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Development of a sensitive direct competitive chemiluminescent enzyme immunoassay for gentamicin based on the construction of a specific single-chain variable fragment-alkaline phosphatase fusion protein

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Abstract

A sensitive chemiluminescent enzyme immunoassay (CLEIA) was established for the determination of gentamicin (GEN) residue levels in animal tissue. This assay is based on a fusion protein of single-chain variable fragment (scFv) and alkaline phosphatase (AP). Initially, V_L and V_H derived from anti-gentamicin monoclonal antibody were linked by a short peptide to construct a scFv. Subsequently, the constructed scFv sequence was accessed into the pLIP6/GN vector, and a soluble scFv-AP fusion protein was generated. The scFv-AP fusion protein was used to develop a direct competitive CLEIA (dcCLEIA) for the determination of gentamicin. In the dcCLEIA,

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

CRediT authorship contribution statement

Weijie Deng: Conceptualization, Methodology, Investigation, Formal analysis, Data Curation, Writing-Original draft preparation
Dan Wang: Investigation, Formal analysis, Methodology, Data Curation, Software, Funding acquisition
Peng Dai: Conceptualization, Methodology, Data Curation, Formal analysis
Yanping Hong: Data Curation, Formal analysis, Funding acquisition
Jianhua Xiong: Data Curation, Formal analysis, Funding acquisition
Luying Duan: Writing-review & editing
Ruimin Lu: Investigation, Methodology, Data Curation, Software
Jianchun Wan: Conceptualization, Methodology, Data Curation, Formal analysis
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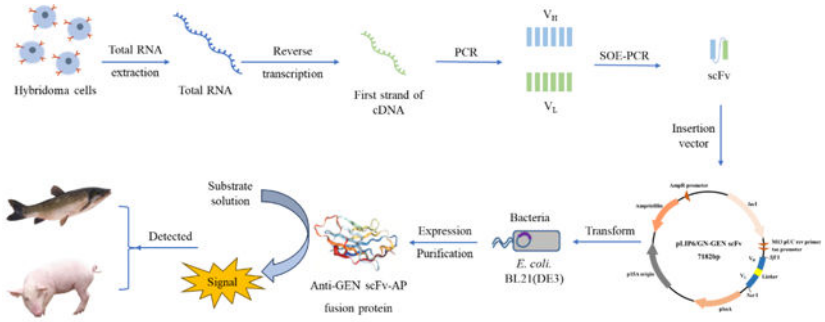
Bruce D. Hammock: Conceptualization, Writing-review & editing, Funding acquisition
Wuying Yang: Conceptualization, Supervision, Validation, Funding acquisition, Resources, Writing-review & editing

the half inhibitory concentration (IC_{50}) and limit of detection (LOD) were 1.073 ng/mL and 0.380 ng/mL, respectively. The average recoveries of gentamicin spiked in animal tissue samples ranged from 78% to 96%. These results showed a strong correlation with ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The above results suggest that the anti-GEN scFv-AP fusion protein is suitable for detecting gentamicin residues in edible animal tissues.

Graphical abstract



Keywords

Gentamicin; Anti-gentamicin scFv; Alkaline phosphatase; ScFv-AP fusion protein; Chemiluminescent enzyme immunoassay

1. Introduction

Gentamicin is a broad-spectrum aminoglycoside antibiotic, because of its affordability and effectiveness against bacteria, it is widely used in agricultural production for treating infections of livestock, poultry and aquatic animals such as pneumonia, mastitis and bacillary diarrhea [1,2]. However, excessive use of gentamicin can cause its accumulation in animal-derived food products [3]. Extensive research has shown that excessive amounts of gentamicin in animal-derived foods cause serious side effects to the human body mainly include nephrotoxicity, ototoxicity and the development of antibiotic resistance in pathogenic bacteria [4,5]. In order to control the misuse of gentamicin, many countries, including China, have already stipulated maximum limits on its use. In the European Union (EU) and China, the maximum residue limits (MRLs) in edible tissues of pigs and bovine range from 50–750 μg/kg and 100–5000 μg/kg, respectively. The MRLs for edible pig tissue in the United States is 100–300 μg/kg. Due to the potential hazard of gentamicin to human health, it is essential to establish prompt and sensitive detection methods for monitoring the overuse of gentamicin in both high throughput laboratory assays and field assays.

At present, the instrumental methods reported for the determination of gentamicin in food include high performance liquid chromatography (HPLC) [6–10] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [11–13]. The traditional instrumental methods mentioned above can provide favourable specificity, prominent sensitivity and desired accuracy, but their high cost and time-consuming characteristics also

play a limiting role in the actual sample detection. Instrumental methods are difficult to adapt for field assays. In contrast, immunoassay methods, which are based on the quick antigen-antibody reaction, have many advantages of quick detection, high sensitivity, good specificity, inexpensive, field assay and high-throughput screening. Some immunoassay methods have already been reported for detecting gentamicin residues [14–16].

Generally, the quality of an antibody directly affects the specificity and sensitivity of the immunoassay. Based on the comparison with polyclonal antibodies (pAb) and monoclonal antibodies (mAb), the recombinant antibodies (rAb) exhibit noticeable advantages, which have led to the rapid development of genetic engineered antibody technology. ScFv is small antibody fragments consisting of the variable region of the heavy (V_H) and the variable region of the light (V_L) chains of an intact antibody in tandem with a nimble linker peptide. If properly constructed, scFv antibodies not only show excellent specificity and affinity, but they can also be cloned into a vector and expressed in large quantities through eukaryotic or prokaryotic expression [17–19]. More importantly, recombinant scFv genes can be easily expressed by fusing them with other genes, which allows the expressed fusion protein to have two different functions [20,21]. For instance, it has been reported that scFv antibodies fused with alkaline phosphatase (AP) or other enzymes using molecular techniques can generate uniform and stable scFv-AP fusion protein that are used for analytes of direct immunoassay [22–24]. The fusion of scFv with the commonly used AP or other enzymes leads to good immunoassay reagents. These fusion proteins can reduce the number of operational steps in detection, shorten the testing time, eliminate the need for chemically conjugated enzyme-labelled secondary antibodies, and greatly improve the efficiency of the immunoassay method. Currently, most of the immunoassay methods reported in the literature for the determination of gentamicin residue are enzyme-linked immunosorbent assay (ELISA) [25,26].

