Immunochromatographic assays for ultrasensitive and high specific determination of enrofloxacin in milk, eggs, honey, and chicken meat

Xianlu Lei,^{1,2} Xinxin Xu,^{1,2} Liqiang Liu,^{1,2*} Hua Kuang,^{1,2} Liguang Xu,^{1,2} and Chuanlai Xu^{1,2*}

¹State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, 214121, People's Republic of China

²International Joint Research Laboratory for Biointerface and Biodetection, and School of Food Science and Technology, Jiangnan University, Wuxi, 214121, People's Republic of China

ABSTRACT

Enrofloxacin, a veterinary antibiotic that persists in food, poses a risk to human health. Here, a monoclonal antibody against enrofloxacin, 1H12, was prepared based on the hapten ENR-1, and showed excellent sensitivity with a 50% inhibitory concentration (IC_{50}) of 0.03 ng/mL. Using this antibody, 2 lateral-flow immunochromatographic assays were developed for determination of enrofloxacin in egg, milk, honey, and chicken meat samples. The detection ranges (IC_{20} – IC_{80}) were 0.16–0.82 ng/g, 0.24–1.8 ng/g, 0.25–3.6 ng/g, and 0.61–3.9 ng/g by colloidal gold-immunochromatographic sensor (CG-ICS) analysis, and 0.022–0.42 ng/g, 0.054–0.42 ng/g, 0.069–1.4 ng/g, and 0.19–2.2 ng/g by Eu-fluorescence-immunochromatographic sensor (EF-ICS) analysis. The intraassay and interassay recovery rates were 88.9 to 108.5% with coefficients of variation of 1.3 to 7.0% by CG-ICS analysis, and 88.6 to 113.6% with coefficients of variation of 1.3 to 8.1% by EF-ICS analysis. Thus, our newly developed ICS are sensitive and reliable, providing an option for rapid quantitative detection of enrofloxacin in food samples.

Key words: antibody, antibiotic, enrofloxacin, immunochromatographic, strip

INTRODUCTION

Enrofloxacin (ENR; Figure 1a) is an important member of the fluoroquinolones and possesses excellent antibacterial effects against gram-positive bacteria, gram-negative bacteria, and mycoplasma by inhibiting DNA-gyrase, which is an essential enzyme in prokaryotes (Zhang et al., 2011; Wang et al., 2014). It is widely used for treatment and prophylaxis of diseases, as well as growth promotion in the livestock and aquaculture industries (Chen et al., 2012; Tochi et al., 2016). Although ENR is relatively less toxic compared with

other antibiotics, drug residues in the bodies of animals caused by unreasonable use may pose a threat to human health via the food chain (Shen et al., 2019a). Some studies have revealed that long-term, low-dose exposure to ENR can induce bacterial resistance, which increases the risk of treatment failures of human diseases (Otero et al., 2016; Li et al., 2019). In addition, allergic reactions and changes in intestinal flora after eating foods containing ENR residues are also issues that cannot be ignored (Wang et al., 2017). Therefore, many countries and organizations have set a maximum residue limit (MRL) owing to the concerns related to ENR residue in animal products (Tochi et al., 2016). Ten years after approving the use of ENR as an antibiotic for poultry, the US Food and Drug Administration (FDA) withdrew this decision in 2005 (Wu et al., 2009). The European Commission has fixed MRL for the sum of ENR plus ciprofloxacin at 100 to 200 μ g/kg in chicken tissue, while Japan has fixed the limit at 10 μ g/kg in all chicken tissues (Pulgarin et al., 2011; Chen et al., 2012; Wang et al., 2014). The People's Republic of China set MRL at 100 to 300 μ g/kg in different animal tissues, and ENR is banned during egg production (Shen et al., 2019a).

The development of analytical technology is an important safeguard to strengthen the regulation of antibiotic residues in biological matrices. Instrumental analysis is currently the most widely used method for monitoring ENR residues due to its high sensitivity and accuracy, with methods including HPLC (Aufartova et al., 2017; Huang et al., 2018), liquid chromatography (LC)-MS (Lolo et al., 2005; Panzenhagen et al., 2016), and surface plasmon resonance (Fernandez et al., 2010). However, these methods not only require expensive equipment, professional skill, and complicated sample pretreatment, but are also low-throughput and time-consuming techniques (Meng and Xi, 2011). Consequently, it is difficult to meet the need for on-site screening of large numbers of samples using these approaches.

