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Development of a giant panda calprotectin-detecting immunoassay for non-invasive intestinal health monitoring

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Abstract

Non-invasive assessment of intestinal health in wildlife species remains a major challenge within One Health surveillance frameworks, particularly for protected or endangered animals. The giant panda (Ailuropoda melanoleuca), which exhibits unique digestive physiology and heightened sensitivity to dietary and environmental changes, lacks species-appropriate tools for monitoring intestinal inflammation. In this study, we developed an immunological framework based on recombinant giant panda calprotectin subunits. Recombinant giant panda S100A8 and S100A9 subunits were individually expressed using a Pichia pastoris system and used to generate monoclonal antibodies via hybridoma technology. Positive P. pastoris transformants were identified by colony PCR, and recombinant proteins were purified and verified prior to immunization. Following antibody screening and isotype characterization, systematic pairing assays were performed to identify compatible capture–detection antibody combinations suitable for sandwich ELISA development. The resulting monoclonal antibodies exhibited strong reactivity toward recombinant giant panda calprotectin, and multiple antibody pairs demonstrated antigen-dependent signals with low background reactivity. Although this study does not present a fully validated diagnostic assay, a semi-quantitative strategy for native calprotectin was established using immunoenrichment combined with SDS–PAGE and ImageJ-based densitometric analysis. Based on this approach, working standards were prepared, and a sandwich ELISA system was constructed. By enabling non-invasive monitoring of intestinal inflammation without reliance on cross-species reagents, this work contributes to the development of wildlife-appropriate health surveillance tools within a One Health framework and provides a reference approach for biomarker development in other non-model species.

Introduction

The giant panda (Ailuropoda melanoleuca) represents one of the most distinctive evolutionary lineages within Carnivora. Although pandas diverged from carnivorous ancestors several million years ago, their transition to an almost exclusively bamboo-based diet occurred without a parallel transformation of the gastrointestinal tract toward a herbivore-like morphology [1, 2]. Consequently, giant pandas retain a relatively short and simple digestive system that lacks specialized fermentation chambers, fundamentally limiting their capacity to efficiently utilize structural plant fiber [3, 4].

These anatomical constraints are closely reflected in panda digestive physiology. Apparent digestibility of bamboo fiber is low, and gastrointestinal transit is rapid, often occurring within hours rather than days. To maintain energy balance on a low-energy, low-digestibility diet, giant pandas rely on a compensatory strategy involving high food intake, selective consumption of different bamboo parts, and exceptionally low daily energy expenditure [5, 6]. As a result, the intestinal environment of giant pandas is particularly sensitive to variations in dietary composition and physical structure.

Accumulating evidence indicates that the panda gut ecosystem responds more strongly to dietary structure than to nutrient composition alone. Bamboo shoots, leaves, and culms differ substantially in

fiber composition, water content, and mechanical properties, and these differences translate into distinct physicochemical conditions within the gastrointestinal tract [7, 8]. For instance, fibrous bamboo culms impose greater mechanical load and provide limited readily available substrates, whereas bamboo shoots contain higher levels of protein and moisture, potentially altering intestinal transit dynamics and microbial activity [3, 9].

Under captive conditions, such sensitivities may be further amplified. Compared with free-ranging individuals, captive giant pandas are exposed to more frequent dietary adjustments, altered feeding schedules, and additional environmental stressors. Gastrointestinal irregularities—including soft stools, increased mucus content, and reduced digestive efficiency—are commonly observed in captive settings and may reflect subclinical intestinal stress or early-stage mucosal disturbance rather than overt gastrointestinal disease [10, 11].

Despite the high prevalence of gastrointestinal disturbances, assessment of intestinal health in giant pandas remains challenging. Invasive diagnostic approaches, such as endoscopy and tissue biopsy, can provide detailed information on mucosal pathology but are impractical for routine or repeated application due to welfare concerns and logistical constraints [12]. In contrast, systemic blood-based indicators often fail to capture localized intestinal inflammation, particularly when inflammatory activity is mild or spatially restricted. This limitation has been widely documented in both human and veterinary medicine, where fecal biomarkers have been shown to better reflect mucosal inflammatory status than systemic markers such as C-reactive protein or leukocyte counts [13–15].

In this context, fecal biomarkers offer a practical and biologically relevant alternative for assessing intestinal health. Because feces originate directly from the intestinal lumen, they integrate signals related to digestion, microbial metabolism, mucosal secretion, and immune cell recruitment. Among available fecal markers, calprotectin—a heterodimer composed of S100A8 and S100A9 proteins—has emerged as one of the most reliable indicators of intestinal inflammation. Calprotectin is highly abundant in neutrophils and monocytes and is released during cellular activation, migration, or cell death, thereby directly linking mucosal immune activity to luminal output [16, 17].

In human medicine, fecal calprotectin is widely used for the diagnosis of inflammatory bowel disease, monitoring disease activity, and assessing therapeutic response. Numerous studies have demonstrated strong correlations between fecal calprotectin concentrations and endoscopic or histological inflammation, often outperforming systemic inflammatory markers such as C-reactive protein or leukocyte counts [14, 18]. Comparable applications have also been reported in veterinary medicine, particularly in dogs with chronic enteropathies, supporting the broader relevance of calprotectin as a marker of intestinal immune activation across mammalian species [19, 20].

