Original Article

Development of a Rapid Multi-residue Assay for Detecting β-lactams Using Penicillin Binding Protein 2x*

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Abstract

Objective To develop a rapid multi-residue assay for detecting 16 demanded by the European Union (EU).

Methods A recombinant penicillin-binding protein (PBP) 2x* from Streptococcus pneumoniae R6 was expressed in vitro and six β-lactams were conjugated to HRP by four methods. A rapid multi-residue assay for β-lactams was established with PBP2x* and HRP-conjugate.

Results PBP2x* was expressed and purified successfully and the ideal HRP-conjugate was identified. The multi-residue assay was developed. After optimization, penicillin G, ampicillin, amoxicillin, cloxacillin, dicloxacillin, oxacillin, nafcillin, cephalexin, ceftiofur, cefalonium, cefquinome, cefazolin, cefoperazone, cephacetrile, and cephapirin can be detected at levels below MRL in milk with simple pretreatment.

Conclusion This assay developed can detect all 16 β-lactams demanded by the European Union (EU). The whole procedure takes only 45 min and can detect 42 samples and the standards with duplicate analysis.

Key words: Penicillin-Binding Protein 2x*; β-lactam; Multi-residue; Milk

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INTRODUCTION

β-lactam antibiotics are a broad class of antibiotics containing β-lactam nucleus in their molecular structure. These antibiotics include penicillins, cephalosporins, monobactams and carbapenems. β-lactams have been widely used for the treatment of diseases in livestock animals, such as bovine mastitis, pneumonia, bacterial diarrhea, bacterial arthritis, and so on[1]. These medicines have also been used to promote growth of food-producing animal[2]. β-lactam antibiotics are administered to animals by intravenous, intramuscular or subcutaneous injections, orally in feed or water, locally on the skin and by intramammary and intrauterine infusions. However, illegal use of β-lactams antibiotics or non-compliance of animal-treatment may cause the antibiotics residues in dairy products and other foods of animal origin that would induce allergic reactions in sensitive individuals, increase resistance of pathogenic bacteria towards antibiotics, and inhibit bacterial starter cultures in dairy production[3].

In order to control such veterinary drugs residues in food-producing animals, the European Union (EU) has established strict regulations (EU Regulation 2377/90) and set up the Maximum Residue Limits (MRLs) as shown in Table 1. The mark residue of these antibiotics are drugs themselves,

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except penethamate, cephapirin and ceftiofur. The mark residue of penethamate is penicillin G, that of cephapirin is the sum of cephapirin and desacetylcephapirin, and that of ceftiofur is the sum of all residues retaining the β-lactm structure called desfuroylceftiofur.

To monitor and control the residues of β-lactam antibiotics in milk, a series of rapid screening methods such as microbiological approach, immunoassay and receptor assay have been developed and commercially used in recent years. The characteristics of these methods were listed in Table 1. Microbiological approaches are very sensitive to β-lactams, and these approaches can also detect other antibiotics such as sulfonamides at or below MRLs set by EU. Also, some commercial microbiological kits have been widely used in antibiotics screening, such as the Delvotest[4], Charm products[5], and the Eclipse 100[6]. However, these methods need to incubate susceptible milk samples and the incubation causes color change due to acid production and takes hours. Immunoassays are based on the specific interaction between antigen and antibody, and have been extensively used in antibiotics tests with the advantages of rapidity, easy operation, high sensitivity and high throughput[7-8]. To develop sensitive immunoassay, it is crucial to obtain the ideal antibody which has sufficient sensitivity and can recognize penicillins and cephalosporins. However, due to the instability of lactam ring in penicillins and as shown previously, antibodies obtained could only react with the open ring form of benzylpencilloic acid and therefore could not recognize the intact structure[9]. On the other hand, it is difficult to develop a broad spectrum antibody that can react with penicillins and cephalosporins simultaneously because of the diversity of molecular structure of these drugs. Though antibodies against intact lactam ring have been reported[10] and commercial enzyme immunoassay kits for penicillins have been available, they are not popularly used due to the disadvantage that they can not detect the whole group of β-lactams. Based on the form of dipstick, receptor assay such as the Charm ROSA, the Unisensor and so on has been widely used in qualitatively screening of antibiotics due to the advantages of convenience, timesaving and broad spectrum property[11]. Here, a bacterium protein was used instead of antibody as a receptor to bind β-lactam antibiotics.