In recent years, immunoassays based on specific binding of antigens and antibodies has aroused great inter-

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*Corresponding authors: murel@163.com and xcl@jiangnan.edu.cn

est in the field of antibiotic residue detection due to the advantages of low cost, portability, high throughput, and time savings, with techniques such as ELISA (Kong et al., 2019), piezoelectric immunosensors (Karaseva and Ermolaeva, 2012), and fluorescence immunoassay (Miao et al., 2016). According to some reports, several immunoassay methods have also been developed for the determination of ENR in animal samples. For example, a highly sensitive and specific ELISA based on a heterologous coating antigen was established by Wang et al. (2014) at a 50% inhibitory concentration (IC_{50}) of 0.07 $\mu\text{g/L}$. Meanwhile, Sheng et al. (2017) reported 2 types of lateral-flow immunochromatographic assays using dyed polymer microspheres and quantum dots for rapid detection of ENR, with visual limits of detection of 1 $\mu\text{g/L}$, 5 $\mu\text{g/kg}$, and 10 $\mu\text{g/L}$ in buffer, animal tissue, and milk, respectively. In a study by Li et al. (2019), either carbon dots or quantum dots were used as donor probes in 2 fluorescence-quenching immunochromatographic assays to monitor ENR with visual limits of detection of 0.5 to 2.5 $\mu\text{g/kg}$ in animal-derived food samples. Other immunoassay methods including indirect competitive-ELISA (**ic-ELISA**; Huet et al., 2006; Hu et al., 2010, 2019; Kim et al., 2015), colloidal gold immunoassay (Zhao et al., 2008), biomimetic enzyme-linked immunosorbent assay (Wang et al., 2017), fluorescence polarization immunoassay (Shen et al., 2019a), and chemiluminescence enzyme immunoassay (Kim and Kim, 2009; Kim et al., 2013; Yu et al., 2014a,b) have also been reported for the determination of ENR. Although the above-mentioned methods are able to meet the qualitative and semiquantitative analysis requirements for some samples, there is still scope for a rapid quantitative analytical test for ENR, especially for egg samples with more sensitive detection requirements.

In this study, an ultrasensitive and highly specific monoclonal antibody against ENR was prepared using a new hapten. Then, immunoassays based on fluorescence and on gold nanoparticles (**GNP**) were developed for rapid quantitative determination of ENR in milk, eggs, honey, and chicken samples. The technologies

developed help simplify the pre-processing of milk and chicken samples and achieve a lower detection limit for quantitative analysis of egg and honey samples.

MATERIALS AND METHODS

Materials and Reagents

The ENR and other quinolone analogs were purchased from Yuanye Bio-Technology Co. Ltd., and 6-aminohexanoic acid was purchased from J&K Scientific Ltd. Horseradish peroxidase-labeled goat anti-mouse was supplied by Kangcheng Bioengineering. Sigma-Aldrich provided keyhole limpet hemocyanin, BSA, polyethylene glycol, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (**EDC**), *N*-hydroxysuccinimide, Freund's complete adjuvant, and Freund's incomplete adjuvant. Gibco BRL supplied cell culture reagents, including RPMI 1640 cell culture medium, fetal bovine serum, and media supplement. All other reagents were obtained from the National Pharmaceutical Group Chemical Reagent Co. Ltd.

Polyvinylchloride backboard, nitrocellulose (**NC**) high-flow-plus membranes (Pura-bind RP), CB-SB08 glass fiber membrane, and SX18 absorbance pad were purchased from Whatman-Xinhua Filter Paper Co. Ltd. An HM3035 strip-cutting instrument was supplied by Kinbio Tech Co., Ltd. Membrane strip readers were designed in our laboratory.

Synthesis and Characterization of Hapten and Antigen

Hapten ENR-1. The ENR-1 (Figure 1b) was synthesized by linking the carboxyl group at position 3 of ENR with the amino group on 6-aminocaproic acid, referring to Wang et al. (2014) and Zhou et al. (2020), with some modifications. In brief, 200 mg of ENR was added to oxalyl chloride (4 mL) and stirred for 2 h. Then, the solution was dried under nitrogen, the residue was re-dissolved in tetrahydrofuran (4 mL), and mixed with 250 mg of 6-aminocaproic acid overnight at room

Figure 1. The structures of enrofloxacin (ENR; a) and hapten ENR-1 (b).

temperature. After the reaction was terminated with 1 mL of H₂O, 15 mL of trichloromethane was used to extract the mixture 3 times. All trichloromethane was collected and evaporated under a stream of nitrogen, then the residue was purified by preparative thin layer chromatography on silica gel plates with an elution solvent of methanol/tetrahydrofuran (3/1) containing 3% acetic acid. Finally, the product, ENR-1, was characterized by LC-MS/MS.

