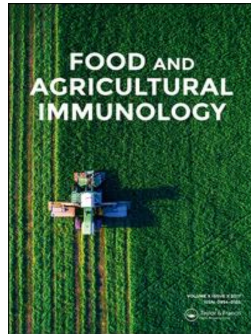

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Development of an immunochromatographic strip assay based on a monoclonal antibody for detection of cimaterol

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ABSTRACT

An anti-cimaterol (cima) monoclonal antibody, 2H8, was prepared and used to develop an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) and an immunochromatographic test strip assay for the detection of cima, a newly described β-agonist. The half-maximal inhibitory concentration of the antibody was 1.56 ng/mL, and no cross-reactivity with other β-adrenergic agonists was observed. The antibody's limit of detection was 0.416 ng/mL, and its linear range of detection was 0.416–5.868 ng/mL. The recovery rates of cima in swine urine ranged from 86% to 92.5%, and the test strip had a cut-off value of 10 ng/mL. Both ic-ELISA and the developed strip assay are effective when detecting cima in swine urine.

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KEYWORDS
Cimaterol; indirect competitive enzyme-linked immunosorbent assay (ic-ELISA); immunochromatographic test strip; urine

Introduction

Cimaterol (cima) is a new generation of β-agonists, following ractopamine (Needham, Hoffman, & Gous, 2017), clenbuterol and salbutamol. On the one hand, β-agonists relax airway smooth muscle and are the most important bronchodilators. Recently, β-agonists have been reported to regulate cardiovascular and cerebrovascular diseases (Noh et al., 2017). These molecules have been widely studied and used in the treatment of chronic airway diseases such as bronchial asthma (Nabil, Elessawy, Hosny, & Ramadan, 2014; Ura, Tanaka, Takahashi, Yamazaki, & Fujimoto, 2016). On the other hand, β-agonists are often illegally added into the feed of food animals to increase the lean meat (Karamolegou, Dasenaki, Belessi, Georgakilas, & Thomaidis, 2018; Li et al., 2018) and to confer advantages such as increased feed efficiency and decreased time to market (Vandenberg, Leatherland, & Moccia, 2015). However, these drugs may remain in the edible parts of those animals, and their residues may threaten human health. Some symptoms caused by these drug residues in meat products have been reported, for example, headaches, dizziness, muscle tremors, and nausea (Junhua et al., 2015; Peng et al., 2017; Zvereva,

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Shpakova, Zherdev, Xu, & Dzantiev, 2018). Cimaterol, as a β-agonist, has all of the advantages and disadvantages stated above. Furthermore, use of all β-agonists including cimaterol as additives for animal feed is prohibited in China (Li et al., 2015). Therefore, establishing a rapid and effective risk assessment for cimaterol is imperative. In our previous study, we established an immunochromatographic strip based on the monoclonal antibody 3G10 for the rapid detection of ten β-agonists; this strip had a cut-off value of 20 ng/mL for cimaterol and showed stability in detecting swine urine samples (Rui et al., 2017).

Several methods have recently been used to detect cimaterol concentrations, such as molecularly imprinted technology (Zhang, Huang, et al., 2016), high-performance liquid chromatography, liquid chromatography-tandem mass spectrometry electrochemical detection (Ding, Qi, & Suo, 2013), ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry (Zhang, Hua, et al., 2016), LC-MS/MS (Mastrianni, Metavarayuth, Brewer, & Wang, 2018), and immunological methods. Instrumental detection methods have the advantage of high accuracy (Montes Nino et al., 2017), but their disadvantages, such as high cost and time-consuming procedures, cannot be ignored.

Compared with instrumental detection methods, the enzyme-linked immunosorbent assay (ELISA) has the advantages of convenience, low cost, and sensitivity (Chen et al., 2017; Zeng, Jiang, Liu, Song, & Kuang, 2017).

Because ELISA is based on antigen–antibody reactions and enzyme-catalyzed reactions, it has high specificity and sensitivity (Chen, He et al., 2019; Dezhao et al., 2019), thus making it a preferable method for rapid determination of β-agonists in food samples. Furthermore, because of its ease of operation, rapidity, and lack of requirements for instruments, lateral-flow immunochromatographic assay (ICA) strips are a widely used tool for on-site screening(Chen, Huang et al., 2019; Fang et al., 2019).

