Gold immunochromatographic assay for trimethoprim in milk and honey samples based on a heterogenous monoclonal antibody

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ABSTRACT
Three heterogenous haptens (3,4,5-Trimethoxybenzoic acid [H1], 3,4,5-Trimethoxyphenylacetic acid [H2], 3-(3,4,5-Trimethoxyphenyl) propanoic acid [H3]) were used to synthesize immunogens, with the aim of screening a more sensitive monoclonal antibody (mAb) for trimethoprim (TMP). H2 proved to be the best heterogenous hapten. The mAb was specific with no cross-reactivity with other structurally related chemicals. The 50% maximal inhibitory concentration of the mAb against TMP was 1.98 µg L⁻¹, which is more sensitive compared with the mAb reported in our previous work. A mAb-based gold immunochromatographic assay (GICA) was established to analyze TMP residues in milk and honey samples. The concentration of coating antigen in test line and concentration of mAb conjugated with gold nanoparticles was 0.3 µg mL⁻¹ and 0.25 µg mL⁻¹, respectively. Under optimized conditions, the visual limit of detection concentration of TMP spiked in milk and honey samples was 10 µg L⁻¹ and 15 µg kg⁻¹, respectively. The proposed GICA provides rapid and simple determination of TMP residues in milk and honey samples.

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Introduction
In veterinary, trimethoprim (TMP) is regarded as a sulfonamide potentiator which is usually used in combination with sulfonamides (Felix et al., 2016; Fisse, Straßburger-Krogias, Gold, & Ellrichmann, 2017). This drug combination has synergistic effects, blocking folic acid metabolism in bacteria through two different mechanisms (Felix et al., 2014; Felix et al., 2016). In addition, sulfonamide potentiators can also enhance the antibacterial activity of other antimicrobials, including fluoroquinolones, aminoglycosides, and β-lactam (Mandal, Pal, Chowdhury, & Debmandal, 2009; Xin, Yizhi, Hong, & Tiesuo, 2011; Zhou et al., 2015). Therefore, antibiotic synergists including TMP can be used as therapeutic agents to cure animal diseases after appropriate drug withdraw time (Tiwari, Tiwari, Singh, Singh, & Ahuja, 2012). However, the abuse of TMP has led to the drug residue in animal foods. The physical and chemical properties of TMP are stable, thus it could be concentrated in humans through the food chain. The TMP residues...
in humans may arouse serious problems, such as antibiotic-resistance, nausea, emesis, headache, pruritus, and rash (Croubels, Wassink, & De Backer, 2002). Therefore, to protect human health from these potential risks, a maximum residue limits (MRLs) for TMP was established in China, the European Union, and other countries for TMP of 50 µg kg\(^{-1}\) in edible tissues of all food-producing species, except eggs, and 100 µg kg\(^{-1}\) in edible tissues of horses (Andrade, de Moraes, Rocha, Fatibello, & Cass, 2009; Yang et al., 2016).

Based on the aforementioned background information of TMP residues in animal foods, the establishment of a fast, sensitive, and simple screening method can help the authorities monitoring food safety problems. To date, various methods have been developed for the analysis of TMP residues in animal foods. These methods mainly relied on instruments: electrophoresis (da Silva, Vidal, do Lago, & Angnes, 2013; Liu, Wan, Xu, Duan, & Yang, 2017), high performance liquid chromatography (HPLC) (Linli, Suxia, Jianzhong, Jinfeng, & Lequn, 2008), liquid chromatography–tandem mass spectrometry (LC/MS) (Economou, Petraki, Tsipi, & Botitsi, 2012; Gao et al., 2014; Li, Sun, Zhang, & Pang, 2013; Luo et al., 2014), and HPLC–MS (Yang et al., 2016; Yu-an et al., 2009). The sensitivity and specificity of these instrumental methods are adequate. However, the drawbacks of these methods are also obvious. The requirement for expensive equipment, highly trained technicians, and time-consuming sample pretreatments mean these methods cannot be implemented for fast screening of high-throughput samples. Meanwhile, the enzyme-linked immunosorbent assay (ELISA) based on a monoclonal antibody (mAb) was also established for detection of TMP residues (Chen, Liu, et al., 2016). Compared with instrumental methods ELISA is simpler and more cost-effective. In our previous work, the working range of indirect and competitive ELISA (ic-ELISA) was 1.83–9.36 µg L\(^{-1}\) the 50% maximal inhibitory concentration (IC\(_{50}\)) was 4.14 µg L\(^{-1}\), the whole assay could be finished in 90 min. The sensitivity of ELISA based on mAb can satisfy the MRLs required by the authorities.