Chemiluminescent enzyme immunoassay (CLEIA) is an efficient format for immunoassays, offering greater specificity and higher sensitivity compared to ELISA. Therefore, the CLEIA procedure offers clear advantages over various alternative detection modes for microtoxic substances [27,28]. Until now, there have been no reports on the preparation of scFv against gentamicin from murine hybridomas or the establishment of the CLEIA for determination of gentamicin residues in food by the anti-GEN scFv-AP fusion protein. In our previous study, positive hybridoma cell 40H6, which can secrete an anti-gentamicin monoclonal antibody, was selected and obtained using the hybridoma technique [29]. In this experiment, total RNA was extracted from 40H6 cell and reverse transcribed into cDNA, which was then used as the formwork to amplify V_H and V_L gene fragments. Then the selected a set of most suitable V_H and V_L were used to form anti-GEN scFv gene fragments through splicing by overlap extension-polymerase chain reaction (SOE-PCR). The anti-GEN scFv gene fragment was inserted into the pLIP6/GN vector, which carries the alkaline phosphatase gene [30], to construct pLIP6/GN-GEN scFv recombinant plasmid. Under induction of isopropyl β-D-thiogalactoside (IPTG), the *E. coli* BL21(DE3) transformed with the recombinant plasmid expressed the fusion protein against GEN. And the fusion protein then was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (WB). Based on the purified anti-GEN scFv-AP fusion protein, a novel direct competitive CLEIA was established for the detection of gentamicin

residue. This method was supported by comparison data from UPLC-MS/MS. To the best of our knowledge, this is the first report on the use of dcCLEIA based on the anti-GEN scFv-AP fusion protein for detecting gentamicin residues in animal tissue.

2. Material and methods

2.1. Materials

All primers used for PCR were synthesized by Invitrogen Biotechnology Co. (Shanghai, China). Penicillin mixture (100×), agarose, 6× DNA Loading Buffer, ampicillin, gentamicin, IPTG, 4-Nitrophenyl phosphate (PNPP), lysozyme, sodium deoxycholate, 2×protein loading buffer and X-gal were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Easypure Quick Gel Extraction Kit, TransZol Up plus RNA Kit, pEASY-T3 vector, EasyScript One-Step Gdna Removal and cDNA Synthesis SuperMix, EasyPure polymerase chain reaction (PCR) Purification Kit, DNA 5000 Marker, GelStain, the plasmid mini preparation kit and 2×TransStart FastPfu PCR SuperMix were purchased from Beijing Transgen Co., Ltd. (Beijing, China). The IPVH00010 PVDF membrane was purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd. (Shanghai, China). SDS-PAGE gel preparation kit was purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). 96-well white chemiluminescence plates and the chemiluminescence substrate solution (aqueous solutions of 1,2-dioxocyclic hexane derivatives) were purchased from Beijing Key-Bio Biotech Co. Ltd. (Beijing, China). The restriction enzymes *Not*I, *Bgl*I, *sfi*I and T4 DNA ligase were purchased from New England Biolabs Inc. (Hertfordshire, U.K). The hybridoma cell 40H6 producing mAb against GEN and the pLIP6/GN expression vector were stored in our laboratory. NHS-Activated Sepharose™ 4 Fast Flow was purchased from Shanghai Yuanye Biotechnology Co., LTD (Shanghai, China).

2.2. Cloning of the V_H and V_L genes

Prior to extracting total RNA, the anti-GEN monoclonal antibody positive hybridoma cells line named 40H6 obtained in previous work was repurified three times by limited dilution method to ensure its purity (100% positive). After culturing positive hybridoma cells 40H6 to a density of approximately 1.0×10^7, total RNA was extracted by the TransZol Up plus RNA kit (Transgen, Beijing, China). Subsequently, the extracted RNA was reverse transcribed using the EasyScript One-Step Gdna Removal and cDNA Synthesis SuperMix kit to synthesize first-strand cDNA. Then the synthesized cDNA was used as a template to amplify the V_L and V_H genes by the universal primers listed in Table S1 [31]. The PCR products amplified successfully were purified by Easypure Quick Gel Extraction Kit (Transgen, Beijing, China) and then inserted into the pEASY-T3 vector for sequencing identification (Sangon, Shanghai, China). The V_H and V_L genes that were successfully sequenced were analysized by V-QUEST in the international ImMunoGeneTics (IMGT) database, and the antibody genes and nonsense genes derived from tumor cells were excluded in order to obtain candidate V_H and V_L genes. After translating the candidate V_H and V_L genes with correct analysis results into amino acid sequences, a homology comparison was performed using BLAST in the National Center for Biotechnology Information (NCBI) database. The three-dimensional structure homology modeling was performed by SWISS-MODEL subroutine of ExPASy Proteomics tools to obtain a three-

dimensional simulation structure of the scFv antibody. The most suitable combination of V_H and V_L was selected by the above steps.

