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Near-infrared fluorescence-based multiplex lateral flow immunoassay for the simultaneous detection of four antibiotic residue families in milk

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ABSTRACT

In this study, we developed a novel near-infrared fluorescence based multiplex lateral flow immunoassay by conjugating a near-infrared label to broad-specificity monoclonal antibody/receptor as detection complexes. Different antigens were dispensed onto separate test zones of nitrocellulose membrane to serve as capture reagents. This assay format allowed the simultaneous detection of four families of antibiotics (β-lactams, tetracyclines, quinolones and sulfonamides) in milk within 20 min. Qualitative and quantitative analysis of target antibiotics were realized by imaging the fluorescence intensity of the near-infrared label captured on respective test lines. For qualitative analysis, the cut-off values of β-lactams, tetracyclines, quinolones and sulfonamides were determined to be 8 ng/mL, 2 ng/mL, 4 ng/mL and 8 ng/mL respectively, which were much lower than the conventional gold nanoparticle based lateral flow immunoassay. For quantitative analysis, the detection ranges were 0.26–3.56 ng/mL for β-lactams, 0.04–0.98 ng/mL for tetracyclines, 0.08–2.0 ng/mL for quinolones, and 0.1–3.98 ng/mL for sulfonamides, with linear correlation coefficients higher than 0.97. The mean spiked recoveries ranged from 93.7% to 108.2% with coefficient of variations less than 16.3%. These results demonstrated that this novel immunoassay is a promising approach for rapidly screening the four families of antibiotic residues in milk.

1. Introduction

Antibiotics are widely used for disease prevention and treatment as well as growth promotion in animal husbandry (Van Boeckel et al., 2015). Unfortunately, the use of antibiotics, especially if not used according to label directions, can result in drug residues in animal-origin food such as milk. β-lactams (BLs), tetracycline (TCs), quinolones (QNs) and sulfonamides (SAs) are the most often used antibiotics in dairy cattle management (Adrian et al., 2009; Song et al., 2015). The residue of these antibiotics in milk may cause allergic reactions in susceptible individuals, result in the rise of drug-resistant bacteria and generate other toxicity in humans (Marshall and Levy, 2011; Conzuelo et al., 2013). To protect the health of consumers, maximum residue limits (MRLs) have been set for these antibiotics in milk (European Commission, 2010).

In an effort to protect consumers, a number of analytical methods have been developed to monitor antibiotic residues in milk. These methods can be broadly categorized as microbiological assays, instrumental analyses and immunoassays (Stolker et al., 2007; Blasco et al., 2007; Cháfer-Pericás et al., 2010). Microbiological assays are time-consuming and have relatively poor sensitivity and specificity (Myllyniemi et al., 2002; Virolainen et al., 2008). Instrumental methods are typically represented by liquid chromatography (LC) (Stolker and Brinkman, 2005) and LC coupled with tandem mass spectrometry (LC-MS/MS) (O'Mahony et al., 2013). These approaches require expensive equipment and professional expertize, and may take hours or days to yield results, which limit their application in the common laboratory or field environment. The requirement for timely monitoring antibiotic residue in dairy industry has demanded the development of more rapid and cost-effective methods. Therefore, various immunoassays, including enzyme immunoassay, lateral flow

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immunoassay (LFA), and other novel immune-sensors have been developed for the detection of various antibiotic residues in milk (Cháfer-Pericás et al., 2010).

