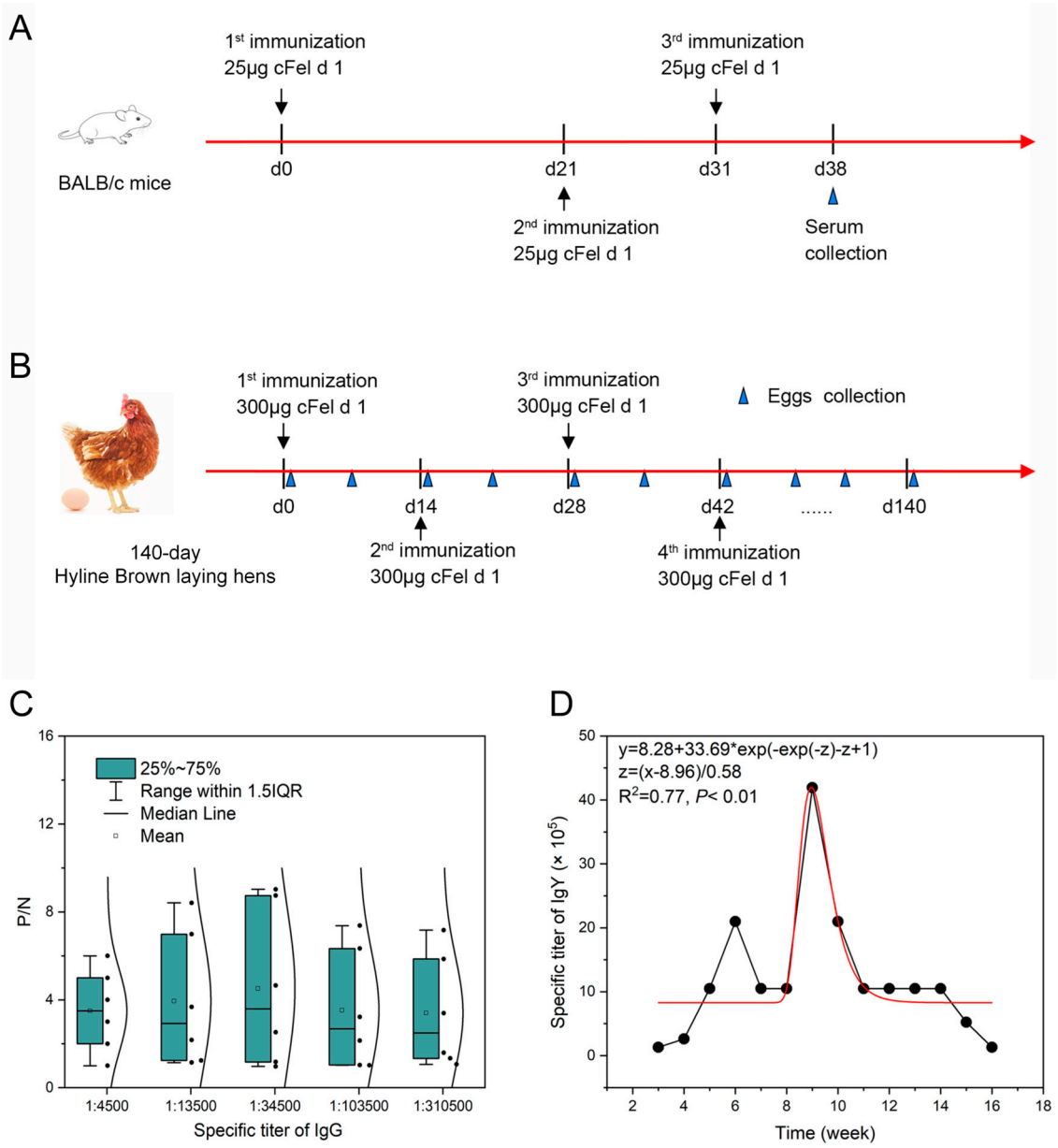

Figure 3. Preparation of anti-cFel d 1 antibody and its titer determination. (**A**) Immunization of mice. Mice were immunized with cFel d 1 protein at different time points, and their serum was taken one week after the last immunization. (**B**) Immunization of laying hens. Laying hens were immunized with cFel d 1 protein at different time points, and eggs were taken every week after the first immunization to detect the titer of specific IgY antibodies. (**C**) Titers of specific IgG antibodies. Black lines indicate the normal distribution curves. (**D**) Titers of specific IgY antibodies. The asterisk (*) indicates the multiplication sign (×).

As shown in Figure S13, anti-cFel d 1 IgY antibodies exhibited high-affinity binding to both recombinant cFel d 1 protein and native salivary Fel d 1. In contrast, control IgY from adjuvant-immunized hens showed no detectable reactivity to either antigen form. These results confirm that immunization with recombinant cFel d 1 elicits a robust and specific IgY antibody response in hens.

2.6. Anti-cFel d 1 IgY Effectively Reduced Salivary Fel d 1 Levels in Cats

To evaluate the efficacy of anti-cFel d 1 IgY in allergen reduction, cats received diets supplemented with 1–3% egg yolk powder containing specific IgY antibodies (Figure 4A).