2.3. Assembly of anti-GEN scFv

The most suitable V_H gene and V_L gene were spliced into the scFv antibody gene using a $(Gly_4Ser)_3$ linker peptide through SOE-PCR [22,32]. The basic principle of SOE-PCR is performed separately with primers having complementary paired fragments to obtain two DNA fragments with overlapping strands, and then the amplified fragments from different sources are overlapped and spliced together by overlapping strand extension in the subsequent amplification reaction. Primer design is one of the key steps in SOE-PCR. Two pairs of primers (V_H primer pair and V_L primer pair) were designed in the SOE-PCR experiment of our study, as shown in Table S2. The V_H primer pair consists of two primers, V_H-Forward-*Sfi*I and V_H-Backward-linker. The V_L primer pair contains V_L-Forward-linker and V_L-Backward-*Not*I. The SOE-PCR experiment of our study included two rounds of PCR reactions. In the first round of the PCR reaction, the V_H gene with *Sfi*I site and a portion of the linker was amplified by primers V_H-forward-*Sfi*I and V_H-backward-linker. The V_L gene with a portion of the linker and *Not*I site was amplified by primers V_L-forward-Linker and V_L-backward-*Not*I. In the second round of the PCR reaction, the complementary sections of primer V_H-Backward-Linker and primer V_L-Forward-Linker overlaped and formed overlapping chains, while the V_H and V_L genes were spliced together by overlapping strand extension in the subsequent amplification reaction. Finally, the recombinant target scFv gene sequence (V_H-Linker-V_L) was obtained through the two rounds of PCR reaction above. The SOE-PCR products were purified by Easypure Quick Gel Extraction Kit.

2.4. Construction of the pLIP6/GN-GEN scFv plasmid

The purified scFv gene was digested with *Not*I and *Sfi*I restriction enzymes, and the vector pLIP6/GN was also using the same method for processing. The two types of digested products were purified once each and mixed proportionally according to the concentration. T4 ligase was used for ligation and the reaction system was passed at 16 °C overnight. The following day the ligation results were transformed into BL21 competent cells through thermal activation and cultured at 37 °C overnight on Luria-Bertani (LB) solid medium, which contained 100 μg/mL of ampicillin. The next day, positive single clones were selected for *Sfi*I and *Not*I enzyme digestion, PCR and sequencing identification.

2.5. Expression and purification of the anti-GEN scFv-AP Fusion Protein

Protein expression was performed on the clones that were positively identified by the sequencing mentioned above. The diagram of recombinant plasmid pLIP6/GN-GEN scFv are shown in Fig. S1. Briefly, the anti-GEN scFv gene was cloned into the pLIP6/GN vector and then the vector carrying the alkaline phosphatase gene were induced with IPTG to express the fusion proteins that specifically recognize GEN and carry AP enzyme [33]. The positive bacteria containing the recombinant plasmid were transferred to LB-Amp medium and cultured with shaking at 37 °C and 180 rpm until the desired absorbance was achieved. Subsequently, IPTG was added at a final concentration of 0.6 mM to induce the production of the soluble fusion protein [34]. The fusion protein was extracted using

ultrasonic extraction. The procedure for ultrasonic extraction was as follows: First, the medium was centrifuged at 9000 g for 18 minutes at 4 °C. The bacterial precipitate was then collected and washed twice with PBS. After that, it was sonicated in an ice-water bath using a sonication power of 400 W for 5 seconds, with a 5-second interval. The resulting solution was centrifuged again at 9000 g for 18 minutes at 4 °C. The supernatant containing the soluble anti-GEN scFv-AP fusion protein was collected and identified by SDS-PAGE and WB using an anti-AP mAb.

The collected crushed supernatant protein was purified using NHS-Activated Sepharose™ 4 Fast Flow, following the instructions provided in the operating manual [35]. The purified antibodies were identified by SDS-PAGE and then stored frozen at −20°C for subsequent immunoassays.

2.6. Direct competitive CLEIA procedure

The optimal concentration of gentamicin-OVA and anti-GEN scFv-AP fusion proteins were determined through a checkerboard titration procedure. The dcCLEIA detection was carried out as follows: First, a chemiluminescent microplate was coated with gentamicin-OVA and incubated at 4 °C overnight. The next day, the microplate with the coated liquid was washed four times with PBST and then blocked with 4% non-fat powdered milk in PBS at 37 °C for 2 hours. After three rounds of PBST oscillation washing, the microplates were placed in the incubator to dry. Fifty microliters of gentamicin standard (or sample) and another fifty microliters of the anti-GEN scFv-AP antibody solution, both dissolved in PBST, were added to each well and incubated at 37 °C for half an hour. After discarding the liquid, the plate was cleaned with four washes of 240 μL PBST. Then, 100 μL of the chemiluminescence substrate solution was added to each well and mixed gently. Finally, the relative light units (RLU) of the wells were measured instantly using the SpectraMax M2 microplate reader (Molecular Devices, USA). The RLU value of control wells without inhibitor is B_0, and the RLU value of wells with different concentrations of inhibitor is B. B/B_0 represents the binding rate, and the concentration of inhibitor at 50% binding rate is referred to as IC_{50}. Generally, the smaller the IC_{50} value, the higher the specificity of the antibody. According to the optimized concentration of coated antigen and anti-GEN scFv-AP fusion protein mentioned earlier, the standard curve of dcCLEIA for gentamicin was fitted with a sigmoidal curve using Origin 8.5 software.

Based on the scFv-AP fusion protein, the dcELISA method was also established. The dcELISA was performed similarly to dcCLEIA. Differently, 100 μL of the freshly prepared ELISA-AP substrate solution (containing 0.5 mM $MgCl_2$, 1 M diethanolamine, and 3 mM p-nitrophenyl phosphate at pH 9.8) was pipetted into each ELISA microplate instead of the chemiluminescence solution. The solution was then incubated at 37 °C for 10–15 minutes. The optical density (OD) values at 405 nm were measured using a SpectraMax M2 microplate reader after terminating the chromogenic reaction with 40 μL of 4 M NaOH. The standard curve of the dcELISA method was also generated using Origin 8.5. The IC_{50} and LOD values of the dcELISA were measured for comparison with dcCLEIA.

2.7. Cross-Reactivity(CR)

Five other aminoglycosides were tested as gentamicin analogues and were assessed for cross-reactivity using the dcCLEIA procedure described above. The IC_{50} values of these structural analogues were determined and compared with those of gentamicin. The cross-reactivity values for gentamicin analogues were measured by the following calculation formula: CR=[IC_{50}(gentamicin)/IC_{50} (analyte)]×100%.