Among these immunoassays, LFA is the most rapid, simplest and cost-effective method. Most LFAs employ colloidal gold nanoparticle (GNP) as reporters for colorimetric detection, which can realize qualitative or quantitative analysis of target chemicals (Cháfer-Pericás et al., 2010). However, some problems remain to be resolved for GNP-based LFA. For example, some colored samples can interfere with the results from lateral flow strips and the test sensitivity needs further improvement (Wang et al., 2011; Goryacheva et al., 2013). As an alternative to GNP, fluorescence labels such as quantum dots (Wang et al., 2011), fluorescence nanosilica (Song et al., 2013) and fluorescence beads (Wang et al., 2015) have been widely used in LFA. Since the excitation and emission wavelengths of most fluorescence labels are in the ultraviolet–visible (UV–vis) wavelength range, the membrane support, biological components and plastics can produce high background due to light scattering and auto-fluorescence (Swanson and D'Andrea, 2013). Thus, the advantage of intense signal intensity contributed by these fluorescence labels is compromised by their high fluorescence background (Cháfer-Pericás et al., 2010; Wang et al., 2011; Song et al., 2013; Wang et al., 2015). Compared to fluorescence labels in UV–vis spectral region, fluorophores with excitation and emission wavelength in the near-infrared (NIR) region are desirable in many applications (Iizumi et al., 2013; Guo et al., 2014). At the NIR wavelength range, both light scattering and auto-fluorescence are dramatically reduced. As a result, NIR fluorescence imaging provides a better signal-to-background ratio and higher sensitivity (Gong et al., 2012; Guo et al., 2014). In recent years, several NIR-based immunoassays have been developed for the detection of human epidermal growth factor (Gong et al., 2012), fetoprotein antigen (Liang et al., 2012), protein G (Iizumi et al., 2013), interleukin and C-reactive protein (Swanson and D'Andrea, 2013). However, few reports are available for applying NIR fluorescence label in LFA for the detection of small molecules. In this study, we introduced a NIR fluorescence-based LFA for the simultaneous determination of BLs, TCs, QNs and SAs in milk. To the best of our knowledge, this is the first study to report an NIR-based immunoassay for antibiotic residue in animal-origin food.

2. Materials and methods

2.1. Chemicals and equipment

Chemical standards of cefquinome (CQN), tetracycline (TC), enrofloxacin (ENR), sulfadimidine (SMD), bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, USA). Other chemical standards of BLs, TCs, QNs, and SAs were purchased from Sigma Aldrich (St. Louis, USA) or Dr. Ehrenstorfer GmBH (Augsburg, Germany). Goat anti-mouse IgG and Tween 20 were obtained from Thermo Fisher Scientific (West Palm Beach, USA). IRDye® 800CW *N*-hydroxysuccinimide (NHS) ester was purchased from LI-COR Biosciences (Lincoln, USA). Other chemical reagents were purchased from Beijing Regent Corporation (Beijing, China). Nitrocellulose membrane HF180 was obtained from Millipore Inc. (Billerica, USA). Absorbent pad CH 37 and adhesive backing card were purchased from Shanghai GoldBio Co. Ltd. (Shanghai, China). Anti-BL receptor, anti-TC monoclonal antibody (mAb), anti-SA mAb, anti-QN mAb and pretreated sample pad (GF2-II) were provided by Beijing ZKHR Biotechnology Inc. (Beijing, China). Coating antigen SA-BSA was provided by Dalian Bioscizone Biotek Co., Ltd. (Dalian, China). Ampicillin-BSA, tetracycline-BSA and norfloxacin-BSA were synthesized in our laboratory (Supplementary material). HM 3030 Dispensing Platform and ZQ 2000 Guillotine Cutting Module used for strip assembly were purchased from Shanghai GoldBio Co. Ltd. (Shanghai, China). Odyssey infrared imaging system was purchased from LI-COR Biosciences (Lincoln, USA).

