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Development of a colloidal gold-based lateral-flow immunoassay for the rapid detection of Phenylethanolamine A in swine urine

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Running title: **colloidal gold-based lateral-flow immunoassay of Phenylethanolamine A**

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Abstract

Phenylethanolamine A (PEAA) is a new emerged phenethanolamine member of the family of β-adrenergic agonists illegally used as feed additives for growth promotion. In this study, a highly sensitive and specific lateral-flow immunochromatographic assay (LFIA) using colloidal gold-labeled monoclonal antibody was developed for the rapid detection of PEAA. The assay procedure could be accomplished within 10 min, and the result of this qualitative one-step assay was evaluated visually according to whether test lines appeared or not. When applied to the swine urines, the half maximal inhibitory concentration (IC_{50}), the detection limit (LOD) and limit of quantification (LOQ) of the test strip under an optical density scanner were calculated to be 0.52 ± 0.11 ng mL^{-1}, 0.188 ng mL^{-1} and 0.263 ng mL^{-1}**,** respectively. The cut-off value of PEAA with the naked eyes was 2.7 ng mL^{-1}. The specificity of the assay was evaluated by the measurement of cross-reactivity (CR) of the monoclonal antibody with PEAA, PEAA-NH_2 and 11 other β-adrenergic agonist compounds. Studies indicated that the monoclonal antibody was highly specific for PEAA and PEAA-NH_2, with negligible cross-reactivity with other β-adrenergic agonists including ractopamine (CR is 0.52%). To investigate accuracy and precision of the assay, swine urine samples were fortified with PEAA at different concentrations and analyzed by using the test strips with the scanner. Acceptable recovery rates of 92-102% and the intra- and inter-assay coefficients of variation (CV) of 8.70-17.65% were achieved. Parallel analysis of spiked swine urine samples with PEAA showed comparable results obtained from the lateral-flow test strip and LC-MS/MS. There was an

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acceptable correlation coefficient of 0.9789 between the two methods. Therefore, the described lateral-flow test strip could be used as a reliable, rapid and cost-effective on-site screening technique for the determination of PEAA residue in swine urine.

Keywords: Phenylethanolamine A; lateral-flow immunochromatographic assay (LFIA); colloidal gold; monoclonal antibody; urine sample

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1. Introduction

β-adrenergic agonists are synthetic phenethanolamine compounds which enhance animal growth and increase feeding efficiency by inhibiting fat synthesis, stimulating lipolysis, increasing protein synthesis and carcass leanness.[1-4] However, the misuse of the growth promoters can lead to the excessive residues in edible meat or tissue. Consumption the food contaminated by β-adrenergic agonists can cause acute intoxication of cardiovascular system, nervous system and respiratory system and has adverse effect on human health.[5-7] There was an increasing concern of the hazards posed to human health by the presence of β-adrenergic agonist residues in animal tissues.[8] Therefore, β-adrenergic agonists except ractopamine, which had been approved as a feed additive for swine and cattle in the United States and some other countries, are now banned as feed additives for growth promotion in food animals in China, the United States and most European countries. Recently a new β-adrenergic agonist named phenylethanolamine A appeared in China as the alternative of common β-adrenergic agonists in order to escape from the supervision .[9-12]

Phenylethanolamine A [PEAA, 2 - (4 - (nitrophenyl) butan -2 - ylamino) - 1 - (4-methoxyphenyl) ethanol, $C_{19}H_{24}N_2O_4$, MW=344.17, Fig.1] was validated to be a phenethanolamine member of the family of β-adrenergic agonists. It was prohibited from being used in animal feeds and drinking water in China since 2010. [13] To reduce the potential risk of PEAA residues for human health and monitor the illegal use of PEAA, the Ministry of Agriculture of China issued a standard analytical