2.8. Analysis of Spiked Samples

Fish and pork were chosen for the spiked recovery experiment. The two kinds of animal sample were acquired from a nearby market and identified as gentamicin free negative sample by UPLC-MS/MS method. A validation study was performed by evaluating recovery from spiked fish and pork samples with gentamicin standard substances at a concentration rate of 0, 375, 750 and 1500 ng/g. The specific pretreatment steps are as follows: two grams wet mass of spiked pork and fish tissue were transferred into a 50 ml polypropylene centrifuge tube. The samples with gentamicin added were extracted with 10 mL of distilled water at 37 °C for 20 minutes. The protein in the samples was then precipitated by adding 20 mL of 3% trichloroacetic acid. After shaking the extraction for 25 minutes, the mixture was centrifuged at 5500 g for 20 minutes. Finally, 3 mL of the supernatant was extracted with 2 mL of *n*-hexane through liquid-liquid extraction. The mixture was then centrifuged at 1000 g for 12 minutes, and the bottom layer of liquid was collected for further analysis. These bottom layers of liquid extracts were divided into two groups and assayed using dcCLEIA, which was developed in our study, and UPLC-MS/MS, respectively. The UPLC-MS/MS analysis was conducted by the Technology Center of Nanchang Customs District, China. The experimental operation procedure of the UPLC-MS/MS method was based on a previous literature published by our research group [29]. The recovery rate of spiked gentamicin were calculated with the following formula: Recovery=(detection amount/additive amount)×100%. To validate the accuracy and reliability of the dcCLEIA method, the gentamicin contents obtained from both methods were compared, and the correlation was calculated.

3. Results

3.1. Selecting of the most sutiable V_H and V_L genes and assembly of anti-GEN scFv

Total RNA was extracted from the anti-GEN monoclonal antibody-positive hybridoma cell 40H6, which was obtained in previous work. The nucleic acid electrophoresis result of the extracted total RNA was shown in Fig. S2. The obtained total RNA was reverse-transcribed into first strand of cDNA. The two genes encoding the variable regions of V_H and V_L were amplified from cDNAs using PCR. The purified V_L and V_H genes were then transferred into the pEASY-T3 vector for sequencing. Within the successfully sequenced V_H genes, four of the gene sequences had a base pair number that was a multiple of 3 and were all 363 bp in size. Within the eight V_L genes that were successfully sequenced, only one met the requirements of being a multiple of three and had a length of 333 bp.

The four V_H and one V_L gene sequences were inputted into the IMGT database for analysis in order to distinguish between the functional and aberrant sequences. The sequence analysis

of four V_H genes (V_HF1B2, V_HF2B2, V_HF6B2 and V_HF8B2) from IMGT/V-QUEST indicated that all four V_H genes were productive IGH rearranged sequences (with no stop codon and an in-frame junction) (Fig. 1A, Table S3, Table S4, Table S5, Table S6). The sequence analysis of the V_L gene from IMGT/V-QUEST revealed that the V_L gene (V_LF2B3) was a productive IGH rearranged sequence (Fig. 1B, Tables S7). The analysis results from NCBI/BLAST showed that the V_H sequence was 79.20% homologous to WGF12958.1 mouse antibody (shown in Table S8), and the V_L sequence was 90.99% homologous to ABV48919.1 mouse antibodies (shown in Table S9), respectively. The amino acid sequences of four V_H genes were compared by DNAMAN software, and the results of the multiple comparison are shown in Fig. S3. The comparison revealed that the four V_H genes are nearly identical, except for two amino acids that differ in the FR1 region and the amino acid homology of the four V_H genes was found to be 99.38%. In the RT-PCR experiment above, the PCR amplification of the V_HF1B2 gene is the most efficient among the four productive V_H genes. So, based on the above results, the V_HF1B2 gene was selected to be assembled with the only productive V_L gene (V_LF2B3) to obtain the anti-GEN scFv gene. The three-dimensional structure simulation diagram of anti-Gen scFv gene (V_HF1B2-linker-v_LF2B3) was obtained after homologous modeling through SWISS-MODEL subroutine of ExPASy Proteomics tools, as shown in Fig. S4. According to Fig. S4A, the heavy and light chains of anti-Gen scFv antibody were connected by linking peptides, and the distribution of 6 complementarity-determining regions (CDRs) formed A “ring bucket” structure. According to Fig. S4B, it can be seen that the complementary determining region bound to the antigen is a hole-like “pocket”, which accords with the spatial structure of typical scFv, and theoretically has good antigenic binding activity.

The most suitable V_H gene (V_HF1B2) and V_L gene (V_LF2B3) were spliced into scFv antibody gene with a $(Gly_4Ser)_3$ linker peptide by SOE-PCR. The products of V_H, V_L and scFv amplification were shown in Fig. 2. The anti-gen scFv gene was approximately was 750 bp. The results suggest that the anti-GEN scFv was assembled successfully.

3.2. Construction of pLIP6/GN-GEN scFv expression plasmid

The anti-Gen scFv gene was cloned into the pLIP6/GN vector and then transformed into BL21 competent cell. The constructed plasmid was identified by PCR, digestion reaction and sequencing. The PCR identification result was shown in Fig. 3A. About 750 bp of expected bands were amplified by the positive clone using primers. The pLIP6/GN vector contains three *BglⅡ* sites, and the anti-Gen scFv gene was inserted between two of these *BglⅡ* sites. Thus, after digestion by *BglⅡ* (Fig. 3B), the pLIP6/GN formed two fragments of approximately 2000bp and one fragment of approximately 3000bp (lane 4), while the recombinant plasmid pLIP6/GN-GEN scFv formed two about 3000bp fragments and one about 2000bp fragment (lane 3). These results indicate that the anti-Gen scFv gene was successfully constructed and inserted into the pLIP6/GN vector. The sequencing identification result of pLIP6/GN-GEN scFv expression plasmid was shown in Fig. S5, the V_H and V_L regions of the anti-GEN scFv gene constructed in our study consisted of three CDRs and four framework regions (FRs), respectively. Additionally, It exhibited a high degree of homology with the mouse-derived antibody (WGF12958.1 mouse antibody and ABV48919.1 mouse antibody, shown in Table S8 and Table S9).

