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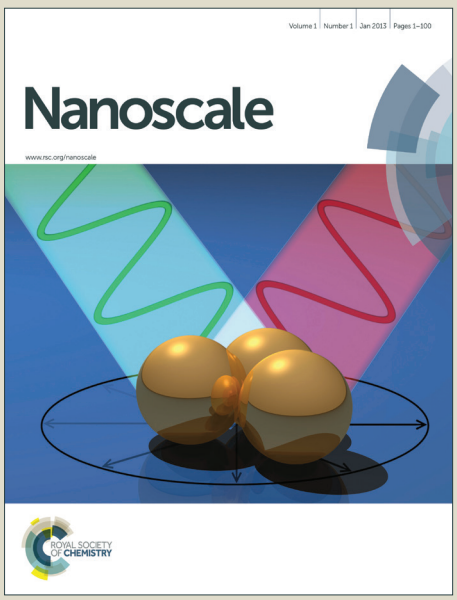

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Gold immunochromatographic assay for rapid and simultaneous detection of fifteen β-lactams

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ABSTRACT

A novel gold immunochromatographic assay (GICA) based on anti-β-lactams receptors was innovatively developed that successfully allowed rapid and simultaneous detection of fifteen β-lactams in milk samples in 5-10 minutes. Replacement of the antibodies used in traditional GICA with anti-β-lactam receptors overcame the difficulty in producing broad specific antibodies against β-lactams. Conjugates of ampicillin with BSA and goat anti-mouse immunoglobulin (IgG) were immobilized onto the test and control lines on nitrocellulose membrane, respectively. Since goat anti-mouse IgG does not combine with receptors, negative serum from mice labelled with gold nanoparticles (GNP) was mixed with GNP-labelled receptors. Results were obtained within 20 min using a paper-based sensor. The utility of the assay was confirmed with the analysis of milk samples. The limits of detection (LOD) for amoxicillin, ampicillin, penicillin G, penicillin V, cloxacillin, dicloxacillin, nafcillin, oxacillin, cefaclor, ceftezole, cefotaxime, ceftiofur, cefoperazone, cefathiamidie, and cefepime were 0.25, 0.5, 0.5, 0.5, 1, 5, 5, 10, 25, 10, 100, 10, 5, 5, 2 ng/mL, respectively, which satisfies the maximum residue limits (MRL) set by European Union (EU). In conclusion, our newly developed GICA-based anti-β-lactams receptors assay provides a rapid and effective method for one-site detection of multiple β-lactams in milk samples.

Keywords: β-lactams, gold immunochromatographic assay, receptors, milk samples

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1 Introduction

In current veterinary, extensive administration of antibiotics to dairy cattle for therapeutic purpose (for instance, treatment of bovine mastitis, pneumonia or bacterial infections) [1] [2] has triggered significant food safety issues because of antibiotics resistance[3], which is transferred to humans through the ingestion of meat and milk products [4, 5]. Furthermore, allergic reaction to antibiotic residues and inhibition of starter cultures used in the production of fermented milk products pose a significant threat to our daily life [6, 7]. At present, the β-lactams, regarded as the oldest and the most important group of antibiotics primarily composed of penicillins and cephalosporins that possess a huge side-chain attached to 6-aminopenicillanic acid and 7-aminocephaloporanic acid nuclei, respectively [8, 9], are the most extensively characterized antimicrobial group in milk owing to their therapeutic efficacy [10, 11] .

To improve the quality of dairy products and ensure consumer safety, many regulatory authorities have established maximum residue limits (MRL) of β-lactams in dairy products. The MRL of penicillin G in milk is 4, which is regulated by the EU[12]. The EU additionally set MRLs of amoxicillin, ampicillin, cloxacillin, dicloxacillin, nafcillin, oxacillin, cefacetrile, and cefoperazone as 4, 4, 30, 30, 30, 30, 125, and 50 μg/kg in milk, respectively [13]. Several analytical methods have been developed to determine the antibiotic residues of β-lactams in food. These procedures have been classified into four main categories [13]: microbial inhibition, chromatographic techniques, biosensors, immunochemical techniques. As the traditional method for antibiotic detection, microbiological approaches based on bacterial growth inhibition have been widely commercialized due to their reliability and cost-effectiveness [14, 15]. The LODs for β-lactams of many commercial microbial inhibition tests range from 2 to 100 μg/kg, which can meet the MRLs set by EU.