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method for the detection of PEAA in feed using high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) in 2010.[14] Recently some chromatographic analytical methods including liquid chromatography tandem mass spectrometry (LC-MS/MS) and high performance liquid chromatography (HPLC) were developed for the detection of PEAA in biological and feed samples.[15-18] Although chromatographic analytical methods are accurate, they are expensive, time-consuming and required personnel with professional training to operate the sophisticated instruments. Therefore, there is an urgent need to develop sensitive, specific, rapid and low-cost screening methods for the detection of PEAA residue. The screening methods were often immunoassays, including enzyme-linked immunosorbent assay (ELISA), lateral-flow immunoassay (LFIA) and so on. ELISA and LFIA had been intensively applied for the detection of β-adrenergic agonists over the past twenty years.[19-42] In the last three years, the ELISA screening method based on the polyclonal and monoclonal antibodies were also developed for the detection of PEAA in urine, tissue and feed samples.[9-12]

The use of membrane based lateral-flow immunoassay tests for on-site screening provides a simple, low-cost, sensitivity, specificity and user-friendly alternative to expensive, laborious and time-consuming instrumental methods and more sophisticated immunoassay formats.[37-42] The primary aim of this paper was to develop a lateral-flow colloidal gold-based technique for the detection of PEAA residues in swine urine.

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2. Materials and methods

2.1 Reagents

Bovine serum albumin (BSA), goat anti-mouse IgG, Tween 20, PEG-20000, Gold(III) chloride trihydrate (ACS reagent), polyvinyl alcohol, sodium azide, EDTA and sucrose (no. S9378) were purchased from Sigma Co. (St. Louis, MO, USA). PEAA was supplied by Hangzhou DNA Sci-Tech Co. (Hangzhou, China). Ractopamine, formoterol, clenbuterol, salbutamol, terbutalin, cimaterol, cimbuterol, clorprenaline, bambuterol, tulobuterol and zilpaterol were purchased from Dr. Ehrenstorfer Co. (Augsburg, Germany). HPLC-grade formic acid, methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Hi-Flow Plus 180 membrane from Millipore (Bedford, MA, USA), conjugate pad grade 8964 and absorbent pad type 133 from Pall (Saint Germain-en-Laye, France), glass fiber grade F075-17 from Whatman (Maidstone, Kent, England) were used. Ultrapure water was generated from a NANO pure system (Thermo, USA).

2.2 Monoclonal antibody to PEAA and coating antigen

Monoclonal antibody 2H8 (IgG2a/κ) specific for PEAA was obtained by immunizing mice with PEAA-BSA as described previously.[11] The monoclonal antibody was purified from ascites using the protein A affinity column (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's manual. The coating antigen of the PEAA-OVA conjugate was prepared by the diazotization method as described previously.[11] Experimental procedures were carried out strictly in accordance with the "Administrative Rules for Laboratory Animals in Zhejiang

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67 Province" (2009), and was approved by Animal Care and Use Committee of
68 Hangzhou Normal University (Hangzhou, China). All efforts were made to minimize
69 the animals' suffering and to reduce the number of animals used.

70 **2.3 Preparation of standard solutions and swine urine samples**

71 Standard solutions of PEAA, PEAA derivative (PEAA-NH_2) and other β-adrenergic
72 agonists were prepared by diluting stock solutions of these compounds (1 mg mL^{-1},
73 in methanol, store at -20 °C). PEAA and derivative stock solutions were diluted in
74 normal swine urine, which was determined to be the negative content of β-adrenergic
75 agonists by LC-MS/MS, at 0, 0.03, 0.10, 0.30, 0.90, 2.70, and 8.10 ng mL^{-1} and other
76 β-adrenergic agonists of ractopamine, formoterol, clenbuterol, salbutamol, terbutalin,
77 cimaterol, cimbuterol, clorprenaline, bambuterol, tulobuterol and zilpaterol at 10, 25,
78 50, 100, 250, 500, 1000, 2000, 4000, 8000 ng mL^{-1}.

79 Swine urine samples were collected manually in glass vials and stored at -20 °C
80 from several locally small farms where swine with mix of genders such as Duroc,
81 Landrace and Yorkshire were bread and fed in Zhejiang Province, China.

82 **2.4 Instruments**

83 Centrifugation was carried out with a Meafuge 11R centrifuge (Thermo, USA).
84 UV-vis data were recorded on a UV-4802S spectrophotometer (Unico, China). The
85 conjugate pads and membrane were spotted on them by using a Quanti 3000 Biojets
86 attached to a XYZ Bioatrip Dispenser (Bio-Dot, CA, USA). The prepared master card
87 was cut into 3.8 mm width strips using a CM 4000 Cutter (Bio-Dot, CA, USA). The
88 test lines were scanned with a BioDot TSR3000 Membrane Strip Reader (BioDot, CA,

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89 USA).

