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Development of Competitive ELISA and CLEIA for Quantitative Analysis of Polymyxin B

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Abstract

Polymyxin B (PMB), a member of polypeptide antibiotics, is widely used for the treatment of infection in animals such as cattle, sheep, pigs, and chickens. However, it is toxic on the kidneys and nervous system, and polymyxin resistance is increasingly reported, which leaves a serious threat to human health. Therefore, it is essential to establish rapid methods for detecting PMB with high sensitivity and specificity. In this study, an anti-PMB polyclonal antibody (pAb) was obtained by immunizing New Zealand white rabbits with PMB conjugated with glycosylated bovine serum albumin (GBSA). Indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) and indirect competitive chemiluminescent enzyme immunoassay (ic-CLEIA) were developed. Under the optimal conditions, inhibitory concentrations (IC_{50}) of PMB were 257.1 ng/mL (ic-ELISA) and 250.8 ng/mL (ic-CLEIA); the limits of detection (LOD) were 17.4 ng/mL (ic-ELISA) and 14.5 ng/mL (ic-CLEIA), respectively. Cross-reactivity of the pAb toward polymyxin E (PME) was 257.1%, and no response was found with other antibiotics. The recovery rates in spiked meat samples were 77.4~106.1% (ic-ELISA) and 84.1~107.1% (ic-CLEIA), respectively.

Keywords Polymyxin B (PMB) · Animal-derived food · Enzyme-linked immunosorbent assay (ELISA) · Chemiluminescent enzyme immunoassay (CLEIA)

Introduction

Polymyxins (PM) are pentacationic polypeptides with a common structure: a cyclic heptapeptide, a linear tripeptide portion, and a fatty acyl (FA) tail which is linked to the N-terminus of the tripeptide part (Storm et al. 1977). They have five different agents, which are described and named as polymyxins A–E (Kadar et al. 2013). They differ from each other in their amino acid sequences and FA chains. Among these molecules, only PMB and PME are available for clinical use (Cai et al. 2015). They are isolated from cultures of various strains of *Bacillus polymyxa* and related species (Liu et al. 2017; Pendela and Adams 2004). PMB has been used for the treatment of infections caused by gram-negative bacteria, especially the *Pseudomonas aeruginosa* and *Escherichia coli* (Gales et al. 2001; Orwa et al. 2000). It is the drug of first choice in the treatment of infections of the urinary tract, eye, meninges, and bloodstream. Many countries have promulgated standards for residue limits of PME. The EU and China both set up PME residue limits of 150 μg/kg in the animal muscle, fat, and liver and 200 μg/kg in the kidney. However, there is no maximum residue limit for PMB in animal-derived foods and animal feed internationally. PMB has been known to show antiendotoxin activities, and it is reported to bind to endotoxin in vitro to suppress many of its activities (Guo et al. 2007; Ronco and Klein 2014). Owing to the emergence of multidrug-resistant (MDR) gram-negative antibiotics and the reduction of newly developed antibiotics, PMB has been increasingly used as a last-resort drug in the treatment of MDR gram-negative bacterial infections (Bergen et al. 2012a; Velkov et al. 2013).

With the increasing use of PM in humans and animals, bacterial resistance has been on the increase (Li et al. 2018;

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Olaitan et al. 2014). In 2010, the European Medicines Agency warned in their report (Bergen et al. 2012b) about the increasing resistance to PME, especially in Southern European countries where PME has been frequently used in agriculture. In the husbandry industry, PMB is used as a feed additive to promote animal growth. Excessive use of PMB gives rise to a large amount of residues in animal tissues, which may transfer to humans and put humans at risk for the acquisition of antibiotic-resistant pathogens (Sitzlar et al. 2012). Therefore, there is a huge need for quantification of PMB in veterinary practices and food safety inspection.