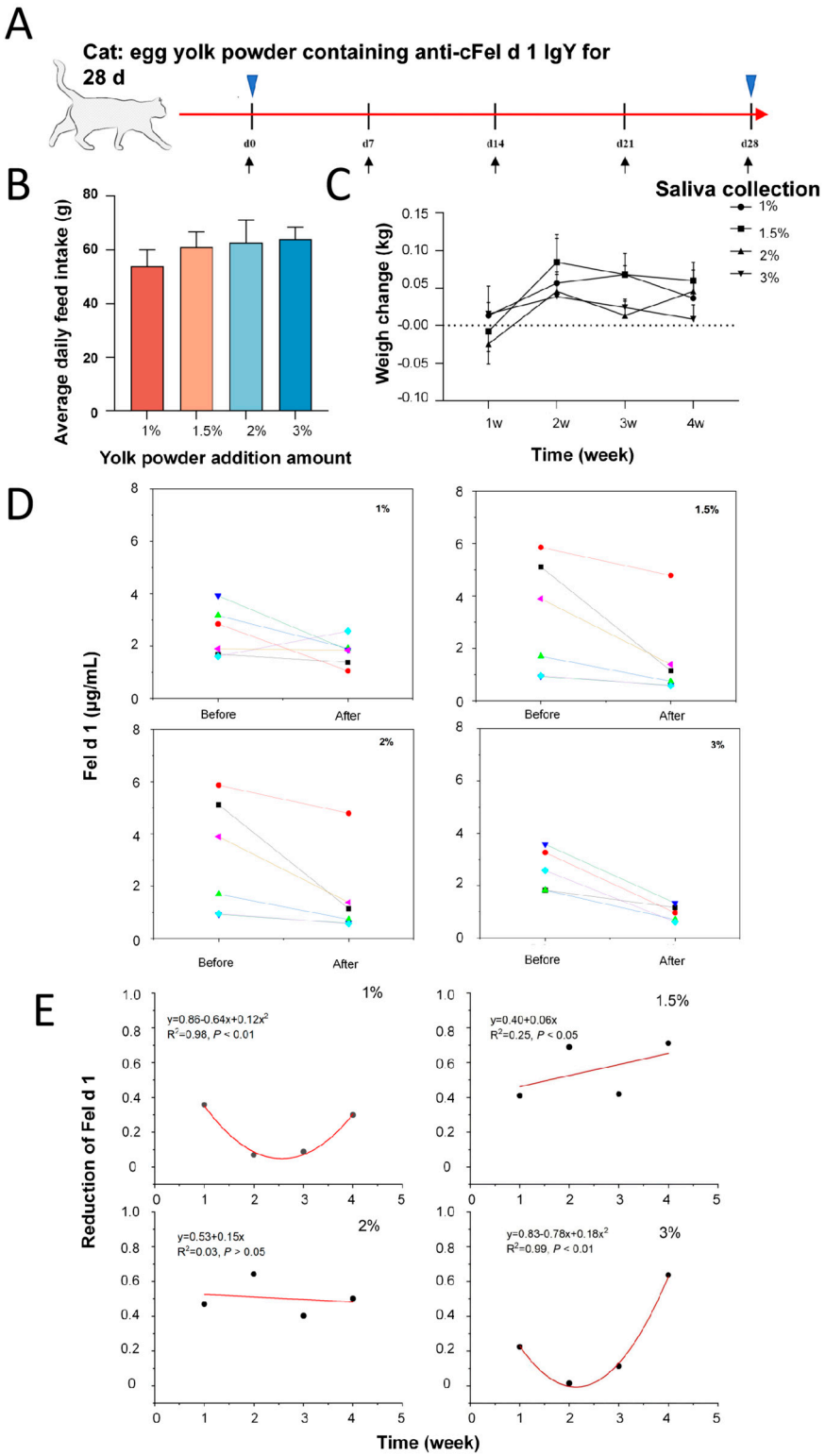

Figure 4. Efficacy of anti-cFel d 1 IgY antibodies in cats. (**A**) Cat feeding experiment. Twenty-three healthy cats were divided into four groups, five or six in each treatment group, fed with 1%, 1.5%, 2% and 3% of egg yolk powder containing anti-cFel d 1 IgY antibodies. (**B**) Mean average daily feed intakes ± standard deviation (SD) of cats in each treatment group. (**C**) The body weights of the cats in each treatment group throughout the study are expressed as mean ± SD. (**D**) Changes in salivary Fel d 1 levels in cats. The average concentration changes in salivary Fel d 1 in cats were detected before and after feeding with 1%, 1.5%, 2%, and 3% anti-cFel d 1 IgY. (**E**) Decrease in the salivary Fel d 1 levels in cats following dietary supplementation with 1%, 1.5%, 2%, and 3% anti-cFel d 1 IgY.

Throughout the 4-week study period, we systematically monitored several parameters—daily feed intake, weekly body weight change, and weekly collection of saliva samples—for Fel d 1 quantification using an indirect enzyme-linked immunosorbent assay (Ielisa). The average daily feed intake of cats fed with egg yolk powder containing anti-cFel d 1 IgY at concentrations of 1%, 1.5%, 2%, and 3% was 53.9 g, 61 g, 62.7 g, and 68 g, respectively (Figure 4B). The feed intake showed an increasing trend with higher levels of egg yolk powder/anti-cFel d 1 IgY in the diet, although no statistically significant differences were observed. The dietary supplementation of egg yolk powder containing anti-cFel d 1 IgY did not significantly affect the body weight of the cats during the feeding period (Figure 4C). Additionally, supplementation with anti-cFel d 1 IgY egg yolk powder significantly reduced salivary Fel d 1: 30% (1%, $p < 0.01$ **), 71% (1.5%, $p < 0.05$ *), 50% (2%, $p < 0.05$ *), and 64% (3%, $p < 0.05$ *) reduction, with the 1.5% dose being the most effective. No significant differences were observed among the 1.5–3% groups (Figure 4D,E).

3. Discussion

Feline allergens represent one of the most prevalent indoor aeroallergens globally, posing significant public health challenges. These allergens predominantly trigger rapid IgE-mediated type I hypersensitivity reactions, characterized by an accelerated immunological cascade [23]. Upon antigen recognition, this mechanism can provoke exaggerated secondary inflammatory responses, potentially leading to severe clinical symptoms including bronchoconstriction, acute airway obstruction, and even life-threatening anaphylaxis. Current management strategies, including drug therapy and conventional vaccination approaches, primarily focus on human patients, but these often result in prolonged treatment periods [7,8]. Few studies have explored interventions directed at the animal source, such as reducing the secretion of active allergen by cats [22,24].