Table1. Detection Limits of Different Methods for Rapid Detection of Antibiotics (ng/mL)

	Commercial Microbial Inhibition Tests			**Commercial Rapid Test Kits Based on Receptor**			**Laboratory Method Based on Receptor**		**EU MRL**
	Delvotest SP	Charm AIM-96	Eclipse 100	Charm ROSA	Twin Sensor	SNAP	Optical Biosensor Assay	Microplate Assay	
Penicillin G	2	2-3	4	2-3	2-3	2-4	2	1	4
Ampicillin	2-3	4	5	3-5	3-5	4-5	2	1	4
Amoxicillin	2	4	5	4-5	3-5	6	2		4
Cloxacillin	15	10-25	40	20-30	6-8	30	15	3	30
Dicloxacillin	10	30	15	15-25	-	30	-	-	30
Oxacillin	5	5-10	25	-	-	35-40	-	-	30
Nafcillin	5	-	10	-	30-40	-	-	-	30
Cephalexin	40-60	-	75	15-30	-	-	50	-	100
Ceftiofur	<50	50-100	75	10-20	10-15	50	-	-	100
Cefalonium	5-10	15-20	-	3-6	-	-	-	-	20
Cefquinome	-	-	-	15-25	-	-	-	1	20
Cefazolin	-	-	-	12-20	18-22	25-27.5	-	7	50
Cefoperazone	40	20-30	-	5-9	3-4	-	25	5	50
Cephapirin	5	8-10	8	6-10	6-8	-	-	-	60
Cefacetrile	20	-	30	8-18	-	-	-	-	125

***Note*.** All above data on commercial product came from the product instruction book. -: no results obtained.

β-lactam antibiotics act by covalently binding to transpeptidases and inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls[12]. These transpeptidases are named penicillin binding proteins (PBPs)[13-14]. The PBPs are broadly classified into high-molecular-weight (HMW) and low-molecular-weight (LMW) categories[15]. Previous research have shown that HMW PBPs are associated with bacteria resistance[16]. *Streptococcus pneumoniae R6* contains five HMW PBPs (la, 92 kD; lb, 89 kD; 2x, 85 kD; 2a, 81 kD; 2b, 77 kD)[17] and one LMW PBP (3, 43 kD)[18], among which PBP2x involves in β-lactam resistance[19]. PBP2x bonds to the cytoplasmic membrane by a short N-terminal hydrophobic peptide[20] and this hydrophobic peptide has been confirmed not to be associated with enzymatic function and penicillin binding activity[21]. Therefore, by deleting this membrane anchor, the protein can be produced and purified in a soluble form, called PBP2x*. Lamar J. once developed a PBP2x*-based microplate assay to detect β-lactam in different food matrices[22]. Cefoperazone, cefquinome, cefazolin, cloxacillin, ampicillin and penicillin G can be detected at levels below MRL in different food matrices, including milk, meat, egg, and honey. Cacciatore G. ever established an optical biosensor for the detection of β-lactams in milk using PBP2x*[23]. Penicillin G, ampicillin, amoxicillin, cloxacillin, cephalexin and cefoperazone can be detected in defatted raw milk samples at concentrations corresponding to EU MRL.

The detection limit of β-lactam antibiotics using different methods like microbiological approach, rapid tests and receptor assay are listed in Table 1 and it is obvious that these methods can detect some β-lactam antibiotics below EU MRL, but no method can detect all 16 β-lactam antibiotics demanded by EU.

This study aimed to develop a direct and rapid competitive receptor assay for the detection of 16 β-lactam antibiotics in milk using recombinant PBP2x*. The principle of this assay is based on the direct competitive inhibition of PBP2x* by free β-lactam antibiotics and HRP-conjugates and the development of the assay and detection of β-lactam antibiotics in different milk samples are described.