There are many different methods used for the determination of polymyxin: chromatography methods (LC-MS/MS) have been widely used and recognized as standard laboratory methods for PMB quantification and subtype identification with high sensitivity and accuracy. PMB_1, PMB_2, PMB_3, and isoleucine-PMB_1 were separated and quantified using stepwise gradient elution of water containing 0.1% of formic acid and 0.1% of trichloroacetic acid (mobile phase A) and 90% acetonitrile with 0.1% formic acid (mobile phase B) (Hee et al. 2017). These PMBs were completely resolved in the analytical run time of 6.5 min. Immunoassay is another approach to sensitive, rapid, and cost-effective detection of antibiotics, especially for field screening of large samples. A highly sensitive ELISA was developed for the quantification of PMB sulfate with the detection limit of 32 pg/mL (Saita et al. 1999). However, this assay was aimed only for the detection of PMB sulfate in human serum samples. Consequently, it is necessary that a sensitive, rapid, and reliable method should be developed for the determination of PMB residues in animal-derived foods.

The present study aimed at developing the most commonly used quantitative immunoassays, namely, ELISA and CLEIA, for PMB residue analysis in animal-derived edible tissues.

Materials and Methods

Synthesis of Polymyxin B Conjugates

The glycosylated bovine serum albumin (GBSA) was synthesized via the carbodiimide (EDAC) method (He et al. 2015). PMB (purity 98%, China Food and Drug Verification Research Institute, Beijing, China) was conjugated with GBSA to obtain an immunogen. For the synthesis of GBSA, 10.09 mg of bovine serum albumin (BSA), 3.30 mg of EDAC, and 10 μL D-gluconic acid (Sigma, St. Louis, MO, USA) were dissolved in 500 μL PBS, and the reaction was carried out for 16 h at 17 °C. Then, the mixture was dialyzed against PBS (0.01 mol/L, pH 7.4) at 4 °C. The artificial antigens were synthesized via the sodium periodate oxidation method (Burkin and Galvidis 2010) (Fig. 1). For the synthesis of the immunizing antigen, 0.5 mL of 0.1 M $NaIO_4$ was added dropwise to 10 mg GBSA, which was dissolved in 3.5 mL PBS, and the mixed solution was stirred for 2 h at room temperature. Then, the mixture was reacted with 0.5 mL of 1 M glycol at RT with continuous stirring for 10 min. When the reaction ended, the solution obtained was added to 4 mL of PMB PBS solution and reacted for 2 h. The pH value of the reaction mixture was adjusted between 9.0 and 9.5 with 5% K_2CO_3 solution. After this reaction, 10 mg $NaBH_4$ was added into the mixture and reacted for 16 h. Finally, the obtained conjugate (PMB-GBSA) was dialyzed against four changes of PBS (0.01 M, pH 7.4) for 3 days.

UV spectra (Specord 50UV-VIS spectrophotometer, Analytik Jena AG, Germany) of PMB, GBSA, and PMB-GBSA were tested at a wavelength ranging from 190 to 400 nm. The conjugations were confirmed again by SDS-PAGE.

Similarly, the coating antigen synthesis PMB-Gelatin (PMB-Gel) was prepared as described above.

Generation of Polyclonal Antibodies

A half milliliter of saline-containing 500 μg of PMB-GBSA was emulsified with 500 μL of Freund's complete adjuvant and administered intradermally to female New Zealand white rabbits at sites along both sides of the spine. After the initial immunization, booster injections were given four times at biweekly intervals, using 300 μg of PMB-GBSA in 500 μL of Freund's incomplete adjuvant (FIA, Sigma, St. Louis, MO, USA) per rabbit per

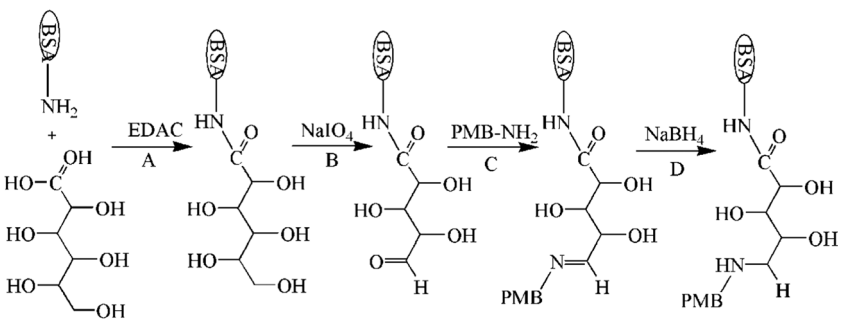


Fig. 1 Synthetic route for the immunogen. (A) Synthesis of GBSA by EDAC; (B) $NaIO_4$ oxidizes the hydroxyl of GBSA; (C) carbonyl coupled to PMB; (D) $NaBH_4$ reduces double-bonded carbonic acid of conjugation