The major feline allergen, Fel d 1, is a tetrameric glycoprotein with a molecular mass of 35–39 kDa, comprising two non-covalently associated heterodimers. Each heterodimer contains two distinct polypeptide chains: a shorter chain of 70 residues and a longer chain with variable length (85, 90 or 92 residues), which are covalently linked through multiple disulfide bonds [25]. Structural analyses have revealed that Fel d 1 shares significant sequence homology (25–50%) with several evolutionarily conserved proteins, including rabbit uteroglobin, the α subunit of mouse salivary androgen-binding protein (ABP), slow loris brachial gland secretion protein, and Clara cell 10 kDa protein family [26–28]. Chain 1 showed >90% sequence identity to uteroglobin and major allergen I polypeptide chain 1 from multiple felid species (*Puma yagouaroundi*, *Lynx rufus*, *Neofelis nebulosa*, *Prionailurus bengalensis*, and *Panthera onca*) (Figure S1). Notably, chain 2 contains an N-glycosylation site and exhibits >85% similarity to corresponding chains from *Prionailurus bengalensis*, *Leopardus geoffroyi*, and *Panthera uncia* (Figure S2). This evolutionary conservation across mammalian species suggests that these structurally related proteins may serve critical biological functions. Indeed, previous research has shown roles for Fel d 1 in epithelial barrier maintenance, immunomodulation, and chemical signaling [3,29,30].

The production of recombinant full-length Fel d 1 may be hindered by technical challenges (e.g., protein misfolding, low expression, and incorrect disulfide bond formation) which diminish its immunogenic potential [21,31,32]. Structural analyses revealed that recombinant protein in *E. coli* exhibits marked conformational differences and approximately 25-fold lower IgE-binding capacity compared to the native protein, likely due to disrupted disulfide bonds (Cys3–Cys73, Cys44–Cys48, and Cys7–Cys70) and impaired chain association [33]. Enhanced immunogenicity was achieved through a multi-epitope design featuring: (1) incorporation of immunodominant CTL epitopes (VKMAETCPI71–79, YTSPLCVKM65–73, AETCPIFYD74–82) within random coil and turn regions (Figures S6–S10), (2) preserved

native conformational epitopes, and (3) optimized Th1/Th2 determinant presentation. This design particularly boosted IgY production (Figure 3), suggesting passive immunotherapy potential. These results indicate that our multi-epitope approach effectively overcomes the limitations of full-length recombinant protein production while preserving a robust immunological response.

T-cell epitopes predominantly consist of continuous linear sequences that can be located throughout antigenic proteins, often corresponding to structurally buried regions in their native three-dimensional conformations [34,35]. Following internalization and proteolytic processing by antigen-presenting cells (APCs), these epitopes become exposed and subsequently loaded onto either MHC class II (for $CD4^+$ T cell recognition) or MHC class I (for $CD8^+$ T cell activation) molecules, ultimately being presented on the APC surface. Specifically, Th epitopes form stable complexes with MHC-II molecules that are recognized by $CD4^+$ T cell receptors, while CTL epitopes associate with MHC-I molecules for $CD8^+$ T cell engagement. Structural analyses by Kaiser et al. [22] identified three immunodominant IgE-binding regions in Fel d 1 (residues 25–38, 46–59, and 15–28), with the 25–38 epitope demonstrating particularly high seroreactivity (46%) in humans. Our antigenicity predictions further identified additional immunogenic sequences: the Th epitope VKMAETCPI71–79 and CTL epitopes YTSPLCVKM65–73, AETCPIFYD74–82, and GEAVQNTVE145–153 (Table 1). Notably, all predicated epitopes were positioned within random coil and turn regions (Figures S6–S10), a structural feature that likely enhances antigenic potential by facilitating proteolytic processing and MHC binding. These predictions were consistent with both the ProPred and CTLpred algorithms (Table 1), further validating the epitope selection strategy.

In multi-epitope vaccine design, the choice of linker is crucial, as it must maintain epitope independence and prevent novel junctional epitopes [36,37]. Optimal linkers not only facilitate proteolytic processing for antigen presentation but also preserve the native conformation of individual epitopes. Velders et al. [38] demonstrated that the "AAY" linker significantly enhances epitope cleavage efficiency as a protease recognition site, while the Livingston et al. [39] group showed that the "GPGPG" linker effectively isolates epitopes without interfering with their immunogenic presentation. Shi et al. [40] confirmed that the "AAA" linker can potentiate CTL epitope responses in HBV vaccines. In this study, we employed four flexible linkers (GGGGS) to connect five immunodominant Fel d 1 epitopes (VAQYKALPVVLENARIL25–41, DAKMTEEDKENALS46–59, FAVANGNELLLDLS85–98, LVMTTISSS132–140, and GEAVQNTVE145–153), generating a 9 kDa chimeric cFel d 1 protein (Figure 2A) with exceptional antigen specificity confirmed by Western blot (WB) analysis (Figure S13). Most notably, supplementing cat diets with 1.5–3% anti-Fel d 1 IgY-enriched egg yolk powder for four weeks reduced salivary Fel d 1 levels by 50–71% (Figure 4E), superior to the 47% reduction reported for full-length Fel d 1-targeting antibodies [18,41]. Notably, the 1.5% dose demonstrated the highest efficacy, which may be related to the prozone—wherein an excess of antibodies may result in steric hindrance that inhibits binding between Fel d 1 and IgY antibodies [42–45]. These results indicate that epitope-targeted IgY antibodies exerted significant neutralization (up to 71% reduction) and enhanced specificity without any adverse side effects being observed. Furthermore, our preliminary data indicated a reduction in environmental Fel d 1 levels in cats following dietary supplementation with anti-Fel d 1 IgY-enriched egg yolk powder. The ability of epitope-targeted IgY antibodies to prevent IgE binding to Fel d 1 and subsequent activation will be further investigated using ex vivo mast cell models in future studies.