However, cross-species application of calprotectin assays is associated with inherent limitations. Differences in protein sequence, immunogenic epitopes, and fecal matrix composition can compromise measurement accuracy and consistency when antibodies developed for one species are applied to another. Although commercially available human or canine assays may provide approximate or

qualitative information, their quantitative reliability in giant pandas remains uncertain, especially for longitudinal monitoring or detection of low-grade inflammatory states.

Within a One Health framework, the development of species-appropriate, non-invasive biomarkers for wildlife health monitoring is increasingly recognized as a critical component of early risk detection and disease prevention at the human–animal–environment interface. To improve the accuracy of calprotectin-based assessment in giant pandas, the present study aimed to establish a calprotectin detection system based on panda-derived antigen and murine hybridoma antibody resources. Recombinant panda S100A8/A9 proteins were expressed, and monoclonal antibodies were generated using hybridoma technology. Through systematic screening and antibody pairing, this study sought to establish a robust immunological foundation for sandwich ELISA development, thereby enabling more reliable and species-appropriate non-invasive monitoring of intestinal inflammation in giant pandas.

Methods

Gene sequence analysis, codon optimization, and recombinant expression of S100A8 and S100A9 subunits

The coding sequences of giant panda S100A8 (NCBI accession no. 100466995 and S100A9 (NCBI accession no. 100474047 were retrieved from the National Center for Biotechnology Information (NCBI) database.Open reading frames were verified using ExPASy Translate, and sequence integrity was confirmed to exclude frame shifts or premature stop codons. To improve heterologous expression efficiency, both genes were codon-optimized for expression in Pichia pastoris while preserving the native amino acid sequences.

The optimized genes were synthesized and cloned into a P. pastoris expression vector. Recombinant plasmids were linearized and transformed into P. pastoris X33 competent cells. Transformants were selected on Zeocin-containing plates (InvivoGen, Cat. No. ant-zn-1). Codon optimization was performed using a commercial gene synthesis platform (e.g., GenScript proprietary optimization algorithm), taking into account codon usage bias, GC content, mRNA secondary structure, and potential repetitive elements to enhance expression efficiency in P. pastoris. The codon-optimized coding sequences of S100A8 and S100A9, excluding signal peptides, affinity tags, stop codons, vector backbone sequences, and non-coding flanking regions, are provided separately in Supplementary Table S1. In this study, S100A8 and S100A9 were designed and expressed as individual recombinant subunits rather than as a single-chain fusion protein or a co-expressed heterodimer. This strategy allowed independent production and purification of each component for subsequent immunization and antibody screening.

PCR identification of recombinant Pichia pastoris transformants

To screen and confirm positive P. pastoris transformants, colony PCR was performed after antibiotic selection. Colony PCR was performed directly using yeast colonies as template without prior genomic

DNA extraction. Two AOX flanking primers (5'AOX and 3'AOX) and two gene-specific primers (102667-1-R and 102667-2-F) were used for PCR-based identification of recombinant clones. Primer sequences are listed in Supplementary Table S2. PCR products were analyzed by 1% agarose gel electrophoresis. Clones showing the expected amplification pattern were selected for subsequent methanol-induced protein expression. The expression construct used for transformation is shown in Fig. S1.

Protein expression, purification, and verification

PCR-positive Pichia pastoris clones were cultured for recombinant protein expression in 500 mL baffled flasks containing 100 mL buffered glycerol-complex medium (BMGY). Cultures were incubated at 28–30°C with shaking at 220 rpm until reaching an OD600 of 2–6. Cells were then harvested by centrifugation and resuspended in buffered methanol-complex medium (BMMY) for induction. Recombinant protein expression was induced by adding methanol to a final concentration of 0.5% (v/v), which was replenished every 24 h. Induction was carried out for 72 h under the same culture conditions. Following induction, the culture supernatant was collected by centrifugation for downstream purification.

Recombinant giant panda S100A8 and S100A9 subunits were expressed separately rather than as a pre-assembled heterodimer. Proteins were purified from the supernatant using Ni–NTA affinity chromatography (Qiagen, Cat. No. 30210) under native conditions according to the manufacturer's instructions. Eluted fractions were analyzed by SDS–PAGE to assess protein purity using a commercial gel preparation kit (Bio-Rad Laboratories, Cat. No. 4561096), and proteins were transferred onto PVDF membranes (Millipore, Cat. No. IPVH00010) for Western blot analysis. Anti-His tag antibody (Abcam, Cat. No. ab18184) and anti-FLAG tag antibody (Sigma-Aldrich, Cat. No. F1804) were used as primary antibodies, followed by HRP-conjugated goat anti-mouse IgG (Abcam, Cat. No. ab205719) as the secondary antibody. Western blot analysis was performed under reducing conditions using SDS sample buffer containing β-mercaptoethanol. In addition, non-reducing SDS–PAGE was conducted for comparison to evaluate potential intermolecular interactions.

Mouse immunization and serum titer determination

Female BALB/c mice (6–8 weeks old) were immunized subcutaneously with 50 μg of purified recombinant calprotectin emulsified in Freund's complete adjuvant for the primary immunization, followed by two booster immunizations with the same antigen dose emulsified in Freund's incomplete adjuvant at two-week intervals. Serum samples were collected one week after each immunization to monitor antibody titers. Female BALB/c mice (6–8 weeks old) were purchased from Beijing HFK Bio-Technology Co., Ltd. (Beijing, China; experimental animal production license no. SCXK (Jing) 2024-0003) and housed under specific-pathogen-free conditions at the Laboratory Animal Center of China Agricultural University. The vendor's production license and the animal quality certificate for this batch are provided as Supplementary Information.

