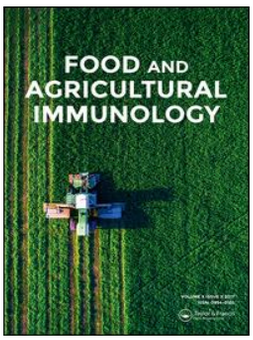

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Gold immunochromatographic assay for kitasamycin and josamycin residues screening in milk and egg samples

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ABSTRACT

In this study, a kitasamycin (KIT) hapten was prepared through the o-carboxymethoxylamine method and used to produce a monoclonal antibody against KIT and josamycin (JOS). The half inhibition concentration for an indirect competitive enzyme-linked immunosorbent assay was 1.49 and 2.67 ng/mL, respectively. Based on this antibody, a gold immunochromatographic assay (GICA) was developed for the detection of KIT and JOS residues in milk and egg samples. A hand-held strip scanner was used for quantitative analysis, with limits of detection for KIT and JOS of 1.51 and 1.91 μg/L in milk, and 3.0 and 2.72 μg/kg in eggs. The proposed GICA was validated by LC–MS/MS, which indicated that the GICA method would be a useful tool for the on-site detection of KIT and JOS residues in milk and egg samples.

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KEYWORDS
Kitasamycin; josamycin; gold immunochromatographic assay; monoclonal antibody

Introduction

Kitasamycin (KIT) and josamycin (JOS) are 16-membered macrolides (Figure 1) (Arsic et al., 2018), which can inhibit protein synthesis and induce toxic effects in the Gram-positive and some Gram-negative bacteria (Leclercq, 2002). Due to their beneficial antibacterial effects, they have been applied in veterinary medicine. However, their abuse or incorrect use can cause the presence of residues in animal-derived food and the environment (Managaki, Murata, Takada, Tuyen, & Chiem, 2007). Through the food chain at certain levels, these residues can enter the human body, and become hazardous to human health (Alban, Nielsen, & Dahl, 2008). This can ultimately result in the emergence of super bacteria (Ferri, Ranucci, Romagnoli, & Giaccone, 2017). Many countries have set maximum residue limit (MRL) for KIT residues in edible animal tissues and eggs (200 μg/kg). In the European Union, the MRL for JOS residues is 200 μg/kg in bovine and chicken tissue and eggs. Therefore, establishing rapid, accurate and sensitive analytical methods for the detection of KIT and JOS residues is necessary.

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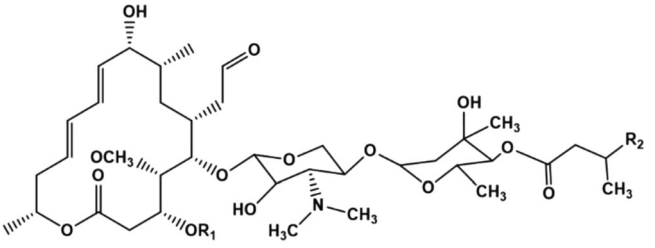

R_1=H, R_2=CH_3, Kitasamycin (KIT);
R_1=CO-CH_3, R_2=CH_2-CH_3, Josamycin (JOS).

Figure 1. Chemical structures of KIT and JOS.

Many instrumental methods have been developed for the detection of KIT and JOS residues, such as high-performance liquid chromatography and liquid chromatography–electrospray mass spectrometry (LC–MS/MS) (Han, Jin, Wu, Shi, & Cui, 2019; Song et al., 2016, 2018; Zheng et al., 2018). Due to their high accuracy and sensitivity, LC–MS/MS methods have been set as the gold standard for the detection of KIT and JOS. However, these methods need expensive equipment, complicated sample pretreatment, long detection time and specialized technical personnel. These requirements, however, limit their application for on-site detection of large quantities of samples.