MATERIALS AND METHODS

Chemicals and Instruments

Penicillin G, ampicillin, amoxicillin, cloxacillin, dicloxacillin, oxacillin, nafcillin, cephalexin, ceftiofur, cefalonium, cefquinome, cefazolin, cefoperazone, cephacetrile, and cephapirin were from Sigma-Aldrich (St. Louis, MO, USA) and National Institutes for Food and Drug Control (Beijing, China). *Streptococcus pneumoniae* R6 was from American Type Culture Collection (ATCC), number 49619. pET28b, T4 DNA Ligase and His•Bind® Columns were from Merk (Darmstadt, Germany). pMD19-T was from Takara (Dalian, China) and BCA Protein Assay Kit was from Pierce Biotechnology (Rockford, USA). Bovine serum albumin (BSA), tetramethylbenzidine (TMB), kanamycin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and glutaraldehyde were from Sigma–Aldrich (St. Louis, MO, USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Roche Diagnostics (Mannheim, Germany). ELISA plates (96 wells) were from Costar (Cambridge, MA). All other reagents were of reagent grade or better and from standard sources. Absorbance were read in dual-wavelength mode (450-630 nm) with a μQuant model microplate reader (Bio-Tek Instruments, Inc. USA).

Buffers and Solution

His•Bind® Columns binding buffer: 20 mmol/L sodium phosphate buffer, 500 mmol/L NaCL, 5 mmol/L Imidazole; His•Bind® Columns wash buffer: 20 mmol/L sodium phosphate buffer, 500 mmol/L NaCL, 20 mmol/L Imidazole; His•Bind® Columns elution buffer: 20 mmol/L sodium phosphate buffer, 500 mmol/L NaCL, 500 mmol/L Imidazole. Phosphate-buffered saline (PBS) buffer contained 10 mmol/L sodium phosphate buffer, pH 7.4, in 0.14 mmol/L NaCL. Coating buffer contained 0.85 mol/L carbonate buffer, pH 9.6. Blocking solution was prepared with coating buffer containing 2% casein. Washing solution (phosphate-buffered saline with Tween-20, PBST) was prepared with PBS containing 0.05% Tween-20. Substrate solution for HRP was 4 μL of 30% H_2O_2 and 400 μL of 0.6% TMB in DMF per 25 mL of acetate buffer, pH 5.5. Stopping solution was 2 mol/L H_2SO_4. Dilution solution was PBS with 3% casein.

Stock Solutions of β-lactams

Stock solutions of β-lactam antibiotics (penicillin G, ampicillin, amoxicillin, cloxacillin, dicloxacillin, oxacillin, nafcillin, cephalexin, ceftiofur, cefalonium, cefquinome, cefazolin, cefoperazone, and cephapirin) were prepared in PBS with concentrations of 1 mg/mL. Before experiments, the stock solution were

freshly diluted in order to obtain the working solution of 100 ng/mL for penicillin G, ampicillin, amoxicillin, nafcillin and ceftiofur, 1000 ng/mL for cloxacillin, dicloxacillin, oxacillin, cephapirin, cefazolin, cefalonium and cefquinome, 5000 ng/mL for cephalexin, cefacetrile, cefoperazone.

*Expression and Purification of Recombinant PBP2x**

A soluble derivative of PBP2x was expressed as a his-tag fusion protein in *E. coli* BL21 (DE3) harboring plasmid pET28b-PBP2x* coding for the PBP2x* fusion protein. The PBP2x* gene encoding the PBP2x derivative of *S. pneumoniae* R6 with a deletion of amino acids 19-48 was obtained according to literature[24]. The PBP2x* gene product was first cloned into pMD19-T, and further subcloned into a pET28b vector. *E. coli* BL21 (DE3) containing pET28b-PBP2x* was first grown overnight with vigorous shaking at 37 °C in LB medium with 30 μg/mL kanamycin. 2 mL overnight cultures were then added into 200 mL fresh LB medium and shaken at 37 °C afterwards until A600 reached 0.6. IPTG was added to a final concentration of 1 mmol/L and incubated for 4 h at 30 °C. Cells were then harvested by centrifugation at 10 000 g for 10 min, washed once with ice cold PBS and then stored at −70 °C. The precipitate was suspended in 20 mL of ice cold binding buffer and broken by 200W ultrasonication until the solution was clear. After centrifugation at 5000 g for 10 min at 4 °C, His-PBP2x* was purified from the supernatant by His•Bind® Columns in accordance with the manufacturer's instructions. Briefly, the column was balanced by 10-column volume of binding buffer and then the lysate were loaded and washed afterwards with 5-column volume of wash buffer and eluted with 5-column volume of elution buffer. All procedures were performed at 4 °C. The flow-through samples were saved for SDS-PAGE analysis. The protein concentration was determined by BCA Protein Assay Kit.