booster dose. Post-immunization blood was collected from the marginal ear vein. Blood was clot for 30 min and centrifuged at 5000*g* for 5 min, and the serum was collected. Serum aliquots were kept at 4 °C for immediate use and at − 20 °C for long-term storage. It was used as anti-PMB antiserum for ELISA and CLEIA.

Indirect Competitive ELISA

The optimal dilutions of the antibody and the coating antigen were determined by checkerboard titration at an absorption value of 1.0. According to conventional protocols (Cui et al. 2016), the ic-ELISA was carried out as follows: 96-well ELISA plates (Costar Inc., Cambridge, MA, USA) were coated with PMB-Gel (100 μL per well) in 0.1 M sodium carbonate buffer (CBS, pH 9.6) at 4 °C overnight. The plates were washed three times with PBST (0.5% Tween-20 in PBS) and blocked with 2% BSA in PBS (200 μL per well) at 37 °C for 2 h. The same washing step was implemented. A series of PMB standards and samples dissolved in PBS were mixed with an equal volume of diluted serum in PBS. One hundred microliters per well of the mixture was added to the plates at 37 °C for 40 min. After washing three times, the plates were incubated with goat anti-rabbit HRP (100 μL per well, diluted 1:8000) dissolved in PBS containing 1% BSA at 37 °C for 40 min. The plates were washed again. Subsequently, 100 μL per well of 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma, St. Louis, MO, USA) substrate solution was added to plates, which were then incubated at 37 °C for 10 min. The reaction was terminated by the addition of 2 M H_2SO_4 (50 μL per well). The absorbance values were measured at 450 nm.

The competition curve was established by plotting the B/B_0 values against the PMB concentrations of 5, 10, 25, 50, 100, 250, 500, 1000, and 2000 ng/mL, where B and B_0 represented the absorbance values of the well containing the competitor and the zero competitor concentrations, respectively, relative to the control OD_{450}. The concentrations of PMB in the samples were quantified using the calibration curve.

Cross-reactivity Assay

Structural analogs and commonly used antibiotics such as PME, kanamycin, gentamicin, enrofloxacin, sulfadimethoxine, and chloramphenicol (Solarbio Science & Technology Co., Ltd., Beijing, China) were studied for cross-reactivity to determine the specificity of the antibodies. Cross-reactivity (CR %) was quantitated as follows: CR % = (IC_{50} for PMB/IC_{50} for analogs) × 100%.

Indirect Competitive CLEIA

The optimal concentrations of PMB-Gel and anti-PMB antibody were selected using ELISA by checkerboard titration. The indirect competitive CLEIA was described as follows: 100 μL/well of PMB-Gel in CBS (pH 9.6) was coated on the 96-well polystyrene microtiter plates and incubated at 4 °C overnight. On the following day, the plate was washed three times using PBST and blocked by adding 200 μL of PBS containing 2% BSA at 37 °C for 2 h. After a further washing step, 50 μL of diluted pAb and 50 μL of PMB standard solution were added to each well and incubated at 37 °C for 40-min incubation. PMB standard solution was prepared by diluting with PBS at a series of concentrations (5, 10, 25, 50, 100, 250, 500, 1000, and 2000 ng/mL). After three washes with PBST, 100 μL/well of goat anti-mouse IgG-HRP was added and incubated at 37 °C for 40 min. Finally, 100 μL of substrate solution prepared freshly was added into each well and incubated for 5 min in the dark. Then, chemiluminescence intensity was monitored on Synergy H1 (BioTek, VT, USA). The standard curve was evaluated by plotting the chemiluminescence intensity against the logarithm of each concentration and was fitted to a logistic equation using the Origin 8.0 program (OriginLab, Hampton, USA).

