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Development of a heterologous enzyme-linked immunosorbent assay for the detection of clindamycin and lincomycin residues in edible animal tissues

Jie He [a,1], Nan Wu [a,1], Pengjie Luo [b], Ping Guo [c], Jianwen Qu [a], Shuying Zhang [a], Xin Zou [a], Feiyao Wu [a], Huijuan Xie [a], Can Wang [a], Wenxiao Jiang [a,*]

[a] Department of Physiology, School of Basic Medical Sciences, Shenzhen University Health Sciences Center, Shenzhen 518060, China
[b] Key Laboratory of Food Safety Risk Assessment, Ministry of Health, China National Center for Food Safety Risk Assessment, Beijing 100021, China
[c] Technology Center of JiangXi Entry-Exit Inspection and Quarantine Bureau, Nanchang 330038, China

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ABSTRACT

In this study, new clindamycin (CLIN) artificial antigens were prepared and used to produce broad-specificity monoclonal antibodies. Based on the as-produced mAbs, a heterologous ELISA was developed to detect CLIN and lincomycin (LIN) residues in edible animal tissues. The IC_{50} values of the developed assay were 0.3 ng/mL (CLIN) and 1.2 ng/mL (LIN) in buffer, respectively. The detection limits were estimated to be 1.8 μg/kg (CLIN) and 6.8 μg/kg (LIN) in bovine, chicken, porcine and fish muscles. In the spike and recovery tests, the mean recovery rate ranged from 76% to 112% at different spiked levels, and the intra−/inter-assay coefficients of variation were in the range of 7.1% to 13.2%. This method was verified using LC − MS/MS with a correlation coefficient >0.97. The developed ELISA is therefore well suited for simultaneous determination of CLIN and LIN residues in bovine, chicken, porcine and fish muscles.

1. Introduction

Veterinary drugs were routinely used to treat and prevent animal diseases in food animal husbandry in order to improve growth performance. Lincosamides are a class of antibiotics which include lincomycin (LIN), clindamycin (CLIN), and pirlimycin. LIN was discovered from the fermentation of *Streptomyces* spp. in 1963. (Giguère, 2013) CLIN, a lincomycin analog, showed distinct advantages over lincomycin for the treatment of a number of bacterial infections. Thus, the use of lincomycin as an antibiotic has been largely superseded by clindamycin. (Batzias, Delis, & Athanasiou, 2005) Pirlimycin, a lincomycin analog, was also approved as an intramammary infusion for the treatment of mastitis in cattle. In veterinary medicine, CLIN and LIN (Fig. 1) are the most commonly used lincosamide antibiotics for treating diverse infections that are caused by gram-positive bacteria (Spížek & Řezanka, 2004), especially those that are penicillin resistant (Morar, Bhullar, Hughes, Junop, & Wright, 2009).

However, the antibiotic residues in food pose hazards for human health, such as allergies, nausea, diarrhea, rash and pseudomembranous enteritis (Batzias et al., 2005; Giguère, 2013). In addition, the widely used antibiotics in animals are associated with the prevalence of antibiotic resistance in bacteria (Apata, 2009; Wagner et al., 2003). To avoid these hazards for public health, maximum residue limits (MRLs) have been set by the European Union (EU), the United States (US) and China. The MRLs for LIN were fixed at 100 μg/kg (muscle), 50 μg/kg (Fat), 500 μg/kg (Liver), 1500 μg/kg (Kidney), 150 μg/kg (Milk) and 50 μg/kg (Eggs), respectively. (European Commission, 2010) The MRLs in chicken and pig muscles were fixed at 200 μg/kg for LIN. (Codex Alimentarius, 2015) The Ministry of Agriculture of China has also set MRLs that range from 100 μg/kg in milk to 1500 μg/kg in kidneys. To the best of our knowledge, no MRLs were established for CLIN residues in edible animal tissues, although CLIN was a good antibacterial agent in veterinary medicine.

During the last two decades, chromatographic methods have been widely used for the detection of lincosamide antibiotic residues in different food matrices (Juan, Moltó, Mañes, & Font, 2010; Luo, Yin, Ang, Rushing, & Thompson, 1996; Thompson, Noot, Calvert, & Pernal, 2005). However, these applications are usable only on a laboratory scale because the chromatographic methods require extensive sample preparation and expensive equipment (Jiang et al., 2015). Immunoassays are widely used as a screening test due to their simplicity, sensitivity and high sample throughput (Jiang et al., 2012; Wang, Xu, Zhang, & He, 2009). Burkin et al. (Burkin & Galvidis, 2010) and Zhou et al. (Zhou et al., 2014) have previously reported the production of an *anti*-LIN

* Corresponding author.
E-mail address: jiangwenxiao@szu.edu.cn (W. Jiang).
[1] Jie He and Nan Wu contributed equally to this work.