In this study, we aimed to establish an indirect competitive ELISA based on a specific monoclonal antibody (mAb) against cimaterol followed by a colloidal gold immunoassay test strip. The mAb is stable in swine urine and therefore is suitable for use in a strip to detect cimaterol in urine samples.

Materials and methods

Chemicals and apparatus

Cimaterol, salbutamol, clenbuterol, ractopamine, brombuterol hydrochloride, and penbuterol were obtained from J&K Scientific (Shanghai, China). Bovine serum albumin (BSA; MW, 67,000), ovalbumin (OVA; MW, 43,000 Da), keyhole limpet hemocyanin (KLH; MW, 5,000,000 Da), incomplete Freund's adjuvant, complete Freund's adjuvant, Tween-20, enzyme-immunoassay-grade horseradish peroxidase (HRP), HRP-labeled goat anti-mouse immunoglobulin G antibody, 3,3′,5,5′-tetramethylbenzidine, and gelatin were were purchased from Sigma Chemical Company (St. Louis, MO, USA). RPMI-1640 cell culture medium, hypoxanthine–aminopterin–thymidine supplement, hypoxanthine–thymidine supplement, and fetal calf serum were purchased from Gibco BRL (Paisley, UK). All were of analytical or high-performance liquid chromatography grade.

The materials used for the strips were from Shanghai Jieyi Biotechnology Co., Ltd. (Shanghai, China), including Ahlstrom 8964 test paper for the conjugate pad, a membrane for immobilizing the coating antigen, a glass fibre membrane for the sample pad, and nitrocellulose (NC) membrane and H5076 filter paper for the absorbent pad. Trisodium citrate, chloroauric acid, and trehalose were purchased from Sigma Chemical Company.

The following instruments were used in this study: UV/Vis scanner (Bokin instruments, Tsushima, Japan), water bath (Shanghai Instrument Group Co., Ltd, Supply & Sales Co., Shanghai, China), Waters MALDI Synapt Q-TOF MS (Waters, Shanghai, China), vortex machine (Shanghai Huxi Analysis Instrument Factory Co., Ltd, Shanghai, China), membrane dispenser (Xinqidian Gene-Technology Co. Ltd, Beijing, China), and Multiskan MKS microplate reader (Thermo Labsystems Company, Beijing, China).

Solutions

Solutions used in this study are as follows: the coating buffer was 50 mM carbonate bicarbonate (CB, pH 9.6), the blocking buffer was CB with 0.2% gelatin, the assay buffer was phosphate-buffered saline (PBS; 0.01 M phosphate, pH 7.4), the washing buffer (PBST) was 0.01 M PBS with 0.05% Tween-20 (v/v); the colour buffer was a mixture of solution A (0.3% H_2O_2, v/v in phosphate buffer) and solution B (0.01% 3,3′,5,5′-tetramethylbenzidine, w/v in glycol) at a 5:1 (v/v) ratio; and the stop solution was 2 M sulfuric acid. Urine was obtained from a local pig farm (Wuxi, China).

Antigen preparation

According to Wang, Guo, Liu, Kuang, and Xu (2018), the hapten was conjugated with carrier protein to prepare the complete antigen. The immunogen and coating antigen (cima-Dia-KLH) were directly coupled to a protein (BSA, KLH, or OVA) through the diazobenzidine method. First, the cima hapten was weighed into 1 mL of N, N-dimethylformamide solution with 40 μL of 1 M hydrochloric acid according to the reaction ratio. The mixture was then stored at 4°C for 30 min. Subsequently, 5 μL of 30% sodium nitrite was added into the mixture and stirred at 4°C for 1 h in the dark. This solution was designated solution I. The protein (10 mg; BSA, KLH, or OVA) was dissolved in 1 mL of 0.1 M CB (pH 9.6). Solution I was added to the protein solution of BSA, KLH, or OVA and stirred for 4 h in the dark. The conjugate was dialyzed against 0.01 M PBS for 2 days, during which the dialysate was changed four to six times. Antigens as prepared above were stored at −20°C before use. All conjugates were characterized by UV/Vis spectroscopy.