ENR-1-BSA. The ENR-1 was connected to carrier BSA using the active ester method, referring to previous studies (Wang et al., 2018, 2020; Lei et al., 2020a). Briefly, 6 mg of ENR-1 and 4.6 mg of *N*-hydroxysuccinimide were dissolved in *N,N*-dimethyl formamide (DMF, 500 µL), and then 7.7 mg of EDC was added to the solution after an interval of 10 min. After stirring continuously for 4 h at room temperature, the activation solution was added dropwise to 15 mg of BSA dissolved in coating buffer solution (0.05 M, pH 9.6, 2 mL). The reaction was completed by stirring the mixture overnight at room temperature. The ENR-1-BSA was obtained by dialyzing the solution against PBS (0.01 M, pH 7.4) for 3 d and it was then stored at 4°C for future use. Finally, ENR-1-BSA was characterized by UV-visible spectroscopy.

Ofloracin-Ovalbumin. The coating antigen ofloxacin (OFL)-ovalbumin (OVA) was obtained by the mixed-anhydride method (Lei et al., 2020b). First, 7.3 mg of OFL was dissolved in 0.7 mL of anhydrous DMF and mixed with 5.7 µL of tributylamine for 15 min in an ice bath, and then 3.2 µL of isobutyl-chloroformate was added into the solution. After stirring continuously for 1 h in an ice bath, the mixture was dripped into 15 mg of OVA dissolved in 2 mL of coating buffer solution (0.05 M, pH 9.6). The reaction proceeded for 6 h in an ice bath. The OFL-OVA was obtained by dialyzing the solution against PBS (0.01 M, pH 7.4) for 3 d and stored at 4°C for future use. The conjugate OFL-OVA was identified by UV-visible spectroscopy.

Preparation and Characterization of the Anti-ENR Monoclonal Antibody

This animal study was approved by the Institutional Animal Care and Use Committee of Jiangnan University [IACUC Issue Number: JN. No 20201130b2000615(338)], and all procedures strictly abided by the relevant laws and regulations for experimental animal welfare and ethics. Female BALB/c mice (SPF, age 6–8 wk, weight 20–24 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. and kept in a barrier environment at the Experimental Animal Center of Jiangnan University. Two immunogens, including ENR-1-BSA and ENR-BSA, were each used to immu-

nize 5 mice by hypodermic injection in the neck and back (Peng et al., 2017; Guo et al., 2018; Lin et al., 2021). Mice were snatched softly and massaged for 2 min before immunization to reduce their tension and pain. The first immunization was executed with 80 µg of immunogen emulsified with Freund's complete adjuvant, and then followed by 4 booster immunizations with 40 µg of immunogen emulsified with incomplete adjuvant, at intervals of 21 d. From the second booster immunization, serum was collected from the tails of the mice on the seventh day after each immunization and tested by ic-ELISA. High affinity for the coating antigen and ENR standard was the main indicator to evaluate the immune response of the mice, and the mice with the best response were selected to be injected intraperitoneally with 20 µg of antigen and killed 3 d later for cell fusion. The other mice were killed with CO₂. For purpose of reducing the experimental interference, immunization and blood collection for all mice were performed at the same time, and serum was tested by another investigator who was unaware of the immunization procedure. The immune response of the mice was evaluated by the following formula: inhibition ratio = $[1 - OD_{450} (1 \text{ ng/mL}) / OD_{450} (0 \text{ ng/mL})] \times 100\%$, where OD₄₅₀ is the optical density at 450 nm.

Spleen cells prepared from the mice were mixed with Sp2/0 myeloma cells and fused using PEG 4000 purchased from Thermo Fisher Scientific Co. Ltd. (Zhou et al., 2021). A pure cell line that stably secreted anti-ENR antibody was selected through 3 rounds of subcloning, and then injected into the abdominal cavity of mice. One week later, ascites were prepared and purified using the saturated ammonium sulfate method, and the characteristics of the mAb were tested by ic-ELISA, as previously described (Fan et al., 2012; Li et al., 2018; Shen et al., 2019b).