As a semiquantitative analytical method, gold immunochromatographic assay (GICA) is more rapid and simple than ELISA. The assay can be completed in 5–10 min and the result can be judged by naked eyes. It is very convenient for high throughput and on-site determination. To the best of my knowledge, there is no related report of a GICA for TMP analysis. In this study, a sensitive, specific, and heterogenous mAb was screened, and a GICA was established based on the selected mAb.

### Materials and methods

#### Chemicals and reagents

TMP, sulfadiazine, sulfamethazine, sulfamethoxazole, sulfamethizole, 25% glutaraldehyde (GA) solution, 3,4,5-Trimethoxybenzoic acid, 3,4,5-Trimethoxyphenylacetic acid, and 3-(3,4,5-Trimethoxyphenyl) propanoic acid were purchased from J&K Scientific Ltd. (Beijing, China). Diaveridine, sulfadimethoxine, sulfathiazole, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide (EDC) N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), ovalbumin (OVA), Freund’s complete and incomplete adjuvant, 3,3′5,5′-tetramethylbenzidine and polyethylene glycol 1500 (PEG 1500) were purchased from
Sigma-Aldrich (St. Louis, MO, USA). Enzyme immunoassay-grade horseradish peroxidase labeled goat anti-mouse immunoglobulin was supplied by Hua Mei Co. (Shanghai, China). All other chemicals and solvents were of analytical grade.

**Apparatus**

A Milli-Q Ultrapure System (Bedford, MA, USA) was used to prepare ultrapure water. Ultraviolet (UV) spectra were scanned by an UV–visible spectrophotometer (Agilent, CA, USA). Polyvinylchloride, absorbance (H5079), and sample (glass-fiber membrane, GL-b01) pads were purchased from JieYi Biotechnology Co., Ltd. (Shanghai, China). Nitrocellulose (NC) membrane (Unistart CN140) was obtained from Sartorius Stedim Biotech GmbH (Goettingen, Germany). The CM4000 guillotine cutting module and dispensing platform were supplied by Kinbio Tech Co., Ltd. (Shanghai, China). Absorbance measurements were performed with a spectrophotometric microtiter plate reader (Thermo Fisher Scientific Inc, MA, USA).

**Synthesis of heterogenous coating antigens and immunogens**

Three different chemicals which possess a common structure (benzene ring with three methoxyl groups) and different linear carboxyl were used as the hetero-hapten to synthesize coating antigens. The carboxyl groups were conjugated with amino group of a protein through EDC/NHS method (Song et al., 2016). Briefly, 15.84 mg (0.075 mmol) of H1 was dissolved in 2 mL of dimethylformamide (DMF), and 17.02 mg (0.15 mmol) of NHS was added to the solution and stirred for 10 min at room temperature. Then, 28.5 mg (0.15 mmol) of EDC was added into the solution and activated for 4 h in room temperature. Finally, 100 mg (0.0015 mmol) of OVA was dissolved in 8 mL of carbonate buffer (0.05M, pH 9.6). The activated H1 solution was dropped into the protein solution and allowed to react overnight. Lastly, the mixture was dialyzed for three days with phosphate-buffered saline (PBS, 0.01 M, pH 7.2) to remove the free H1 and obtain pure coating antigen (H1–EDC–OVA). The synthesis of H2–EDC–OVA and H3–EDC–OVA was similar to generate H1–EDC–OVA. The characterization of the conjugates was confirmed by UV–visible spectroscopy. The immunogens (TMP–GA–BSA) were synthesized by GA method referred to our previous work (Chen, Liu, et al., 2016).