3.3. Expression and purification of anti-GEN soluble scFv-AP fusion protein

Expression was induced by IPTG in pLIP6/GN bacteria and pLIP6/GN-GEN positive bacteria, while uninduced positive bacteria were used as controls. The presence of the anti-GEN fusion protein in the supernatant was identified by SDS-PAGE (Fig. 4A) and WB (Fig. 4B). The fusion protein against gentamicin appeared as a distinct band (about 75 kDa) on SDS-PAGE and WB. Typically, the molecular weight of a scFv is about 30 kDa, while the AP enzyme is about 50 kDa. These results indicated that the size of the fusion protein was consistent with the experimental expectations. The expressed proteins were purified using NHS-Activated Sepharose™ 4 Fast Flow. The purification results of the recombinant protein confirmed that the purified fusion protein was very pure (Fig. 4C).

3.4. Direct competitive CLEIA procedure

A sensitive direct competitive CLEIA based on the scFv-AP fusion protein purified above was established in this work. In order to enhance the sensitivity and efficiency of the dcCLEIA, we conducted a checkerboard titration to screen the concentration of the GEN-OVA and the purified fusion protein. The results of the checkerboard titration showed that the most suitable match between the concentration of GEN-OVA and the purified fusion protein was 0.125 μg/mL and 25 μg/mL, as shown in Table 1. A fitted standard curve of the dcCLEIA for GEN was generated using the suitable concentration of coated antigen and anti-GEN scFv-AP fusion protein (Fig. 5A). The fitted standard curve showed an IC_{50} of 1.073 ng/mL and an LOD of 0.380 ng/mL, with a linear range of 0.546–3.428 ng/mL. Compared with dcELISA based on scFv-AP fusion protein for gentamicin (IC_{50}=7.693 ng/mL, LOD=1.029 ng/mL) (Fig. 5B), the dcCLEIA had lower IC_{50} and LOD, which indicated that dcCLEIA had better sensitivity and was more suitable for the detection of gentamicin residues in edible animal tissue. Herein, the aqueous solutions of 1, 2-dioxocyclic hexane derivatives were used as the chemiluminescent substrate for AP in the dcCLEIA for gentamicin.

3.5. Specificity of the assay

Five other aminoglycosides including kanamycin, strepomycin, ampicillin, chloramphenicol and neomycin were tested for cross-reactivity by the dcCLEIA procedure described in our study. The procedures for the determination of these drugs were consistent with those for gentamicin. The results of the cross-reaction experiment show that the scFv antibody had no significant specific reaction (less than 0.1%) with the structural analogues of gentamicin (Table 2). Similar to parental mAb, the anti-GEN scFv-AP fusion protein exhibits the desired selectivity for GEN [29]. The above results show that the fusion protein has good specificity to gentamicin.

3.6. Spiked sample analysis

The accuracy of dcCLEIA was determined by measuring the recovery from spiked pork and fish samples. These two kinds of samples were sourced from nearby markets and the UPLC-MS/MS results showed that they did not contain gentamicin. During the tests, we utilized the dcCLEIA method developed in our study to detect gentamicin in both the spiked and non-spiked samples. Additionally, we employed the UPLC-MS/MS method for confirmation.

The results of spiked sample analysis by dcCLEIA and UPLC-MS/MS are shown in Table 3. All samples spiked with gentamicin exhibited excellent agreement between the detection quantity and the additive quantity. In the dcCLEIA method, the recoveries of gentamicin in pork range from 78% to 93% with coefficient variations (CVs) of 2.9–4.1%. Similarly, the recoveries of gentamicin in fish range from 80% to 96% with CVs of 1.7–7.4%. In the UPLC-MS/MS method, the recoveries of gentamicin in pork range from 96% to 106% with CVs of 0.2–5.1%, recoveries of gentamicin in fish range from 100% to 105% with CVs of 0.8–3.2%. As shown in Fig. S6, the results of dcCLEIA correlated well with the UPLC-MS/MS results. The dcCLEIA thus satisfied the accuracy requirement for detecting residues in edible animal tissues.

4. Conclusion

Compared to traditional mAb and pAb, genetically engineered antibodies like scFv offer several advantages. It can be expressed rapidly and in large quantities using cost-effective and scalable prokaryotic expression systems. Additionally, It can be modified for various purposes, such as genetic modification or labeling with fluorescent substances or enzymes. For the past few years, there has been a increasing number of studies focusing on genetically engineered antibodies.

In our previous study, a traditional positive hybridoma cell 40H6 which secretes an anti-gentamicin monoclonal antibody was prepared by immunizing BALB/c mice with gentamicin-BSA and using the hybridoma cell fusion screening technique. Based on the positive hybridoma cell 40H6, the anti-GEN scFv gene was successfully constructed using genetic engineering technology in this study. The scFv gene was inserted into the pLIP6/GN and subsequently expressed and purified. A sensitive and specific scFv-AP fusion protein against gentamicin was prepared successfully.

Based on the anti-GEN scFv-AP fusion protein, we established a sensitive dcCLEIA for the detection of gentamicin in animal tissues. The IC_{50} of the assay was determined to be 1.073 ng/mL, with a LOD of 0.380 ng/mL in our study. Pork and fish were used as samples for spiked recovery experiments, and the comparison with the results of UPLC-MS confirmed that the established method mentioned above demonstrates good accuracy and feasibility. Compared with other quantitative assays, the dcCLEIA method established in our study demonstrated short detection time, low limit of detection, high sensitivity and specificity. Therefore, these findings indicate that the dcCLEIA reported here is a suitable and powerful tool for the rapid analysis of gentamicin in a good deal of animal food samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

1. This is the first report on the preparation of anti-GEN scFv antibody from murine positive hybridoma cells.
2. Traditionally, scFv genes are screened and obtained by phage display antibody library technology. In our study, the anti-GEN scFv gene was first constructed directly by splicing overlap extension-polymerase chain reaction (SOE-PCR) from 100% positive hybridoma cell.
3. The anti-GEN scFv-AP fusion protein was expressed for the first time through the pLIP6/GN vector, which not only had the characteristics of scFv, but also had the chromogenic activity of the labeled enzyme.
4. For the first time a sensitive direct competitive CLEIA for gentamicin was developed based on the anti-GEN scFv-AP fusion protein, which has the desired sensitivity and specificity.