Preparation of Immunochromatographic Strips

Colloidal Gold-Labeled mAb and Eu Fluorescence-Labeled mAb. Nanogold and fluorescent materials, with excellent optical properties, biocompatibility, and long-term stability, are widely used as probes for immunoassays in various fields (Han et al., 2018; Fang et al., 2020). Colloidal gold nanoparticles (GNP) with a diameter of approximately 15 nm were synthesized as described in a previous study (Zhang et al., 2015; Lei et al., 2020a; Suryoprabowo et al., 2021), and Eu-fluorescent microspheres (FMP) with a diameter of approximately 210 nm were purchased from Microdetection Company. The GNP and FMP were bound to the mAb through electrostatic adsorption (Zeng et al., 2020) and a condensation reaction between the carboxyl and amino groups (Natarajan et

al., 2019; Suryoprabowo et al., 2021; Zhou et al., 2021), respectively. Briefly, 6 mL of GNP was transferred into a 15-mL centrifuge tube and the pH was adjusted to 8.0 with 0.2 M K_2CO_3 , and then 48 μ g of mAb diluted to 0.2 mg/mL with 0.05 M HEPES was added to the solution and mixed. After standing for 45 min at room temperature, 300 μ L of 10% BSA solution was added and allowed to stand for another 2 h to block the surface of the GNP where no mAb was bound. Finally, the mixture was centrifuged at $7,200 \times g$ ($4^\circ C$) for 45 min, then the pellet was re-dissolved in 600 μ L of resuspension buffer (0.02 M PBS, containing 0.5% PEG 6000 purchased from Thermo Fisher Scientific Co. Ltd., 5% sucrose, 0.1% Tween, and 0.02% NaN_3 , pH 7.4) and stored at $4^\circ C$ for further use.

The process of labeling antibodies with FMP was as follows: 100 μ L of FMP was mixed with 400 μ L of borate buffer solution (BBS; 0.05 M, pH 8.0) in a 2-mL centrifuge tube. Then, 30 μ L of EDC (10 mg/mL) was added to the solution and the mixture was shaken continuously at room temperature for 15 min. After centrifuging at $18,900 \times g$ ($4^\circ C$) for 10 min, the sediment was re-dissolved in BBS (0.5 mL, 0.05 M, pH 8.0) and sonicated for 1 min at 100 W. Next, 70 μ g of mAb was added into the solution and shaken for 2 h at $20^\circ C$, then 55 μ L of 10% BSA was added and shaken

for another 2 h at $20^\circ C$. The solution was then centrifuged at $18,900 \times g$ ($4^\circ C$) for 10 min and washed twice with BBS (0.5 mL, 0.05 M, pH 8.0). Finally, the pellet was re-dissolved with stock solution (0.05 M BBS, 5% Tween-20, 2% BSA) and stored at $4^\circ C$ for further use.

Immunochromatographic Sensor Assembly.

The immunochromatographic sensor (ICS) was fabricated as previously reported (Lei et al., 2020a) and consisted of 5 parts (Figure 2a): support plate, absorbent pad, NC membrane, conjugate pad, and sample pad. Before assembly, the coating antigen and goat anti-mouse antibody were separately used to create a control line (C line) and a test line (T line) in the central area of the NC membrane with an interval of 5 mm. The conjugate pad was sprayed with colloidal gold (CG)-labeled mAb or Eu fluorescence (EF)-labeled mAb, and dried at $37^\circ C$ overnight. The NC membrane was attached to the middle of the support plate, and other components were layered as shown in Figure 2(a). Finally, the board was cut into 4-mm strips and loaded into the card case.

Principle and Performance of the ICS

The ICS was developed as previously described (Lei et al., 2020a). Briefly, the coated antigen and the

Figure 2. Schematic diagram (a) and illustration (b) of the immunochromatographic sensors [colloidal gold-immunochromatographic sensor (CG-ICS) and Eu-fluorescent-immunochromatographic sensor (EF-ICS)]. ENR = enrofloxacin; NC = nitrocellulose; S = sample hole.

target analyte competitively bind to the specific mAb labeled with GNP or FMP. Figure 2(b) shows roughly the reaction process of the ICS: the sample solution is dropped into the sample hole and flows horizontally toward the absorbent pad under capillary action; the labeled mAb on the conjugate pad is dissolved in the solution and specifically binds to the antigen. After a complete reaction, any remaining mAb will be captured by the coated antigen on the T line to create color, whereas both the antigen-antibody conjugate and the mAb can bind to the goat anti-mouse antibody on the C line to confirm the effectiveness of the ICS. The color intensity of both the T line (T value) and the C line (C value) are closely related to the amount of labeled mAb bound and can be measured with a handheld reader. Quantitative analysis of the drug is performed based on the relationship between the antigen concentration and the color intensity of the T line and the C line.

