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Short communication

Development of a quantitative immuno-affinity test column assay for on-site screening of clindamycin residues in milk

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ABSTRACT

A sensitive and specific monoclonal antibody against clindamycin (CLIN) was produced and used to develop an immuno-affinity test column (IATC) assay for on-site screening of CLIN residues in milk. The qualitative limit of detection of the IATC assay, estimated as 1.0 μg L^{-1} by visual detection, was sufficient to measure maximum residue levels for lincosamide antibiotics in milk. The quantitative IATC assay resulted in a lower detection limit (0.11 μg L^{-1}) by evaluating the colour intensity of the test layers, which was approximately 9 times greater sensitivity than visual detection. During the spike and recovery test, the average recoveries ranged from 76% to 114% at different spiked levels, and the intra-/interday coefficients of variation were in the range 9.6–16.7%. The detection time was shortened to 20 min, whereas the sensitivity was comparable with a traditional ELISA. The IATC assay shows promise for on-site screening of CLIN residues in milk.

1. Introduction

Clindamycin (7-chloro-7-deoxy-lincomycin, CLIN), a semi-synthetic derivative of lincomycin (LIN), is among the most important lincosamide antibiotics in clinical use (Spížek & Řezanka, 2004). In veterinary medicine, CLIN has been widely administered to dairy cattle for the prevention and treatment of mastitis. However, the presence of CLIN residues in milk could cause allergic or toxic reactions in humans (Giguère, 2013). Moreover, CLIN and other antibiotics used for treatment of animals may be linked to the development of antibiotic-resistant bacterial strains in humans (Lewis & Jorgensen, 2005). To protect consumers from exposure to these harmful residues, maximum residue limits (MRLs) have been set for lincosamide antibiotics ranging from 50 μg kg^{-1} to 1500 μg kg^{-1} in different food matrices (Codex Alimentarius, 2015), but no MRLs have been set for CLIN residues in milk and dairy products.

Chromatographic separation combined with mass spectrometric detection is the widely accepted method for determining lincosamide antibiotics residues in different food matrices (Cherlet, Croubels, & De Backer, 2002; Martens-Lobenhoffer & Banditt, 2001). However, these chromatographic techniques are not suitable for on-site screening purposes since they require expensive instruments, skilled professional technicians and time-consuming sample treatment procedures.

Immunoassays have advantages over chromatographic procedures in simplicity, sensitivity, and capability of high sample throughput (Jiang et al., 2013a). To date, few immunoassays have been described for CLIN residue analysis. Recently, enzyme linked immunosorbent assay (ELISA), lateral-flow tests, and other analytical methods have been developed for the rapid screening of veterinary drug residues. However, lateral-flow tests have low sensitivity, ELISA is time-consuming, and immunosensors require special equipment.

Thus, it is urgent to develop rapid and sensitive analytical methods for screening purposes. Immuno-affinity chromatography (IAC) is a widely used sample clean-up technique in the field of food safety. Beloglazova et al. (2008) reported a novel IAC-based non-instrumental immunochemical test for detecting mycotoxin residues in food. The analytical results (blue colour development on test layers) were visual to the naked eye after the addition of chromogenic substrate, hence making it suitable for on-site screening purpose. In this paper, sepharose 4B gel beads were used as a carrier media to develop a rapid and sensitive immuno-affinity test column (IATC) assay for the on-site screening of CLIN residues in milk. Furthermore, the quantitative detection of the

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CLIN assay was also investigated by evaluating the colour intensity of the test layers. Compared with conventional strategies, the developed IATC assay could be a useful on-site screening technique for CLIN residue analysis in milk.

2. Materials and methods

2.1. Reagents and materials

Clindamycin (CLIN) was purchased from Sigma–Aldrich (St. Louis, MO, USA). CNBr-activated sepharose 4B was purchased from GE Healthcare (Pittsburgh, PA, USA). Goat-anti-mouse IgG and goat-anti-HRP IgG were purchased from Jackson Immuno-Research Inc. (West Grove, PA, USA). Bond Elut reservoir (1 mL) and polyethylene frits were purchased from Agilent Technologies (Santa Clara, CA, USA).

The following buffers and reagents were prepared: phosphate-buffered saline (PBS, 0.01 M, pH 7.4), coating buffer (0.05 M sodium carbonate buffer, pH 9.6), coupling buffer ($NaHCO_3$ buffer containing 0.5 M NaCl, pH 8.3), blocking buffer (coupling buffer containing 0.2 M of glycine, pH 8.0) and acetate buffer (pH 4.0, containing 0.5 M NaCl), and chromogenic substrate (0.1% TMB, H_2O_2 in 0.05 M citrate buffer, pH 4.5).

2.2. Antigen synthesis and monoclonal antibody production

The CLIN derivative with a carboxylic group was synthesised in accordance with previous research (Wang et al., 2010). The CLIN derivative was covalently linked to KLH, BSA and OVA using the active-ester method. Moreover, CLIN was conjugated to HRP using a sodium periodate ($NaIO_4$) reduction method in accordance with the method of Burkin and Galvidis (2010). The preparation of the conjugates and characterisation of monoclonal antibody (MAb) against CLIN were conducted according to our previous research (Jiang, Beier, Wang, Wu, & Shen, 2013b).