Monoclonal antibody preparation

Female BALB/c mice (6–8 weeks of age) were immunized with 0.1 mg immunogen mixed with complete Freund's adjuvant at the ratio of 1:1 (v/v) per mouse through subcutaneous multisite injections, and booster immunizations were administered after 1 month (Guo et al., 2019; Lei, Xu, Song, Liu, & Kuang, 2018). Subsequently, booster immunizations were given over a period of 3 weeks. Each mouse was injected with incomplete Freund's adjuvant mixed with immunogen (50 μg per mice). After the third immunization, tail blood samples were collected 6–8 days after each booster and examined by

indirect competitive ELISA (ic-ELISA) (Guo et al., 2018). Here, we selected the mouse with the highest inhibitory rate and titre and then gave it a final immunization with 25 μg of immunogen through intraperitoneal injection on day 21. After 3 days, spleen cells from the immunized mouse were fused with Sp2/0 myeloma cells to produce hybridoma cells (Wang et al., 2018). After three rounds of subcloning and selection, the eligible cell lines were expanded and injected into BALB/c mice to produce ascites. The ascites were purified by caprylic acid and ammonium sulfate precipitation, and then dialyzed against PBS for 72 h at 4°C, with four to six changes of dialysate (Li, Liu, Song, & Kuang, 2017). The antibodies were stored at −20°C before use, and their concentrations were measured with UV spectrophotometry. All animal studies in this work were performed according to institutional ethical guidelines and were approved by the Committee on Animal Welfare of Jiangnan University.

Characterization of mAbs

An ic-ELISA was used to screen for the mouse sera with the highest affinity and sensitivity for cimaterol. The bimodal matrix method was used to evaluate the mouse sera in this study. After dilution with coating buffer, the coating antigen (cima-BSA) was added into 96-well plates with 100 μL per well and incubated at 37°C for 2 h. After the plates were washed three times, blocking buffer was added to the plates at 200 μL per well and incubated at 37°C for 2 h. After the plates were washed, they were placed in a 37°C incubator for 30 min to dry. The dried plates were kept at 4°C before use. Then 50 μL/well standard and diluted monoclonal antibody was added to the wells and incubated at 37°C for 30 min. After the plates were washed and patted dry with absorbent paper, 100 μL/well of goat anti-mouse HRP (1:3000 in PBST with gelatin) was added. The plates were incubated at 37°C for 30 min and then washed three times and patted dry again. Substrate solution (100 μL) was added to each well and incubated at 37°C for 15 min in the dark. The reaction was terminated by the addition of 50 μL/well stop solution. The absorbance at 450 nm of each well was measured with a microplate reader. A standard curve of mAb 2H8 against cimaterol concentration is as shown.

Cross-reactivity

Cross-reactivity (CR) was used to determine the specificity of the mAb. Salbutamol, clenbuterol, ractopamine, cimaterol, brombuterol hydrochloride, and penbuterol were tested with an ic-ELISA (Kong, Liu, Song, Kuang, & Xu, 2017).

$$\mathrm{CR}(\%) = \left(\frac{\mathrm{IC}_{50}\,\text{of cima}}{\mathrm{IC}_{50}\,\text{of the tested analogue}}\right) \times 100\%.$$

Screening CIMA-negative pig urine samples

In this study, we aimed to produce a high quality product meeting the requirements of the market. Therefore, the product was required to have sensitivity and stability in the presence of pig urine. Cima-negative urine samples were collected from slaughterhouses in

Wuxi and Nanjing and used for testing. The pig urine samples were sent to the Jiangsu Province Entry–Exit Inspection and Quarantine Bureau (Nanjing, Jiangsu) for testing, and a Beta-Agonists ELISA test kit (Randox Food Diagnostics, Crumlin, UK) was used to screen the samples for cima-negative pig urine. The urine was purified by centrifugation (8000 rpm, 10 min) and stored at −20°C before analysis. Different concentrations of cima standard were added to the urine, and the ic-ELISA was used to analyse the effectiveness of the mAb in urine detection.

Preparation of gold nanoparticles

The preparation of colloidal gold-labeled mAbs by the reduction of gold chloride with sodium citrate has been previously described (Fujii, Uto, Nomura, & Shoyama, 2017). First, 200 mL of 0.01 g/L chloroauric acid was heated to boiling under stirring at 300°C. Subsequently, 2 mL of freshly prepared trisodium citrate (1%, w/v) was added under constant stirring. After 1 min, the solution, whose colour had become wine red, was boiled for 15 min, and its volume was adjusted to 100 mL with ultrapure water. Finally, The solution was cooled to room temperature and stored at 4°C for subsequent use (Wu, Wu, Zheng, Xu, & Kuang, 2018).