The sensitivity of the ICS was tested and evaluated as previously reported (Shen et al., 2019b; Lei et al., 2020a). An ENR standard stock solution was prepared by dissolving 1 mg of ENR in 10 mL of DMF, and this was then diluted to form a series of gradient standard solutions. When 100 μ L of standard solution was dripped into the sample hole of the ICS and incubated at 37°C for 6 min, the resulting colors of the T line and C line could be observed by the naked eye and detected by a handheld reader. For qualitative analysis, the visual colorimetric value (**vCMV**) was defined as the minimum concentration causing a distinct visible color difference compared with the negative group. The visual cut-off value was defined as the minimum concentration causing a colorless T line. For quantitative analysis, the T/C value was defined as the ratio of color intensity value on the T line to that on the C line, and a calibration curve was plotted by fitting the T/C value against the concentration of ENR under the logistic mode. The sensitivity of the ICS was evaluated by analyzing the IC_{50} of the negative sample signal and the quantitative range (IC_{20} – IC_{80}).

Sample Analysis

Four negative samples of milk, honey, egg, and chicken meat were obtained from the Entry-Exit Inspection and Quarantine Bureau of Jiangsu Province. These were added to ENR standards at different concentrations and used to analyze the actual detection ability of the strips. Based on the consideration of simplified procedures and shortened detection time, the milk and honey samples were directly used for analysis, either undiluted or after being diluted 5 times, respectively, whereas the chicken sample was extracted with a 4-fold volume of PBS (1:4, g/mL) by rotating for 5 min fol-

lowed by centrifuging at $7,200 \times g$ (4°C) for 5 min. Egg samples were extracted with acetonitrile for the purpose of reducing matrix interference and improving detection sensitivity. Briefly, the sample was extracted with a 3-fold volume of acetonitrile (1:3, g/mL) by rotating for 5 min, and then centrifuged for 5 min at $7,200 \times g$ (4°C). Then, the upper organic solution was collected and dried under nitrogen flow at 50°C. The residue was re-dissolved with the same amount of PBS as the sample and used for further analysis.

RESULTS AND DISCUSSION

Characterization of the Hapten and Antigen

Hapten ENR-1. In this study, the carboxy-terminal arm of ENR (molecular weight 359.4; Figure 1a) was extended by linking to 6-aminohexanoic acid with the purpose of better exposing the characteristic groups. The result of ENR-1 identified by LC-MS/MS is shown in Figure 3. A selective ion chromatogram of m/z 473.25 was generated, and the corresponding product ion spectra at a retention time of 3.71 min revealed the MS/MS spectrum of target haptens. Therefore, ENR-1 was synthesized successfully.

Antigen ENR-1-BSA and OFL-OVA. Small molecules (<5,000 Da) cannot be used directly for antibody preparation and immunoassay due to their weak immunogenicity. In this study, ENR-1 and OFL were coupled to BSA and OVA to prepare the immunogen and coating antigen, respectively. Figure 4(a) shows the absorption peaks of ENR-1 and BSA at 282 and 279 nm, respectively, whereas ENR-1-BSA appeared as a wider peak than BSA at 279 nm and showed the same linear trend as ENR-1 from 282 to 500 nm by the influence of ENR-1 groups bound to the BSA. Figure 4(b) shows that the absorption peaks of OFL appeared at 285 and 332 nm, whereas OVA appeared at 278 nm. The absorption curve of OFL-OVA obviously differed from OVA and appeared to have the characteristic absorption of OFL groups. Therefore, the immunogen ENR-1-BSA and the coating antigen OFL-OVA were prepared successfully.