2.2. Preparation of detection reagent

The detection reagent consisted of one NIR dye-receptor (anti-BL) and three NIR dye-mAbs (anti-TC, anti-QN and anti-SA). The preparation protocol is described as follows. Briefly, 1 mg of the NHS ester of 800CW dye was dissolved in 1 mL dimethyl sulphoxide. Fifty microliter of the 1 mg/mL NIR dye solution was respectively mixed with 0.5 mL of 1 mg/mL each mAb (anti-TC, anti-QN and anti-SA) in 0.1 M phosphate buffer (pH 7.5). For anti-BL receptor, 100 μL of 1 mg/mL NHS-800CW solution was mixed with 0.5 mL of 1 mg/mL receptor in 0.1 M phosphate buffer (pH 7.5). All the mixtures were then incubated for 2 h at room temperature in the dark. Excessive unreacted 800CW dye was removed by Hitrap™ desalting column (GE healthcare Bio-Sciences Corp, NJ, USA). The purified 800CW dye-mAb/receptor conjugates were then diluted to 0.2 μg/mL with 0.01 M phosphate buffer saline (PBS) containing 1% BSA, 3% sucrose and 0.5% Triton-100. Next, the four diluted 800CW dye-mAb/receptor conjugates were mixed at a ratio of 1.5:4:3:5, and 60 μL per well of the mixture was added into a micro-well plate. Finally, the solution in the micro-wells was freeze-dried and the lyophilized detection reagent was ready for use.

2.3. Immobilization of capture reagent

The goat anti-mouse polyclonal antibody (pAb, 0.6 mg/mL) and rabbit anti-receptor pAb (0.8 mg/mL) were equally mixed in 0.01 M PBS (pH 7.4) and applied onto nitrocellulose membrane as the control line. The coating antigens, ampicillin-BSA (0.6 mg/mL), tetracycline-BSA (1.0 mg/mL), norfloxacin-BSA (0.8 mg/mL) and SA-BSA (0.8 mg/mL) were then dispensed onto nitrocellulose membrane as test lines (Fig. 1). The dispensed volumes were 1 μL/cm line. Finally, the nitrocellulose membrane was dried at 37 °C for 1 h and stored under dry conditions at 4 °C until use.

2.4. Assembly of strip

The NIR lateral flow strip had four components: sample pad, nitrocellulose membrane, absorbent pad and adhesive backing card (Fig. 1). The strip assembly procedure referred to the normal protocol in our laboratory (Chen et al., 2008). Briefly, the nitrocellulose membrane (Millipore HF 180) lined with coating antigens was pasted onto the center of plastic backing card; the pretreated sample pad was pasted with one end partly covering (2 mm) the nitrocellulose membrane. Next, the absorbent pad (CH 37) was pasted onto the other side of the backing card and it also laid-over (2 mm) the nitrocellulose membrane. Finally, the assembled plate was cut to 4 mm width for each strip and stored under dry conditions at 4 °C until use.

2.5. Test procedure

A micro-well format of LFA was performed. Briefly, milk was diluted 2-fold with 0.02 M PBS (pH 7.4) containing 0.05% Tween 20 (Supplementary material). Then, 200 μL of the diluted sample was added into microplate well with pre-coated detection reagent, and the sample solution dissolved the detection reagent in the well. After incubation for 5 min, the lateral flow strip was immersed into the sample well, and the solution would flow towards the absorbent pad. After 10 min, the lateral flow strips were imaged by an Odyssey Li-COR Imaging system at the 800 nm channel. The