Immunoassays, especially colloidal gold immunochromatographic assays (GICAs) have recently been widely applied in food safety monitoring (Li et al., 2019), such as in pesticides (Verma & Bhardwaj, 2015; Ye, Wu, Xu, Zheng, & Kuang, 2018; Zhang et al., 2019), veterinary drugs (Peng et al., 2017; Tochi et al., 2016), environmental contaminants (Xing et al., 2015), harmful microorganisms (Wang et al., 2016, 2017a) and food allergens (Zeng et al., 2019). Immunoassays have the advantage of being simple, rapid, time-saving and low cost. Therefore, they are suitable for high-throughput screening and on-site detection. With the development of photoelectric technology, GICA methods can become quantitative and portable. Some immunoassays have been developed previously for the rapid detection of macrolide residues (Galvidis, Lapa, & Burkin, 2015; Li et al., 2015a, 2015b; Wang, Beier, & Shen, 2017b; Wang et al., 2015). However, there are few reports on the establishment of immunoassays for the detection of KIT and JOS residues in food from animal-origin.

In this work, we prepared a monoclonal antibody (mAb) for the simultaneous screening of KIT and JOS. Based on this antibody, GICAs with hand-held strip scanner were established for the quantitative detection of KIT and JOS residues in spiked milk and egg samples. These results were further validated by LC–MS/MS.

Materials and methods

Chemicals and materials

The following standards: KIT, JOS, spiramycin (SPI), tilmicosin(TIL), tylosin (TYL), azithromycin (AZI), erythromycin (ERY), clarithromycin (CLA) and doramectin (DOR), were purchased from J&K Scientific Co., Ltd. (Shanghai, China). Cross-linkers containing O-carboxymethoxylamine (CMO), N-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethylarbodiimide (EDC); carrier proteins including keyhole limpet

haemocyanin (KLH), bovine serum albumin (BSA), ovalbumin (OVA); and immune adjuvants including Freund's complete adjuvant and Freund's incomplete adjuvant were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-mouse immunoglobulin (IgG) was obtained from Jackson ImmunoResearch Laboratories. Cell fusion reagents, including RPMI 1640 medium, foetal bovine serum, polyethylene (PEG, MW 1450), hypoxanthine-aminopterin-thymidine (HAT), and hypoxanthine-thymidine (HT) were attained from Thermo Fisher Scientific Inc. (Shanghai, China). Sample pad (GL-b01), nitrocellulose (NC) membrane, absorbance pad (H5079), and polyvinylchloride (PVC) sheets were purchased from JieYi Biotechnology Co., Ltd. (Shanghai, China).

Apparatus

A microplate reader for ELISA analysis was provided by Molecular Devices (Shanghai, China). The dispensing platform and CM4000 guillotine cutting module for strip preparation were purchased from Kinbio Tech (Shanghai, China). NanoDrop™ One for protein concentration analyses were purchased from Thermo Fisher Scientific Inc. (Shanghai, China).

The mAb preparation

Hapten and antigen synthesis

The hapten was synthesized using the CMO method (Figure 2) (Jiang et al., 2013). One-hundred mg of KIT and 20 mg of CMO were added to a mixture of 5 mL methanol and 5 mL 0.02 M sodium bicarbonate solution. The mixture was kept stirring at 60°C for 12 h. Then the reactant was dried using a rotator with blowing nitrogen. The dry residue was dissolved in methanol and the undissolved fraction was removed by centrifugation at 5000 rpm for 10 min. The supernatant was dried again using nitrogen at 50°C to obtain the hapten KIT-CMO.

Hapten KIT-CMO was conjugated with carrier proteins using the active ester method (EDC/NHS method, Chen et al., 2017; Guo, Wu, Liu, Kuang, & Xu, 2018a). Briefly, a mixture of 15 mg KIT-CMO, 2.68 mg NHS and 4.44 mg EDC was dissolved in dimethylformamide, and stirred continuously at room temperature. After 12 h, this activating solution was divided equally and added to 10 mg BSA (or OVA) solution (5 mg/mL in carbonate buffer solution, pH 9.6). Twenty-four later, the reaction solution was dialysed using 0.01 M phosphate buffer (PBS, pH 7.4) for 5 days. The conjugate concentration was determined using a NanoDrop™ One. The KIT-CMO-BSA was used as an immunogen, and the KIT-CMO-OVA was used as coating antigen.