However, these protocols are time-consuming and non-specific, which are unsuitable for rapid and high throughput detection. Chromatographic assays have traditionally been employed as the reference method in antibiotic residue researches [16-19]. Although this procedure is accurate and sensitive, expensive instruments and skilled operators are needed. What's more, the pretreatment of samples is complex. In recent years, studies on biosensors have become increasingly prevalent. Various biosensors have been developed based on different transduction: enzymes, proteins, and so on [11, 20-22]. In general, the detection based on biosensors is sensitive and specific. Nevertheless, it is unpractical for the one-site detection and large batches. Immunochemical methods mainly comprise enzyme-linked immunoassay (EIA) [23], gold immunochromatographic assays (GICA) [24], fluorescence-polarization immunoassay (FPIA) [25] and other immunosensors based on different transduction elements. To a degree, the biosensors have always been utilized together with immunoassay [26]. However, it is quite difficult to produce sensitive antibodies against β-lactams, due to the instability of the ring structure (the common structure of β-lactams) [27, 28]. Even though some papers reported that antibodies against one or several β-lactams were produced, they failed to develop the assay that can detect the both penicillins and cephalosporins. Recently, the researches of β-lactams detections based on receptors have become the hot topic[8, 29, 30]. A receptor-based enzyme linked immunosorbent assay (ELISA) was developed to detect β-lactams, but the total time of ELISA was too long compared with GICA, which can be quickly achieved in 5-10 minutes. In addition, there is no need for trained persons and specific apparatus for high throughput one-site determination by GICA.

In such a case, GICA based on receptors recognizing multi-β-lactams (both penicillins and cephalosporins) can not only solve the bottleneck problem for

traditional immunoassay, but also achieve the fast, sensitive, and high throughput detection. To the best of our knowledge, we have established a novel GICA based on class-specific anti-β-lactam receptors that can simultaneously recognize fifteen β-lactams for the first time. This newly developed procedure which can be fulfilled in 5-10 minutes, effectively avoids the challenge of class-specific antibodies, supporting its utility in multiple β-lactams identification.

2 Materials and methods

2.1 Reagents and apparatus

Ampicillin (AMP) sodium，penicillin G potassium salt, nafcillin sodium salt monohydrate, and dicloxacillin sodium hydrate were purchased from J&K Scientific Ltd. (Beijing, China). Penicillin V potassium salt, amoxicillin, cloxacillin sodium salt monohydrate, oxacillin sodium salt were purchased from Aladdin Scientific Ltd. (Shanghai, China). Cefaclor, ceftezole, cefotaxime, ceftiofur, cefoperazone, cefathiamidine, and cefepime were acquired from National Standard Substances Center (Beijing, China). Goat anti-mouse immunoglobulin (IgG) antibody was purchased from Jackson ImmunoResearch Laboratories (PA, USA). Bovine serum albumin (BSA), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2-(4-Morpholino) ethanesulfonic acid (MES) were purchased from J & K Scientific Ltd (Beijing, China). The anti-β-lactams receptors were purchased from Wuxi Determine-Bio Scientific Ltd (Wuxi, China).

All buffer solutions were prepared with pure water produced using the milli-Q ultrapure system (Bedford, MA). Polyvinylchloride (PVC) pads, absorbance pad

(H5079), and the sample pad (glass-fiber membrane, GL-b01) were obtained from JieYi Biotechnology Co., Ltd. (Shanghai, China). The nitrocellulose (NC) membrane (Unistart CN140) was obtained from Sartorius Stedim Biotech GmbH (Goettingen, Germany). The CM4000 Guillotine Cutting Module and the Dispensing Platform were purchased from Kinbio Tech Co., Ltd. (Shanghai, China). The electrophoresis apparatus was supplied by Bio-Rad Laboratories Co., Ltd (CA, USA).

2.2 Preparation of AMP-BSA conjugates

Conjugation of AMP-BSA was achieved by EDC/NHS method on account of carboxyl group of AMP. The detailed procedure was based on several earlier reports, with some modifications [31, 32]. Briefly, 142 mg of EDC and 89 mg of NHS were added into100 mg of AMP dissolved in 5 mL of MES (0.01M, pH 6.5) and allowed to react for 1 h under room temperature with constant stirring. The mixture solution was slowly dropped into 330 mg of BSA dissolved in HEPES (0.01 M, pH 7.0) and reacted for 4 h under room temperature with constant stirring. The final solution was dialyzed for 3 days under 4 ℃ to obtain pure AMP-BSA conjugates. Electrophoretograms of AMP-BSA and BSA (Figure S1) were used to confirm the success of conjugates.

2.3 Preparation of anti-β-lactam receptors and negative serum labelled with GNPs

The productions of receptors are as follows [29]: (1) the gene of receptors was cloned by polymerase chain reaction (PCR); (2) the amplified gene was inserted into the carrier in *Escherichia coli* (*E. coli*); (3) the inserted gene expressed into receptor proteins; and (4) the proteins were extracted via ultrasonic treatment and purified using the

affinity column. Appropriate receptor generation was confirmed via electrophoresis.

GNPs were synthesized in our laboratory with the sodium citrate reduction method as previously described with some modifications [31, 33, 34]. Briefly, 50 mL of $HAuCl_4 \cdot 4H_2O$ (0.01%, w/v) was boiled thoroughly with constant stirring and rapidly mixed with 2 mL of freshly prepared 1% (w/v) trisodium citrate. The mixture was sequentially boiled for 6 min until the solution color changed to wine-red. The solution was cooled to room temperature and stored at 4℃ for future use. GNPs were characterized by transmission electron microscopy (TEM) and UV-vis spectrometry.