HPLC Analysis

The method was performed as the report described with some modification (Chepyala et al. 2015; Wan et al. 2006). The liquid chromatographic system (Agilent 1260, USA) for PMB sulfate was equipped with an LC pump delivering the mobile phase (V_{ACN}:V_{H2O} = 90:10) at a flow rate of 0.8 mL/min and with an automatic injector with a loop of 60 μL. The stationary phase was packed in a stainless steel column (100 × 4.6 mm, id 3.5 μm). The column temperature was maintained at 25 °C. An integrator was connected to the diode array detector (DAD) to record the signals at 220 nm. A series of different concentrations of PMB were prepared in PBS. The calibration curve was generated via linear regression, and the linear range of PMB was calculated using the peak area ratio of PMB to the external standard (PMB).

Spiked Sample Preparations and Analysis

To verify the developed methods, the chicken, pork, and beef samples were purchased from the local market in Yangling, Shaanxi Province, China. The samples were extracted in reference to a previous report (Yu et al. 2017); briefly, each sample (1-g wet mass) was homogenized and spiked with PMB standards at different concentrations (100, 200, and 500 ng/g). Four milliliters of 0.1% formic

acid and 2 mL of methanol were mixed with the tissue sample. The mixture was sonicated for 30 min at 60 °C, and the suspension was centrifuged (Eppendorf 5804R instrument, Eppendorf, Hamburg, Germany) at 4000*g* for 15 min at 4 °C. The supernatants were transferred to a solid-phase extraction column and washed with 10 mL H_2O. The analyte was eluted from the column by 3 mL methanol and then dried under nitrogen. At last, dried samples were re-dissolved in 1 mL PBS and tested using the developed ELISA and CLEIA. The recovery rates were calculated on the basis of the standard curve constructed by ic-ELISA and ic-CLEIA.

Results

Characterization of the Antigen

Three absorbance curves (GBSA, PMB, and PMB-GBSA) were observed after the UV screening from 190 to 400 nm. PMB did not have an absorption peak over 230 nm. PMB and GBSA showed a similar characteristic absorption peak at 220 nm. For this reason, reducing PAGE was performed to further confirm the conjugation of PMB-GBSA (Fig. 2). PMB-GBSA showed larger molecular weight.

Optimization of PMB-Gel and Antiserum Work Concentrations

The antibody working titer was determined by the checkerboard method (Fig. 3). The OD_{450} value 1.0 indicated that the best concentrations of the antiserum and the coated PMB-Gel were fixed on 1:3000 and 1 μg/mL, respectively.

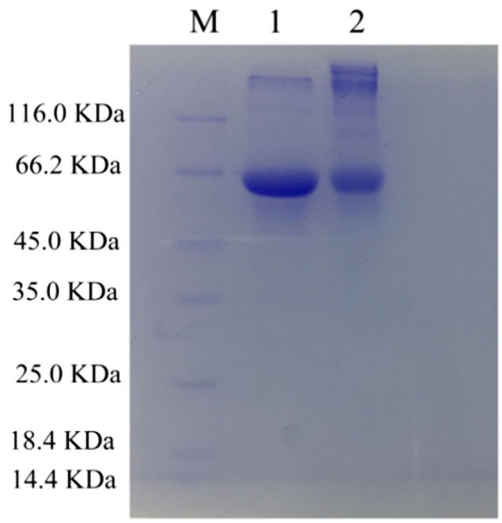


Fig. 2 Reduced PAGE analysis on GBSA (lane 1) and PMB-GBSA conjugate (lane 2)