V_H	<----------FR1-IMGT--------><--CDR1-IMGT--><--- FR2-IMGT-->
HF1B2	**Q**VQL**Q**QSGAELIKPGASVKISCKAN**GYTFSNSW**IEWVKQRPGHGLEWIG
HF2B2	**Q**VQL**V**QSGAELIKPGASVKISCKAN**GYTFSNSW**IEWVKQRPGHGLEWIG
HF6B2	**E**VQL**Q**QSGAELIKPGASVKISCKAN**GYTFSNSW**IEWVKQRPGHGLEWIG
HF8B2	**E**VQL**Q**QSGAELIKPGASVKISCKAN**GYTFSNSW**IEWVKQRPGHGLEWIG
	<-CDR2-IMGT-><---------- FR3-IMGT----------><---
HF1B2	E**ILPGSYRT**HYNEKFRGKATVTADVSSNTVYMQLSSLTSEDSAIYYC**TRV**
HF2B2	E**ILPGSYRT**HYNEKFRGKATVTADVSSNTVYMQLSSLTSEDSAIYYC**TRV**
HF6B2	E**ILPGSYRT**HYNEKFRGKATVTADVSSNTVYMQLSSLTSEDSAIYYC**TRV**
HF8B2	E**ILPGSYRT**HYNEKFRGKATVTADVSSNTVYMQLSSLTSEDSAIYYC**TRV**
	- CDR3-IMGT--><--FR4-IMGT----------
HF1B2	**GIYYDSDWFPY**WGQGTMVTVSA----------(productive sequence)
HF2B2	**GIYYDSDWFPY**WGQGTMVTVSA----------(productive sequence)
HF6B2	**GIYYDSDWFPY**WGQGTMVTVSA----------(productive sequence)
HF8B2	**GIYYDSDWFPY**WGQGTMVTVSA----------(productive sequence)

A

V_L	<----------FR1-IMGT--------><-CDR1-IMGT-><---- FR2-IMGT---->
LF2B3	DIVLTQSPVSLAVSLGQRATISCRAS**ESVDSYGNSF**INWYQQKPGQPPKV
	<-CDR2-IMGT-><----------FR3-IMGT----------><--CDR3-IMGT-->
LF2B3	LIY**RAS**NLESGIPARFSGSGSRTDFTLTINPVEADDVATYYC**QQSYADFPT**
	<-FR4-IMGT----------
LF2B3	FGAGTKLELK ----------(productive sequence)

B

Fig. 1.

The protein sequence analysis of four unique V_H genes (A) amplified by PCR using HF/HB primers and one unique V_L gene (B) amplified by PCR using LF/LB primers from IGMT/V-QUEST database. The CDR region was marked by black font. The CDR3/FR4 junction region in V_L sequences are labeled with yellow color.

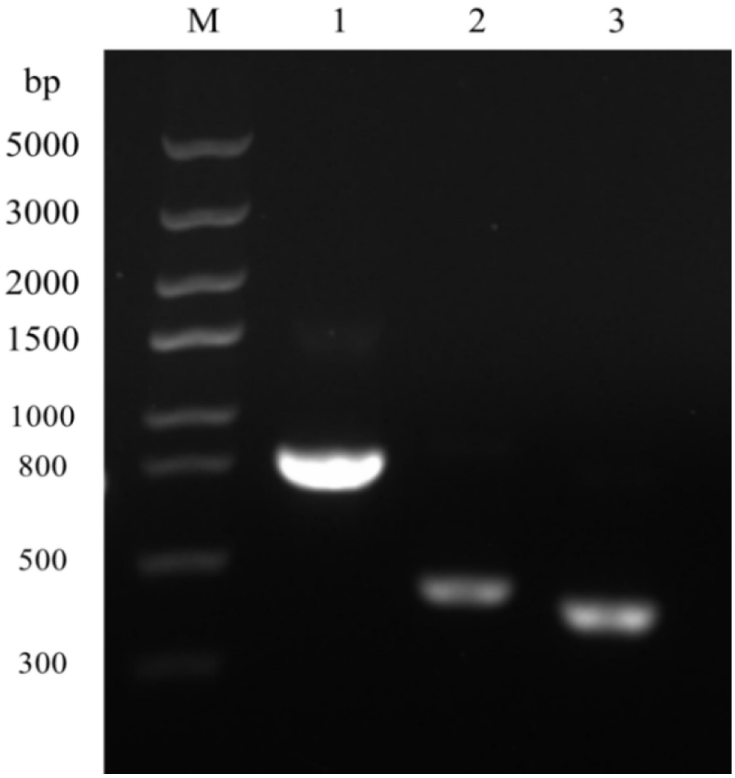


Fig. 2.

The results of V_H, V_L and scFv amplification. Lane M, 5000 Marker; Lane 1: scFv gene; Lane 2: V_H gene; Lane 3: V_L gene.