Labeling of β-lactams with HRP

Penicillin G, ampicillin, amoxicillin, cloxacillin, cefalexin, and cefquinome were labeled with HRP and different coupling methods were used according to the active site of β-lactams.

Physiological Approach Penicillin G (10 mg) and carrier (10 mg) were dissolved in 5 mL coating buffer. The solution was stirred for 24 h at 37 °C and dialyzed in PBS afterwards. The same approach was conducted for ampicillin, amoxicillin and cloxacillin.

Glutaraldehyde Method HRP (100 mg) was dissolved in 5 mL PBS with a final concentration of 0.5% glutaraldehyde. The mixture was then stirred for 24 h at 4 °C and the activated HRP was separated by Sephadex G-75. Ampicillin (10 mg) dissolved in 5 mL PBS reacted with 10 mg activated HRP by glutaraldehyde at a final concentration of 0.5%. The mixture was then incubated overnight at room temperature and dialyzed in PBS afterwards. The same approach was conducted for amoxicillin, cefalexin and cefquinome.

EDC Method Penicillin G (10 mg) dissolved in 5 mL PBS were mixed with 10 mg HRP in 5 mL PBS and 10 mg EDC in 2 mL PBS. The mixture was then stirred for 24 h at 4 °C and dialyzed in PBS afterwards. The same approach was conducted for ampicillin, amoxicillin, cloxacillin, cefalexin, and cefquinome.

EDC/NHS Method Penicillin G (10 mg), EDC (10 mg) and NHS (8 mg) were dissolved in 5 mL PBS and stirred for 60 min. Then HRP (50 mg) was added and the mixture was stirred for 24 h at 4 °C and dialyzed in PBS. The same approach was conducted for ampicillin, amoxicillin, cloxacillin, cefalexin, and cefquinome.

The protein concentration of all HRP-conjugates was tested by BCA Protein Assay Kit and aliquots of these conjugates were stored at -20 °C.

Development of a Direct Competitive Receptor Assay to Detect β-lactams

A direct competition microplate format assay was condcuted to measure β-lactams. PBP2x* protein diluted in coating buffer was immobilized in ELISA plate for 18 h at 4 °C, 100 μL per well. The plate wells were then blocked with 200 μL per well of blocking solution at 37 °C for 2 h. 50 μL standard buffer and 50 μL HRP-conjugates diluted in PBS were added into each well in turn. Incubations were performed for 30 min at 37 °C and then the plate was washed for five times with washing solution. The peroxidase activity was revealed with freshly prepared substrate solution. Fifteen minutes later, the reaction was stopped by adding 50 μL stopping solution per well. The absorbance was immediately read at 450 nm with a reference wavelength at 630 nm.

Optimization of the Microplate Assay

HRP-conjugates synthesized previously were used to combine with PBP2x*. According to the absorbance and sensitivity to penicillin G, the chosen conjugate was used to develop a direct competitive

receptor assay. The concentration of PBP2x* and HRP-conjugates were then optimized to obtain ideal sensitivity using the chessboard method.

Milk Samples Analysis

Processed milk samples were obtained from local supermarket and raw milk samples were from a dairy farm selected by our laboratory. Milk samples were spiked with β-lactams at the MRL. Processed milk samples were then centrifuged at 10 000 g for 15 min at 4 °C. Raw milk samples were analyzed without treatment. 21 processed milk samples and 33 raw milk samples were analyzed at the Reference Laboratory of Veterinary Drug Residues (Beijing, China) and the results were compared with the confirmation of LC/MS-MS (SN/T 2050-2008).

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and total recovery of the device were determined using the following equations[25]:

$$\text{Sensitivity} = TP/(TP+FN)\times 100\% \quad (1)$$

$$\text{Specificity} = TN/(TN+FP)\times 100\% \quad (2)$$

$$PPV = TP/(TP+FP)\times 100\% \quad (3)$$

$$NPV = TN/(TN+FN)\times 100\% \quad (4)$$

True positive (TP) is defined as to be positive by both the reference method and the test method, false positive (FP) is defined as to be negative by the reference method and positive by the test method, true negative (TN) is defined as to be negative by both the reference method and the test method, and false negative (FN) is defined as to be positive by the reference method and negative by the test method.

RESULTS

*Expression and Purification of Recombinant PBP2x**

PBP2x* was expressed in *E. coli* BL21 (DE3) and purified with His•Bind® Columns successfully. The data from SDS-PAGE and western-blot analysis are in good consistency with the expected size of 78 kD for PBP2x* in Figure 1.