4. Materials and Methods

4.1. Bacterial Strains, Plasmids, and Reagents

The *E. coli* BL21(DE3) competent cells and pET-28a expression vector were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Skim milk powder (Blotting Grade) was obtained from Beyotime Biotechnology (Shanghai, China). 3,3′,5,5′-Tetramethylbenzidine (TMB) single-component substrate solution, Tween-20, and polyethylene glycol 6000 (PEG-6000) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Mouse anti-chicken IgY-HRP conjugate was acquired from Beijing Bersee Science and Technology Co., Ltd. (Beijing, China). All other chemicals used were of analytical-grade purity.

4.2. Sequence Homology and Structural Characterization of Fel d 1 Allergen

The amino acid sequences of Fel d 1 chain 1 (NCBI accession P30438) and chain 2 (NCBI accession NP_001041619) were retrieved from the NCBI protein database. A multiple sequence alignment was performed using the top 50 homologous sequences with the highest similarity scores. The phylogenetic tree was constructed using the maximum likelihood method and visualized through the iTOL web platform [46].

4.3. Structural Characterization of Fel d 1 Allergen

The secondary structure of Fel d 1 was predicted using the SOPMA method. The SWISS-MODEL workspace (http://swissmodel.expasy.org/) (accessed on 2 March 2023) was used to predict the tertiary structure modeling; the model quality was evaluated using QMEAN and GMQE scoring systems [47–49].

4.4. Cell Epitope Analysis and Chimeric Protein Design of Fel d 1 Allergen

The immunoinformatic analysis of Fel d 1 was performed using an integrated bioinformatics approach. Antigenic determinants were comprehensively evaluated through multiple parameters, including antigenicity, hydrophilicity, β-turn, surface accessibility and molecular flexibility, using the antigenic determinant prediction workspace of Nanjing Detai bioinformatics platform (http://www.detaibio.com/peptide-antigen-prediction (accessed on 4 March 2023).

The T-helper (Th) cell epitopes of the Fel d 1 protein were predicted using the ProPred-I program, while cytotoxic T lymphocyte (CTL) epitopes were identified through IEDB (https://nextgen-tools.iedb.org/) or CTLpred workspace (http://crdd.osdd.net/raghava/ctlpred/) [50,51]. Dominant core epitopes were selected based on a stringent threshold score of 0.51.

Selected dominant epitopes were assembled into a cFel d 1 construct using a flexible linker (GGGGS) to maintain epitope independence and structural integrity. The engineered protein was subsequently characterized through secondary and tertiary structure prediction using SOPMA molecular modeling system (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa%20_sopma.html) (accessed on 2 March 2023) and Swiss-Model workspace (http://swissmodel.expasy.org/) (accessed on 2 March 2023).

4.5. Recombinant Expression and Purification of cFel d 1 Protein

The cFel d 1 gene sequence was codon-optimized for *E. coli* expression using the ExpOptimizer tool and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) [52]. The optimized gene was cloned into the pET-28a expression vector, and the recombinant pET-28a-cFel d 1 plasmid was transformed into *E. coli* BL21(DE3). Transformants were selected on Luria–Bertani (LB) plates (containing 50 μg/mL kanamycin) at 37 °C for 16–18 h. Positive clones were verified by colony PCR and Sanger sequencing (Supplementary Materials).

Recombinant cFel d 1 protein production was scaled from a shake flask to a 5 L bioreactor system. Briefly, a single positive colony of recombinant *E. coli* BL21(DE3)/pET-28a-cFel d 1 was inoculated into LB medium (containing 50 μg/mL kanamycin) and grown overnight at 37 °C. Subsequently, the culture was diluted 1:100 in fresh LB medium and incubated until optical density ($OD_{600\ nm}$) reached 0.6–0.8. Protein expression of cFel d 1 was induced with 0.4 mM IPTG at 30 °C (Supplementary Materials).

Following 4–6 h of induction, cells were harvested by centrifugation (10,000 rpm, 15 min, 4 °C) and resuspended in lysis buffer (25 mM Tris, 150 mM NaCl, pH 7.5). The cells were sonicated using a JY92-II N ultrasonic homogenizer (Ningbo Scientz Biotechnology Co., Ltd.) (Ningbo, China) under optimized conditions (15% amplitude, 5 s pulse/10 s rest cycles for 20 min). The lysate was clarified by centrifugation (10,000 rpm, 15 min, 4 °C), and the supernatant was sterile-filtered (0.22 μm) before loading onto a Ni^{2+} affinity chromatography column. Stepwise elution with imidazole gradient (50–500 mM) yielded purified cFel d 1, which was subsequently characterized by 16.5% Tris-Tricine-SDS-PAGE and matrix-assisted laser desorption/ionization Time-of-Flight mass spectrometry (MALDI-TOF-MS) to confirm molecular mass and purity.

4.6. Immunization of cFel d 1 Protein

The immunization experiments were conducted using purified cFel d 1 protein (500 μg/mL in PBS, pH 7.4) following standardized protocols. Female BALB/c mice (6-week-old, n = 5) received subcutaneous immunizations with 25 μg cFel d 1 antigen on day 0 (primary) and boosters on days 21 and 31 [19]. Complete Freund's adjuvant (1:1 emulsion) was used for the primary immunization, and incomplete adjuvant was used for the subsequent boosters. The control group received PBS/adjuvant mixtures.