90 **2.5 Colloidal gold-based lateral-flow immunoassay**

91 **2.5.1 Preparation of colloidal gold labeled monoclonal antibody.** Colloidal
92 gold with an average diameter of 40 nm were prepared by controlled reduction of
93 gold chloride with 1% sodium citrate according to the procedure described by Hayat.
94 [43] Briefly, 100 mL of 0.2% gold chloride trihydrate solution in super purified water
95 was heated to boil, and then 1.5 mL of 1% sodium citrate solution was added while
96 stirring. After the color changed from light yellow to brilliant red, the solution was
97 boiled for another 5 min, and then cooled to and stored at room temperature with
98 0.05% sodium azide added.

99 The colloidal gold labeled monoclonal antibody against PEAA (2H8) was
100 prepared as described by Yokota et al. with some modification.[44] Briefly, 1 mL of
101 anti-PEAA mAb at the optimum concentration of 1 mg mL^{-1} was incubated with 10
102 mL of colloidal gold solution (pH 8.9) for 30 min at room temperature. Blocking
103 with 1 mL 10% BSA solution in 0.02 M sodium borate buffer (pH 8.9) at room
104 temperature for another 10 min, the mixture was centrifuged at 4 °C, 20000×g for 30
105 min and then the labeled anti-PEAA mAb washed by repeated centrifugation
106 (20000×g) with 1% BSA in 0.02 M sodium borate buffer (pH 8.9) at 4 °C for 30 min.
107 The precipitates were resuspended with 1 mL PBS (0.05M, pH 7.4) containing 1%
108 BSA and 0.05% sodium azide and stored at 4 °C for use.

109 **2.5.2 Preparation of the conjugate pad.** The conjugate pad (300 × 8 mm) was
110 dispensed with 300 μL of the optimum mixture of colloidal gold labeled anti-PEAA

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111 mAb (300 μL) diluted with 700 μL PBS containing 5.0% (w/v) sucrose, 5.0% (w/v)
112 BSA, 0.8% (w/v) NaCl, 0.1% (w/v) EDTA, 0.3% (v/v) Tween 20 and 0.05% (w/v)
113 sodium azide by using a Quanti 3000 Biojets attached to a XYZ Bioatrip Dispenser.
114 After dispensing, the pad was dried at 37 °C for 2 hrs and then stored in a desiccator
115 at room temperature.

116 **2.5.3 Preparation of the membrane.** Test and control lines were spotted on the
117 Hi-Flow Plus 180 membrane (300×25mm) using a Quanti 3000 Biojets attached to a
118 XYZ Bioatrip Dispenser (Bio-Dot, CA, USA). The test line was separately coated
119 with PEAA-OVA conjugate at the bottom of the membrane. Goat anti-mouse IgG
120 was dispensed on the top of the membrane as the control line. The distance between
121 the lines was 70 mm. The PEAA-OVA conjugate and goat anti-mouse IgG were
122 separately diluted in PBS containing 5% methanol (v/v) to the optimum
123 concentration of 0.6 and 1.2 mg mL^{-1}, respectively, and applied in the form of dots at
124 50 dots/μL/cm to form the test and control lines. After drying at 37 °C for 60 min,
125 the membrane was blocked with PBS (0.05M, pH 7.4) containing 1% (w/v) casein at
126 room temperature for another 60 min. Then the membrane was dried at 37 °C for 2
127 hrs, vacuum-packaged in plastic bag containing silica as moisture absorbent and
128 stored under dry condition at room temperature for use.

129 **2.5.4 Preparation of sample pad and absorbent pad.** Glass fiber grade F075-17
130 from Whatman (Maidstone, Kent, England) was used as the sample pad. The sample
131 pad (300 × 20 mm) was saturated with sodium borate buffer (0.02M, pH 9.2)
132 containing 2.0% (w/v) sucrose, 1.0% (w/v) BSA, 0.8% (w/v) NaCl, 0.2%(w/v)

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polyvinyl alcohol, 1.0%(w/v) PEG20000 and 0.05% (w/v) sodium azide at room temperature for 30 min. Then the sample pad was dried at 37 °C for 2 hrs and stored as described above. The absorbent pad was cut to 300 × 30 mm for use.