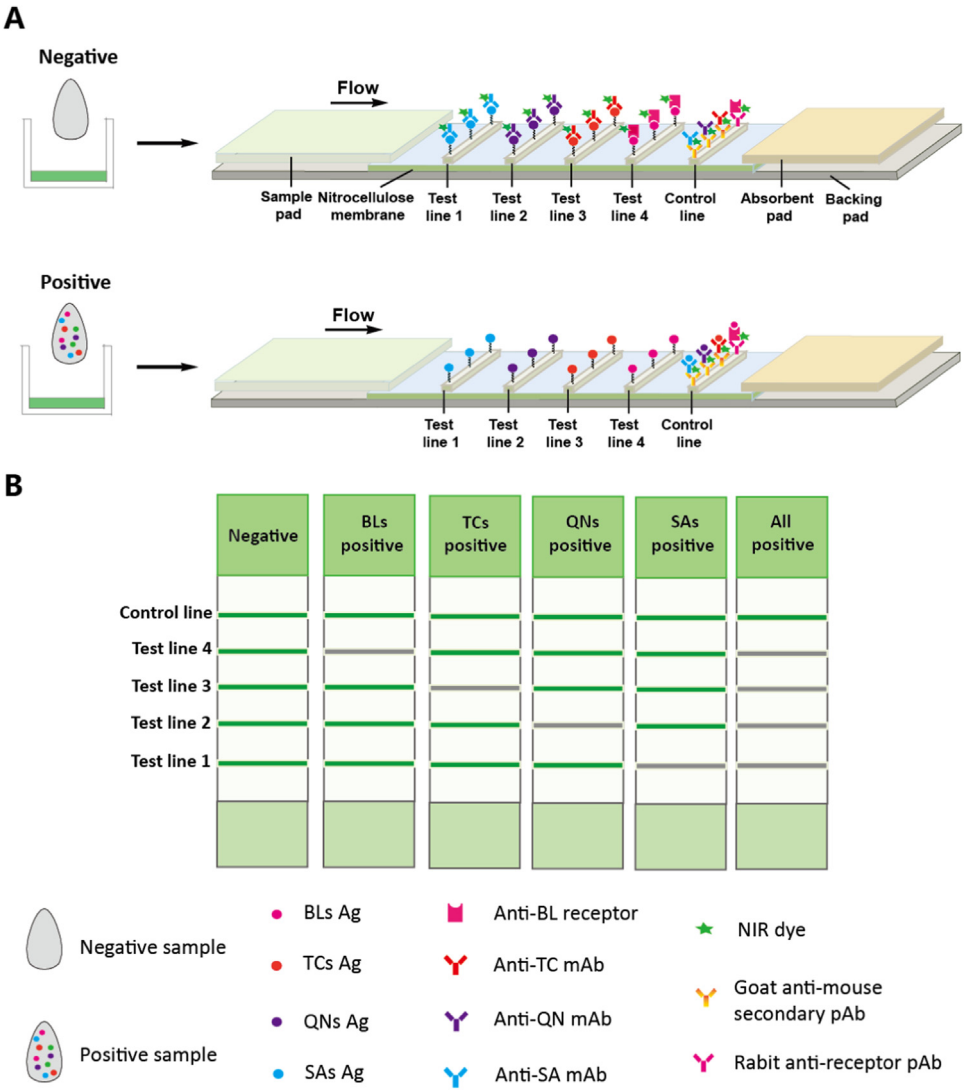

Fig. 1. Schematic illustration of multiplex NIR-based LFA.

focus offset was set at 3.9-mm and the resolution was set at 100-μm. The fluorescence intensity of each test line and control line was analyzed by Image J software (NIH, USA).

3. Results and discussion

3.1. Assay principle of NIR-based LFA and result judgment

The assay principle of multiplex NIR-based LFA is shown in Fig. 1. Four different antigens were coated onto separate locations of nitrocellulose membrane as capture reagents. Four different mAbs and receptor were respectively conjugated with NIR dye as detection reagents. If the milk sample was free of antibiotic residue, specific NIR dye-antibody/receptor conjugate would bind with respective coating antigen on nitrocellulose membrane to form a visible green test line. However, if one of the antibiotics were present in the milk sample, the antibiotic residue would compete with the immobilized capture antigen for the limited amount of NIR dye-antibody/receptor conjugate. Thus less NIR dye-antibody/receptor would be captured by the corresponding coating antigen on nitrocellulose membrane, and the fluorescence intensity of respective test line would be diminished. If there was sufficient antibiotic residue, they would completely hinder detection reagents from combining with respective capture reagents, thus a positive sample would have no visible test line on the corresponding position of the nitrocellulose membrane. If the test procedure was properly performed, the control line would always be visible. For qualitative analysis, the fluorescence image was visually inspected for result judgment, while for quantitative analysis, calibration curves were constructed by plotting the ratios between fluorescence intensity of spiked and blank sample (B/B_0) against the logarithm concentrations of antibiotic residues. The fluorescence intensity of unknown sample was then compared to respective calibration curve to determine analyte concentration.