Hybridoma generation and screening

Splenocytes isolated from immunized BALB/c mice were fused with Sp2/0 myeloma cells using polyethylene glycol–mediated fusion. Fused cells were cultured in hypoxanthine–aminopterin–thymidine (HAT) selection medium and distributed into 96-well plates. One week after the final booster, mice with the highest serum antibody titers were euthanized by cervical dislocation (without prior anaesthesia), and spleens were aseptically removed for splenocyte preparation. Euthanasia was performed in accordance with the AVMA Guidelines for the Euthanasia of Animals.

Hybridoma culture supernatants were screened by indirect ELISA using recombinant calprotectin as the antigen. Positive clones were identified based on ELISA optical density values and further subjected to subcloning by limiting dilution to obtain stable monoclonal antibody–producing cell lines.

Monoclonal antibody isotyping, purification, and pairing

Stable hybridoma clones were expanded, and monoclonal antibodies were produced using the ascites fluid method. Antibody isotypes were determined using a commercial mouse monoclonal antibody isotyping kit. Monoclonal antibodies were purified by Protein A affinity chromatography (GE Healthcare, Cat. No. 17-1279-01), and antibody purity was assessed by SDS–PAGE analysis.

Antibody pairing assays were performed using an indirect sandwich ELISA format. Recombinant calprotectin protein was used as the primary antigen during initial screening. In selected experiments, enriched fecal extracts were also included to evaluate antibody performance in a complex biological matrix.

Capture antibodies were coated onto 96-well plates (Corning Inc., Cat. No. 3590) at a concentration of 1–5 μg/mL in carbonate buffer (pH 9.6), Detection antibodies were applied at optimized dilutions, and signal intensity was measured as optical density at 450 nm. Pairing efficiency was evaluated based on signal-to-noise ratio (S/N), defined as the ratio between antigen-containing wells and blank controls.**Estimation of native calprotectin concentration and preparation of working standards**

To enable semi-quantitative analysis in the absence of a commercially available standard for giant panda calprotectin, native calprotectin was enriched from fecal samples. Fecal samples (~ 2 g) were suspended in PBS with protease inhibitors, homogenized, and centrifuged (10,000 rpm, 15 min, 4°C). Supernatants were concentrated 5–10× by dialysis (10 kDa cut-off) against PEG. Calprotectin was enriched via immunoaffinity capture using immobilized anti-calprotectin monoclonal antibody #10. After incubation (4°C, 1–2 h), the column was washed, bound proteins eluted with glycine-HCl (pH 2.7), neutralized, and clarified by centrifugation for SDS-PAGE analysis. The target band corresponding to approximately 33 kDa was selected for densitometric analysis. Band intensity was quantified using ImageJ software, and relative protein abundance was estimated based on grayscale values. To improve accuracy, the estimated concentration of the target protein was further corrected by incorporating purity measurements derived from SDS–PAGE analysis.

A series of samples with known total protein concentrations (0.2, 0.1, 0.05, 0.025, and 0.0125 mg/mL) were analyzed. Based on purity correction, the corresponding concentrations of the target protein were calculated and used to determine the actual amount of target protein loaded per lane.

A standard relationship between grayscale intensity and target protein loading amount was established using a four-parameter logistic (4PL) model. This model was subsequently used to estimate the concentration of native calprotectin in enriched samples and to prepare working standards for downstream ELISA assay development.

Establishment of sandwich ELISA and standard curve construction

Based on antibody pairing results, monoclonal antibody #10 (capture) and #16 (detection) were selected. Under optimized conditions (checkerboard titration), capture antibody was used at 1:9,000, detection antibody at 1:243,000, and HRP-streptavidin at 1:3,000. Microplates were coated with capture antibody (overnight, 4°C), washed (PBST), and blocked (1% BSA, 0.1% Tween-20, 2 h, 37°C). Standards (0.33–27 μg/mL, triplicate) or diluted fecal samples (100 μL/well) were added and incubated (1 h, 37°C). After washing, biotinylated detection antibody was added (1 h, 37°C), followed by HRP-streptavidin (30 min, 37°C). Plates were washed, TMB substrate added (10–15 min, dark), reaction stopped with 2 mol/L H_2SO_4, and absorbance read at 450 nm. A four-parameter logistic (4PL) model was used to fit the standard curve.**Limit of detection (LOD) calculation**

The limit of detection (LOD) of the ELISA system was determined based on blank controls. Three replicate blank wells were measured, and the mean OD450 value and standard deviation (SD) were calculated. The OD-based detection threshold was defined as the mean blank value plus three times the standard deviation (Mean + 3SD). The corresponding concentration was then interpolated from the standard curve using the fitted 4PL model.

Results

PCR identification of recombinant Pichia pastoris transformants

Colony PCR analysis was performed to identify P. pastoris transformants carrying the recombinant calprotectin expression constructs. Among 20 screened colonies, multiple clones produced specific amplification bands consistent with the positive control, whereas no amplification was observed in the negative control. Approximately half of the screened colonies were PCR-positive and were selected for subsequent methanol-induced expression experiments. Representative PCR results are shown in Fig. 1.