**Immunization schedule**

The immunization schedule was performed according to reference (Peng, Song, et al., 2016). After the third immunization, the sera of mice were collected and analyzed by ic-ELISA (Gu, Liu, Song, Kuang, & Xu, 2016). Three heterogenous coating antigens were used to screen the sera. Meanwhile, the best coating antigen was also screened. After the fifth immunization, the mouse with the highest titer and lowest IC₅₀ was screened for spleen donation for cell fusion. A final booster intraperitoneal injection (20 µg of immunogen directly dissolved in 100 µL of physiological saline) was performed three days before cell fusion.
Cell fusion and hybridoma screening

The procedure of cell fusion and hybridoma screening was performed as previously reported (Kong, Liu, Song, Kuang, & Xu, 2017). To obtain the pure cell line, the hybridoma was subcloned three times. Seven days after each subclone, ic-ELISA was fulfilled to screen the best cell line. The best cell line was cultured to allow the cells to proliferate, following with intraperitoneal administration to mice primed with paraffin to obtain ascites. The saturated ammonium sulfate method was used to purify the ascites to obtain the mAb. The concentration of mAb was measured by UV–visible spectroscopy at 278 nm. The mAb was labeled and stored at −20°C.

Sensitivity and specificity

A bidimensional titration assay was performed to determine an appropriate combination of mAb concentration and coating concentration. Then, ic-ELISA was used to determine the IC_{50} of mAb, which represents the concentration of competing compound that produced a 50% inhibition of antibody binding to the coating antigen (Jiang et al., 2011).

In our previous work, the homogenous mAb showed no cross-reactivity (CR) with other sulfonamide synergistic agents and other sulfonamides. In this work, the CR of heterogenous mAb was also determined (Chen, Kong, et al., 2016).

CR% = (IC_{50} value of TMP)/(IC_{50} value of related compound) × 100.

Seven analogues (diaveridine, sulfadiazine, sulfamethazine, sulfadimethoxine, sulfathiazole, sulfamethoxazole, and sulfamethizole) were used to evaluate the specificity of mAb. These compounds, previously dissolved in DMF at 1 mg mL\(^{-1}\), were diluted to 5, 10, 20, 50, and 100 µg L\(^{-1}\) with PBS.

Gold immunochromatographic assay

Synthesis of gold nanoparticles

Gold nanoparticles (GNPs) were synthesized through the sodium citrate reduction method with slight modification (Chen et al., 2015; Peng, Liu, Kuang, Cui, & Xu, 2017). Briefly, 2 mL of freshly prepared trisodium citrate (1%, w/v) was added into 50 mL of boiling HAuCl\(_4\)-H\(_2\)O (0.01%, w/v) under vigorous stirring. The mixture solution was boiled until the color changed to wine red, then left to cool to room temperature, and stored at 4°C. The GNPs form a stable colloidal state under the effect of electrostatic forces. GNPs were characterized by transmission electron microscopy and UV–visible spectrometry.

Preparation of mAb labeled with GNPs

The antibody combined with the GNPs through electrostatic interaction (Xing, Liu, Zhang, Kuang, & Xu, 2014). Briefly, pH of GNPs solution (20 mL) was adjusted to 8.2 by adding with K\(_2\)CO\(_3\) (0.1 M). Antibody (0.25 mg) was mixed with GNPs solution for 50 min at room temperature under continuous stirring. Subsequently, BSA (100 mg
dissolved in 5 mL ultrapure water) was slowly added to the GNPs–mAb solution and centrifuged at 875 g for 25 min to remove free mAb and blocking agent. The supernatant was pooled and the precipitate was washed with re-suspension buffer (borate buffer, 0.02 M, containing 1% BSA, 5% sucrose, and 0.5% PEG 6000, pH 8). Finally, the precipitate was dissolved in 5 mL of borate buffer.