3.2. Specificity of mAbs and receptor

The specificities of the three mAbs and one receptor were evaluated by concentration of 50% inhibition (IC_{50}) and cross-reactivity. The IC_{50} values of all the four families of antibiotics were determined by competitive indirect ELISA and the cross-reactivity values of each receptor/mAb were calculated (Supplementary material). As shown in Table S1, the receptor and mAbs could separately recognize 14 BLs, 4 TCs, 11 QNs and 12 SAs, with corresponding IC_{50} values ranging from 0.31 to 8.23 ng/mL for BLs, 0.68–0.99 ng/mL for TCs, 0.41–2.09 ng/mL for QNs and 0.66–5.79 ng/mL for SAs. Using CQN, TC, ENR and SDM as reference

antibiotic analytes, the cross-reactivity values ranged from 22% to 588% for BLs, 69–100% for TCs, 44–226% for QNs, 29–253% for SAs, respectively. As such, the broad specificity of the mAbs/receptor is potentially beneficial for the development of a broad-spectrum LFA for simultaneously screening the four families of antibiotic residues in milk.

3.3. Optimization of NIR dye-antibody/receptor conjugates

In this study, IRDye® 800CW, with its maximum 774/789 nm excitation/emission wavelength spectra (Gong et al., 2012) was used as NIR fluorescence label. For the preparation of dye-antibody/receptor conjugate, the NHS ester form of 800CW dye was used. The NHS ester reactive group can react with the amine groups of protein to form a covalently linked dye–protein conjugate. The final dye/protein ratio can be estimated by measuring the absorbance of 800CW dye at 774 nm and the absorbance of protein at 280 nm (Swanson and D'Andrea, 2013). By varying the amount of dye and protein for labeling, 800CW-antibody/receptor conjugates with coupling ratios ranging from 0.4 to 7.2 were obtained (Table S2). These conjugates were then applied to the NIR-based LFA and their assay performances were compared. The result indicated that the optimal coupling ratios were 1.6:1, 4.8:1, 4.3:1, and 5.1:1 for anti-BL receptor, anti-TC mAb, anti-QN mAb and anti-SA mAb, respectively.

3.4. Qualitative analysis of antibiotic residue in milk

Based on the optimal conditions (Supplementary material), serially diluted antibiotic solutions (CQN, TC, ENR and SMD) were spiked into blank milk samples and analyzed by the NIR-based LFA. The cut-off values were defined as the lowest antibiotic concentration resulting in invisible test lines. As shown in Fig. 2A, the cut-off values were determined to be 8 ng/mL for CQN, 2 ng/mL for TC, 4 ng/mL for ENR and 8 ng/mL for SMD, respectively. All these cut-off values were much below the MRLs set by the European Union (European Commission, 2010). Based on the same mAb and receptor, a multiplex GNP-based LFA was also developed and the cut-off values were determined to be 35, 15, 40 and 50 ng/mL for CQN, TC, ENR and SMD, respectively (Fig. S1). This comparison indicated the cut-off values of NIR- based LFA were improved about 4–10 fold over the GNP-based LFA. The increase on the assay sensitivity may be attributed to two factors: first, at the NIR spectral region, the fluorescence background from nitrocellulose membrane and sample matrix can be reduced to a minimal level (Gong et al., 2012). Second, NIR light has strong penetration ability due to its low light absorption and scattering (Guo et al., 2014), hence all the NIR dye captured at the test line may be excited and the emitted fluorescence can be detected by the imaging system. In contrast, for GNP-based LFA, only the Ab–GNP conjugate in the top 10 μm of nitrocellulose membrane surface can be detected (Technical Manual of Millipore Corporation, 2002). Therefore, with the decreased fluorescence background and the increased depth of signal source, the signal/noise ratio of NIR-based LFA can be significantly improved. Benefiting from the higher signal/noise ratio, less antibody consumption for NIR-based LFA was observed as compared to that for GNP-based LFA. The optimal amounts of antibody/receptor for each test of NIR-based LFA ranged from 1.3 to 4.4 ng (1.3 ng of anti-BL receptor, 3.6 ng of anti-TC mAb, 2.6 ng of anti-QN mAb, 4.4 ng of anti-SA mAb). On the other hand, for each GNP-based LFA test, the optimal amounts ranged from 36 to 92 ng (36 ng of anti-BL bioreceptor, 92 ng of anti-TC mAb, 68 ng of anti-QN mAb, 89 ng of anti-SA mAb). This indicated that the amount of mAb/receptor for NIR-based LFA was less than 1/20th of that for GNP-based LFA. Less antibody/receptor consumption consequently produced higher sensitivity for this competitive LFA.