Lane M, DL5000 DNA marker (100–5000 bp); lanes 1–20, PCR products from individual colonies; lane +, positive control; lane −, negative control (nuclease-free water). 5'AOX/102667-1-R: ~550–600 bp; 102667-2-F/3'AOX: ~450–500 bp

Development of recombinant giant panda calprotectin subunits for immunoassay development

PCR-positive P. pastoris clones were successfully induced for recombinant protein expression. Following methanol induction, the target proteins were predominantly detected in the culture supernatant, indicating efficient secretion of recombinant giant panda calprotectin subunits.

As shown in Fig. 2A, SDS–PAGE analysis revealed distinct protein bands at approximately ~ 15 kDa and ~ 18 kDa, consistent with the expected molecular weights of S100A8 and S100A9, respectively. The banding patterns were comparable under reducing and non-reducing conditions, with no evidence of a stable higher-molecular-weight complex.

Protein identity was further validated by Western blot analysis (Fig. 2B), in which the recombinant proteins were specifically recognized by anti-His and anti-Flag tag antibodies at the expected molecular sizes, confirming successful expression and tag integrity.

During purification, SDS–PAGE analysis of sequential fractions (Fig. 2C) demonstrated progressive enrichment of the target proteins, with the dominant bands becoming increasingly prominent and background bands correspondingly reduced, indicating effective purification by Ni–NTA affinity chromatography.

Because S100A8 and S100A9 were expressed as separate recombinant subunits, the observed bands correspond to individual proteins rather than a pre-assembled heterodimer. Under non-reducing conditions, slight band variations may reflect transient intermolecular interactions but do not support the formation of a stable heterodimer prior to downstream applications.

Immune response and hybridoma screening

All immunized BALB/c mice developed strong antigen-specific immune responses against recombinant giant panda calprotectin. Indirect ELISA revealed detectable serum antibody titers up to a dilution of 1:64,000 (Table S3). Mice with the highest serum titers were selected for splenocyte–myeloma cell fusion.

Hybridoma fusion generated approximately 1,400 primary clones. Initial ELISA screening identified 74 clones producing antibodies with strong reactivity to recombinant calprotectin. After subsequent subcloning by limiting dilution, 25 stable monoclonal antibody–producing hybridoma cell lines were obtained. A summary of the generated monoclonal antibody clones and their basic characteristics is provided in Supplementary Tables S4–S6.

Characterization of monoclonal antibodies and antibody pairing

Isotype analysis showed that the majority of monoclonal antibodies belonged to the IgG class, with IgG1 and IgG2b as the predominant subclasses, and all antibodies carried κ light chains. Purified monoclonal antibodies exhibited high purity as assessed by SDS–PAGE and remained stable during continuous cell culture.

The reactivity of monoclonal antibodies against recombinant giant panda calprotectin subunits was initially evaluated by ELISA, showing clear antigen-dependent signals with minimal background in blank controls. Systematic antibody pairing screening was then performed using a sandwich ELISA-based format to identify compatible capture–detection combinations. The results demonstrated that only a subset of antibody combinations produced strong and reproducible signals with low background reactivity. Among these, antibody #10 used as the capture antibody in combination with antibody #16 as the detection antibody consistently showed the highest signal intensity and favorable signal-to-noise characteristics(Tables S7). These results indicate that effective antibody pairing is highly dependent on epitope compatibility, and only selected monoclonal antibody combinations are suitable for subsequent development of a sandwich ELISA system.

Estimation of native calprotectin concentration using densitometric analysis

To establish a working reference for quantitative analysis, native calprotectin enriched from fecal samples was subjected to SDS–PAGE separation, and the band corresponding to approximately 33 kDa was selected for densitometric analysis(Fig. S2). Quantitative analysis based on ImageJ grayscale measurements showed a strong correlation between band intensity and target protein loading amount. After purity correction, the estimated amounts of target protein ranged from 0.0143 to 0.2749 μg across the tested concentration gradient.

Among multiple observed bands (15 kDa, 18 kDa, 24 kDa, 33 kDa, and 50 kDa), the 33 kDa band exhibited the best fitting performance. A four-parameter logistic (4PL) model demonstrated an excellent fit (R^2 = 0.9999), indicating that grayscale intensity could reliably reflect the relative abundance of the target protein(Fig. 3). Therefore, the 33 kDa band was selected as the primary basis for estimating native calprotectin concentration and preparing working standards for subsequent ELISA analysis.

A four-parameter logistic (4PL) regression curve was established based on grayscale intensity measurements of the ~ 33 kDa band obtained from SDS–PAGE analysis of enriched native calprotectin. Band intensities were quantified using ImageJ and plotted against the estimated protein loading amounts after purity correction.

Establishment of ELISA standard curve

Under optimized assay conditions, a sandwich ELISA system was established using the selected antibody pair (#10/#16). The assay exhibited a clear concentration-dependent increase in absorbance across the tested range (0.33–27 μg/mL). The standard curve fitted using a four-parameter logistic

(4PL) model showed a high correlation coefficient (R^2 = 0.9994), indicating good fitting performance and quantitative potential within the tested range(Fig. 4).