Labeling of β-lactams with HRP

β-lactams were conjugated to HRP by four methods, from which total 20 conjugates were obtained. The main features of these conjugates were listed and evaluated in Table 2, including the affinity against PBP2x* and the sensitivity to detect β-lactams. As the most extensive application in β-lactams, Penicillin G was applied as a standard. Among these conjugates, AMP-EDC-HRP showed the highest affinity to PBP2x* and the highest sensitivity for detection of benzylpenicillin, and was therefore used for the development and optimization of the direct competitive assay.

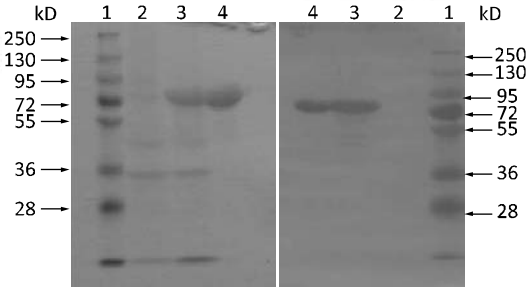

Figure 1. SDS-PAGE and Western-Blot analysis of the purification of PBP2x*. Left is the result of SDS-PAGE and right is that of Western-Blot. Lane 1: protein marker; Lane 2: total cellular protein without induction; Lane 3: total cellular protein 4 h after the addition of IPTG; Lane 4: PBP2x* purified by His•Bind® Columns.

Development and Optimization of Microplate Assay to Detect β-lactams

Based on the principle of direct competitive enzyme linked immunosorbent assay, a new direct competitive receptor assay was developed. The fusion protein PBP2x* was immobilized on a polyvinylchloride (PVC) microtiter plate and the blank sites were blocked by some other macromolecular materials. The same amounts of samples or standards treated by HRP-conjugation were then dropped into the wells and combined with PBP2x* competitively. After unbound HRP-conjugates were washed, the retained peroxidase activity was determined as described. The more β-lactams in the samples or standards, the less HRP-conjugates retained in the well and the weaker the signal, and vice versa. According to the chessboard results, the working concentration of PBP2x* and Amp-EDC-HRP was 2 μg/mL and 1:500 (original concentration was 1 mg/mL).

The parameters of standard curve were shown in Table 3, including the range of detection, LOD, linear fit and relativity. The LOD was defined as detectable concentration equivalent to 15% decline of zero binding. Since the mark residue of penethamate is penicillin G, the data for penethamate has not been listed individually.

Table 2. Characteristics of HRP-conjugates Synthesized with Different Methods

Couple Method	Standards	Name	Absorbance	LODc (ng/mL)
EDC Method	Penicillin G	Pen-EDC-HRP	0.73	1.89
	Ampicillin	Amp-EDC-HRP	2.42	0.81
	Amoxicillin	Amox-EDC-HRP	2.21	0.96
	Cloxacillin	Clox-EDC-HRP	0.67	3.22
	Cefalexin	Cef-EDC-HRP	0.24	\[b]
	Cefquinome	Ceq-EDC-HRP	0.95	2.34
EDC/NHS Method	Penicillin G	Pen-NHS-HRP	0.57	2.34
	Ampicillin	Amp- NHS -HRP	2.24	1.58
	Amoxicillin	Amox- NHS -HRP	2.48	1.67
	Cloxacillin	Clox- NHS -HRP	0.77	3.34
	Cefalexin	Cef- NHS -HRP	0.11	\[b]
	Cefquinome	Ceq- NHS -HRP	0.59	2.89
Glutaraldehyde Method	Ampicillin	Amp-GA-HRP	0.38	\[b]
	Amoxicillin	Amox-GA-HRP	0.24	\[b]
	Cefalexin	Cef-GA-HRP	0.31	\[b]
	Cefquinome	Ceq-GA-HRP	0.22	\[b]
Physiological Method	Penicillin G	Pen-Phy-HRP	\[a]	\[b]
	Ampicillin	Amp-Phy-HRP	\[a]	\[b]
	Amoxicillin	Amox-Phy-HRP	\[a]	\[b]
	Cloxacillin	Clox-Phy-HRP	\[a]	\[b]

***Note*.** a: the absorbance was below 0.1; b: the LOD was not calculated when the absorbance under 0.5. c: the LOD detected using penicillin G as standard.