**Principle of GICA**

As shown in Figure 1, the GICA strip consists of four sections: an NC membrane, an absorbent pad, a conjugate pad, and a sample pad assembled in layers. Goat anti-mouse IgG and coating antigen were sprayed onto control line and test line on the NC membrane, respectively. The absorbent pad was attached to the NC membrane close to the control line, whereas the sample pad was attached on the other side close to the test line (Guo et al., 2014).

The principle of GICA is similar to the one-step competitive ELISA (Peng, Wang, et al., 2016). The sample solution was mixed with GNPs–mAb and allowed to react for 5 min, then dropped onto the sample pad. Due to the capillary action, the mixture solution quickly spreads on the NC membrane. The goat anti-mouse IgG on control line can capture the excess GNPs–mAb, which means that the control line will always appear red regardless of the presence of target analyte. The appearance of the red color on control line proves the validity of the assay. If the sample is target negative, the GNPs–mAb can combine with both goat anti-mouse IgG and coating antigen, resulting in red bands on control line and test line, respectively. If the sample is target positive, the GNPs–mAb will have conjugate with target before addition to sample band and cannot conjugate with coating antigen, resulting in only one red band on the control line. The visual limit of detection (vLOD) of the GICA is determined by the concentration of target when the test line is colorless than the control line (Chen et al., 2017).

![Figure 1](image_url). Schematic illustration of GICA.
Analysis of milk and honey samples using GICA

Negative milk samples and honey samples, kindly donated by the Jiangsu Entry-Exit Inspection and Quarantine Bureau, were confirmed by HPLC/MS. TMP was dissolved in DMF to the concentration of 1 mg mL\(^{-1}\). Firstly, TMP was spiked in PBS directly, to determine a probable working range, which be referred to the TMP concentration spiked in milk and honey samples. For milk samples, the final concentration of TMP was 0, 0.5, 1, 2, 5, 10 µg L\(^{-1}\). For honey samples, the final concentration was 0, 1, 2, 5, 10, 15 µg kg\(^{-1}\).

The \(T/T_0\) value of each concentration for TMP was determined by a hand-held strip scan reader. The standard curve for TMP was developed for GICA based on the \(T/T_0\) value. The \(T_0\) value and \(T\) value is the parameter that shows the chromaticity of control line and test line. The \(T/T_0\) value shows the difference between control line and test line, evaluating the vLOD of GICA.

Results and discussions

Synthesis of immunogens

As shown in Figure 2, the two amino groups on pyrimidine ring of TMP were easy to conjugate with carrier proteins via GA method. Therefore, the immunogen and coating antigen were synthesized through the same method for screening a homogenous mAb. In our previous work, the homogenous mAb possessed an IC\(_{50}\) of 4.14 µg L\(^{-1}\) for ic-ELISA. To improve the sensitivity of mAb, heterogenous haptens were considered for synthesis of coating antigen. Because that the amino groups were chosen to conjugate with carrier protein, the left section (benzene ring with three methoxyl groups) of TMP molecular structure was much exposed. Therefore, the mAb was inclined to recognize the left section. We concluded that the heterogenous hapten should contain the same structure to the left section of TMP. Depended on these opinions, three chemicals, all

![Figure 2. The structures of TMP and three heterogenous haptens.](image)
of them possess benzene ring with three methoxyl groups and a carboxyl group, were chosen to conjugate with the carrier protein through the carboxyl group. The spectra of the coating antigen were shown in Figure 3, which indicated the success of the conjugation. The difference among three haptens was the length of the chain which ended up with a carboxyl group. As the results showed, the H2 was the best hapten.