Anti-β-lactam receptors were characterized by SDS-PAGE. Molecular weights of receptors were determined as 67-96 kDa. The procedure of labelling anti-β-lactam receptors with GNPs was similar to that of GNP labelling of monoclonal antibodies, with some modifications [35]. Briefly, 80 μL of K_2CO_3 (0.1 M) was added into 20 mL of GNPs for adjustment of pH to 9. Receptors (0.2 mg) were slowly added to the GNPs solution under continuous stirring for 30 min at room temperature, followed by treatment with BSA (50 mg) dissolved in 1 mL of ultrapure water under stirring for 30 min and centrifugation at 875 rpm for 40 min to remove free blocking agent and excess receptor. The resulting precipitate was resuspended twice in borate buffer (0.002 M, pH 8, containing 1% (w/v) sucrose and 0.01% Tween-20) to ~5% of its original volume.

Anti-β-lactams receptors produced via gene expression and could not conjugate with goat anti-mouse IgG, resulting in no color change in the control line to red. To resolve this issue, negative serum was collected and labelled with GNPs using a similar procedure to that described above.

2.4 Principle and assembly of GICA

As shown in Figure 1, a gold immunochromatographic assay strip consist of four sections (absorbent pad, conjugate pad, sample pad and NC membrane), which are sequentially assembled onto a plastic backing sheet. The NC membrane is coated with AMP-BSA conjugate on the test line to capture the anti-β-lactams receptor labelled with GNPs, which allowed colloidal gold to aggregate and form a visible line. The control line was coated with goat anti-mouse IgG for conjugation with GNP-labelled negative serum, allowing the aggregation of colloidal gold. One red band consistently appears on the control line regardless of the presence of analytes, certifying the validity of the test. In cases where visible red lines appear in both the control and test zone, the sample is negative. Conversely, the presence of only one visible red band in the control zone indicates a positive sample .

(Figure 1)

In our study, the sample pad was firstly imbued with buffer solution (0.01M PBS containing 0.2% BSA and 0.2% Tween-20) and air-dried overnight for future use. Next, the NC membrane was coated with two conjugate types (AMP-BSA and goat anti-mouse IgG) on the test and control lines, respectively, using the rapid test dispenser platform at a jetting rate of 1 μL/cm. The NC membrane was dried at 37^0C for 3 h and stored in a desiccator. Optimal volumetric ratios of receptor-GNP and negative serum-GNP conjugates were added to a microwell and freeze-dried in a vacuum freeze-dryer. The strips and freeze-dried mixtures were stored in a desiccator at room temperature until use.

2.5 Analysis of milk sample using GICA based on anti-β-lactam receptors

Negative milk samples confirmed by LC/MS/MS, which were supported by Jiangsu Entry-Exit Inspection and Quarantine Bureau, were spiked with fifteen β-lactams at a range of concentrations. Amoxicillin, ampicillin, penicillin G, penicillin V, oxacillin, dicloxacillin, cloxacillin, nafcillin, cefaclor, ceftezole, cefotaxime, ceftiofur, cefoperazone, cefathiamidine, cefepime, cefalexin, cefadroxil, cefradine, cefuroxime, and cefodizime were prepared at a concentration of 1 mg/mL using HEPES (0.01 M, pH 7.0), and further diluted 100 times to a concentration of 1 μg/mL to obtain stock solution. Fortified milk was diluted 10 times with HEPES (0.01M, pH 7.0) to obtain different final concentrations as follows: amoxicillin (0, 0.25, 0.5, 1, 2, and 5 ng/mL), ampicillin (0, 0.5, 1, 2, 5, and 10 ng/mL), penicillin G (0, 0.25, 0.5, 1, 2, and 5 ng/mL), penicillin V (0, 0.25, 0.5, 1, 2, and 5 ng/mL), cloxacillin (0, 1, 2, 5, 10, and 25 ng/mL), dicloxacillin (0, 1, 2, 5, 10, and 20 ng/mL), nafcillin (0, 2, 5, 10, 25, and 50 ng/mL), oxacillin (0, 4, 10, 20, 50, and 100 ng/mL), cefaclor (0, 10, 25, 50, 100, and 200 ng/mL), ceftezole (0, 5, 10, 25, 50, and 100 ng/mL), cefotaxime (0, 50, 100, 250, 500, and 1000 ng/mL), ceftiofur (0, 10, 25, 50, 200, and 200 ng/mL), cefoperazone (0, 1, 2, 5, 10, and 25 ng/mL), cefathiamidine (0, 5, 10, 25, 50, and 100 ng/mL) and cefepime (0, 2, 5, 10, 25, and 50 ng/mL).

2.6 Live subject statement

This article does not contain any studies involving human subjects. All animal studies were carried out under the guidance of animal welfare committee of Jiangnan University.

3 Results and Discussion

3.1 Preparation of AMP-BSA conjugates

Successful generation of AMP-BSA conjugates was confirmed via SDS-PAGE. As

shown in Figure S2, the apparent shift band between the same concentration of BSA and AMP-BSA was indicative of successful conjugation.