These results demonstrate that the developed ELISA system provides a reliable basis for semi-quantitative detection of giant panda fecal calprotectin. Together with the densitometric standard preparation strategy, this establishes a preliminary but functional workflow for quantitative assay development in the absence of commercially available standards.

A sandwich ELISA was established using the selected antibody pair (#10/#16) under optimized assay conditions.

Precision analysis

To evaluate repeatability, enriched fecal matrix samples containing 0.15 μg/mL (low), 61.68 μg/mL (medium), and 230.92 μg/mL (high) calprotectin were prepared and measured in triplicate within the same assay run. The coefficient of variation (CV%) was calculated as SD/mean × 100%. The CV values were 4.84% for the low, 1.75% for the medium, and 0.75% for the high concentration, indicating good repeatability of the assay under complex biological matrix conditions.

Spike-recovery analysis

For spike-recovery analysis, fecal matrix samples with a baseline calprotectin concentration of 0.016 μg/mL were spiked with working-standard calprotectin to final concentrations of 16.87 μg/mL (low), 22.5 μg/mL (medium), and 33.75 μg/mL (high). Each level was measured in triplicate. Recovery was calculated as (measured spiked sample – measured unspiked matrix) / spiked amount × 100%. The mean recoveries were 72.10% (low), 129.26% (medium), and 127.50% (high), indicating acceptable accuracy with some matrix effect at low concentrations.

Limit of detection of the ELISA system

The limit of detection (LOD) of the ELISA system was determined based on blank measurements. The mean OD450 value of blank wells was 0.07735, with a standard deviation of 0.00178. Based on the criterion of mean blank value plus three times the standard deviation (Mean + 3SD), the OD detection threshold was calculated to be 0.0827. Using the standard curve, this value corresponded to a concentration of 0.0071 μg/mL. These results indicate that the developed ELISA system has a relatively low detection limit and is suitable for detecting calprotectin in fecal samples.**Discussion**

In this study, monoclonal antibodies targeting giant panda calprotectin were generated through a workflow combining recombinant protein expression and hybridoma-based antibody production. Recombinant S100A8 and S100A9 proteins were successfully expressed in Pichia pastoris and purified to a level suitable for immunization, enabling subsequent antibody generation and screening. Systematic antibody pairing further identified combinations appropriate for sandwich ELISA development.

The use of a recombinant expression strategy was both necessary and practical in this context. For endangered species such as the giant panda, access to native tissues or inflammatory exudates is extremely limited, rendering direct purification of endogenous calprotectin impractical [21, 22]. Recombinant expression provides a stable and renewable antigen source and allows immunization to be conducted with proteins of defined composition and reproducibility. In the present study, successful expression and secretion of recombinant panda calprotectin in Pichia pastoris established a reliable foundation for downstream antibody development.

It should be noted that S100A8 and S100A9 were expressed as separate recombinant subunits rather than as a native heterodimer. Although calprotectin exists physiologically as a S100A8/A9 heterodimer, the use of individual subunits as immunogens represents a practical compromise under recombinant expression conditions. In addition, recombinant proteins produced in yeast systems may differ from native mammalian proteins in post-translational modifications, which could affect epitope structure and antibody binding characteristics. Therefore, further validation using native calprotectin derived from fecal samples will be necessary to fully evaluate assay performance in biologically relevant contexts.

An important outcome of this work was the generation of monoclonal antibodies with confirmed reactivity toward giant panda calprotectin. Although calprotectin is a conserved inflammatory marker across mammals, interspecies differences in amino acid sequence and epitope structure can influence antibody recognition [23, 24]. This limitation is particularly relevant when antibodies developed for human or canine antigens are applied to phylogenetically distant species such as the giant panda. These results suggest that the use of panda-derived antigens provides a suitable basis for assay development in this species, although direct comparative studies with cross-species antibodies were not performed in the present work.

The hybridoma screening process yielded 25 stable monoclonal antibody–producing cell lines from an initial pool of approximately 1,400 fusion clones. Subsequent pairing experiments demonstrated that only a subset of antibody combinations produced strong antigen-dependent signals with low background reactivity, underscoring the importance of empirical pairing in sandwich ELISA development [25]. Even antibodies with high standalone binding activity may fail to function effectively as pairs if they recognize overlapping or sterically constrained epitopes.

Based on selected antibody pairs, a preliminary sandwich ELISA system was established and evaluated. By combining immunoenrichment with SDS–PAGE-based densitometric analysis, a practical strategy was developed to estimate native calprotectin concentration in the absence of commercially available standards. The resulting ELISA system demonstrated good fitting performance, repeatability, and acceptable recovery in fecal samples. Notably, both precision and recovery evaluations were performed in enriched fecal matrices rather than in simple buffer systems, providing a more realistic assessment of assay performance under complex biological conditions.

Importantly, the assay exhibited a limit of detection (LOD) of 0.0071 μg/mL, indicating sufficient sensitivity to detect low concentrations of calprotectin. This level of sensitivity is particularly relevant for

identifying mild or early-stage intestinal inflammation. However, matrix effects and biological variability in fecal samples may influence assay performance, and further validation using a larger number of biological samples will be necessary.