Preparation of colloidal gold-labeled mAbs

After purification, the mAbs was mixed with K_2CO_3 (0.1 M) and a colloidal gold solution, and stirred at room temperature for 2 h. Then 100 mL of BSA (10%) was added, stirred for 2 h, and then centrifuged (5100 × g, 20 min). BSA aqueous solution (100 mL, 10%) was added to block the free colloidal gold, stirred for 2 h, and then centrifuged (5100 × g, 20 min). The precipitate was collected, reconstituted into 1 mL ultrapure water and stored at 4°C (Guo et al., 2015).

Immunochromatographic strip preparation

Five parts comprising a polyvinyl chloride backsheet, sample pad, absorbent pad, conjugation pad (glass fibre), and NC film constituted the “dry” immunochromatographic strip. Appropriate concentrations of goat anti-mouse immunoglobulin G and coating antigen were sprayed onto the NC membrane, then dried at 37°C to form a control line (C) and a test line (T). The end of the C wire was connected to the adsorption pad, the end of the T wire was connected to the sample pad, and then strips of 3.8 mm were cut and sealed at room temperature for storage.

Immunochromatographic strip assay

After the liquid sample was added to the sample pad, it migrated upward via capillary action. If the T and C lines became red after several minutes, the sample was considered negative. If the C line was darker than the T line, the sample was considered positive.

Recovery

In this study, different concentrations of cima standards were added to the negative urine according to the half-maximal inhibitory concentration (IC_{50}) value and linear range of the mAb, and the measured values were calculated from the standard curve.

The equation for the recovery rate was as follows:

$$C\ (\text{recovery rate}) = \frac{\text{detected concentration}}{\text{added concentration}} \times 100\%.$$

When 80% < C < 120%, the antibody was suitable for detecting actual samples.

Results and discussion

Conjugated antigen preparation

Cima is not able to evoke an immune response, owing to its small molecular weight. Because of their stronger immunogenicity, BSA and KLH were used to prepare the immunogen. Conversely, given its weaker immunogenicity, OVA was chosen as the carrier protein for coating antigen preparation. We coupled KLH with cima to obtain the immunogen, and BSA with cima to prepare the coating antigen. Identification of each complete antigen was performed by UV spectrophotometry.

The cima complete antigen UV spectrum is shown in Figure 1. Cima had an absorption peak near 320 nm, and the proteins KLH and BSA showed characteristic peaks at 280 nm; the coupled products were in the wavelength range of 280–300 nm. The absorbance values and peak shapes changed significantly. An absorption peak was observed for the diazo bond at 340 nm with the antigen produced with the diazotization method. As is shown in Figure 1(a), one of the characteristic peaks of KLH was at 340 nm; this was the same absorption peak as the diazo bond, but the absorption peak of the coupling product was much larger at the same concentration.

In the characterization diagram of the immunogen of Figure 1(a), the absorbance of the coupled product at 280 nm was significantly enhanced, and as the reaction ratio increased

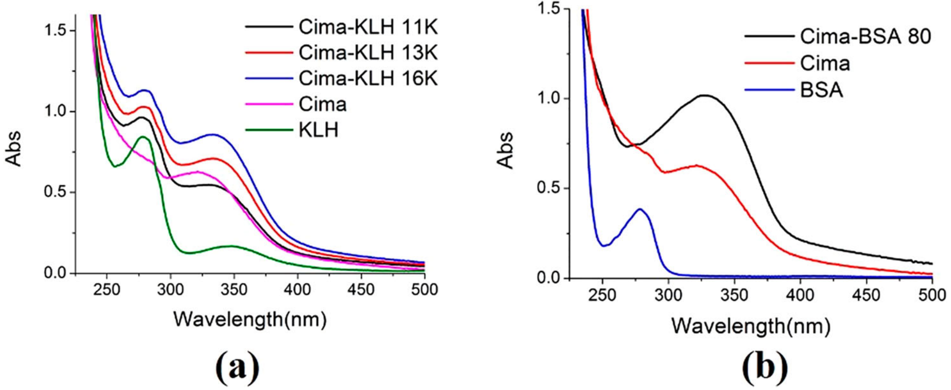

Figure 1. The ultraviolet-visible absorption spectra of artificial antigen: (a) Cima-Dia-KLH 11 K/13 K/16 K; (b) Cima-Dia-BSA 80.

from 11k to 13k, the enhancement increased. Under the influence of the cima small molecule, the absorption peak at 280 nm showed a slight redshift. In Figure 1(b), owing to the dual action of cima and the diazo peak, the peak of the coupled product (cima-Dia-BSA 80) showed a significant redshift. The above findings indicated that the complete antigen preparation was successful.