3.2 Preparation of anti-β-lactam receptors labelled with GNPs

The charge and stability of conjugates are determined by solution pH. Therefore, pH plays a crucial role in the preparation of receptor-GNP conjugates. Colloidal gold carries a negative charge and therefore combines efficiently with positively charged proteins through electrostatic bonds. Receptor-GNP conjugates are formed through electrostatic interactions between IgG and negatively charged GNPs. Colloidal gold must be stabilized with BSA or polyethylene glycol (PEG) after conjugation with receptors. The conjugation between negative serum and GNPs was similar to that between receptors and GNPs. K_2CO_3 was used to adjust the pH of colloidal gold. The K_2CO_3 volume and final receptor concentrations were optimized. Our results indicate that 6 μL of 0.1 M K_2CO_3 for each mL GNP solution is optimal for the mixture of receptor-GNP and negative serum-GNP conjugates.

In total, 50 μL of 10% BSA (used as a stabilizer) was added dropwise with constant stirring to 1 mL of conjugates to reduce non-specific reactions.

3.3 Optimization of the GICA

Different concentrations of anti-β-lactam receptors (0.1, 0.2, 0.4 and 0.8 μg/mL), AMP-BSA (0.05, 0.1, 0.2, and 0.4 μg/mL), and GNPs (2, 4, and 8 nM) were examined, with a view to optimizing the results. The optimal conditions were determined as 0.2 μg/mL anti-β-lactam receptor, 0.1 μg/mL AMP-BSA, and 4 nM GNPs. Under these conditions, receptor-GNP and negative serum-GNP solutions were stable after centrifuging twice and resuspension.

3.4 Validation of GICA based on anti-β-lactam receptors with milk samples

Spiked milk samples were pretreated with simple dilution, with a view to eliminating matrix interference. As shown in Figures S3-9, the strip performed well at a sequence of concentrations. This biosensor can therefore be applied for one-site detection for fifteen β-lactams by untrained persons with no need for specific apparatus. The test line becomes less intense or colorless relative to the control line depending on the concentration, which can be judged by the naked eye. Figures 2 and 3 depict four representative penicillin and cephalosporin compounds in milk samples, respectively. For detection of amoxicillin, the LOD was 0.25 ng/mL, which was at the advanced level. Analyses of the remaining seven compounds are shown in Figures S3-9. A summarized image for all 15 chemicals is shown in Figure 4. The test line of each strip is obviously less intense in color than the control line. The concentration is defined as visual LOD. The cut-off values for the GICA, defined as the concentration producing no color on the test line, present another parameter for semi-quantitative assessment. Cut-off values and LOD obtained for β-lactams in the milk sample confirm that the method meets the MRLs set by EU (Table 1). Moreover, 20 independent milk samples were analyzed to ascertain the reproducibility of the method. Stability experiments indicate that neither reaction intensity nor sensitivity are influenced during the course of storage for six months.

(Figure 2)

(Figure 3)

(Figure 4)

(Table 1)

4 Conclusions

In this paper, a novel biosensor was developed that allowed successful detection of fifteen β-lactams in milk samples for the first time. Rapid, semi-quantitative detection by the naked eye was achieved using simple GICA based on anti-β-lactam receptors in 5-10 minutes, facilitating high-throughput testing for β-lactams in milk samples. In recent years, the usage of β-lactams in animal husbandry and dairy products has drastically increased leading to an urgent requirement for rapid, simple, and high-throughput detection methods that assist in company surveillance and safeguarding consumer rights.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article was provided.

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Captions

Fig. 1. The principle of GICA based on anti-β-lactams receptors

Fig. 2. The images of GICA based anti-β-lactams receptors for four kinds of representative penicillins in milk samples: (A) ammocillin: 0, 0.25, 0.5, 1, 2 and 5 ng/mL; (B) ampicillin: 0, 0.5, 1, 2, 5, and 10 ng/mL; (C) penicillin G: 0, 0.25, 0.5, 1, 2, and 5 ng/mL; (D) penicillin V: 0, 0.25, 0.5, 1, 2, and 5 ng/mL, respectively.

Fig. 3. The images of GICA based anti-β-lactams receptors for four kinds of representative cephalosporins in milk samples: (A) cefaclor: 0, 10, 25, 50, 100, and 200 ng/mL; (B) ceftezole: 0, 5, 10, 25, 50, and 100 ng/mL; (C) cefoperazone: 0, 1, 2, 5, 10, and 25 ng/mL; (D) cefathiamidine: 0, 5, 10, 25, 50, and 100 ng/mL, respectively.