Characterization of the mAb

High-quality monoclonal antibodies are critical to the development of immunoassay methods, and ic-ELISA was used to select and evaluate antibodies in this study. The immune effects based on different immunogens (ENR-1-BSA and ENR-BSA) and coating antigens (ENR-1-OVA, ENR-OVA, PEF-OVA, and OFL-OVA) were tested. The results (Supplemental Table S1, <https://doi.org/10.6084/m9.figshare.14823606>; Lei, 2021)

revealed that ENR-1-BSA (#2) and OFL-OVA demonstrated the best IC_{50} in mouse serum, whereas there were small differences in immune response among mice immunized with same antigens. The monoclonal cell line 1H12 was prepared by cell fusion and subcloning, and Figure 5(a) shows that the mAb produced by the cell line 1H12 belonged to the IgG1 subtype. A standard curve was created based on 0.1 $\mu\text{g/mL}$ of OFL-OVA and 0.05 $\mu\text{g/mL}$ of the mAb (Figure 5b), with the corresponding regression equation $y = 0.0992 + 1.55/[1 + (x/0.0303)^{1.22}]$. The IC_{50} and linear range were 0.0303 ng/mL and 0.00969–0.0945 ng/mL (IC_{20} to IC_{80}), respectively. The specificity of the mAb was evaluated by testing 20 quinolones (Supplemental Table S2, <https://doi.org/10.6084/m9.figshare.14823606>; Lei, 2021), and cross-reactivity (CR) analysis showed that the mAb was barely able to recognize other quinolones (CR <0.01%) except for ofloxacin (0.13%), pefloxacin (0.037%), and ciprofloxacin (0.014%). Therefore, mAb 1H12 showed high sensitivity and specificity for ENR. Compared with the current best polyclonal antibody prepared by Wang et al. (2014), our mAb 1H12 not only achieved slightly higher sensitivity but also can be produced permanently.

Sensitivity of the ICS

The size of colloidal GNP are usually chosen from 10 to 60 nm in immunoassays. Although large-sized GNP can produce a deep color that helps to improve the sensitivity of the detection method to some degree, they show poor uniform shape and broad size distribution that affect the stability of the detection method (Cui et al., 2015). In this study, the colloidal GNP with a diameter of 15 nm was synthesized and applied based on the consideration of the excellent stability of the ICS. The GNP and purchased FMP were characterized by high-resolution transmission electron microscopy, and both of them had good uniformity and dispersion as shown in Supplemental Figure S1 (<https://doi.org/10.6084/m9.figshare.14823606>; Lei, 2021).

The CG-ICS was prepared using 0.3 mg/mL of goat anti-mouse antibody on the C line and 0.02 mg/mL of the antigen OFL-OVA on the T line, whereas the EF-ICS was prepared using 0.3 mg/mL of goat anti-mouse antibody and 0.05 mg/mL of antigen OFL-OVA. The sensitivity of both ICS was tested based on a concentration series of ENR standards (0, 0.078, 0.16, 0.31, 0.63, 1.3, 2.5, and 5.0 ng/mL for CG-ICS, and 0, 0.031,

Figure 3. The liquid chromatography (LC)-MS/MS spectrum of enrofloxacin (ENR)-1.

0.063, 0.13, 0.25, 0.5, 1.0, and 2.0 ng/mL for EF-ICS) dissolved into 0.05 M PBS (3% ON-870). As shown in Figure 6(a) and Figure 7(a), the color intensity on the T line faded away with the increase in the concentration of the ENR standard solution added into the sample hole, and disappeared completely at 2.5 ng/mL (CG-ICS) and 1 ng/mL (EF-ICS). The vCMV value was 0.16 ng/mL for CG-ICS and 0.063 ng/mL for EF-ICS. The color intensity value of the T line and C line was read, and the corresponding calibration curves for ENR were constructed by plotting T/C (y-axis) against the concentration of ENR (x-axis). The IC_{50} was 0.17 ng/mL by CG-ICS and 0.045 ng/mL by EF-ICS, and the detection range (IC_{20} – IC_{80}) was 0.057–0.48 ng/mL and 0.013–0.16 ng/mL, respectively. In previous studies, Kim and Kim (2009) designed an immuno-strip biosensor to detect ENR in a range of 100 to 10,000 ng/

Figure 4. The UV-visible absorption spectra of immunogen ENR-1-BSA (a) and coating antigen OFL-OVA (b). OD = optical density; ENR-1 = enrofloxacin hapten; OFL = ofloxacin; OVA = ovalbumin.