Although the present study focused on antibody generation and preliminary assay establishment, it provides a methodological foundation for non-invasive intestinal health monitoring in giant pandas. The monoclonal antibodies described here may be applied to multiple immunological platforms, including sandwich ELISA and immunochromatographic assays, and potentially extended to other applications such as immunohistochemical analyses [26]. From a One Health perspective, the framework established in this study may be adapted for biomarker assay development in other wildlife species, contributing to broader wildlife health surveillance efforts without compromising animal welfare.

Conclusions

In conclusion, recombinant giant panda S100A8 and S100A9 subunits were successfully expressed and purified using a Pichia pastoris expression system, enabling the generation of monoclonal antibodies targeting panda calprotectin.Through hybridoma screening and systematic antibody pairing, multiple monoclonal antibodies with high specificity toward panda calprotectin were obtained, and suitable combinations for sandwich ELISA development were identified. In addition, a preliminary sandwich ELISA system was established and evaluated, demonstrating good repeatability and acceptable recovery in fecal samples. These findings indicate that the developed approach provides a feasible strategy for semi-quantitative detection of calprotectin in giant pandas. Together, these results establish both an immunological and methodological foundation for the development of species-appropriate immunoassays for wildlife health monitoring within a One Health framework. The strategy presented here also provides a practical reference for biomarker assay development in other wildlife species with limited sample accessibility.

Abbreviations

BMGY
buffered glycerol-complex medium
BMMY
buffered methanol-complex medium
BSA
bovine serum albumin
cv
coefficient of variation
ELISA
enzyme-linked immunosorbent assay
HAT
hypoxanthine–aminopterin–thymidine

HRP
horseradish peroxidase
IgG
immunoglobulin G
LOD
limit of detection
NCBI
National Center for Biotechnology Information
Ni–NTA
nickel–nitrilotriacetic acid
OD
optical density
PBS
phosphate-buffered saline
PBST
phosphate-buffered saline containing Tween-20
PCR
polymerase chain reaction
P. pastoris
Pichia pastoris
SD
standard deviation
SDS–PAGE
sodium dodecyl sulfate–polyacrylamide gel electrophoresis
S/N
signal-to-noise ratio
TMB
3,3',5,5'-tetramethylbenzidine
4PL
four-parameter logistic.

Declarations

Ethics approval and consent to participate

All animal care and experimentation involving laboratory mice were approved by the Animal Ethics Committee of China Agricultural University, Beijing (approval no. Aw72305202-2-01), and were performed in accordance with the approved guidelines. BALB/c mice were purchased from Beijing HFK Bio-Technology Co., Ltd. (Beijing, China; experimental animal production license no. SCXK (Jing) 2024-0003) and housed under specific-pathogen-free conditions; euthanasia was performed by cervical

dislocation (without prior anaesthesia), in accordance with the AVMA Guidelines for the Euthanasia of Animals. The vendor's production license and the animal quality certificate are provided as Supplementary Information.

Fecal samples from giant pandas were collected non-invasively, without any intervention with the animals, with the oral permission and cooperation of the holding institutions (Beijing Zoo, Chengdu Research Base of Giant Panda Breeding, Shanxi Wildlife Park, Shanxi Rare Wildlife Rescue and Breeding Center, Foping Panda Valley, and Foping National Nature Reserve). No additional field or animal-use permit was required for collection of fecal material under the terms agreed with these collaborating institutions.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

J.W. contributed to the study design, sample collection, laboratory experiments, data analysis and interpretation, and drafted the manuscript. L.M. and Y.Q. contributed to sample collection and laboratory experiments. Y.W. and Z.L. contributed to sample processing and data collection. W.S. and Z.H. contributed to data analysis and interpretation. H.X. contributed to the study design and provided technical support. Y.J. conceived and supervised the study, contributed to the study design and interpretation of the results, and critically revised the manuscript. All authors read and approved the final manuscript.

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Data Availability

All data generated or analysed during this study are included in this published article and its supplementary information files. Full-length, unprocessed gel and blot images are provided as Supplementary Fig. S3; supplementary tables S1–S7 are available from the corresponding author on reasonable request.