Table 3. Range of Detection and LOD of β-lactams

Standards	LOD of β-lactms (ng/mL)	Range of Detection (ng/mL)	Linear Fit	R
Penicillin G	0.75	1-16	y=77.89-49.65 ln(x)	-0.9975
Ampicillin	1.22	1-16	y=90.00-52.56 ln(x)	-0.9957
Amoxicillin	1.15	1-16	y=88.27-59.94 ln(x)	-0.9992
Cloxacillin	8.23	1-81	y=142.00-61.78 ln(x)	-0.9998
Dicloxacillin	1.24	1-81	y=88.87-39.22 ln(x)	-0.9905
Naficillin	1.77	1-16	y=81.75-47.49 ln(x)	-0.9958
Oxacillin	0.83	1-81	y=96.86-46.89 ln(x)	-0.9986
Cephalexin	27.45	10-810	y=160.78-52.39 ln(x)	-0.9848
Ceftiofur	0.08	0.1-8.1	y=45.43-36.50 ln(x)	-0.9973
Cefalonium	0.99	1-81	y=85.65-35.63 ln(x)	-0.9929
Cefquinome	1.07	1-81	y=86.68-43.57 ln(x)	-0.9990
Cefazolin	1.37	1-81	y=89.74-33.38 ln(x)	-0.9967
Cefoperazone	20.52	10-810	y=152.49-50.95 ln(x)	-0.9993
Cephapirin	5.29	2-32	y=108.15-31.28 ln(x)	-0.9829
Cefacetrile	18.34	10-810	y=148.78-42.53 ln(x)	-0.9910

Milk Samples Analysis

The receptor assay established above was applied to detect β-lactam residue in milk samples. The influence of the different types of milk on the assay performance is shown in Figure 2 and penicillin G was used as standard spiked in samples. The parameters of standard curves were shown in Table 3, including the range of detection, LOD, linear fit and relativity. The limit of detection was defined as detectable concentration equivalent to 15% decline of zero binding. Since the mark residue of penethamate is benzylpenicillin, the data for penethamate has not been listed individually.

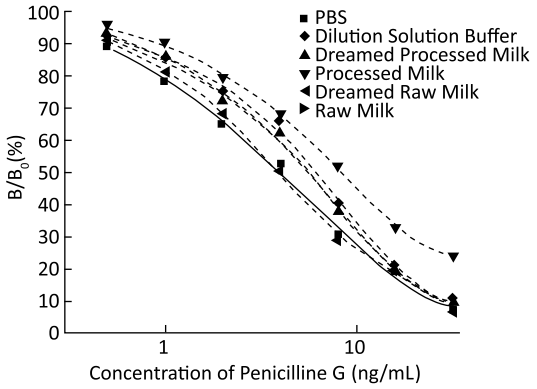

Figure 2. Curves of penicillin G in different matrix.

As a multi-residue screening assay, the calibration curve was developed based on ampicillin standardization. The samples spiked every β-lactam at MRL were then tested and calculated as shown in Table 4. The LOD of this method was 1.22 ng/mL and samples spiked with β-lactam at MRL could be detected as positive.

Table 5 shows the results of 54 samples analyzed with both the assay developed as mentioned above and LC-MS/MS. For the 13 positive samples detected by receptor assay, 11 were positive and 2 were negative detected by. LC-MS/MS. For the 41 negative samples detected by receptor assay, there was none of false negative. According to the equations above, the sensitivity of receptor method was 100%, specificity was 95.3%, PPV 84.6%, and NPV 100%.

Table 4. Detection of β-lactams at MRL Using Ampicillin as Standard

Standards	Concentration Spiked (ng/mL)	Calculated Results (ng/mL)	Results Interpretation*
Penicillin G	4	8.74	+
Ampicillin	4	4.00	+
Amoxicillin	4	4.55	+
Cloxacillin	30	3.10	+
Dicloxacillin	30	15.25	+
Naficillin	30	42.83	+
Oxacillin	30	23.39	+
Cephalexin	100	1.79	+
Ceftiofur	100	295.82	+
Cefalonium	20	22.04	+
Cefquinome	20	21.34	+
Cefazolin	50	15.97	+
Cefoperazone	50	3.88	+
Cephapirin	60	77.15	+
Cefacetrile	125	13.84	+

Note. *: The LOD of ampicillin (1.22 ng/mL) was set as the critical line. When calculated data was greater than 1.22 ng/mL, the results were judged as positive.