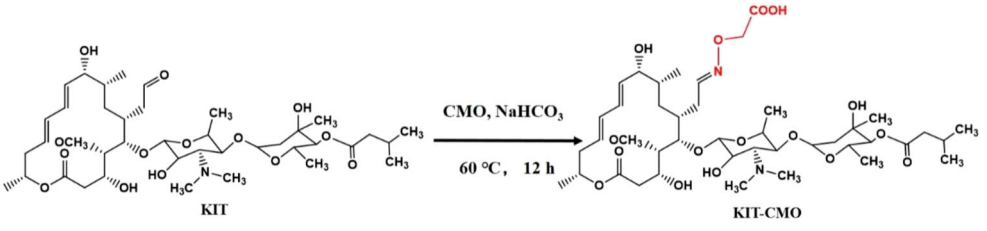

Figure 2. The synthesis route for KIT-CMO.

Immunization

Female BALB/c mice aged between 5 and 6 weeks were immunized with KIT-CMO-BSA. The immunization procedure was similar to that of our previous work (Kong et al., 2019). For the first immunization step, each mouse was immunized subcutaneously using 100 μg KIT-CMO-BSA emulsified with an equivalent amount of Freund's complete adjuvant. Every 3 weeks, a booster immunization was performed with half the dose, and the adjuvant was changed to Freund's incomplete adjuvant. Seven days after the third immunization, the mouse serum was screened using ic-ELISA. The mouse with the highest titre and inhibition against KIT was injected intraperitoneally with 25 μg KIT-CMO-BSA in the absence of immune adjuvant at the fifth immunization. After 3 days, this mouse was sacrificed for cell fusion.

Cell fusion

Mouse splenocytes were fused with SP 2/0 murine myeloma cells using the PEG method and the details of this procedure have been described by ourselves previously. After HAT medium screening, the unfused SP 2/0 murine myeloma cells were killed and the cell supernatant screened by ic-ELISA. The cells with the highest titre and inhibition against KIT were sub cloned using the limiting dilution method. After three rounds of sub-cloning and indirect competitive (ic)-ELISA screening, a purified cell line was obtained, and cryopreserved in liquid nitrogen.

Ascites preparation and purification

The screened cell line was large scale cultured, and then collected by centrifugation at 1200 rpm for 8 min. These cells were injected into BALB/c mice intraperitoneally (2×10^5 cells for each mouse) and after 7–10 days, the ascites were collected and purified using AKTA™ pure protein purification system (GE Healthcare Bio-Sciences, USA) to obtain the mAb and its concentration was determined using a NanoDrop™ One.

The mAb characterization

A checkerboard assay was applied to obtain the working concentration of mAb and coating antigen (KIT-CMO-OVA) (Lei et al., 2018). The ic-ELISA procedure was carried out as described in our previous work. The standard dilution buffer (PBS solution) was optimized using different sodium chloride concentrations (0.4%, 0.8%, 1.6%, 3.2%, and 6.4% by mass fraction), methanol concentrations (0, 10%, 20%, 30%, and 40% by volume fraction), and different pH values (pH 4.7, pH 6.0, pH 7.4, pH 8.8, and pH 9.6). Under optimized conditions, a series of KIT (0, 0.2, 0.5, 1, 2, 5, and 10 ng/mL) and JOS standards (0, 0.2, 0.5, 1, 2, 5, and 10 ng/mL) were tested. An inhibition curve was fitted using a four-parameter logistic function based on absorbance value at 450 nm ($A_{450\ nm}$) and KIT or JOS concentrations. The half-maximum inhibition concentration (IC_{50}) was obtained using the standard curve.

The IC_{50} of the structural analogues including SPI, TIL, TYL, AZI, ERY, CLA, and DOR were also determined. Cross-reactivity (CR) was calculated with the following equation: CR% = (IC_{50} value of KIT)/(IC_{50} value of related analogue).

The affinity constant (Ka) of the mAb was evaluated using ELISA. This is detailed in our previous publication (Guo, Xu, Song, Liu, & Kuang, 2018b). Furthermore, the mAb subtype was tested using the mouse mAb isotyping ELISA kit (Wang et al., 2019).