Cross-reactivity

In our previous work, the homogenous mAb was specific to TMP. The reason was that the benzene ring with methoxyl groups were exposed and considered as the antigenic determinant. In this experiment, all three heterogenous haptens contain the same structure of a benzene ring with three mexyol groups, which is a part of TMP structure. In theory, based on the structure of coating antigen, the mAb should be inclined to recognize the benzene ring of TMP. In other words, the mAb only recognize the different part between TMP and diaveridine, but not the common pyridine structure. Therefore, as shown in Table 1, the heterogenous mAb also was specific to TMP.

Optimization of GICA

Because GNPs (negatively charged) and mAb (positively charged) conjugate via electrostatic interaction, influenced by charge, the pH of colloidal gold was crucial. $\text{K}_2\text{CO}_3$ was
used to adjust the pH of colloidal gold. Finally, BSA or PEG was used to stabilize GNP–mAb conjugates.

Different concentrations of coating antigen (0.03, 0.1, 0.3, and 1 µg mL\(^{-1}\)), mAb (0.125, 0.25, 0.5, and 1 µg mL\(^{-1}\)), and GNPs (2, 4, and 8 nM) were evaluated to screen the optimal conditions for GICA. It proved that 0.3 µg mL\(^{-1}\) of coating antigen, 0.25 µg mL\(^{-1}\) of mAb, and 4 nM of GNPs were the best conditions.

**Analysis of milk and honey samples spiked with TMP**

The milk samples and honey samples were diluted 10 and 20 times, respectively, to eliminate matrix interference. The assay was simple and rapid even for untrained personnel. With concentration of TMP spiked in samples increasing, the T line becomes paler than the C line until becoming colorless and disappearing. Figure 4 shows the results for milk and honey samples. The vLOD of GICA for milk and honey samples were 10 µg L\(^{-1}\) and 15 µg kg\(^{-1}\). Figure 5 shows the standard curves of GICA for TMP spiked in milk and honey samples. The assay can improve the fast screening of TMP residue.

| Compound          | \(IC_{50} \) (µg L\(^{-1}\)) | CR (%) |
|-------------------|-------------------------------|--------|
| TMP               | 1.98                          | 100    |
| Diaveridine       | >20                           | <10    |
| Sulfadiazine      | >20                           | <10    |
| Sulfamethazine    | >20                           | <10    |
| Sulfadimethoxine  | >20                           | <10    |
| Sulfathiazole     | >20                           | <10    |
| Sulfamethoxazole  | >20                           | <10    |
| Sulfamethizole    | >20                           | <10    |

Figure 4. The images of GICA for TMP spiked in PBS, milk samples, and honey samples. The concentration of TMP in 0.01 M PBS and milk sample was 0, 0.5, 1, 2, 5, 10 µg L\(^{-1}\) and in honey samples was 0, 1, 2, 5, 10, 15 µg L\(^{-1}\). (A) and (B): 1 = 0 µg L\(^{-1}\), 2 = 0.5 µg L\(^{-1}\), 3 = 1 µg L\(^{-1}\), 4 = 2 µg L\(^{-1}\), 5 = 5 µg L\(^{-1}\), 6 = 10 µgL\(^{-1}\); (C) 1 = 0 µg kg\(^{-1}\), 2 = 1 µg kg\(^{-1}\), 3 = 2 µg kg\(^{-1}\), 4 = 5 µg kg\(^{-1}\), 5 = 10 µg L\(^{-1}\), 6 = 15 µg kg\(^{-1}\).
Conclusions

A heterogenous specific mAb against TMP was developed, and a GICA for TMP residues in milk and honey samples was established based on the mAb. The semiquantitative assay can be completed in 5–10 min and evaluated by the naked eye, improving the rapid and high-throughput detection for TMP residues in food. Undoubtedly, the establishment of a GICA for TMP residue will greatly assist with the surveillance carried out by a company or authority and protect consumer rights.

Disclosure statement

No potential conflict of interest was reported by the authors.

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