DISCUSSION

PBP2x binds to the cytoplasmic membrane by a short N-terminal hydrophobic peptide. In order to enhance the solubility of PBP2x, the membrane anchor was deleted and the new soluble protein produced was called PBP2x*. Lower temperature may improve the solubility of recombinant protein[26] and in our experiment, PBP2x* protein was produced at 30 °C with satisfactory results.

In this study, pET28b was used as an expression vector, not the pEGX-6p-1 as used before. There are three advantages of using pET28b as the expression vector. First, compared with the GST tag in pEGX-6p-1, his-tag in pET28b has a smaller size and hardly impacts the fold of fusion protein or the function of the proteins. Second, his-tag does not need to be cleaved after purification due to its small size, thus reducing the risk of activity loss of the target protein. Third, the vector of pEGX-6p-1 contains a β-lactamase gene, which allows the selection with ampicillin. In order to avoid using β-lactams in a PBP-overproducer, a new antibiotics resistance gene must be imported to replace the original one, such as a tetracycline resistance cassette[22-23]. The vector of pET28b is kanamycin

resistant that avoids reforming the vector, thus simplifying the procedure and making it easy to operate. Like the GST tag which can promote the solubility of the fusion protein[27], his-tag also contributes to the production of abundant soluble protein by our method.

Due to the widespread use in veterinary medicine, six β-lactams (penicillin G, ampicillin, amoxicillin, cloxacillin, cefalexin, and cefquinome) were applied to conjugate with HRP. Physiological method, EDC, EDC/NHS, and glutaraldehyde method were used to couple β-lactams with proteins. Among these methods, EDC, EDC/NHS, and glutaraldehyde method could be adopted due to the active amino group or carboxyl group in most β-lactams molecular. And the characteristic β-lactam ring could be hydrolyzed at neutral and alkaline solution system and form open ring so that to couple with protein, which was the main reason that penicillin induce the allergic reactions. Different method was adopted to connect with different β-lactams according to the different active groups. These four methods were also widely used in producing antibodies against β-lactams antibiotics[28-29].

Physiological approach is a widely used coupling method in developing specific antibodies. Owing that the lactam rings in cephalosporins are more stable than those in penicillins, only penicillins are chosen to couple with HRP in this study. However, our analysis showed that the absorbance was very low (OD<0.1), when the HRP-conjugates developed by this method were used. This finding indicates that these conjugates hardly bind to PBP2x*. The failure might come from two reasons: poor conjugation efficiency and open ring in molecular structure. It was reported previously that PBP2x* can recognize the intact lactam ring instead of the open ring form[22]. And the interaction between conjugates and PBP2x* was so week, causing the absorbance below 0.1. It was therefore inferred that open ring might be the main reason leading to the results.

Glutaraldehyde (GA) is a homobifunctional cross linker that generally couples with proteins through amino groups, including lysine ε-amino and free α-amino groups. Penicillin G and cloxacillin have not free amino group and they can not be connected with protein using GA method. GA technique has been widely used in protein coupling because of its simplicity, rapidity and effectiveness. However, during the reaction, the protein may polymerize and become large molecules, which may lead to the reduction of protein activity. In this consideration, we tried to separate the activated HRP from the polymerized HRP and excess glutaraldehyde by Sephadex G-75. Even so all conjugates produced by this method still showed low absorbance (OD<0.5). The data implied that the efficiency was not satisfied and this method may be unsuitable for connecting HRP with ampicillin, amoxicillin, cefalexin, and cefquinome.