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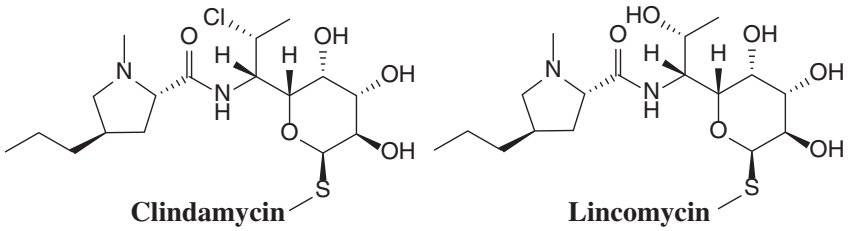

Fig. 1. Chemical structures of clindamycin and lincomycin.

antibody and the development of ELISA and lateral flow assays for the detection of the LIN residues. However, these reports focus only on LIN-specific antibody production and the detection of the LIN residues in different food matrices. In our previous research, anti-pirlimycin antibody was produced and a biotin–streptavidin-amplified ELISA was developed for rapid screening pirlimycin residue in some food matrices. (Jiang et al., 2016)

In this study, CLIN derivative with a carboxyl group was synthesized using the succinic anhydride method. The new CLIN haptens were conjugated with carrier proteins to immunize mice for monoclonal antibodies (mAbs) production. Besides, a heterologous ELISA with a generic coating antigen (LIN-OVA) was performed to screen the hybridomas to choose the broad-selective mAbs, and the produced mAb could simultaneously recognize CLIN and LIN. Based the produced mAb, a sensitive and reliable heterologous ELISA was developed for detecting CLIN and LIN residues in bovine, chicken, porcine and fish muscles. In addition, a simple sample pretreatment procedure was optimized to eliminate the complicated food matrix effects, allowing the entire extraction procedure to be completed within 10 min. Finally, the developed ELISA was verified by a well-established LC — MS/MS method, and the results demonstrated that it could be a feasible tool for detecting CLIN and LIN residues.

2. Materials and methods

2.1. Chemicals and equipment

CLIN, LIN, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), ovalbumin (OVA) and Freund's incomplete and complete adjuvant were purchased from Sigma-Aldrich (St. Louis, MO, USA). Peroxidase-conjugated goat *anti*-mouse IgG was purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA). The optical density (OD) value was measured with a multi-mode microplate reader from BioTek Instruments Inc. (Winooski, VT, United States). The LC — MS/MS 1260-6410B was purchased from Agilent Inc. (San Diego, CA, USA) and was used for the final validation of the developed ELISA method.

2.2. Antigen synthesis

The CLIN derivative with a carboxyl group was synthesized using the succinic anhydride method (Wang et al., 2010a). The CLIN derivative was conjugated to KLH, BSA and OVA using an active ester method. Briefly, the CLIN derivative (0.05 μmol), NHS (0.15 μmol) and EDC (0.1 μmol) were dissolved in 500 μL of *N*, *N*-dimethylformamide, and the mixture was allowed to react at room temperature for 2 h. Then, the reaction mixture was added dropwise to 1 μmol of the carrier protein under magnetic stirring for 4 h at 25 °C. The hapten-protein conjugates were dialysed against distilled water and stored at −20 °C before use.

LIN was conjugated to KLH, BSA and OVA using the sodium periodate ($NaIO_4$) reduction method (Burkin & Galvidis, 2010) with some modifications. Briefly, LIN (10 μmol) and $NaIO_4$ (10 μmol) were combined in 1 mL of water and stirred for 20 min. Then, 500 μL of periodate oxidized LIN (50-fold molar excess of hapten over the carrier protein) was added to the protein solutions (0.1 μmol) in 0.05 M carbonate buffer. After 2 h of stirring, 0.1 mL of $NaBH_4$ (2 mg/mL) was added, and the reaction mixtures were incubated for another 2 h. The synthesized conjugates were dialysed against distilled water and stored at −20 °C before use.

2.3. MAb production

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee of Shenzhen University. Balb/c mice were subcutaneously immunized with a 100 μg/mouse dose of CLIN-KLH. For the first immunization, the artificial antigens were emulsified in Freund's complete adjuvant; subsequent injections were performed in Freund's incomplete adjuvant every two weeks. After the fifth immunization, the mouse exhibiting the highest antibody titre was sacrificed, and the spleens were used for the hybridoma production (Jiang, Beier, Wang, Wu, & Shen, 2013; Kohler & Milstein, 2005). The supernatant of the positive hybridoma was collected for mAb production.

2.4. Heterologous ELISA development

ELISA was conducted as follows: MaxiSorp immunoplates were coated with LIN-OVA in coating buffer (100 μL/well). After incubation at 37 °C for 2 h, the plates were washed with PBST. The plates were blocked for 1 h with 1% casein in PBS (150 μL/well) and then washed twice with PBST. After washing, 50 μL of the diluted standard solution and 50 μL of the diluted antibody were added, and the plates were incubated at 37 °C for another 30 min. After a subsequent washing step, 100 μL of the goat *anti*-mouse IgG-HRP was added, and the plates were incubated at 37 °C for 30 min. After washing, 100 μL of the substrate solution was added to each well, and the plates were incubated at 37 °C for 15 min. The reaction was terminated by adding 50 μL of the stopping solution, and the optical density (OD) value was measured using a microplate absorbance reader.

ELISA was optimized using CLIN as the analyte. The effect of organic solvents on assay performance was tested by preparing a standard solution using various percentages of methanol. To evaluate the effects of assay buffers, both analyte and mAb were diluted in PBS with varying pH values and NaCl concentrations. The maximum OD (OD_{max}) and IC_{50} was used as the primary criterion to evaluate the assay performance.

2.5. Sensitivity and specificity

The sensitivity of the ELISA was evaluated by IC_{50} values (50% inhibition concentration), and a lower IC_{50} value indicated a higher sensitivity. Sigmoid calibration standard curves were obtained by plotting the mean OD values against the logarithm of CLIN concentrations through a four-parameter logistic equation (Jiang et al., 2013):

$$Y = (A–B)/[1 + (X/\times_0)^P] + B.$$

where A = response at high asymptotes of the curve, B = response at low asymptotes of the curve, P = the slope at the inflection point of the sigmoid, $\times_0$ = the concentration of analyte resulting in 50% inhibition of tracer binding and X = the calibration concentration.