2.5.5 Assembly of the test strip. On a plastic baking plate (300 × 80 mm), the conjugate pad was attached to the bottom of the membrane with 1-2 mm overlapping on the membrane, and then the sample pad was attached to the bottom of the conjugate pad in a similar manner. The absorbent pad was attached to the top of the membrane with 1~2 mm overlapping on the membrane also. The prepared master card was cut to 3.8 mm width strips using a CM 4000 Cutter (Bio-Dot, CA, USA). The strips were then sealed in the aluminum foil bag containing desiccant gel and stored under dry conditions at room temperature until use.

2.5.6 Assay procedure and principle. The principle of test strips was illustrated as in Fig.2. The test strips were inserted into 80~100 μL of standard or swine urine samples for 20 s and then put flatwise to allow the liquid to migrate. The specific colloidal gold-labeled anti-PEAA mAb, which was redissolved from the conjugate pad, reacted with PEAA (if it was present in the urine samples). On the mean while, excess of colloidal gold-labeled anti-PEAA mAb was trapped by the PEAA-OVA immobilized on the membrane forming red test line and further trapped by the goat anti-mouse IgG antibody forming the control line while the whole complex were migrating along the membrane. After 10 min, the test result was evaluated visually or test line was scanned with a BioDot TSR3000 Membrane Strip Reader (BioDot, CA, USA).[37, 40] G/Peak and G/D × Area of the relative optical (ROD) decreased as

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155 the PEAA concentration in the standard samples increased. The concentration of
156 PEAA and the ROD (%) produced a sigmoidal dose-response curve that fits to a
157 four-parameter logistic curve pattern indicating the classical competition. The
158 negative test resulted in two red lines (test and control lines). The more PEAA
159 present in the sample, the weaker appeared the test line. The positive sample gave
160 only one red line (the control line). If no control line was present, the test was
161 considered to be invalid.

162 **2.5.7 Immunochromatographic time of the test strip.** The test strips were
163 inserted into 80~100 μL of the blank swine urine sample (0 ng mL^{-1}) and spiked
164 swine urine samples with PEAA at the concentration of 0.15, 0.30, 0.45 ng mL^{-1} for
165 20 s and put flatwise to allow the liquid to migrate for 2~16 min, respectively. And
166 then, the test line of every strip was investigated with a BioDot TSR3000 Membrane
167 Strip Reader.

168 **2.5.8 Sensitivity and specificity of the test strip.** The test strip of PEAA was
169 based on the competitive principle, the inverse relationship between concentrations
170 of PEAA in sample and development of red color on the test lines. Therefore, the
171 sensitivity of the test strip should be determined by testing the PEAA standard
172 samples. The relative optical densities (ROD) decreased as the PEAA concentration
173 in the standard samples increased. Similar to the ELISA assay, the half maximal
174 inhibitory concentration (IC_{50}) with the strip was quantitatively defined as the
175 amount of PEAA in the standard samples that caused 50% decrease of the ROD than
176 that produced by the 0 ng mL^{-1} sample in the present study. By using strip reader, the

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sensitivity of test strip was characterized by IC_{50} value with the PEAA standard concentration range of 0.033-8.1 ng mL^{-1} under optimized conditions.

To evaluate the specificity of the test strip, cross-reactivity (CR) experiments were conducted by measuring the IC_{50} values of PEAA, PEAA-NH_2 and the 11 other β-agonist compounds (ractopamine, fenoterol, clenbuterol, salbutamol, terbutalin, cimaterol, cimbuterol, clorprenaline, bambuterol, tulobuterol and zilpaterol) as competitors. As a quality control, the PEAA calibration curve was generated in every experiment. The CR values were obtained by calculating the ratio of IC_{50} values produced by the competitors and PEAA using the following equation:

$$CR\ (\%) = (IC_{50} \text{ of PEAA}) / (IC_{50} \text{ of competitors}) \times 100\ \%.$$