EDC and EDC/NHS method have been successfully used in antibody production of β-lactams. EDC links the carboxyl-group and amine-group of targets with zero-length and therefore all six β-lactams chosen can couple with protein using these two methods because they have active carboxyl-group or amine-group. The intermediate product in EDC reaction is unstable in aqueous solutions, and therefore, the two-step conjugation procedures require NHS for stabilization. Excitingly, all conjugates from these two methods showed significant interaction with PBP2x*. In particular, ampicillin and amoxicillin conjugates showed strong signal reflecting strong binding to PBP2x* and the absorbance exceeded 2.0. Penicillin G, cloxacillin, and cefquinome conjugates could obtain absorbance over 0.5. But cefalexin conjugate reacted with PBP2x* poorly and the absorbance was below 0.5. The instability of Penicillin G in aqueous solution may be the main reason for the failure of its conjugate development. For cloxacillin, cefalexin and cefquinome, it is likely that PBP2x* has low affinity and sensitivity to these drugs relative to ampicillin and amoxicillin. This hypothesis was confirmed by the following data from the limit of detection (LOD). The LODs of cloxacillin, cefalexin and cefoperazone were 8.75 ng/mL, 9.35 ng/mL, and 11.24 ng/mL respectively, compared with 0.92 ng/mL for ampicillin and 1.03 ng/mL for amoxicillin. Amp-EDC-HRP, Amox-EDC-HRP, Amp-NHS-HRP and Amox- NHS-HRP had similar affinity to PBP2x* and the absorbance was almost the same, but the sensitivity of Amp-EDC-HRP and Amox-EDC-HRP (0.81 ng/mL and 0.96 ng/mL) were a little higher than that of Amp- NHS -HRP and Amox- NHS -HRP (1.58 ng/mL and 1.67 ng/mL). The difference between the data from Amp-EDC-HRP and Amox-EDC-HRP was not significant. Amp-EDC-HRP was chosen for the following experiment.

As shown in Table 3, LODs for 15 β-lactams were far below MRL set by EU. And the method developed here was more sensitive than other tests in detecting major β-lactams except cloxacillin and cefoperazone compared with microplate assay.

Lamar J. had ever developed a similar microplate assay for detecting β-lactams, involving DIG-AMP and anti-digoxigenin Fab fragments labeled with HRP. The whole analysis process lasted about 120 min, compared with 45 min by our method[22]. The SPR assay based on PBP2x* is unavailable in practical application duo to the considerable high costs of instruments[23]. However the method established in this study is easy to operate and timesaving, and thus more suitable for rapid quantitative detection and for high throughput analysis.

Milk is composed of water, carbohydrate, fat, proteins, minerals and vitamins, among which fat and protein are considered to be the main factor to disturb the interaction between receptor and ligand[22,30]. In the present study, fat was removed by centrifugation at 4 °C. Besides, protein is a major element in milk with the average content 3% and dilution buffer containing 3% casein was therefore used to dilute the standards to imitate the matrix effect. The influence of the different types of milk on the assay performance is shown in Figure 2 and penicillin G was used as standard spiked in samples. The standard curves from defated milk and raw milk nearly accorded with that from dilution solution. But the curve from processed milk had a rightward departure from standard curve, while the curve from defated raw milk and PBS had a leftward departure from the standard one. Therefore in the experiments afterwards, the processed milk samples were centrifuged before analysis and the raw milk samples were analyzed directly.

A multi-residue screening assay was developed based on an ampicillin standardization. The LOD of ampicillin was 1.22 ng/mL and the lowest calculated results was 1.79 ng/mL (cephalexin), greater than 1.22 ng/mL, and was considered as positive. The LOD of this screening method was therefore set at 1.22 ng/mL. If the calculated results exceeded 1.22 ng/mL, it was considered that the samples contained some β-lactam and needed further confirmation; otherwise, the samples met the requirement of EU.

The screening method based on receptor was compared with confirmatory method in analyzing real milk samples. In the present study, the confirmatory method was LC-MS/MS according to SN/T 2050-2008. For this receptor method, NPV was 100% and PPV was 84.6%, indicating that the positives would not be undetected and the rate of FP was lower than 20%. It was concluded therefore that this receptor method had good consistency with confirmatory method and could be used as a rapid screening method for the detection of β-lactams in milk.

It is the first report that all β-lactams demanded by EU can be detected. It's worth noting that this method can recognize 16 antibiotics and couldn't determine every antibiotic respectively as all other screening methods (including strip tests) for β-lactam compounds. And real samples may contain more than one β-lactam. Hence, the method is favorable to be used as a screening method to determine the total quantity of all β-lactams in milk like MaxSignal® Beta-Lactam ELISA Test Kit and Beta-Lactam ELISA Kit from RANDOX LIFE SCIENCES. The whole procedure takes only 45 min and it can detect 42 samples and the standard curve with duplicate analysis without complicated pretreatment, which savings time and costs for farmers and enterprises. In the future work, efforts will be made to shorten the detection time by using other assay models, such as lateral flow assay, filtration assay and so on.

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