Establishment of the GICA

Preparation of gold nanoparticle-labelled mAb

Gold nanoparticles of 20 nm diameter were prepared using the citrate reduction method. Briefly, 5 mL of trisodium citrate solution was added quickly to 100 mL of boiled chloroauric acid solution (0.01%, w/v) under continuous stirring, this rapidly changed to red wine colour. Fifteen min later, the solution was cooled to room temperature for further use.

The mAb was labelled with GNPs as follows: 10 mL of GNP solution was firstly adjusted to pH 8.0 using a potassium carbonate solution before the addition of the mAb. Then the mixture was kept at room temperature and 45 min. After this, 1 mL of BSA solution (10% by mass ratio) was added to the mixture for 1 h to block the unconjugated GNPs. The unlabelled mAb was removed by centrifugation at 8000 rpm for 45 min and the precipitate (GNP-labelled mAb) was resuspended in 1 mL of 0.02 M PBS containing 0.1% Tween-20.

Production of the immunochromatographic strip

A schematic diagram of the strip is shown in Figure 3. The strip is made up of an absorption pad, NC membrane, sample pad and PVC sheet (Wang, Guo, Liu, Kuang, & Xu, 2018). The NC membrane is located in the middle of the PVC sheet, on which 0.1 mg/mL of goat anti-mouse IgG was spayed to form the control zone (C line), and an appropriate concentration of KIT-CMO-OVA was spayed to form the test zone (T line). The sample pad and absorption pad were glued onto both ends of the PVC sheet. The assembled card was put into a 37°C oven for 2 h, and then cut into 2.87 mm wide strips. These strips were stored in a drier until used.

Principle of the GICA

One-hundred and fifty μL of standard or sample solution and 50 μL GNP-labelled mAb were mixed and incubated for 3 min on a microplate at room temperature. Then the strip was put in the well. Due to capillary action, the mixture flowed quickly from the sample pad to the absorption pad. The result was able to be seen with the naked eye within 5 min, and could be used for qualitative analysis. A series of KIT or JOS standards (0, 1, 2.5, 5, 10, 25, and 50 ng/mL) were detected using this strip. The colour intensity ratio of the T line to the C line (T/C value) was recorded using a hand-held strip scanner and a quantitative curve could be established by plotting (X-axis) KIT or JOS concentration against T/C values (Y-axis).

The principle of this strip test is based on the competition between the free KIT and the fixed KIT-CMO-OVA for binding to the CNP-labelled antibody (Guo et al., 2015). If the sample is positive, it will conjugate with the GNP-labelled mAb. When the solution reaches the T line, residual GNP-labelled mAb would conjugate with the KIT-CMO-OVA. If the sample is negative, all of the GNP-labelled mAb would combine with KIT-CMO-OVA which had been sprayed onto the T line. Therefore, the T line colour of the positive samples would be lighter than that of the negative samples. Whereas the C line