Similarly, 140-day-old Hyline Brown laying hens purchased from a local company were randomly divided into three treatment groups: antigen group (n = 6, 0.3 mg of antigen), adjuvant group (n = 6), and negative control (n = 6). Intramuscular injections were administered at two-week intervals (four total) under controlled housing conditions, and eggs were collected weekly and pooled by treatment group.

4.7. Extraction and Immunochemical Validation of Anti-cFel d 1 IgY Antibodies

Anti-cFel d 1 IgY antibodies were isolated from eggs using an optimized PEG-6000 precipitation method with some modifications [53,54]. Antibody titers (mouse serum IgG and egg IgY) were quantified by iELISA (Supplementary Materials) [55,56].

Antibody specificity was systematically determined by Tris-Tricine-SDS-PAGE and WB. Briefly, both recombinant cFel d 1 protein (1.44–2.89 μg) and native Fel d 1 present in cat saliva (5–10 μL) were electrophoresed on a Tris-Tricine-SDS-PAGE gel, followed by transfer to PVDF membranes (100 V, 50 min). Membranes were then blocked with 5% skim milk in PBST (PBS containing 0.05% Tween-20) at 37 °C for 1.5 h with constant agitation (60 rpm). Subsequently, membranes were incubated overnight at 4 °C with anti-cFel d 1 IgY antibodies or adjuvant IgY antibodies (diluted 1:5,000 in blocking buffer), washed extensively with PBST, then incubated with HRP-conjugated mouse anti-chicken IgY secondary antibodies (Bersee, BIR798) at 37 °C for 1.5 h (60 rpm). Finally, specific antigen–antibody interactions were visualized using enhanced chemiluminescence (ECL) substrate and documented using a digital imaging system.

4.8. Efficacy of Anti-cFel d 1 IgY in Egg Yolk in Cats

Twenty-three adult healthy cats (11 males and 12 females; mean age, 3.5 years; weight, mean 3–4 kg; breed, Ragdoll, British Shorthair, Maine Coon and Chinese Calico) were included in this study. They were individually housed in cages (162 cm × 68 cm × 189 cm) at the pilot base of the Chinese Academy of Agricultural Sciences (CAAS). The cages were

cleaned once daily and the average daily feed intake of the cats was monitored, as well as their body weights throughout the study. All procedures were conducted in compliance with the Animal Welfare Act guidelines and were approved by the Laboratory Animal Ethical Committee at CAAS (IFR-CAAS20231226). Egg yolks containing anti-cFel d 1 IgY antibodies were lyophilized using a vacuum freeze dryer (LGJ-10C, Beijing, China) and incorporated into the cat's diets. All cats initially received a control diet and maintenance requirements for a one-week baseline period. They were subsequently randomly divided into four groups (n = 6 per group, with the 3% group having n = 5) for a four-week dietary intervention, during which each group was fed a maintenance diet enriched with 1%, 1.5%, 2%, or 3% anti-cFel d 1 IgY. Saliva samples were collected weekly, and salivary Fel d 1 levels were quantified using a commercial ELISA kit (Fel d 1 ELISA 2.0, Indoor Biotechnology).

4.9. Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism software (version 9.5.1) (GraphPad Software, La Jolla, Calif) and RStudio (4.3.2; RStudio: Integrated Development for R, Boston, MA, USA), with $p < 0.05$ being considered statistically significant. Comparisons between two independent groups were conducted using Student's *t*-test.

5. Conclusions

In summary, cFel d 1, designed based on Fel d 1 epitopes, was successfully expressed in *E. coli*. The resulting specific anti-cFel d 1 antibodies showed a strong immune response. Epitope-targeted anti-cFel d 1 IgY antibodies significantly reduced salivary Fel d 1 levels in cats. The antibody demonstrates potent specificity and safety. The allergen epitope-targeted IgY strategy paves the way for novel preventive and therapeutic approaches against allergic diseases.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms27052500/s1.

Author Contributions: Conceptualization, X.T., C.L. and X.W.; methodology, W.A., Y.W., J.W. (Jiaxue Wang), X.F., B.H. and H.T.; writing—review and editing, X.W., Z.W. and A.J.C.; funding acquisition, J.W. (Jinquan Wang). All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Fundamental Research Funds for the Institute of Feed Research, Chinese Academy of Agricultural Sciences (No. 1610382023021) and the Agricultural Science and Technology Innovation Program (ASTIP, No. 2023-IFR-14) from Chinese Academy of Agricultural Sciences.

Institutional Review Board Statement: All animal experiments (involving mice and laying hens for immunization, as well as cat efficacy studies) were performed in accordance with the Animal Care and Use Committee of Institute of Feed Research (IFR) at the Chinese Academy of Agricultural Sciences (CAAS), and they received approval from the Laboratory Animal Ethical Committee and its Inspection of the Institute of Feed Research of CAAS (IFR-CAAS20231101, 1 November 2023 and IFR-CAAS20231226, 26 December 2023).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

WHO	World Health Organization
IUIS	International Union of Immunological Societies
IgE	immunoglobulin E
AIT	allergen-specific immunotherapy
IgG	immunoglobulin G
IgY	Immunoglobulin Y
cFel d 1	chimeric protein
TMB	3,3′,5,5′-Tetramethylbenzidine
Th	T-helper
CTL	cytotoxic T lymphocyte
IPTG	isopropyl-β-D-thiogalactoside
Tris-Tricine-SDS-PAGE	tris-tricine sodium dodecyl-sulfate polyacrylamide gel electrophoresis
MALDI-TOF-MS	matrix-assisted laser desorption/ionzation time of Flight mass spectrometry
iELISA	indirect enzyme-linked immunosorbent assay
WB	Western blot
ECL	chemiluminescence