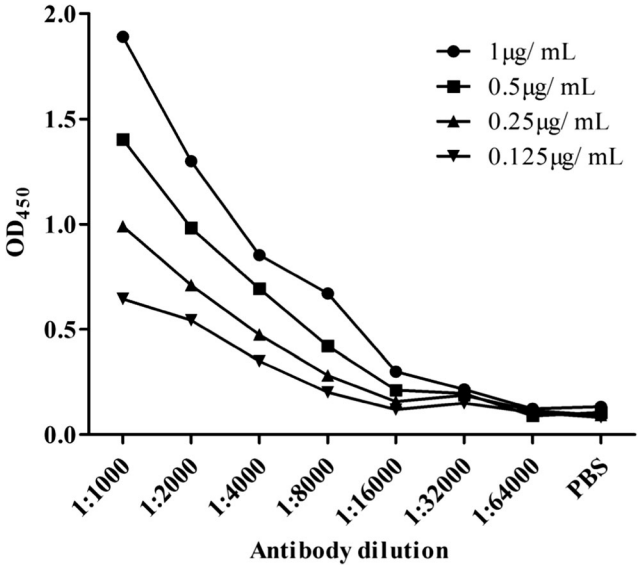


Fig. 3 Checkerboard curve of antigen-antibody of PMB. Different symbols represent increasing amount of the coated antigen

Competitive ELISA

The sensitivity of ELISA was determined under optimal conditions. In the representative competitive inhibition curve for PMB (Fig. 4), the regression curve equation of the anti-PMB pAb was $Y = -0.340X + 1.322$ ($R^2 = 0.984$, $n = 3$), with the IC_{50} value of 257.1 ng/mL and the limit of detection (IC_{10} value) of 17.4 ng/mL. The IC_{50} value demonstrated that the antibody could be used for PMB detection with a linear range from 25 to 2000 ng/mL.

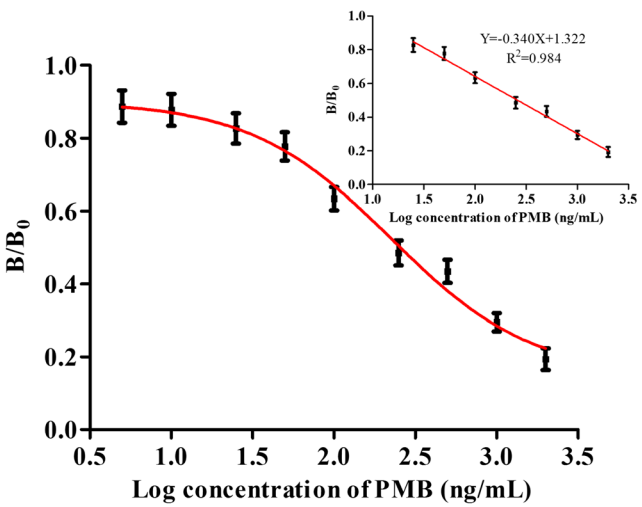


Fig. 4 Standard curve of the competitive ELISA for PMB

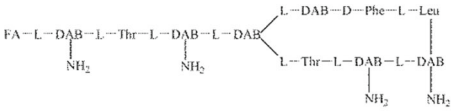
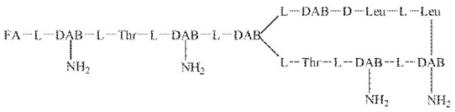
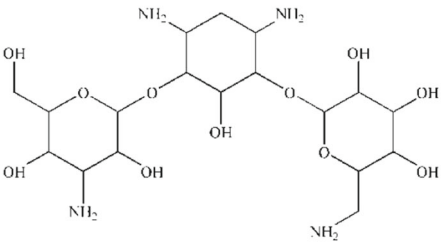
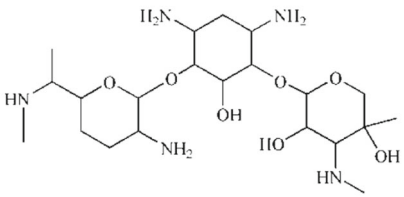
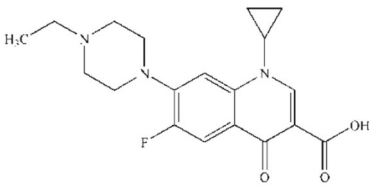
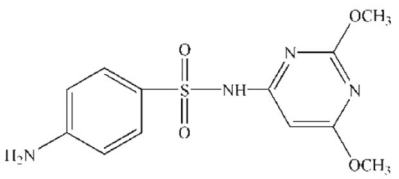
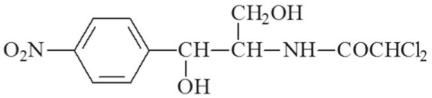
Cross-reactivity and Sensitivity