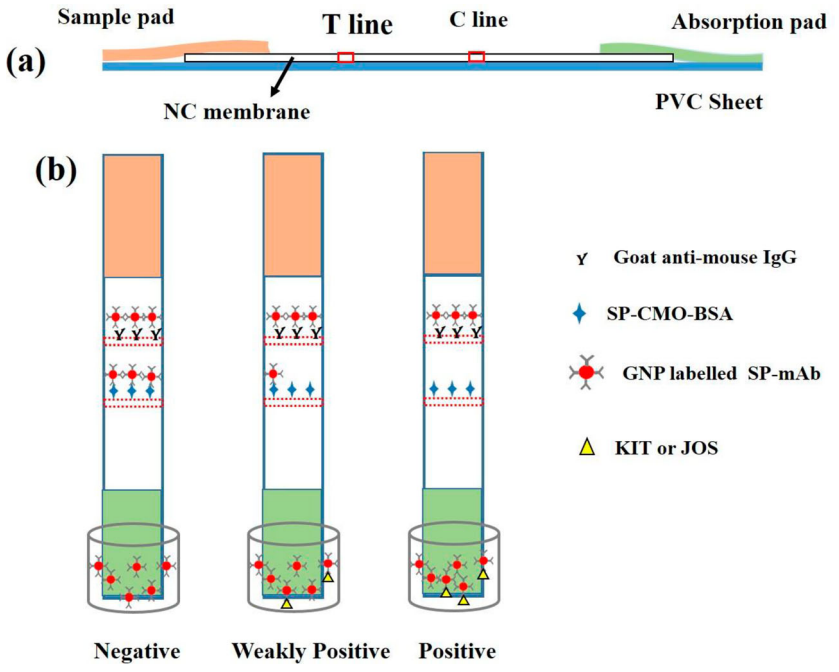

Figure 3. The schematic diagram of strip for GICA. (a) The cross-sectional view of the strip; (b) The schematic illustration in negative, weakly and positive sample.

colour would always be present due to the GNP-labelled mAb conjugating with the goat anti-mouse IgG. If the C line is colourless, the strip is invalid.

Sample analysis

Milk and egg samples were purchased from a supermarket. Milk samples can be analysed directly using strip without any pretreatment. For egg samples, egg white and yolk were stirred well firstly. Then this emulsion (1 ± 0.01 g) was diluted four times using PBS (0.01 M, pH 7.4) before testing. Different concentrations of KIT or JOS standards were spiked into the milk and egg samples for GICA analysis. The different concentrations of spiked milk or egg samples can be formed quantitative curve with T/C value.

To assess the repeatability and accuracy of this proposed GICA, recovery test was conducted. Three-level fortified KIT or JOS concentrations in milk and egg were detected. Besides, LC–MS/MS analysis was also performed to validate the GICA results. Each test was repeated three times.

Results and Discussion

Hapten design and characterization

As both KIT and JOS are all small molecules (below 10 kDa), they cannot cause a mouse immune response directly, and therefore, need to be conjugated to a carrier protein to induce an immune response and produce antibodies against KIT (Liu et al., 2017). It is usual for small molecule compounds to be coupled to carrier proteins

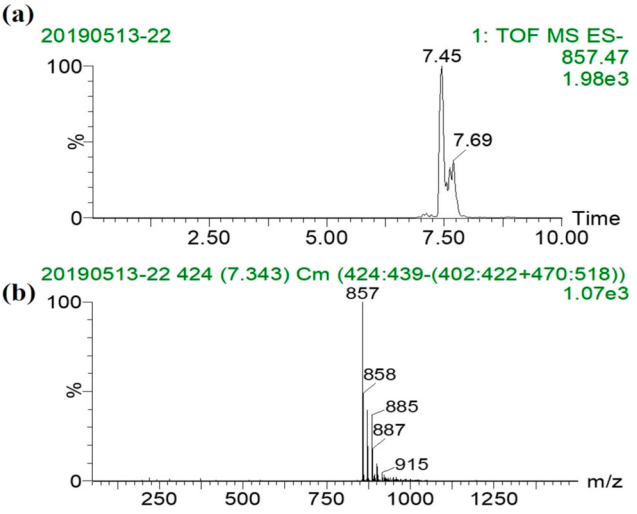

Figure 4. LC–MS/MS analysis of KIT-CMO. A. The negative ions LC spectrum of KIT-CMO; B. The mass spectrum of KIT-CMO.

through active groups such as NH_2 or COOH. In this study, we derived the C=O of KIT to C=N–O–CH_2COOH through the oxime-forming reaction (CMO method). The COOH of KIT-CMO can then be conjugated with the NH_2 group of BSA directly using the EDC/NHS method. Furthermore, CMO can introduce a spacer arm, which is good for antigenic determinant exposure to stimulate antibody generation. According to previous research, the OH can also be derived to COOH using the succinic anhydride method. However, KIT has three hydroxyls, and therefore the derived product would be a mixture and difficult to purify and thereby influence antibody inhibition against KIT.

The structure of KIT-CMO was confirmed by LC–MS/MS and as shown in Figure 4, the retention time of KIT-CMO was 7.45 and an *m*/*z* ratio of 857 at 7.45 min confirmed that the structure of KIT-CMO was $C_{42}H_{70}N_2O_{16}$ (MW = 858.47 Da). The hapten KIT-CMO was conjugated with BSA or OVA using the EDC/NHS method.