2.5.9 Fortification experiment of test strip. The colloidal gold immunoassay validation was carried out using the limit of detection (LOD), the limit of quantification (LOQ), the recovery (%) of the fortified PEAA and coefficients of variation (CVs). The 20 blank swine urine samples, obtained by 20 different animals and certified as free of PEAA using the liquid chromatography tandem mass spectrometry (LC-MS/MS) previously, were analyzed in 6 replicates for PEAA by using the test strips with the scanner. The concentrations of PEAA in the blank samples were calculated according to the standard curve (0, 0.033, 0.1, 0.3, 0.9, 2.7, and 8.1 ng mL^{-1}), as well as the mean value for 20 blank urine samples. The LOD and LOQ were calculated as the mean of the measured content of blank different samples (n = 20) plus three standard deviations (mean + 3SD) and six standard deviations (mean + 6SD) (Commission Decision 87/410/EEC), respectively. [25, 40]

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199 To test accuracy and precision of the strip, the blank swine urine samples were
200 spiked with PEAA at the concentrations of 0.25, 0.50, 1.00, and 2.00 ng mL^{-1} and
201 analyzed in 6 replicates by using the test strips with the scanner. Sample recoveries
202 were determined from the standard curve and calculated as the following equation:
203 recovery rate (%) = measured concentration / fortified concentration × 100 %. The
204 precision of the test strip was analyzed by repeated determination of the intra- and
205 inter-assay CVs of the spiked samples at the PEAA concentrations of 0.25, 0.50, 1.00,
206 and 2.00 ng mL^{-1}. Intra-assay variation was calculated as the mean value of six
207 replicates on one single day. Inter-assay variation was determined by analyzing six
208 replicates carried out on three different days.

209 **2.6 LC-MS/MS analysis of PEAA in swine urine**

210 In parallel with the strip tests, LC-MS/MS analysis of PEAA was performed with a
211 Shimadzu HPLC instrument (Shimadzu; Kyoto, Japan) and a Micromass Quattro
212 Premier XE system (Waters; Manchester, UK) equipped with an electrospray
213 ionization (ESI) source in this study. Chromatographic separations were performed
214 on an Acquity BEH C_{18} column (100 mm×2.1 mm, 1.7 μm) maintained at 30 °C.
215 Solvent A (0.1% formic acid) and solvent B (acetonitrile) constituted the mobile
216 phase. The gradient program was set as the follows: 0~1.0 min, 5% B; 1.1~3.0 min, a
217 linear gradient from 5% to 80% B; 3.1~5.0 min, 80% B; 5.1~6.0 min, 5% B. The
218 flow rate during the whole process was 0.30 mL/min and the injection volume was
219 10 μL.

220 Mass spectrometric detection was conducted on a Micromass Quattro Premier XE

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system (Waters; Manchester, UK) equipped with an electrospray ionization (ESI) source. Positive mode and multiple reaction monitoring (MRM) were selected for the detection experiment. The parameters were set as follows: capillary voltage, 3000V; source temperature, 150 °C; desolvation temperature, 400 °C; cone gas (N_2) flow rate, 60 L/h; desolvation gas (N_2) flow rate, 750 L/h; collision cell pressure, 4×10^{3} mbar. The selected MRM transitions for PEAA were m/z 345.3~327.0 and 345.3~150.0 with a dwell time of 250 ms. The transition chosen for quantification was 345.3~150.0. The optimized collision energies for the transitions of 345.3～327.0 and 345.3~150.0 were 20 eV and 33 eV, respectively.

Comparison was made using linear regression analysis with the line modeled having a zero intercept. The resulting correlation coefficients served as measures of assay variability between test strip and LC-MS/MS method, whereas slopes of the correlations served as indicators of differences in assay responsiveness.