The structural analogs and other antibiotics, including PME, kanamycin, sulfadimethoxine, enrofloxacin, gentamicin, and chloramphenicol, were used to evaluate the specificity of the antibody obtained. The data showed that the pAb exhibited high cross-reactivity for PME, where the cross-reactivity was 257.1% (Table 1). It was found that the pAb did not recognize the other five commonly used in veterinary antibiotics.

Development of Competitive CLEIA

The sensitivity of CLEIA was determined under the same conditions as ELISA. The representative competitive inhibition curve (Fig. 5) revealed the regression curve equation of

Table 1 Cross-reactivity of anti-PMB IgG with other antibiotics

Antibiotic	Structure	IC_{50} (ng/mL)	Cross-reactivity (%)
Polymyxin B		257.1	100
Polymyxin E		100.0	257.1
Kanamycin		NR	<0.01
Gentamicin		NR	<0.01
Enrofloxacin		NR	<0.01
Sulfadimethoxine		NR	<0.01
Chloramphenicol		NR	<0.01

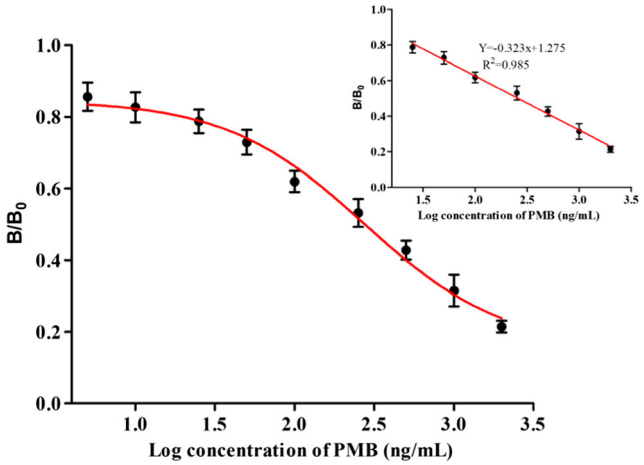


Fig. 5 Standard curve of the competitive CLEIA for PMB

$Y = -0.323X + 1.275$ ($R^2 = 0.985$, $n = 3$) with the IC_{50} value of 250.8 ng/mL and the limit of detection (IC_{10}) value of 14.5 ng/mL. The linear range was 25~2000 ng/mL.

PMB Sulfate Analysis in HPLC

A reversed-phase HPLC was developed for PMB determination by analyzing its major metabolite, PMB sulfate. The retention time of PMB was 0.772 min. The regression curve equation was $Y = 4.572X - 63.683$ ($R^2 = 0.996$, $n = 3$). The linearity ranged from 5.0 to 500.0 μg/mL. The LOQ, where the signal-to-noise ratio equaled 10, was 5 μg/mL. The LOD, where the signal-to-noise ratio equaled 3, was 2 μg/mL.

Precision and Recovery in the Sample Test

ELISA and CLEIA are broadly used as tools of qualitative determination. To evaluate the accuracy and precision of the developed test system in matrices, the spiked chicken, pork, and beef samples containing different concentrations of PMB (100, 200, and 500 ng/g, respectively) were detected using the proposed ic-ELISA and ic-CLEIA, respectively, and both methods showed high recoveries (Table 2) and low coefficients of variation (Table 2).