The mAb characterization

Assay buffer plays an important role in ic-ELISA analysis, and can affect binding between antibody and antigen (Kuang et al., 2013). Methanol as co-solvent can enhance solubility for analysis. Whereas excessive methanol content in the assay buffer can affect the activity of the mAb. As shown in Figure 5(a), the methanol content had little effect on the A_{450nm}, but the IC_{50} value was increased with increasing methanol content. The highest A_{450nm} /IC_{50} value was attained when the methanol content was zero in the dilution buffer. Optimum NaCl content of the buffer can reduce the non-specific absorption of the antibody, and improve its sensitivity and the binding to antigen. From Figure 5(b), it is seen that the A_{450nm} value was clearly decreased when the NaCl content was equal or greater than 3.2%. The highest A_{450nm}/IC_{50} value was found when the NaCl content was 0.8% in the dilution buffer. The pH value was also a crucial factor for the ic-ELISA performance.

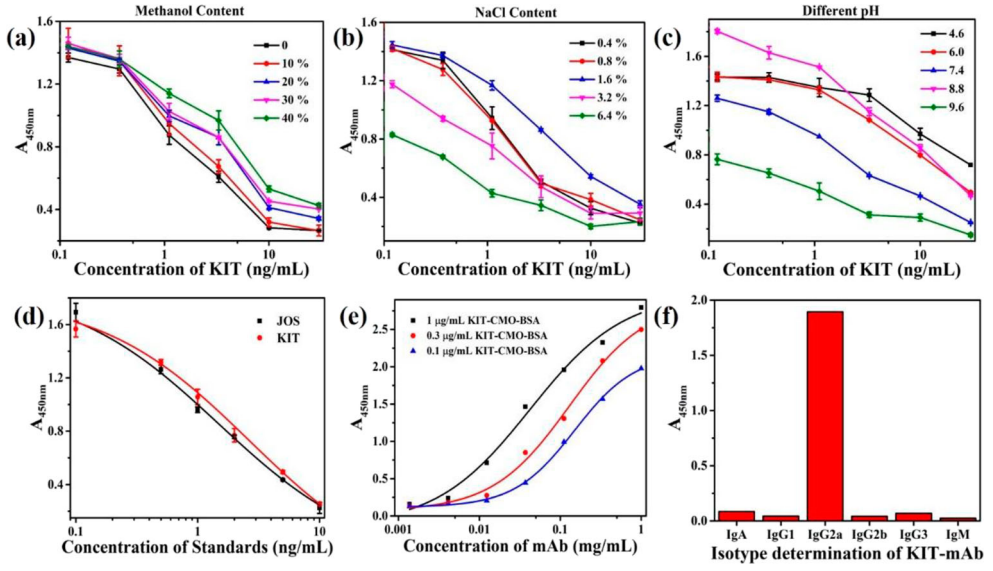

Figure 5. Characterization of mAb. Optimization of assay buffer for ic-ELISA, (a) Methanol content, (b) NaCl content, (c) Different pH; (c) Standard curve for KIT and JOS with ic-ELISA; (e) Affinity detection; (f) Subtypes determination.

This can influence the solubility of KIT or JOS and the antibody conformation. In Figure 5 (c), the A_{450nm} was less than 1.0 when the pH value of the assay buffer was 9.6. The highest A_{450nm} /IC_{50} value was obtained when the pH value of the assay buffer was 7.4. Based on these optimization results, an ic-ELISA standard curve for KIT and JOS was established (Figure 5 (d)), with an IC_{50} for KIT of 1.49 ng/mL, and an IC_{50} of 2.67 ng/mL for JOS.

Cross-activity evaluation is important for actual sample analysis. The cross-reaction with unknown components in the sample can induce a false-positive result. From Table 1, we can see that this mAb has no cross-reactivity with other common macrolide antibiotics used in veterinary medicine including SPI, TIL, TYL, AZI, ERY, CLA, and DOR.