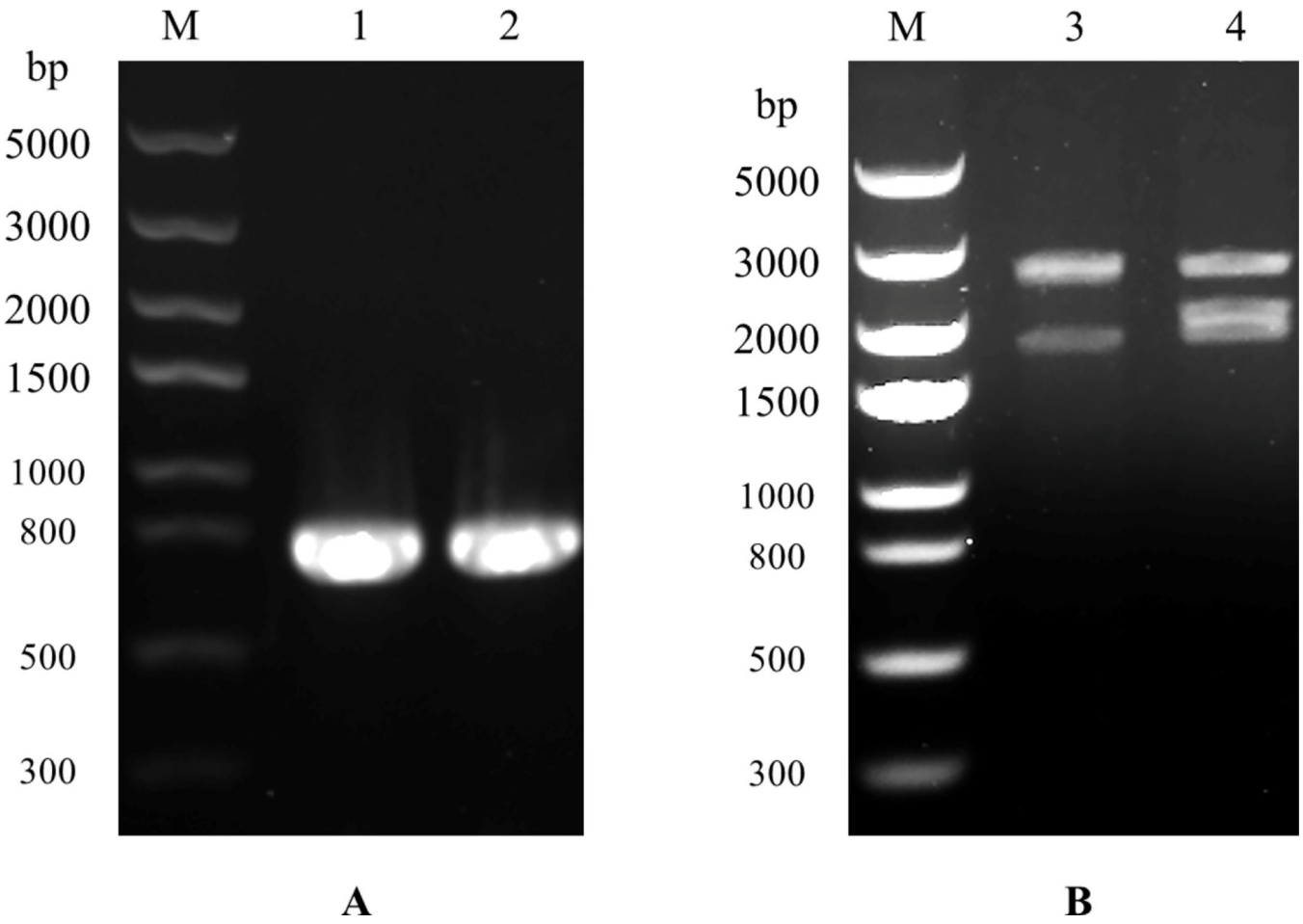


Fig. 3.

The characterization of the pLIP6/GN-GEN scFv plasmid by PCR (A) and *Bgl*I digestion (B). Lane M, 5K Marker; Lane 1–3: pLIP6/GN-scFv; Lane 4: pLIP6/GN.

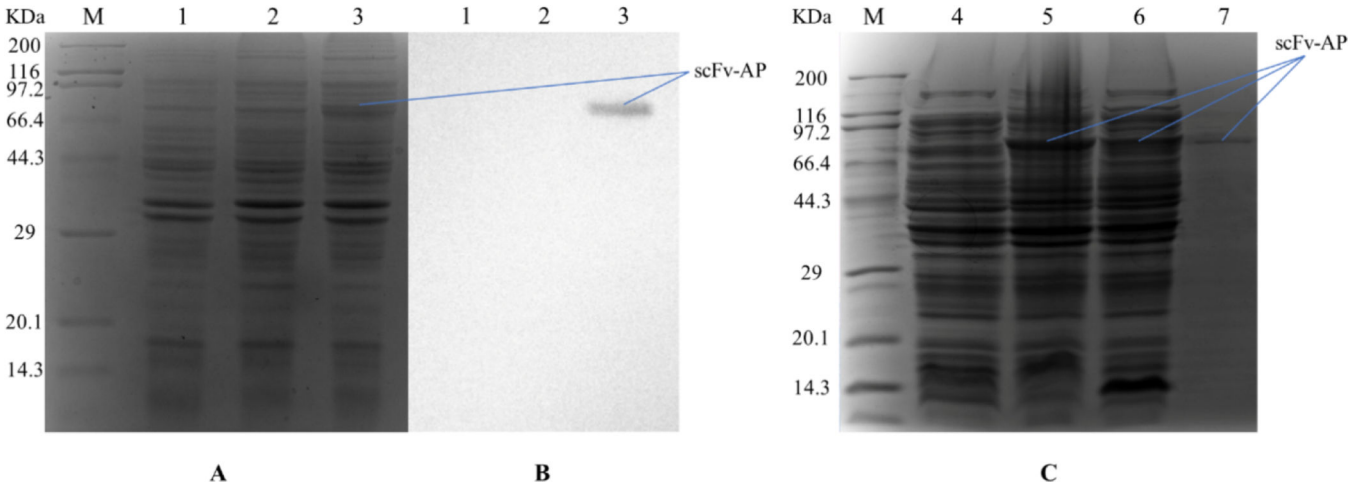


Fig. 4.