Figure 5. Characterization of the mAb 1H12 by indirect competitive-ELISA: isotype determination (a) and standard inhibition curve (b) for enrofloxacin (ENR). OD_{450nm} = optical density at 450 nm.

mL. An ENR immunosensor based on electrochemistry was developed by Wu et al. (2009) with a detection limit of 1 ng/mL. Two fluorescence-quenching immunoassay strips for the detection of ENR were prepared by Li et al. (2019), with visual cut-off values (limit of detection) of 0.1 and 0.25 ng/mL in standard solutions. Compared with these immunoassay assays, our 2 ICS, especially for EF-ICS, had a significant improvement in sensitivity. As we all know, high-sensitivity strips are very helpful for simplifying pre-processing when used in real samples.

Determination of ENR in Samples

Four negative samples (milk, honey, egg, and chicken) were selected to evaluate the detection ability of the strips in actual samples. The samples were spiked with

Figure 6. Test pictures of the colloidal gold-immunochromatographic sensor (CG-ICS) and corresponding calibration curves for determination of enrofloxacin (ENR) in PBS (a), eggs (b), honey (c), chicken meat (d), and milk (e). T/C value = the ratio of color intensity value on the test line to that on the control line. Error bars represent the SD of 3 repetitions.

Figure 7. Test pictures of the Eu-fluorescent-immunochromatographic sensor (EF-ICS) and corresponding calibration curves for determination of enrofloxacin (ENR) in PBS (a), eggs (b), honey (c), chicken meat (d), and milk (e). T/C value = the ratio of color intensity value on the test line to that on the control line. The red line near the upper part of the strip window is the control line, and the lower red line is the test line. Error bars represent the SD of 3 repetitions.

Table 1. Analysis of enrofloxacin in eggs, milk, chicken, and honey using a colloidal gold-immunochromatographic sensor (CG-ICS) and an Eu-fluorescent-immunochromatographic sensor (EF-ICS; n = 6)

Sample	GC-ICS					EF-ICS				
	Recovery rate ¹ (intra-assay)			Recovery rate (interassay)		Recovery rate (intra-assay)			Recovery rate (interassay)	
	Spiked (µg/kg)	Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)	Spiked (µg/kg)	Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)
Eggs	0.25	105.1 ± 2.6	2.5	103.3 ± 3.0	2.9	0.05	105.8 ± 4.5	4.3	102.5 ± 4.7	4.6
	0.5	93.7 ± 1.2	1.3	94.6 ± 1.6	1.7	0.1	98.5 ± 2.7	2.7	96.4 ± 3.9	4.0
	1	98.9 ± 3.2	3.2	95.1 ± 6.3	6.6	0.2	90.6 ± 2.9	3.2	91.3 ± 3.3	3.6
Milk	0.25	108.5 ± 5.4	5.0	105.4 ± 5.6	5.3	0.05	102.0 ± 4.2	4.1	107.5 ± 4.6	4.3
	0.5	94.6 ± 2.2	2.3	95.2 ± 4.3	4.5	0.1	93.5 ± 5.8	6.2	95.1 ± 7.7	8.1
	1	97.3 ± 3.0	3.1	95.4 ± 4.5	4.7	0.2	98.3 ± 1.3	1.3	98.1 ± 1.9	1.9
Chicken	0.75	98.0 ± 5.3	5.4	96.1 ± 6.7	7.0	0.25	99.1 ± 2.8	2.8	98.5 ± 3.2	3.2
	1.5	88.9 ± 2.6	2.9	92.2 ± 2.8	3.0	0.5	92.8 ± 4.1	4.4	88.6 ± 4.7	5.3
	3	102.2 ± 4.8	4.7	98.9 ± 4.1	4.1	1	110.2 ± 7.4	6.7	113.6 ± 8.1	7.1
Honey	0.75	95.4 ± 3.1	3.2	95.8 ± 3.5	3.7	0.25	103.6 ± 4.8	4.6	100.2 ± 5.2	5.2
	1.5	93.7 ± 1.9	2.0	90.1 ± 2.7	3.0	0.5	95.8 ± 4.1	4.3	93.1 ± 4.9	5.3
	3	99.6 ± 3.6	3.6	96.6 ± 5.9	6.1	1	98.4 ± 2.9	2.9	96.6 ± 2.5	2.6

¹Recovery rate = (detection concentration/spiked concentration) × 100%.

different amounts of ENR standard solution (0.1 mg/mL) before pretreatment. The concentrations of ENR added into egg and milk were 0, 0.078, 0.16, 0.31, 0.63, 1.3, 2.5, and 5.0 ng/g when used for CG-ICS analysis, and 0, 0.031, 0.063, 0.13, 0.25, 0.50, 1.0, and 2.0 ng/g for EF-ICS analysis. The concentrations of ENR added into honey and chicken were 0, 0.31, 0.63, 1.3, 2.5, 5.0, 10.0, and 20.0 ng/g for CG-ICS analysis, and 0, 0.13, 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 ng/g for EF-ICS analysis. Three replicates were created for each sample.