Fig. 4. The detection of fifteen β-lactams in milk sample using GICA based on anti-β-lactams receptors. The final concentrations of fifteen β-lactams in ten time diluted milk sample: (1) 0 ng/mL, (2) 1 ng/mL of amoxicillin, (3) 2 ng/mL of amoxicillin, (4) 2 ng/mL ampicillin, (5) 5 ng/mL ampicillin, (6) 0.5 ng/mL penicillin G, (7) 1 ng/mL penicillin G, (8) 1 ng/mL penicillin V, (9) 2 ng/mL penicillin V, (10) 5 ng/mL cloxacillin (11) 10 ng/mL cloxacillin, (12) 10 ng/mL dicloxacillin, (13) 20 ng/mL dicloxacillin, (14) 5 ng/mL nafcillin (15) 10 ng/mL nafcillin, (16) 20 ng/mL oxacillin, (17) 50 ng/mL oxacillin, (18) 50 ng/mL cefaclor, (19) 100 ng/mL cefaclor, (20) 10 ng/mL ceftezole, (21) 25 ng/mL ceftezole, (22) 250 ng/mL cefotaxime, (23) 500 ng/mL cefotaxime, (24) 25 ng/mL ceftiofur, (25) 50 ng/mL ceftiofur, (26) 5 ng/mL cefoperazone, (27) 10 ng/mL cefoperazone, (28) 25 ng/mL cefathiamidine, (29) 50 ng/mL cefathiamidine, (30) 10 ng/mL cefepime, (31) 25 ng/mL cefepime, respectively.

Table 1. Cut-off values of β-lactams in milk sample using GICA based on anti-β-lactams receptors.