Preparation and sensitivity of mAbs

After immunizing mice with the above immunogen five times, we obtained high titre polyclonal serum. After urine sample tests, mice with IC_{50} values of 15 µg/L were selected, and their spleen cells were hybridized with Sp2/0 murine myeloma cells. After three rounds of subcloning, strains capable of producing antibodies with high titres and high sensitivity and rapid growth – cima-2H8, cima-2D6, cima-3D2, and cima-4G8 – were obtained. The cell line cima-2H8 was used for immunochromatographic strip assays (Figure 2).

Cross-reactivity

CR of mAbs with other similar substances was determined by indirect ELISA to determine the specificity of the antibody. A CR <10%, was considered to be specific. We measured the CR with salbutamol, clenbuterol, ractopamine, bromobuterol, and penbutolol (Table 1). Cima-2H8 was found to be sensitive only to cima.

Cimaterol detection with immunochromatographic strip assays

As shown in Figure 3, of the four cell lines (2H8, 2D6, 3D2 and 4G8) obtained in this study, 2H8 was the best and was used in the next experiment.

The optimized cima test strip comprised 1 mL of gold nanoparticles containing 4 µL of K_2CO_3 and 8 µg/mL of antibody at a coating concentration of 0.8 mg/mL. The results

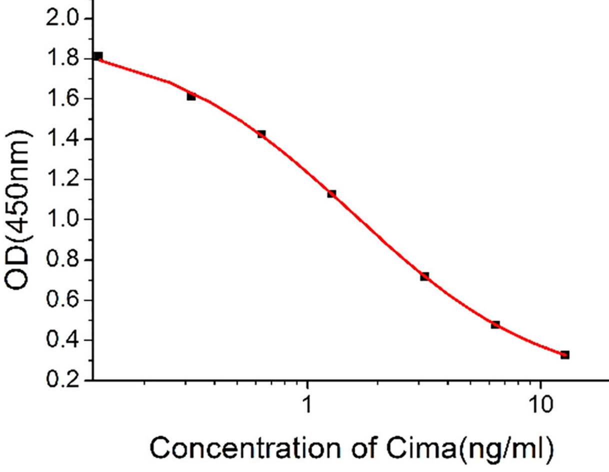

Figure 2. Standard curve obtained by indirect competitive ELISA based on antibody 2H8.

Table 1. Cross-reaction results of mAb 2H8.

Chemicals	Structure	IC_{50} (ng/ml)	Cross-reaction (%)
Cimaterol		1.56	100
Clenbuterol		>150	<1.0
Ractopamine		>150	<1.0
Salbutamol		>150	<1.0
Brobuterol		>150	<1.0

obtained with the dry colloidal gold immunoassay test paper based on mAb cima-2H8 are shown in Figure 4. Cima was spiked into negative pig urine samples to produce positive samples with different concentrations (0, 0.25, 0.5, 1, 2.5, 5, and 10 ng/mL). As the standard concentration increased, the T-line colour weakened and disappeared completely at a cima concentration of 10 ng/mL, consistent with current market standards. Furthermore, the strip we developed here can specifically identify cimaterol and have no cross-reaction with other β-agonists.

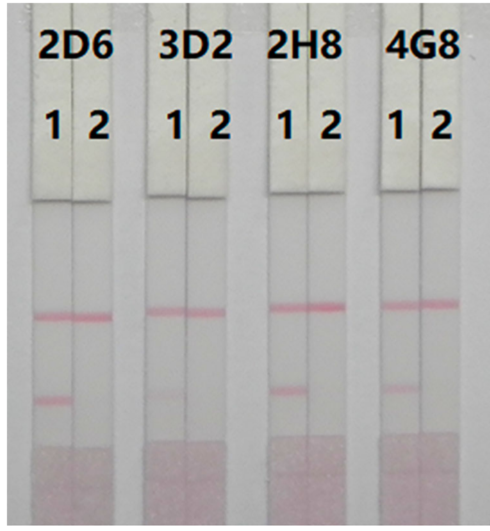

Figure 3. The detection of four anti-cima cell lines 2H8, 2D6, 3D2 and 4G8 by strip in PBS sample (1 = 0 ppb,2 = 10 ppb).