The test pictures of the strips and corresponding calibration curves for CG-ICS and EF-ICS are shown in Figure 6 and Figure 7, respectively, and the performance of the strips in milk, honey, egg and chicken samples was as follows. For qualitative analysis, the results showed that the vCMV from CG-ICS analysis was 0.31 ng/g for egg, milk, and honey samples, and 1.3 ng/g for chicken. The vCMV from EF-ICS analysis was 0.13 ng/g for egg, milk, and chicken samples, and 0.50 ng/mL for honey. The visual cut-off values in egg, milk, honey, and chicken were 2.5, 2.5, 10, and 20 ng/g, respectively, for CG-ICS analysis, and 0.50, 1.0, 8.0, and 4.0 ng/g, respectively, for EF-ICS analysis. All these results indicate that the 2 developed methods can make a good qualitative judgment on samples when read by the naked eye. For quantitative analysis, the results of CG-ICS analysis of egg, milk, honey, and chicken samples produced IC₅₀ values of 0.36, 0.66, 0.95, and 1.6 ng/g, respectively, and the detection ranges were 0.16–0.82, 0.24–1.8, 0.25–3.6, and 0.61–3.9 ng/g. The result of EF-ICS analysis of egg, milk, honey, and chicken samples produced IC₅₀ values of 0.095, 0.15, 0.31, and 0.65 ng/g, respectively, and the detection ranges were 0.022–0.42, 0.054–0.42, 0.069–1.4, and 0.19–2.2 ng/g.

The sensitivity of our ICS was affected by complex sample matrices that interfere with the combination of analytes and antibodies. Egg extracted with acetonitrile have the least effect on sensitivity of ICS according to IC₅₀ values, followed by milk, honey, and chicken samples with sample pretreatments. Two fluorescence-quenching immunoassay strips prepared by Li et al. (2019) show the cut-off values (limit of detection) were 0.5 to 2.5 ng/mL in animal-derived food samples (crucian, perch, pork, beef, chicken, and milk). Zhao et al. (2008) developed a lateral-flow colloidal gold immunoassay strip for the detection of ENR with the IC₅₀ of 0.935 ng/mL in the standard samples. Compared with the above studies and other immunoassay methods, our 2 methods demonstrated excellent detection sensitivity, which will be able to meet the needs of market detection.

The recovery test was performed on 4 samples spiked with 3 different concentrations of ENR, and the results are shown in Table 1. Six replicates were analyzed for

each sample. The recovery rate in 4 samples using CG-ICS was 88.9 to 108.5% with coefficients of variation (CV) of 1.3 to 5.4% for intraassay variation, and 90.1 to 105.4% with CV of 1.7 to 7.0% for interassay variation. Meanwhile, the recovery rates using EF-ICS were 90.6 to 110.2% with CV of 1.3 to 6.7% for intraassay, and 88.6 to 113.6% with CV of 1.9 to 8.1% for interassay. Therefore, the developed CG-ICS and EF-ICS were reliable and sensitive for the determination of ENR in real samples.

CONCLUSIONS

In this study, we prepared a highly specific and sensitive mAb, 1H12, against ENR based on the hapten ENR-1, and OFL-OVA was selected as the perfect coating antigen with an IC_{50} of 0.03 ng/mL in ic-ELISA. Then, 2 lateral-flow immunochromatographic strips, CG-ICS and EF-ICS, were developed for the determination of ENR in milk, honey, egg, and chicken meat samples. Calibration curves were plotted by fitting the T/C values against the concentration of ENR, and the results showed that both ICS achieved low detection limits with simple sample pretreatment. Furthermore, good stability and reliability were demonstrated by testing the recovery rate of ENR from ENR-spiked samples. All in all, the developed CG-ICS and EF-ICS exhibited the advantages of simplicity, time savings, sensitivity, and reliability, and they have potential for the rapid on-site screening of ENR in large-scale samples.

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ORCIDS

Chuanlai Xu <https://orcid.org/0000-0002-5639-7102>