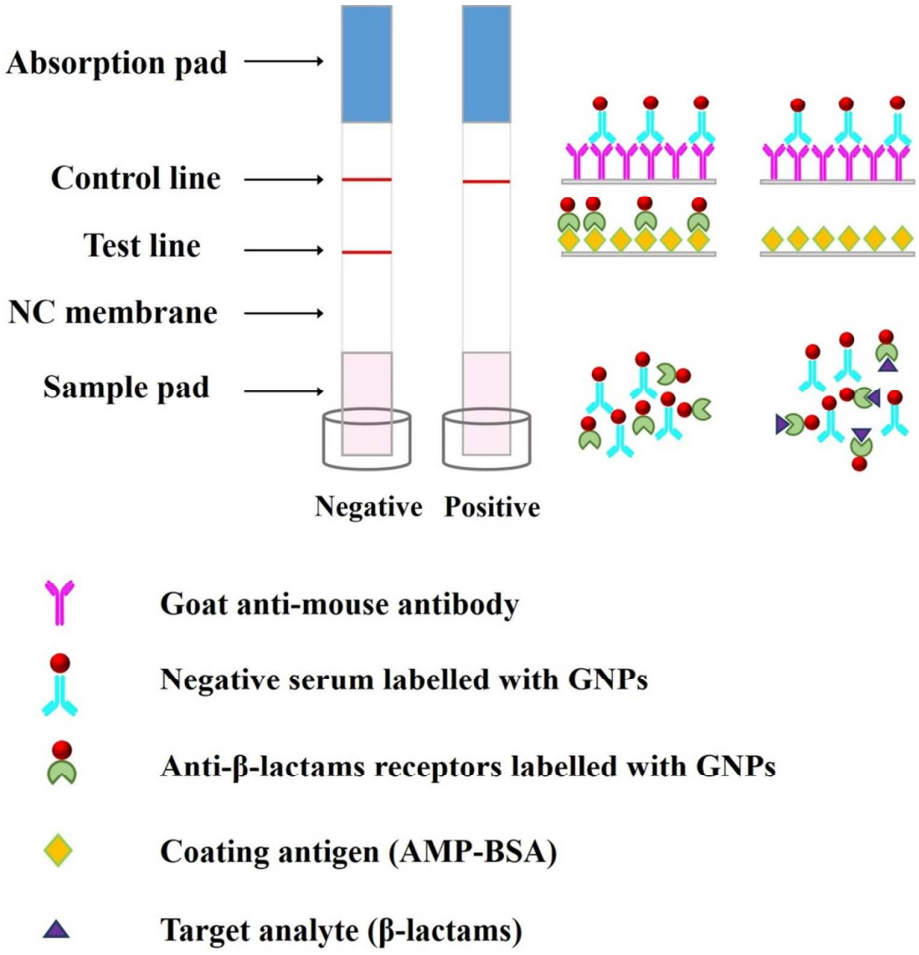


Fig. 1. The principle of GICA based on anti-β-lactams receptors

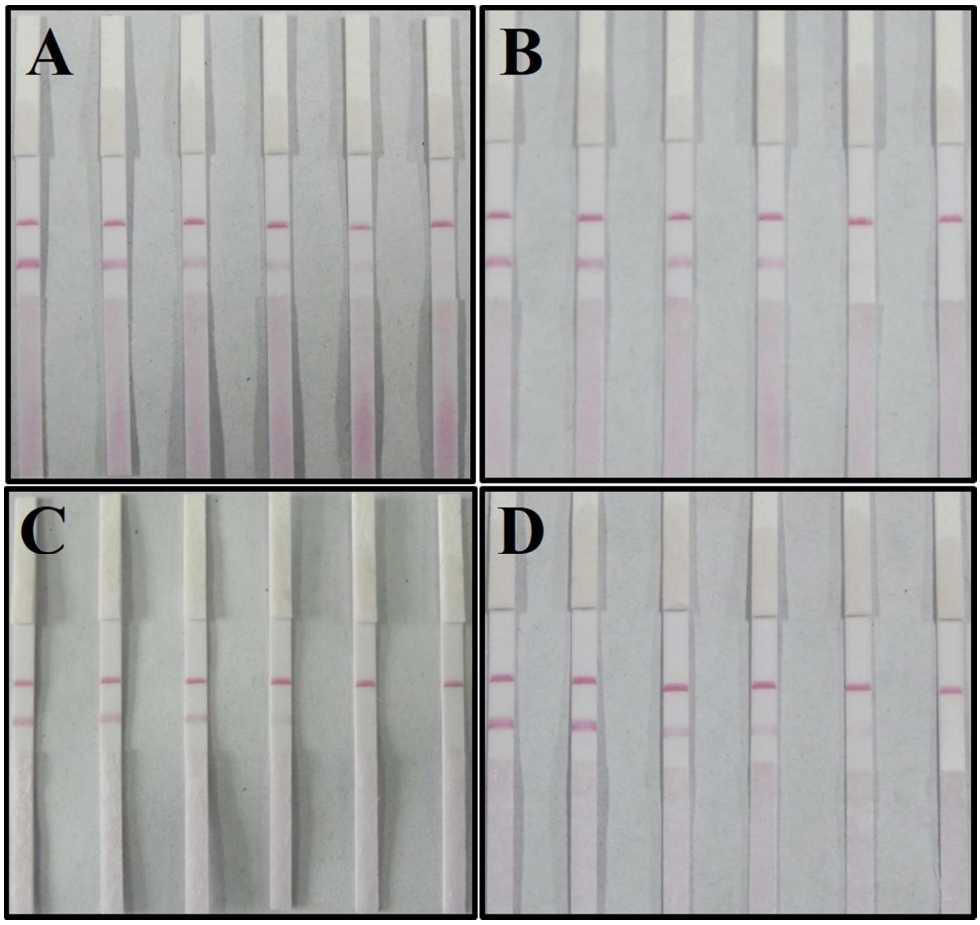


Fig. 2 The images of GICA based anti-β-lactams receptors for four kinds of representative penicillins in milk samples: (A) ammocillin: 0, 0.25, 0.5, 1, 2 and 5 ng/mL; (B) ampicillin: 0, 0.5, 1, 2, 5, and 10 ng/mL; (C) penicillin G: 0, 0.25, 0.5, 1, 2, and 5 ng/mL; (D) penicillin V: 0, 0.25, 0.5, 1, 2, and 5 ng/mL, respectively.