According to our calculation, the Ka of the mAb was 1.19×10^9 L/mol (Figure 5(e)). This high-affinity mAb can be applied in the development of the immunoaffinity chromatography for KIT and JOS. Different mAb subtypes have different antibody purification methods (Bergmann-Leitner et al., 2008). The subtype of mAb prepared in our work was an IgG2a isotype (Figure 5(f)).

Table 1. The cross-activity of mAb.

Analytes	IC_{50} (ng/mL)	CR (%)
Kitasamycin	1.49	100
Josamycin	2.67	55.8
Spiramycin	>1000	< 0.2
Tilmicoin	>1000	< 0.2
Tylosin	>1000	< 0.2
Azithromycin	>1000	< 0.2
Erythromycin	>1000	< 0.2
Clarithromycin	>1000	< 0.2
Doramectin	>1000	< 0.2

GICA

As discussed in our previous article (Peng et al., 2017), a GNP solution of 20 nm diameter produced a uniform and stable red wine colour, which was suitable for labelling the mAb. The optimal labelling pH value was 8.2, as the conjugation between mAb and GNPs was stable when the pH value was greater than or equal to the isoelectric point of the mAb.

Due to the visibility of the GNPs, the strip results can be seen directly with the naked eye. Usually, when the T line became colourless, the analysis concentration was defined as cut-off value. The lowest detectable concentration when the T line colour became light represented the visual limit of detection (vLOD). The cut-off and vLOD values represent two indicators of qualitative determination for the strip assay. From Figure 6(a), in spiked milk samples, we can see that the cut-off value and vLOD of our strip was 25, and 5 μg/L respectively for KIT and JOS. The egg samples were diluted three times before the strip test was used, and the cut off value was 75 μg/kg for KIT and 150 μg/kg for JOS; and the vLOD was 15 μg/kg both for KIT and JOS (Figure 6(b)).

Hand-held strip scanner results can also be used for quantitative analysis. The calculated LOD (cLOD) was defined as the concentration with 20% inhibition of the blank sample signal. A series of KIT or JOS standards spiked into milk and egg samples were detected. As shown in Figure 6, the standard curves were fitted using a four-parameter logistic function. The cLOD was 1.51 μg/L for KIT, and 1.91 μg/L for JOS in milk, and 3.0 μg/kg for KIT, and 2.73 μg/kg in egg.