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Figures

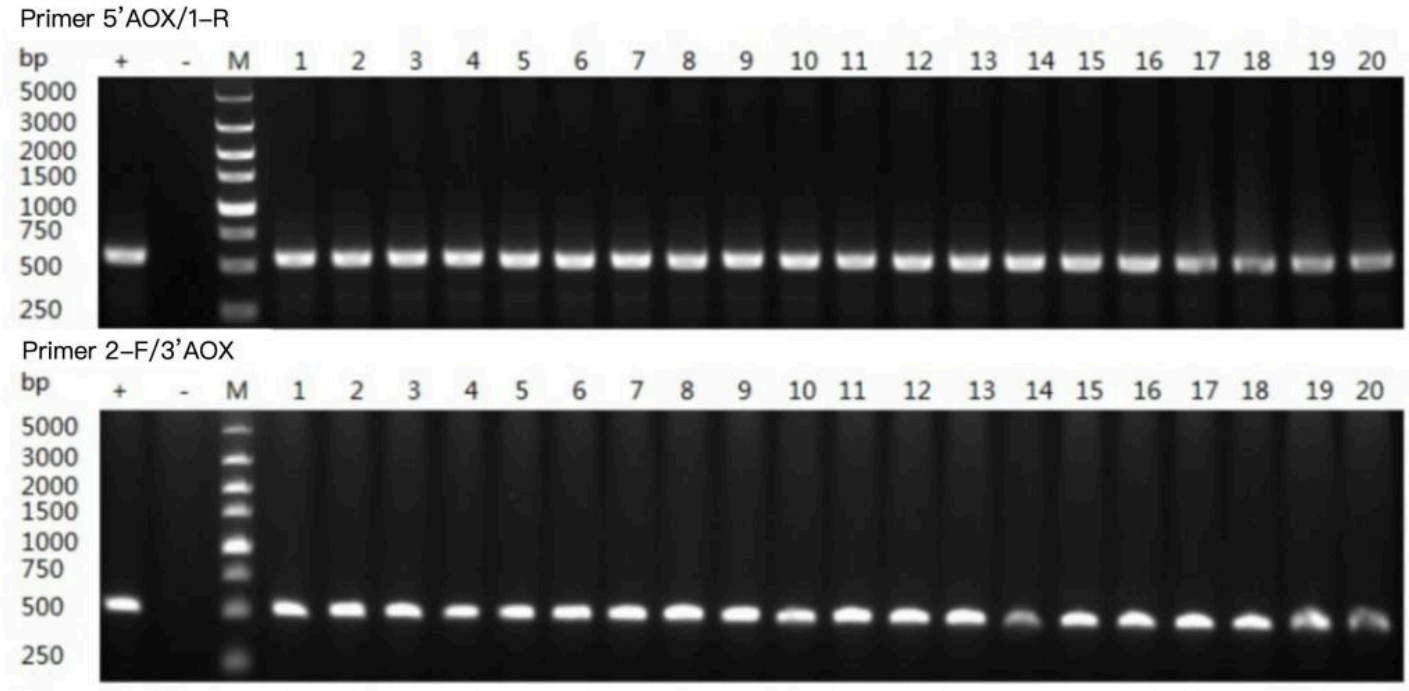


Figure 1

PCR identification of recombinant Pichia pastoris transformants.

Lane M, DL5000 DNA marker (100–5000 bp); lanes 1–20, PCR products from individual colonies; lane +, positive control; lane −, negative control (nuclease-free water). 5'AOX/102667-1-R: ~550–600 bp; 102667-2-F/3'AOX: ~450–500 bp

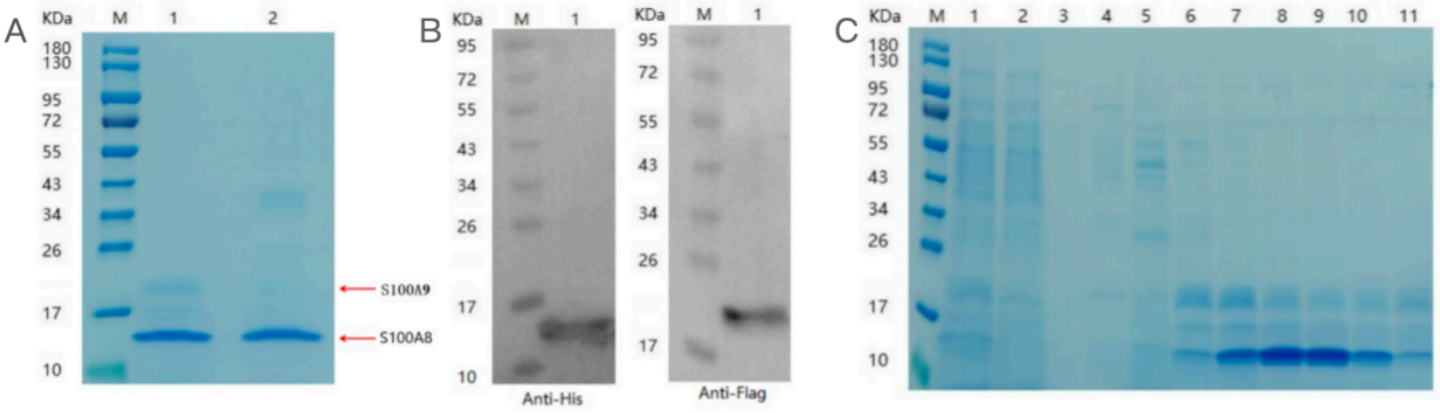


Figure 2

Expression, validation, and purification of recombinant giant panda calprotectin subunits (S100A8 and S100A9). (A) SDS–PAGE analysis of purified recombinant S100A8 and S100A9 proteins under reducing and non-reducing conditions. Lane M, protein molecular weight marker; lane 1, purified recombinant protein under reducing conditions; lane 2, purified recombinant protein under non-reducing conditions.

(B) Western blot analysis of recombinant proteins using anti-His and anti-Flag antibodies, confirming successful expression and tag-specific recognition of S100A8 and S100A9. (C) SDS–PAGE analysis of the purification process of recombinant calprotectin subunits. Lane M, protein molecular weight marker; lane 1, medium sample; lane 2, effluent sample; lane 3, washing solution sample; lane 4, elution solution sample; lanes 5–11, purified product fractions. Full-length, unprocessed versions of the SDS–PAGE gels and Western blots shown in (A)–(C), together with molecular-weight markers, are provided as Supplementary Fig. S3.