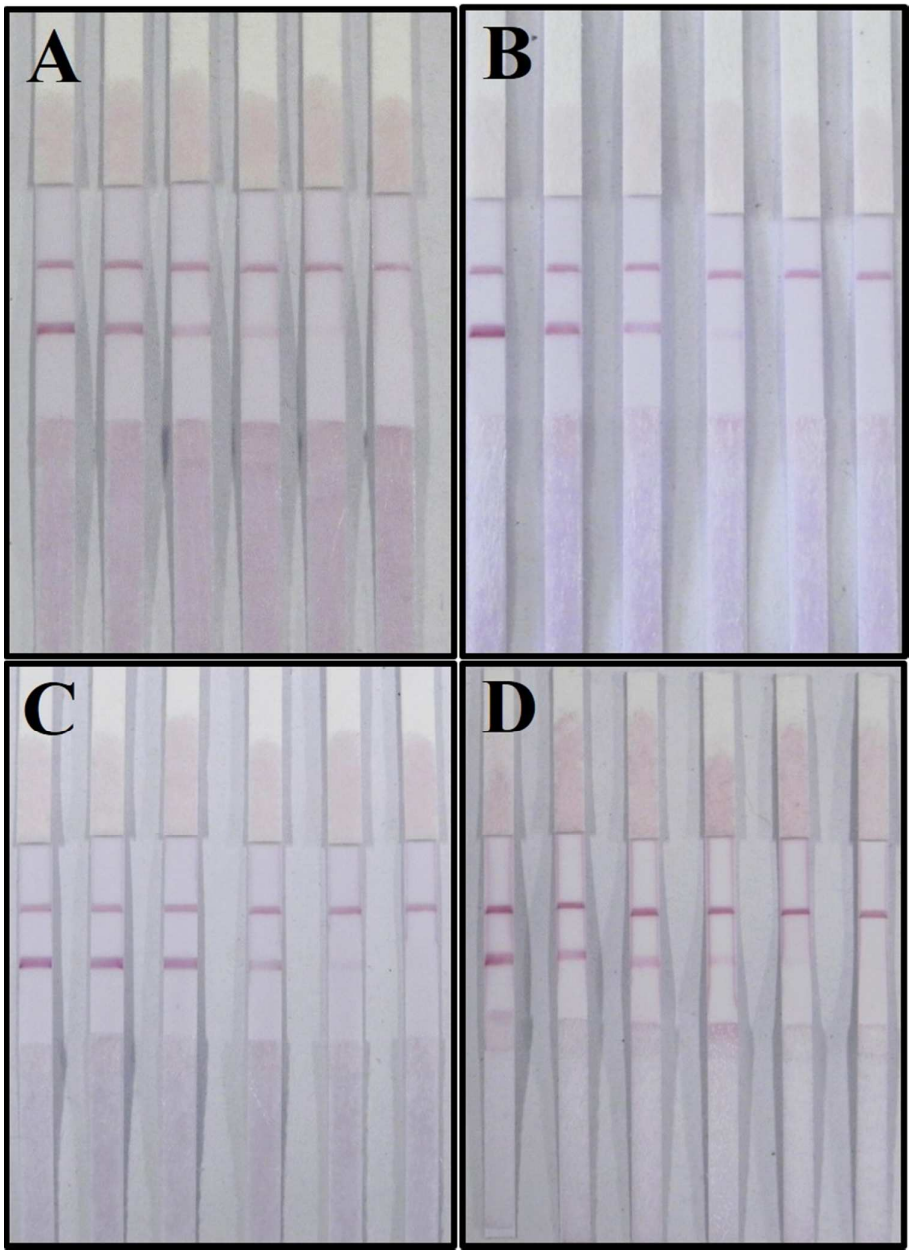


Fig. 3 The images of GICA based anti-β-lactams receptors for four kinds of representative cephalosporins in milk samples: (A) cefaclor: 0, 10, 25, 50, 100, and 200 ng/mL; (B) ceftezole: 0, 5, 10, 25, 50, and 100 ng/mL; (C) cefoperazone: 0, 1, 2, 5, 10, and 25 ng/mL; (D) cefathiamidine: 0, 5, 10, 25, 50, and 100 ng/mL, respectively.

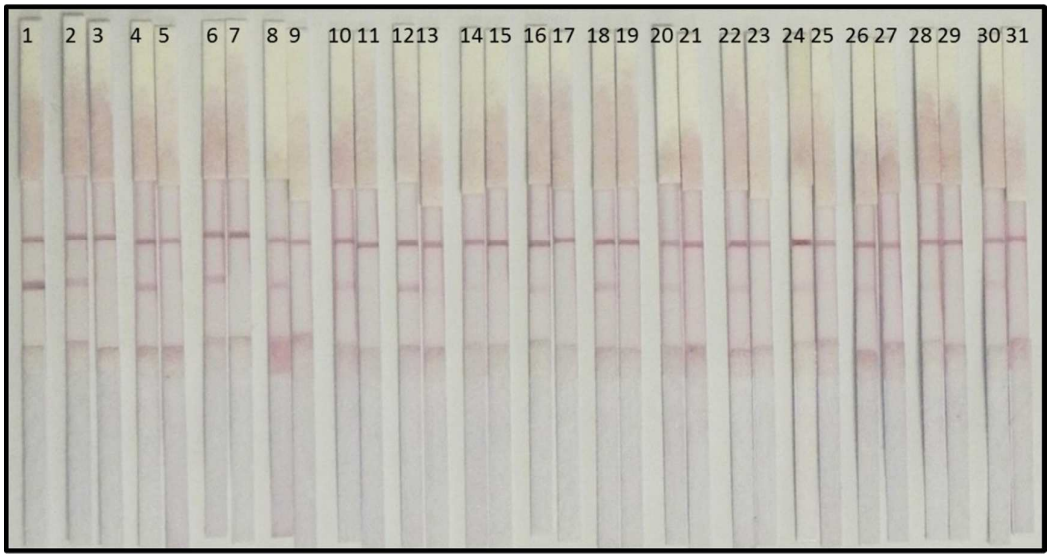
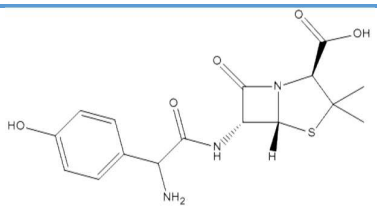
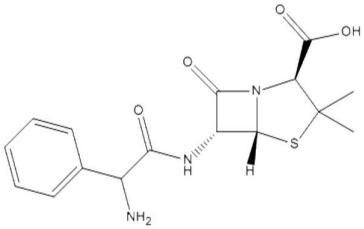
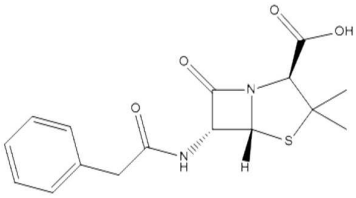
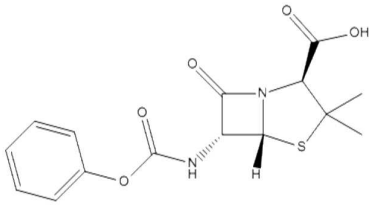
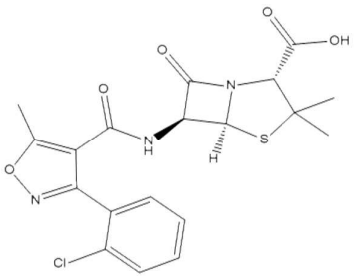
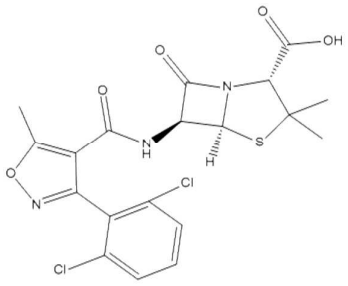
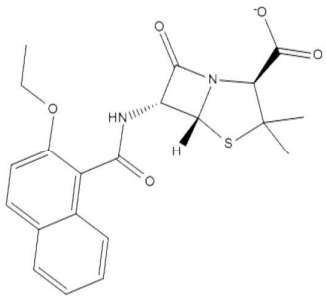
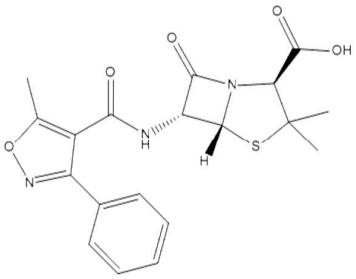
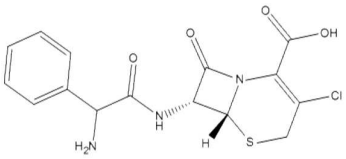
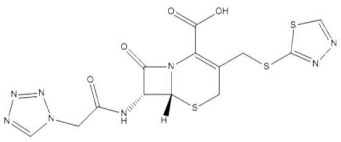
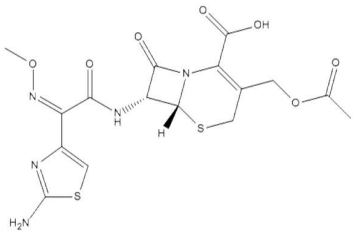
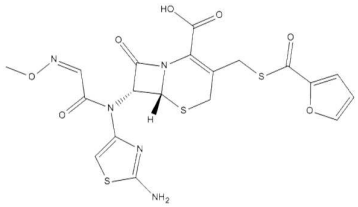
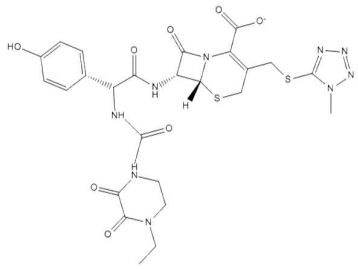
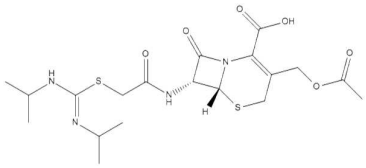
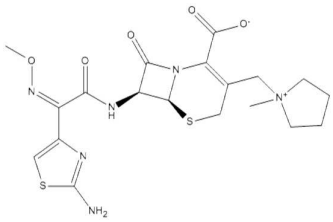