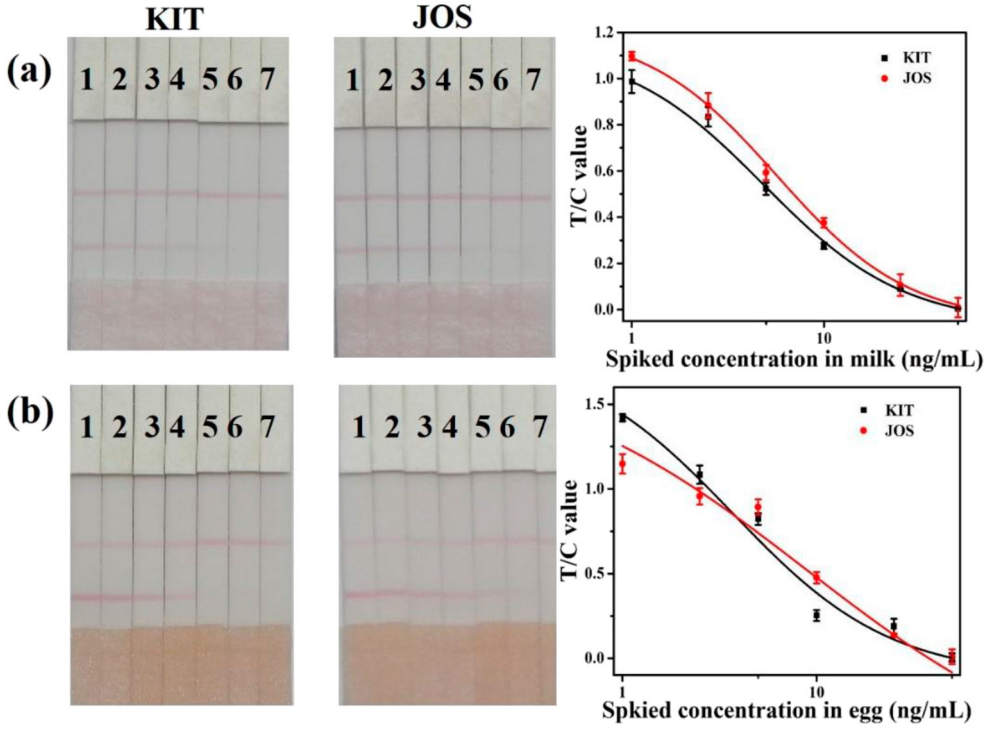

Figure 6. The strip results for KIT and JOS in spiked samples: (a) milk: 1 = 0, 2 = 1.0 μg/L; 3 = 2.5 μg/L; 4 = 5 μg/L; 5 = 10 μg/L; 6 = 25 μg/L; 7 = 50 μg/L. (b) egg: 1 = 0, 2 = 3.0 μg/kg; 3 = 7.5 μg/kg; 4 = 15 μg/kg; 5 = 30 μg/kg; 6 = 75 μg/kg; 7 = 150 μg/kg.

Table 2. Analysis of KIT and JOS in spiked milk, egg sample by GICA and LC–MS/MS ($n = 3$).

			GICA		LC–MS/MS	
Samples	Analysis	Spiked level (μg/L or μg/kg)	Recovery rate (%) ± SD	CV (%)	Recovery rate (%) ± SD	CV (%)
Milk	KIT	1	106 ± 10.1	9.5	95.0 ± 5.4	5.7
		4	92.3 ± 8.3	9	90.2 ± 3.3	3.7
		16	98.8 ± 4.9	5	101 ± 11.3	11.2
	JOS	1	91.5 ± 8.7	9.5	88.3 ± 7.2	8.2
		4	79.3 ± 9.2	11.6	104 ± 4.8	4.6
		16	86.9 ± 5.4	6.2	94 ± 7.3	7.8
Egg	KIT	3	93.6 ± 5.2	5.6	102 ± 6.4	6.3
		12	86.7 ± 12.0	13.8	107 ± 3.9	3.6
		48	95.2 ± 5.8	6.1	98.5 ± 9.2	9.3
	JOS	3	96.4 ± 7.1	7.4	89.2 ± 9.4	10.5
		12	110 ± 4.7	4.3	102 ± 2.8	2.7
		48	95 ± 4.5	4.7	90.4 ± 4.3	4.8

The qualitative and quantitative analysis of this strip met the requirement of the detection levels for KIT and JOS residues in milk and egg samples.

Comparison GICA with LC–MS/MS in spiked samples

A spiked and recovery test was conducted to evaluate the feasibility of our GICA (Jiang et al., 2016). The matrix standard curves established for GICA minimize matrix interferences in the actual test. To validate the GICA results, the spiked samples were detected by LC–MS/MS as a control. Each sample was tested three times. The detection results are shown in Table 2. The mean recovery rate of GICA ranged from 79.3 ± 9.2% to 110 ± 4.7%, with a CV value ranging from 4.3% to 13.8%. Whereas, the recovery rate of the LC–MS/MS was 88.3 ± 7.2% to 107 ± 3.9%, with a CV value ranging from 3.7% to 11.2%.

The GICA results were consistent with those of the LC–MS/MS. Compared with the LC–MS/MS, the GICA can be completed within 15 min, and samples need no complicated pretreatment process. Therefore, the GICA proposed in this work can be applied to screen KIT and JOS residues in milk and egg samples.

Conclusion

In this work, we prepared a sensitive monoclonal antibody which can recognize KIT and JOS with IC_{50} values of 1.49 and 2.67 ng/mL. Based on this mAb, a GICA was established for KIT and JOS residues for both qualitative and quantitative detection, which met the MRL requirements set by the relevant authorities. Furthermore, the GICA results were confirmed by LC–MS/MS. Therefore, the developed GICA can be used as a rapid screening method for KIT and JOS residue detection in milk and egg samples.

Compliance with ethical standards

Ethical approval

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

Disclosure statement

No potential conflict of interest was reported by the authors.

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