Discussion

PMB and PME are commonly used to control and treat animal disease as well as promote fast and more efficient growth of livestock. Improper use of them leads to the development of antibiotic-resistant bacteria and puts animals and humans at risk for the acquisition of antimicrobial drug–resistant pathogens. PME residue determination in animal-derived food products or animal feed is already reported. Still, there is little research about PMB residue in these. A rapid and semi-quantitative nanoparticle (AuNP)–based immunochromatographic test (ICT) strip for PMB residues has been established (Li et al. 2018). Nevertheless, for quantitative detection of PMB, high specificity of the anti-PMB antibody was generated from rabbits immunized with PMB-GBSA in this study. A high cross-reactivity of the pAb with PME may be attributable to their very similar molecular structure. The difference between PMB and PME is the sixth amino acid: PMB has a D-phenylalanine in its

Table 2 Recovery rates of PMB from fresh chicken, pork, and beef samples ($n = 4$)

Sample	ELISA				CLEIA		
	Spiked (ng/g)	Measured (ng/g)	Recovery (%)	RSD (%)	Measured (ng/g)	Recovery (%)	RSD (%)
Chicken	0	< LOD	–	–	< LOD	–	–
	100	77.4	77.4	4.8	84.1	84.1	8.4
	200	175.5	87.8	6.3	184.3	92.2	9.0
	500	451.5	90.3	9.7	429.5	85.9	4. 0
Pork	0	< LOD	–	–	< LOD	–	–
	100	84.1	84.1	7.0	84.8	84.8	12.1
	200	212.1	106.1	7.2	214.2	107.1	9.3
	500	429.6	85.9	8.4	448.1	89.6	8.7
Beef	0	< LOD	–	–	< LOD	–	–
	100	81.5	81.5	7.3	88.8	88.8	7.0
	200	189.6	94.8	4.6	202.5	101.3	5.0
	500	519.9	104.0	7.7	427.3	85.5	9.2

RSD relative standard deviation

No HPLC results listed, because of its low sensitivity

cyclic portion, whereas in PME, there is a D-leucine in the same position (Landman et al. 2008; Vaara 2010). The pAb could be used for preliminary screening of PME and quantitative analysis of PMB. During sample preparation, high protein content in meat products occurs with PMB through hydrophobic interactions and hydrogen bonds, which could interfere with the determination of PMB using the developed assay. Hence, organic solvent was picked to precipitate protein and extract PMB during the pretreatment process of samples, which achieved good extraction effect and reduced the matrix interference.

As an alternative to instrumental analytical methods, ELISA and CLEIA have been considered as promising choices for large sample screening in the detection of antibiotic residues. As a fundamental and classical immunoassay, the ELISA has been applied widely with the advantages of simplicity, specificity, and low cost. Nevertheless, because the enhanced chemiluminescence reaction offers the possibility of increasing the sensitivity of CLEIA compared with conventional immunoassay detections, the CLEIA has gained more attention in recent years (Lu et al. 2011; Yu et al. 2014). Besides, the light intensity of CLEIA can remain stable in 5 min after substrate addition, thus providing rapid detection of the analytical signal. The features of ic-ELISA and ic-CLEIA employed for the detection of PMB have been compared. The ic-ELISA and ic-CLEIA possess the same wide detection range (25~2000 ng/mL). The sensibility of ic-ELISA is lower than that of ic-CLEIA. Furthermore, the two developed assays showed higher sensitivity than HPLC with DAD.

Conclusion

A polyclonal antibody against PMB was raised by immunizing New Zealand white rabbits with PMB-GBSA. The LOD of ic-ELISA and ic-CLEIA were 17.4 ng/mL and 14.5 ng/mL, respectively. They were sensitive for quantitative analysis of PMB and the large sample screening of PME in meat samples. The two methods demonstrated their potential as alternatives to the commonly used chromatography methods.

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Compliance with Ethical Standards

Conflict of Interest Long Xu declares that he has no conflict of interest. Maksim Burkin declares that he has no conflict of interest. Sergei Eremin declares that he has no conflict of interest. Alberto C. P. Dias declares that he has no conflict of interest. Xiaoying Zhang declares that he has no conflict of interest.

Ethical Approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed Consent Informed consent was obtained from all individual participants included in the study (in case humans are involved).

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