The identification of the recombinant protein by SDS-PAGE (A), Western blotting (B) and purification of the scFv protein (C). Lane M, Protein Marker; Lane 1 and 4: pLIP6/GN; Lane 2: non-induced positive bacteria; Lane 3: induced positive bacteria; Lane 5 and 6: induced positive bacteria supernatant and crushing supernatant; Lane 7: The purified fusion protein.

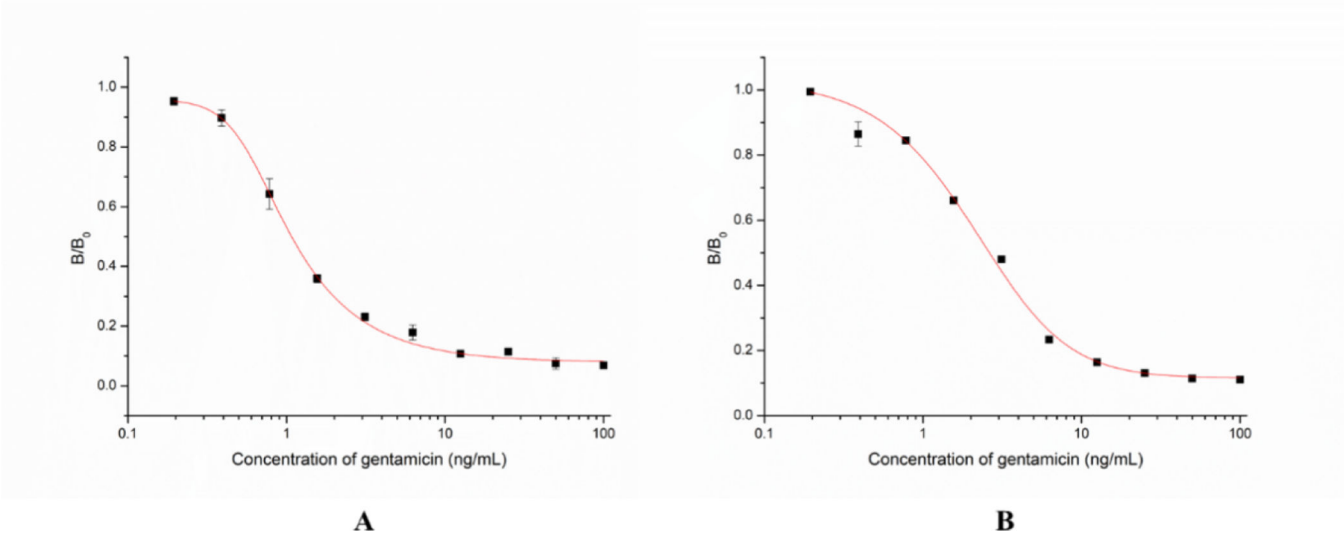


Fig. 5.

The standard curve of dcCLEIA (A) and standard curve of dcELISA (B) for GEN based on the scFv-AP fusion proteins (n=3).

Table 1

The checkerboard titration experiment measured by dcCLEIA.

Concentration of purified scFv-AP (μg/mL)	Concentration of gentamicin-OVA (μg/mL)									
	1		0.5		0.25		0.125		0.0625	
	B_0	B/B_0	B_0	B/B_0	B_0	B/B_0	B_0	B/B_0	B_0	B/B_0
100	37471	0.58	32492	0.55	20888	0.49	10539	0.42	5162	0.45
50	20937	0.32	18954	0.25	13655	0.21	7746	0.18	3517	0.23
25	10012	0.28	9414	0.27	6993	0.18	4954	0.18	2568	0.23
12.5	5060	0.30	4644	0.32	3295	0.22	2598	0.19	1798	0.39
6.25	2173	0.27	1850	0.58	1788	0.26	1454	0.32	1079	0.28
3.125	1504	0.23	1099	0.47	682	0.51	841	0.38	714	0.68
1.5625	749	0.65	756	0.96	408	0.63	900	0.64	702	0.64
0.7813	500	1.04	133	1.08	487	0.96	496	0.80	476	0.85

Table 2

The results of specificity experiments measured by dcCLEIA.

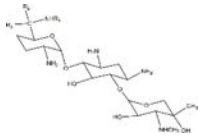
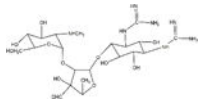
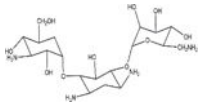
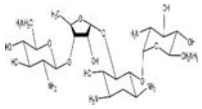
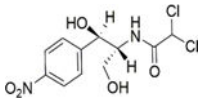
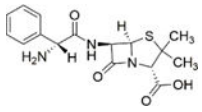
Analytes	Chemical structure	IC_{50}(ng/mL)	Cross-reactivity(%)
Gentamicin		1.073	100
Streptomycin		>3000	<0.10
kanamycin		>3000	<0.10
Neomycin		>3000	<0.10
Chloramphenicol		>3000	<0.10
Ampicillin		>3000	<0.10

Table 3

The recovery rate of gentamicin in spiked animal tissue samples measured by dcCLEIA and UPLC-MS/MS (n=3).

Sample	Spiked (μg/g)	dcCLEIA			UPLC-MS/MS		
		measured ± SD	Recovery (%)	CV (%)	measured ± SD	Recovery (%)	CV (%)
Pork	0	ND	–	–	ND	–	–
	0.375	0.350±0.014	93	3.9	0.396±0.020	106	5.1
	0.750	0.588±0.017	78	2.9	0.737±0.003	98	0.5
	1.500	1.388±0.057	93	4.1	1.434±0.003	96	0.2
Fish	0	ND	–	–	ND	–	–
	0.375	0.344±0.026	92	7.4	0.394±0.003	105	0.8
	0.750	0.719±0.020	96	2.8	0.750±0.024	100	3.2
	1.500	1.193±0.020	80	1.7	1.572±0.022	105	1.4

ND: Not detectable; –: Not data.