2.3. Establishment of an ELISA for CLIN residue analysis

Prior to the development of IACT assay, a direct competitive ELISA needed to be established. Ninety-six well microtitre plates were coated with 100 μL per well of anti-CLIN antibody in coating buffer by incubating overnight at 4 °C. The plates were washed three times, and then blocked for 2 h at 37 °C. After washing 3 times, CLIN standard solution (50 μL per well) was added simultaneously with the tracer, and the plate was incubated for 30 min at 37 °C. Then, 100 μL per well of substrate solution was added after washing 3 times, and the plates were incubated at 37 °C for 15 min. After the addition of 2 M H_2SO_4 (50 μL per well), the absorbance was measured at 450 nm using a microplate reader.

The sensitivity was evaluated based on the IC_{50} (the analyte concentration at 50% inhibition) value, which is an important sensitivity index for ELISA. Competition curves were fitted to a four-parameter logistic equation, from which the IC_{50} values were calculated using Origin Pro 8.0 software. To assess the specificity of the ELISA method, the cross-reactivities (CRs) were obtained using analogues as competitors in the developed ELISA. The IC_{50} values of each analogue were calculated, and the CRs were obtained by comparing the IC_{50} values of the analytes using the following equation: $CR\ (\%) = [IC_{50}\ (CLIN)/IC_{50}\ (analyte)] \times 100$.

For the ELISA, the optimal dilutions of the anti-CLIN MAb and the tracer CLIN-HRP were 1:5000 (0.3 μg mL^{-1}) and 1:2000 (0.2 μg mL^{-1}), respectively. The direct competitive ELISAs for CLIN determination were developed, and the IC_{50} value was calculated to be 2.4 μg L^{-1}. The anti-CLIN antibody was highly specific towards CLIN, and showed low cross-reactivity with lincomycin (5.3%) and pirlimycin (0.3%). Based on these data, the immunoassay developed in this work is capable of detecting CLIN residues in milk.

Milk samples were analysed by the developed ELISA after a simple sample treatment (Jiang et al., 2013a). Briefly, 5 mL of milk samples were transferred into 10-mL centrifuge tubes. The samples were vigorously vortexed for 1 min after the addition of 0.2 mL of 0.36 M sodium nitroprusside and 0.2 mL of 1.04 M zinc sulphate. After centrifugation at 3000 × *g* for 10 min at 4 °C, the supernatant was diluted 10 times with assay buffer for determination by the ELISA. The limit of detection (LOD) was defined as the concentration corresponding to the mean value of 20 blank samples, plus three times the standard deviation. The detection limit was 7.5 μg L^{-1} based on the results of 20 blank samples.

2.4. Preparation of the test columns

CNBr-activated sepharose 4B was activated and coupled with goat-anti-mouse IgG for the preparation of coupled gel according to our previous research (Beloglazova et al., 2012; see Supplementary material). The anti-CLIN antibody immobilised gel and anti-HRP antibody immobilised gel were prepared by adding the relevant MAbs to the coupled gels.

The test columns of the IATC assay consist of one test layer and one control layer, and each layer was placed in the plastic columns between two polyethylene frits. The first frit was placed on the bottom at the 1 mL Bond Elut Reservoir, and 200 μL of anti-HRP MAb coupled gel for quality control was added. The second frit was used to cover the control layer, and the third frit was added to the column to produce an air gap layer (0.3 cm). Then, 200 μL of anti-CLIN MAb coupled gel for the detection of CLIN residues was added to the column above the third frit, and the fourth frit was used to cover the CLIN test layer.

The prepared columns were stored at 4 °C until use.

2.5. Development of the IATC assay

The analysis procedure of the IATC assay includes 4 steps (sample passing, tracer addition, washing to remove unbound tracer, substrate addition and detection), and the analytical signal was observed to be visible to the naked eye (Jiang et al., 2015). Briefly, 1 mL of the standard solution or sample extract was passed through the test columns at the speed of 1 drop per second. Then, 200 μL of the HRP tracers was added, and the test columns were incubated for 5 min at room temperature. To remove an excess of tracers, the test columns were washed with 6 mL of PBS containing Tween 20 (0.05%, w/v; PBST). Finally, 200 μL of a TMB substrate solution was added, and then pushed away by a syringe to immerse both layers. The visual detection was carried out after 10 s of incubation (Fig. S1).

2.6. Matrix effect examination and sample preparation

Milk samples were analysed by the IATC assay after a simple sample treatment as reported previously (Jiang et al., 2013a). Briefly, 5 mL of milk samples were transferred into 10-mL centrifuge tubes. The samples were vigorously vortexed for 1 min after the addition of 0.2 mL of 0.36 M sodium nitroprusside and 0.2 mL of 1.04 M zinc sulphate. After centrifugation at 3000 × *g* for 10 min at 4 °C, 1 mL of supernatant was loaded on the test columns for the detection of CLIN residues.

2.7. Sample analysis and assay validation

Milk samples were purchased from retail markets and tested by the developed IATC assay. The qualitative limits of detection (LOD)