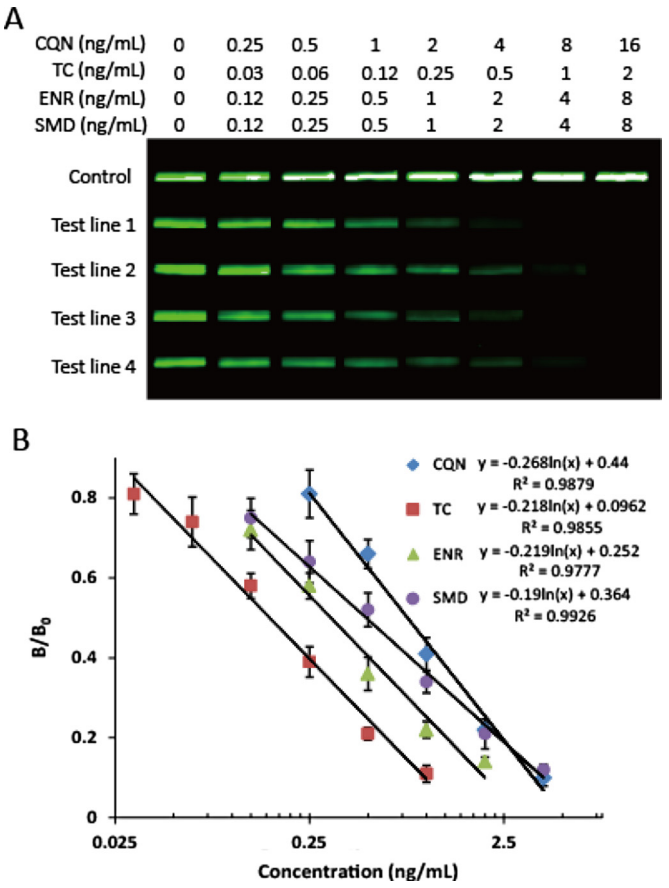

Fig. 2. (A) Typical photo image of NIR lateral flow strips for the detection of four different kinds of antibiotics using CQN, TC, ENR and SMD as reference analytes. (B) Calibration curves of the four antibiotics by plotting B/B_0 against the logarithm concentration of analyte.

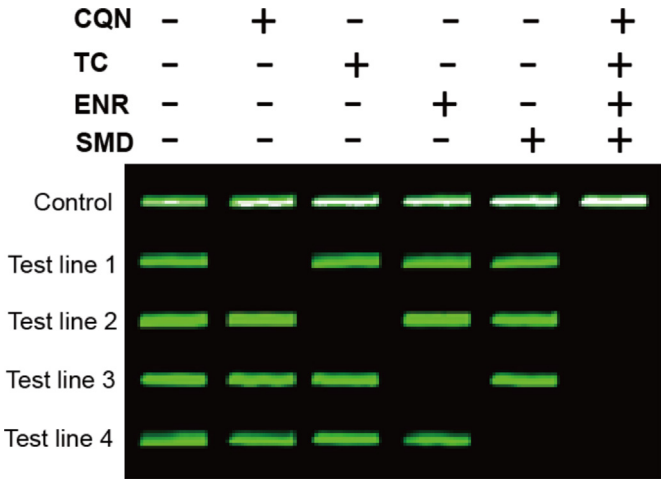

Fig. 3. The photo image of NIR lateral flow strips for the evaluation of assay specificity.