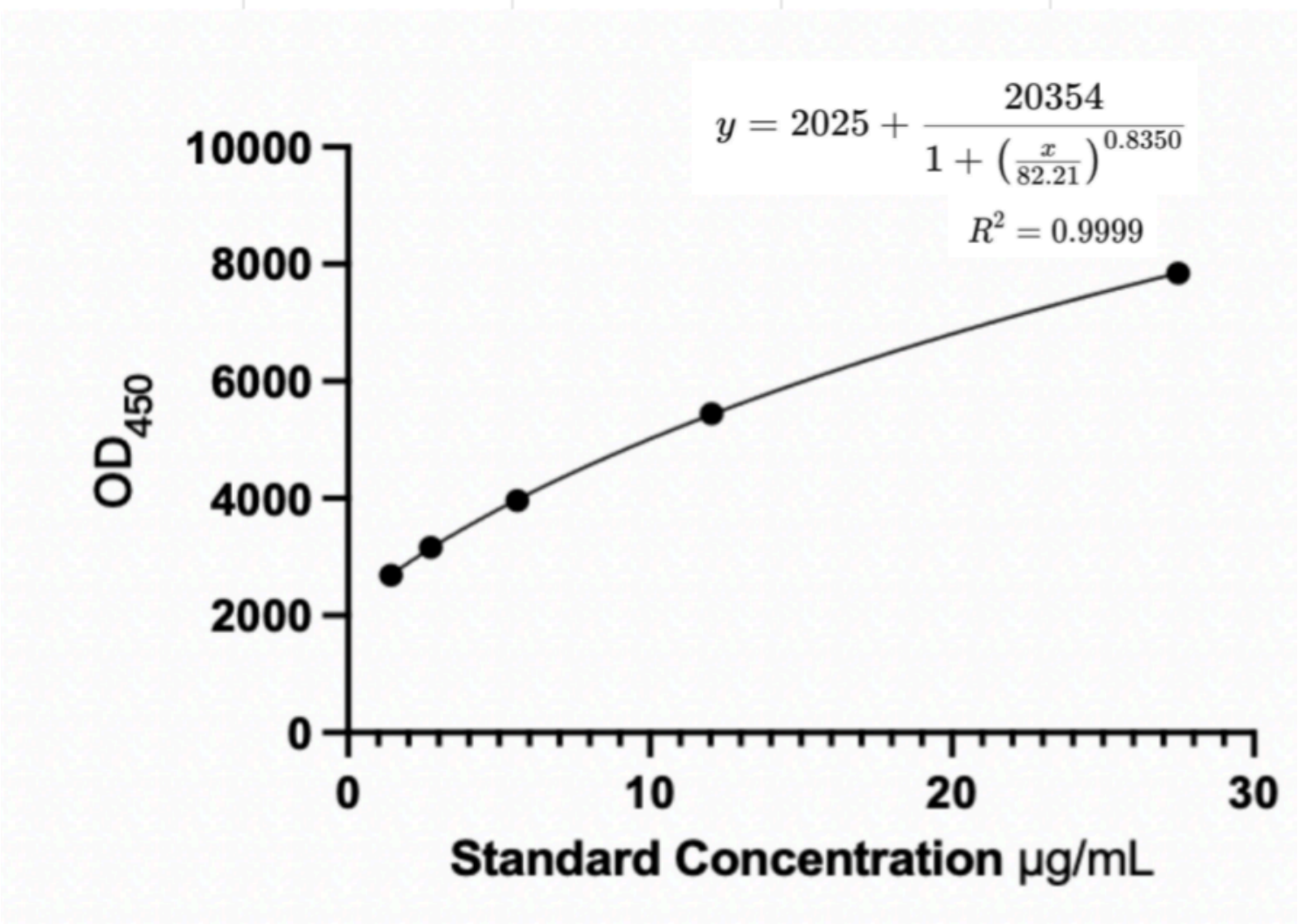


Figure 3

Densitometric calibration curve for estimating native calprotectin concentration

A four-parameter logistic (4PL) regression curve was established based on grayscale intensity measurements of the~33 kDa band obtained from SDS–PAGE analysis of enriched native calprotectin. Band intensities were quantified using ImageJ and plotted against the estimated protein loading amounts after purity correction.

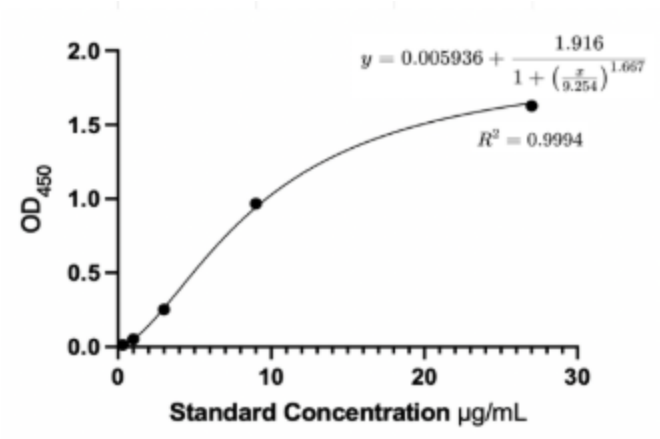


Figure 4

Standard curve of the sandwich ELISA for calprotectin detection

A sandwich ELISA was established using the selected antibody pair (#10/#16) under optimized assay conditions.

Supplementary Files

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