Fig. 4 The detection of fifteen β-lactams in milk sample using GICA based on anti-β-lactams receptors. The final concentrations of fifteen β-lactams in ten time diluted milk sample: (1) 0 ng/mL, (2) 1 ng/mL of amoxicillin, (3) 2 ng/mL of amoxicillin, (4) 2 ng/mL ampicillin, (5) 5 ng/mL ampicillin, (6) 0.5 ng/mL penicillin G, (7) 1 ng/mL penicillin G, (8) 1 ng/mL penicillin V, (9) 2 ng/mL penicillin V, (10) 5 ng/mL cloxacillin (11) 10 ng/mL cloxacillin, (12) 10 ng/mL dicloxacillin, (13) 20 ng/mL dicloxacillin, (14) 5 ng/mL nafcillin (15) 10 ng/mL nafcillin, (16) 20 ng/mL oxacillin, (17) 50 ng/mL oxacillin, (18) 50 ng/mL cefaclor, (19) 100 ng/mL cefaclor, (20) 10 ng/mL ceftezole, (21) 25 ng/mL ceftezole, (22) 250 ng/mL cefotaxime, (23) 500 ng/mL cefotaxime, (24) 25 ng/mL ceftiofur, (25) 50 ng/mL ceftiofur, (26) 5 ng/mL cefoperazone, (27) 10 ng/mL cefoperazone, (28) 25 ng/mL cefathiamidine, (29) 50 ng/mL cefathiamidine, (30) 10 ng/mL cefepime, (31) 25 ng/mL cefepime, respectively.

Table 1. Cut-off values of β-lactams in milk samples using GICA based on anti-β-lactams receptors

Compounds	Structures	Cut-off values (ng/mL)	LOD (ng/mL)
Amoxicillin		5	0.25
Ampicillin		10	0.5
Penicillin G		5	0.5
Penicillin V		5	0.5
Cloxacillin		100	1

Dicloxacillin		20	5
Nafcillin		50	5
Oxacillin		25	10
cefaclor		200	25
ceftezole		100	10
cefotaxime		1000	100

ceftiofur		200	10
cefoperazone		25	5
cefathiamidie		100	5
cefepime		25	2