The specificity of this multiplex NIR-based LFA was then evaluated by testing each analyte separately to exclude false-positive results. As shown in Fig. 3, the NIR dye-antibody/receptor conjugates exhibited high specificity to their respective coating antigens. Additionally there was no mutual interference between the four kinds of antibiotics, even when the concentration of each antibiotic (100 ng/mL) was much higher than their respective cut-off values.

3.5. Quantitative analysis of antibiotic residue in milk

As shown in Fig. 2, with the increased concentrations of the four reference analytes (CQN, TC, ENR and SMD), the fluorescence intensities of respective test lines decreased. Calibration curves were then constructed by plotting the ratios between fluorescence intensities of spiked and blank sample (B/B_0) against the logarithm concentrations of analytes (Fig. 2B). These data were then fitted by linear equations. The sensitivity of this NIR-based LFA was evaluated by the limit of detection (LOD), which was defined as the analyte concentration that produces 20% decrease in line intensity as compared to blank sample (Jiang et al., 2015). The result indicated that the calculated LOD values were 0.26, 0.04, 0.08 and 0.1 ng/mL for CQN, TC, ENR and SMD, respectively (Table S3). These LOD values were far below the MRLs set by the European Union (European Commission, 2010). The assay sensitivity of this analysis is comparable to or better than that of other LFAs (Table S4) and most immunoassays (Table S5) for antibiotic residues in milk. The only methodology with significant higher sensitivity was reported by Song et al. (2015), however, a time-consuming sample preparation and much longer assay time were required in their study. Furthermore, the developed NIR-based LFA herein can simultaneously monitor more antibiotic residues than all the other LFAs and most immunoassays currently reported in literature (Tables S4 and S5). Only one immunoassay based on wavelength-interrogated optical biosensor was reported to have the capability of detecting four families of antibiotic residues in milk (Adrian et al., 2009). As compared to this method, the developed NIR-based LFA herein presented much lower LOD values for all the antibiotics.

The linear ranges of this assay were set to be the concentrations resulting in 10–80% B/B_0 values, and were measured to be 0.26–3.56 ng/mL for CQN, 0.04–0.98 ng/mL for TC, 0.08–2.0 ng/mL for ENR, 0.1–3.98 ng/mL for SMD respectively (Table S3). All the linear correlation coefficient (R^2) values were higher than 0.97 (Fig. 2B), suggesting that the NIR-based LFA was suitable for quantitative analysis.

The accuracy and precision of this assay were also evaluated by the spiked recovery experiment. Blank raw milk samples were spiked with different concentrations of analytes and then measured by the NIR-based LFA. As shown in Table S6, at the spiked concentrations of 0.1–1.6 ng/mL, the mean recoveries of the four reference analytes (CQN, TC, ENR and SMD) ranged from 93.7% to 108.2% with the coefficient of variation (CV) values less than 16.3% ($n=4$). In order to further validate the developed method, 20 milk samples collected from local markets were measured by both NIR-based LFA and LC-MS/MS method (Han et al., 2015). Seventeen samples were determined as negative by NIR-based LFA, and the same 17 samples were confirmed as negative by LC-MS/MS. This indicated that no false negative result was obtained by NIR-based LFA. Three samples were determined as positive by both methods, and the detection values obtained by NIR-based LFA were in good agreement with that of LC-MS/MS method (Table S7). These results demonstrated that the developed method could be applied in real sample analysis.

4. Conclusions

In this study, NIR fluorescence label as a promising alternative reporter was successfully integrated with LFA to detect four families of antibiotics including 41 structurally different chemicals in milk. This novel immunoassay has the advantage of rapidity, low cost and high sensitivity. The cut-off values for qualitative detection and the LODs for quantitative detection were both far below the MRLs set by the European Union (European Commission, 2010). The accuracy and precision of this assay also met the requirements for quantitative analysis. Thus, NIR-based LFA could be an effective tool for antibiotic residue monitoring. The multiplexing capability of this assay was achieved by using separate test lines. Our future work will further extend this multiplexing capability by detecting different kinds of chemicals on individual test lines.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.12.062.

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