References

1. Dotterud, L.K.; Van, T.D.; Kvammen, B.; Dybendal, T.; Elsayed, S.; Falk, E.S. Allergen content in dust from homes and schools in northern Norway in relation to sensitization and allergy symptoms in schoolchildren. *Clin. Exp. Allergy* **1997**, *27*, 252–261. [CrossRef]
2. Dávila, I.; Domínguez-Ortega, J.; Navarro-Pulido, A.; Alonson, A.; Antolín-Amerigo, D.; González-Mancebo, E.; Martín-García, C.; Núñez-Acevedo, B.; Prior, N.; Reche, M.; et al. Consensus document on dog and cat allergy. *Allergy* **2018**, *73*, 1206–1222. [CrossRef]
3. Heinzerling, L.M.; Burbach, G.J.; Edenharter, G.; Bachert, C.; Bindslev-Jensen, C.; Bonini, S.; Bousquet, J.; Bousquet-Rouanet, L.; Bousquet, P.J.; Bresciani, M.; et al. GA(2)LEN skin test study I: GA(2)LEN harmonization of skin prick testing: Novel sensitization patterns for inhalant allergens in Europe. *Allergy* **2009**, *64*, 1498–1506. [CrossRef]
4. Bonnet, B.; Messaoudi, K.; Jacomet, F.; Michaud, E.; Fauquert, J.L.; Caillaud, D.; Evrard, B. An update on molecular cat allergens: Fel d 1 and what else? Chapter 1: Fel d 1, the major cat allergen. *Allergy Asthma Clin. Immunol.* **2018**, *14*, 14. [CrossRef]
5. Liccardi, G.; D'Amato, G.; Russo, M.; Canonica, G.W.; D'Amato, L.; Martino, M.D.; Passalacqua, G. Focus on cat allergen (Fel d 1): Immunological and aerodynamic characteristics, modality of airway sensitization and avoidance strategies. *Int. Arch. Allergy Immunol.* **2003**, *132*, 1–12. [CrossRef]
6. Liccardi, G.; Russo, M.; Barber, D.; Gilder, J.A.; DiPerna, F.; Parmiani, S.; D'Amato, M.; D'Amato, G. Efficacy of dry-cleaning in removing Fel d 1 allergen from wool fabric exposed to cats. *Ann. Allerg. Asthma Immunol.* **2002**, *88*, 301–305. [CrossRef]
7. Sparke, A.H. Human allergy to cats: A review for veterinarians on prevalence, causes, symptoms and control. *J. Feline Med. Surg.* **2021**, *24*, 31–42. [CrossRef] [PubMed]
8. Liccardi, G.; Martini, M.; Bilò, M.B.; Cecchi, L.; Milanese, M.; Brussino, L.; Motta, E.; Rogliani, P. Why is pet (cat/dog) allergen immunotherapy (AIT) such a controversial topic? Current perspectives and future directions. *Eur. Ann. Allergy Clin. Immunol.* **2024**, *56*, 188–191. [CrossRef] [PubMed]
9. Chapman, M.D.; Wood, R.A. The role and remediation of animal allergens in allergic diseases. *Allergy Clin. Immunol.* **2001**, *107*, S414–S421. [CrossRef] [PubMed]
10. Bousquet, J.; Gherasim, A.; de Blay, F.; Mathieu-Dupas, E.; Batot, G.; Laune, D.; Sousa-Pinto, B.; Zuberbier, T.; Pham-Thi, N.; MASK-Cat Study Group. Proof-of-concept study of anti-Fel d 1 IgY antibodies in cat food using the MASK-air® app. *Clin. Transl. Allergy* **2024**, *14*, e12353. [CrossRef]
11. da Silva, W.D.; Tambourgi, D.V. IgY: A promising antibody for use in immunodiagnostic and in immunotherapy. *Vet. Immunol. Immunopathol.* **2010**, *135*, 173–180. [CrossRef]
12. Akbari, M.R.; Ahmadi, A.; Mirkalantari, S.; Salimian, J. Anti-*Vibrio cholerae* IgY antibody inhibits mortality in suckling mice model. *J. Natl. Med. Assoc.* **2018**, *110*, 84–87. [CrossRef] [PubMed]
13. Pereira, E.; Tilburg, M.F.; Florean, E.O.P.T.; Guedes, M.I.F. Egg yolk antibodies (IgY) and their applications in human and veterinary health: A review. *Int. Immunopharmacol.* **2019**, *73*, 293–303. [CrossRef] [PubMed]