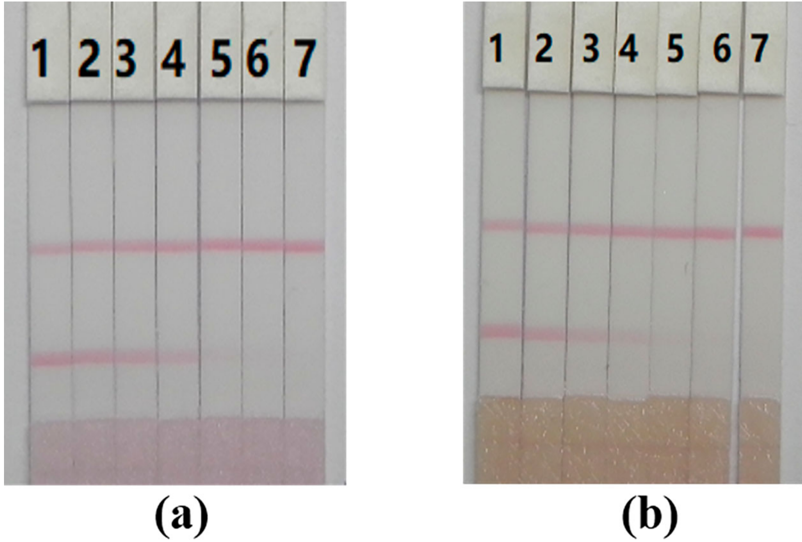

Figure 4. Cima detection by immunochromatographic strip in PBS sample (a) and swine urine sample (a) 1 = 0 ng/mL, 2 = 0.25 ng/mL, 3 = 0.5 ng/mL, 4 = 1 ng/mL, 5 = 2.5 ng/mL, 6 = 5 ng/mL and 7 = 10 ng/mL; The cut-off value for cima was 10 ng/mL. (b) 1 = 0 ng/mL, 2 = 0.25 ng/mL, 3 = 0.5 ng/mL, 4 = 1 ng/mL, 5 = 2.5 ng/mL, 6 = 5 ng/mL and 7 = 10 ng/mL; The cut-off value for cima was 10 ng/mL.

Table 2. Recovery of cima in swine urine by ic-ELISA ($n = 3$).

Samples	Spiked level (ng/mL)	Detection level (ng/mL) Mean ± SD	Recovery rate (%)	CV (%)
Swine urine	0.5	0.43 ± 0.03	86.0 ± 2.0	6.9
	1	0.91 ± 0.05	91.0 ± 5.0	5.5
	1.5	1.37 ± 0.07	91.0 ± 5.0	5.1
	2	1.85 ± 0.11	92.5 ± 5.5	5.9
	3	2.74 ± 0.10	91.3 ± 3.3	3.6

Recovery test with ic-ELISA

Cima at concentrations of 0.5, 1, 1.5, 2, and 3 ng/mL was added to the negative pig urine samples and analysed by ic-ELISA (Table 2). The recovery rate was 86–92.5%, and the coefficients of variation were 3.6–6.9%, thus indicating that the analytical method established with mAb cima-2H8 has good stability and accuracy and can be used for the analysis and detection of actual pig urine samples.

Conclusions

In our study, complete antigens, including immunogens and coating antigens, were obtained by coupling cima directly to proteins. Construction of hybridoma cells yielded a mAb against cima with an IC_{50} of 1.56 ng/ml and a linear range of 0.416–5.868 ng/mL. On the basis of this mAb, we developed a colloidal gold test strip with a cutoff value of 10 ng/ml in swine urine. mAb cima-2H8 showed little CR with other β-agonists, thus indicating high specificity of the antibody. The test strip does not require complex sample pretreatment before testing, thereby shortening the detection time and making this method suitable for rapid detection of cima residues.

Disclosure statement

No potential conflict of interest was reported by the authors.

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