14. Mendoza, J.C.; Vivas, D.; Rodríguez, E.; Inga, R.; Sandoval, G.; Lazo, F.; Yarlequé, A. Experimental efficacy of IgY antibodies produced in eggs against the venom of the Peruvian snake *Bothrops atrox*. *Rev. Peru. Med. Exp. Salud. Publica* **2012**, *29*, 69–75. [CrossRef]
15. Artman, C.; Idegwu, N.; Brumfield, K.D.; Lai, K.; Hauta, S.; Falzarano, D.; Parreño, V.; Yuan, L.; Geyer, J.D.; Goepp, J.G. Feasibility of polyclonal avian immunoglobulins (IgY) as prophylaxis against human norovirus infection. *Viruses* **2022**, *14*, 2371. [CrossRef]
16. Carrara, G.M.P.; Silva, G.B.; Faria, L.S.; Nunes, D.S.; Ribeiro, V.S.; Lopes, C.A.; Gonçalves-Pires, R.F.M.; Santos, M.M.; Borges, I.P.; Ferreira-Junior, Á.; et al. IgY antibody and human neurocysticercosis: A novel approach on immunodiagnosis using *Taenia crassiceps* hydrophobic antigens. *Parasitology* **2020**, *147*, 240–247. [CrossRef]
17. Han, S.; Wen, Y.; Yang, F.; He, P. Chicken egg yolk antibody (IgY) protects mice against enterotoxigenic *Escherichia coli* infection through improving intestinal health and immune response. *Front. Cell. Infect. Microbiol.* **2021**, *11*, 662710. [CrossRef]
18. Satyaraj, E.; Garfner, C.; Filipi, I.; Cramer, K.; Sherrill, S. Reduction of active Fel d1 from cats using an antiFel d1 egg IgY antibody. *Immun. Inflamm. Dis.* **2019**, *7*, 68–73. [CrossRef]
19. Matulka, R.A.; Thompson, L.; Corley, D. Multi-level safety studies of anti Fel d 1 IgY ingredient in cat food. *Front. Vet. Sci.* **2020**, *6*, 477. [CrossRef]
20. Hedrick, E.D.; Matulka, R.A.; Conboy-Schmidt, L.; May, K.A. Evaluation of anti-Fel d 1 IgY ingredient for pet food on growth performance in kittens. *Front. Vet. Sci.* **2024**, *11*, 1355390. [CrossRef] [PubMed]
21. Rogers, B.L.; Morgenstern, J.P.; Garman, R.D.; Bond, J.; Kuo, M. Recombinant Fel d I: Expression, purification, IgE binding and reaction with cat-allergic human T cells. *Mol. Immunol.* **1993**, *30*, 559–568. [CrossRef]
22. Kaiser, L.; Velickovic, T.C.; Badia-Martinez, D.; Adedoyin, J.; Thunberg, S.; Hallén, D.; Berndt, K.; Grönlund, H.; Gafvelin, G.; Hage, M.; et al. Structural characterization of the tetrameric form of the major cat allergen Fel d 1. *J. Mol. Biol.* **2007**, *370*, 714–727. [CrossRef] [PubMed]
23. Morris, D.O. Human allergy to environmental pet danders: A public health perspective. *Vet. Dermatol.* **2010**, *21*, 441–449. [CrossRef] [PubMed]
24. Satyaraj, E.; Li, Q.; Sun, P.; Sherrill, S. Anti-Fel d1 immunoglobulin Y antibody-containing egg ingredient lowers allergen levels in cat saliva. *J. Feline Med. Surg.* **2019**, *21*, 875–881. [CrossRef] [PubMed]
25. Charpin, C.; Mata, P.; Charpin, D.; Lavaut, M.N.; Allasia, C.; Vervloet, D. Fel d I allergen distribution in cat fur and skin. *J. Allergy Clin. Immun.* **1991**, *88*, 77–82. [CrossRef]
26. Karn, R.C. The mouse salivary androgen-binding protein (ABP) alpha subunit closely resembles chain 1 of the cat allergen Fel dI. *Biochem. Genet.* **1994**, *32*, 271–277. [CrossRef]
27. Morgenstern, J.P.; Griffith, I.J.; Brauer, A.W.; Kuo, M.C. Amino acid sequence of Fel dI, the major allergen of the domestic cat: Protein sequence analysis and cDNA cloning. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 9690–9694. [CrossRef]
28. Scheib, H.; Nekaris, K.A.; Rode-Margono, J.; Ragnarsson, L.; Baumann, K.; Dobson, J.S.; Wirdateti, W.; Nouwens, A.; Nijman, V.; Martelli, P.; et al. The toxicological intersection between allergen and toxin: A structural comparison of the cat dander allergenic protein Fel d1 and the slow loris brachial gland secretion protein. *Toxins* **2020**, *12*, 86. [CrossRef]
29. Ichikawa, K.; Vailes, L.D.; Pomés, A.; Chapman, M.D. Identification of a novel cat allergen--cystatin. *Int. Arch. Allergy Immunol.* **2001**, *124*, 55–56. [CrossRef]
30. Brackett, N.F.; Davis, B.W.; Adli, M.; Pomés, A.; Chapman, M.D. Evolutionary biology and gene editing of cat allergen, Fel d 1. *CRISPR J.* **2022**, *5*, 213–223. [CrossRef]
31. Bienboire-Frosini, C.; Durairaj, R.; Pelosi, P.; Pageat, P. The major cat allergen Fel d 1 binds steroid and fatty acid semiochemicals: A combined in silico and in vitro study. *Int. J. Mol. Sci.* **2020**, *21*, 1365. [CrossRef]
32. Slunt, J.B.; Rogers, B.L.; Chapman, M.D. IgE antibodies to recombinant forms of Fel d I: Dichotomy between fluid-phase and solid-phase binding studies. *J. Allergy Clin. Immun.* **1995**, *95*, 1221–1228. [CrossRef]
33. Grönlund, H.; Bergman, T.; Sandström, K.; Alvelius, G.; Reininger, R.; Verdino, P.; Hauswirth, A.; Liderot, K.; Valent, P.; Spitzauer, S.; et al. Formation of disulfide bonds and homodimers of the major cat allergen Fel d 1 equivalent to the natural allergen by expression in *Escherichia coli*. *J. Biol. Chem.* **2003**, *278*, 40144–40151. [CrossRef]
34. Dall Antonia, F.; Pavkov-Keller, T.; Zangger, K.; Keller, W. Structure of allergens and structure based epitope predictions. *Methods* **2014**, *66*, 3–21. [CrossRef]
35. Zheng, B.; Zhang, H.; Shen, W.; Wang, L.; Chen, P. Core epitope analysis of 16 kDa allergen from tartary buckwheat. *Food Chem.* **2021**, *346*, 128953. [CrossRef]
36. Naveed, M.; Tehreem, S.; Arshad, S.; Bukhari, S.A.; Shabbir, M.A.; Essa, R.; Ali, N.; Zaib, S.; Khan, A.; Al-Harrasi, A.; et al. Design of a novel multiple epitope-based vaccine: An immunoinformatics approach to combat SARS-CoV-2 strains. *J. Infect. Public Health* **2021**, *14*, 938–946. [CrossRef] [PubMed]
37. Zaib, S.; Akram, F.; Liaqat, S.T.; Altaf, M.Z.; Khan, I.; Dera, A.A.; Uddin, J.; Ahmed, A.; Al-Harrasi, A. Bioinformatics approach for the construction of multiple epitope vaccine against omicron variant of SARS-CoV-2. *Sci. Rep.* **2022**, *12*, 19087. [CrossRef]

38. Velders, M.P.; Weijzen, S.; Eiben, G.L.; Elmishad, A.G.; Kloetzel, P.M.; Higgins, T.; Ciccarelli, R.B.; Evans, M.; Man, S.; Smith, L.; et al. Defined flanking spacers and enhanced proteolysis is essential for eradication of established tumors by an epitope string DNA vaccine. *J. Immunol.* **2001**, *166*, 5366–5373. [CrossRef]
39. Livingston, B.; Crimi, C.; Newman, M.; Higashimoto, Y.; Appella, E.; Sidney, J.; Sette, A. A rational strategy to design multiepitope immunogens based on multiple Th lymphocyte epitopes. *J. Immunol.* **2002**, *168*, 5499–5506. [CrossRef] [PubMed]
40. Shi, T.D.; Wu, Y.Z.; Zhou, W.; Jia, Z.Y.; Zou, L.Y. Study on antigen-specific cellular immune response induced by therapeutic peptides based on CTL epitope of HBV core antigen in vivo. *Acad. J. Second Mil. Med. Univ.* **2002**, *10*, 1169–1172.
41. Satyaraj, E.; Sun, P.; Sherrill, S. Fel d1 blocking antibodies: A novel method to reduce IgE-mediated allergy to cats. *J. Immunol. Res.* **2021**, *2021*, 5545173. [CrossRef]
42. Mina, A.; McNeice, L.; Banukumar, S.; Vazquez, S. Prozone and postzone effect: Unravelling the issues and designing protocol to address hook effect inimmunoassays. *LabMed Discov.* **2024**, *1*, 100030. [CrossRef]
43. Sandoval, M.N.; McClellan, S.P.; Pont, S.J.; Ross, J.A.; Swartz, M.D.; Silberman, M.A.; Boerwinkle, E. Prozone masks elevated SARS-CoV-2 antibody level measurements. *PLoS ONE* **2024**, *19*, e0301232. [CrossRef]
44. Lee, G.H.; Arthur, I.; Leung, M. False-negative serum cryptococcal lateral flow assay result due to the prozone phenomenon. *J. Clin. Microbiol.* **2018**, *56*, e01878-17. [CrossRef]
45. Stewart, T.W., Jr.; Parnell, D. Postzone v prozone. *JAMA* **1982**, *248*, 646–647. [CrossRef]
46. Kumar, S.; Stecher, G.; Michael, L.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
47. Geourjon, C.; Deleage, G. SOPMA: Significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Bioinformatics* **1995**, *11*, 681–684. [CrossRef]
48. Waterhouse, A.; Bertoni1, M.; Bienert, S.; Studer, G.; Tauriello, G.; Gumienny, R.; Heer, F.T.; Beer, T.A.P.; Rempfer, C.; Bordoli, L.; et al. SWISS-MODEL: Homology modelling of protein structures and complexes. *Nucleic Acids Res.* **2018**, *46*, W296–W303. [CrossRef] [PubMed]
49. Patel, G.B.; Rakholiya, P.; Shindhal, T.; Varjani, S.; Tabhani, N.M.; Shah, K.R. Lipolytic *Nocardiopsis* for reduction of pollution load in textile industry effluent and SWISS model for structural study of lipase. *Bioresour. Technol.* **2021**, *341*, 125673. [CrossRef] [PubMed]
50. Singh, H.; Raghava, G.P.S. ProPred: Prediction of HLA-DR binding sites. *Bioinformatics* **2001**, *17*, 1236–1237. [CrossRef]
51. Bhasin, M.; Raghava, G.P.S. Prediction of CTL epitopes using QM, SVM and ANN techniques. *Vaccine* **2004**, *22*, 3195–3204. [CrossRef]
52. Zhang, C.; Recacha, R.; Ruddock, L.W.; Moilanen, A. Efficient soluble production of folded cat allergen Fel d 1 in *Escherichia coli*. *Protein Expr. Purif.* **2021**, *180*, 105809. [CrossRef]
53. Adrizal, A.; Patterson, P.H.; Cravener, T.; Hendricks, G.L. Egg yolk and serum antibody titers of broiler breeder hens immunized with uricase and or urease. *Poul. Sci.* **2011**, *90*, 2162–2168. [CrossRef] [PubMed]
54. Day, M.J. Introduction to antigen and antibody assays. *Top. Companion Anim. Med.* **2015**, *30*, 128–131. [CrossRef] [PubMed]
55. Pauly, D.; Chacana, P.A.; Calzado, E.G.; Brembs, B.; Schade, R. IgY technology: Extraction of chicken antibodies from egg yolk by polyethylene glycol (PEG) precipitation. *Jove-J. Vis. Exp.* **2011**, *51*, 3084.
56. Madera-Contreras, A.M.; Solano-Texta, R.; Cisneros-Sarabia, A.; Bautista-Santos, I.; Vences-Velázquez, G.; Vences-Velázquez, A.; Cortés-Sarabia, K. Optimized method for the extraction of contaminant-free IgY antibodies from egg yolk using PEG 6000. *MethodsX* **2022**, *9*, 101874. [CrossRef]