3. Results and Discussion

3.1 Optimization of colloidal gold immunoassay for PEAA

The colloidal gold based and competitive immunoassay was developed as a rapid visual qualitative test which gave a simple yes/no response to the levels of target analyte. Therefore, the optimal conditions for the negative test which gave the most intensely red colored test line and the smallest amount of PEAA that resulted in no red color development at the test line should be studied. In addition, the difference between positive and negative samples should be easily distinguished within a

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243 reasonably short immunochromatographic time. For these purposes, the optimal
244 condition experiments for the lateral-flow assay for PEAA were tested similar to the
245 "checkerboard titration" in competitive ELISA (shown in the Electronic
246 Supplementary Information). Using urine samples spiked with PEAA at 0-8.1 ng
247 mL^{-1}, the optimal conditions were selected for the further experiments under the
248 following conditions: PEAA-OVA conjugate and goat anti-mouse IgG
249 concentrations of 0.6 and 1.2 mg mL^{-1}, respectively, forming the test and control
250 lines, 300 μL of the mixture of colloidal gold labeled anti-PEAA mAb (300 μL)
251 diluted with 700 μL PBS dispensing on conjugate pad.

252 In accordance with the upon optimal conditions, the performance of test lines were
253 investigated with a BioDot TSR3000 Membrane Strip Reader to test the
254 immunochromatographic time by using a blank swine urine sample and spiked urine
255 samples with PEAA at 0.15, 0.30 and 0.45 ng mL^{-1}. The relative optical density
256 (ROD) increased simultaneously during 10 min, and not increased obviously after 10
257 min (Fig.3). The results showed after 10 min, almost of colloidal gold-labeled
258 antibodies would bind to the PEAA-OVA and goat anti-mouse IgG coated on the
259 nitrocellulose membrane, if the PEAA levels in the urine samples are negative or
260 below the particular level. At the immunochromatographic time of 10 min, the
261 difference between positive and negative samples could be also easily distinguished
262 with the naked eye. So the immunochromatographic time of 10 min was selected for
263 further experiments.

264 **3.2 Sensitivity of the test strip**

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265 The sensitivity of the test strip was determined by testing the spiked urine samples
266 with PEAA at 0~8.1 ng mL^{-1}. Scanned with the BioDot TSR3000 Membrane Strip
267 Reader, the relative optical densities (ROD) decreased as the PEAA concentrations
268 in the urine samples increased. The relationship between the concentrations of PEAA
269 and the ROD/ROD_0 (%) showed the sigmoidal dose-response curves which fit to a
270 four-parameter logistic curve pattern indicating the classical competition (Fig.4). In
271 the present study, the IC_{50} of PEAA was calculated to be 0.52 ± 0.11 ng mL^{-1}. As
272 compared to the previous reports on the detection of PEAA using ELISA, the IC_{50} of
273 PEAA in this assay was at the similar level.[9-12]

274 The colloidal gold immunoassay was studied as rapid visual qualitative test
275 which gave a simple yes or no response to the levels of the target analytes. The
276 cut-off value with the naked eye was defined here as the amount of PEAA in the
277 standard samples that resulted in no red color development at the test lines. In
278 accordance with visual evaluation, the cut-off value of PEAA was about 2.7 ng mL^{-1}
279 (Fig. 5).

280 In this work, the LOD for PEAA in swine urine samples was 0.188 ng mL^{-1} and
281 the LOQ was 0.263 ng mL^{-1}, which is comparable with that of detection of PEAA
282 using both the LC-MS/MS and ELISA method.[9-12, 16] The LOD and LOQ of the
283 present study could meet the requirement of rapid screening detection for PEAA
284 residues in swine urine samples.

285 **3.3 Specificity of the test strip**

286 The cross reactivity of PEAA test strip with PEAA derivative ($PEAA-NH_2$) and 11

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287 other β-adrenergic agonist compounds were examined at 25 °C. The IC_{50} and the
288 cross-reactivities of the test strip to PEAA, PEAA-NH_2 and other β-adrenergic
289 agonists were analyzed with the four parameter logistic equation and shown in Table
290 1. The result was showed that the anti-PEAA mAb was highly specific to PEAA and
291 its derivative PEAA-NH_2 with IC_{50} of 0.49±0.09 ng mL^{-1} (106%) (n=6). No
292 cross-reactivity of anti-PEAA mAb to other tested β-adrenergic agonists except
293 Ractopamine for concentrations up to 100 ng mL^{-1} was observed. The specificity
294 will reduce the possibility of false positive result.

295 **3.4 Validation**

296 LC-MS/MS analysis, which was considered as one of confirmatory methods for
297 identification and quantification of β-adrenergic agonists, was performed to quantify
298 the amount of PEAA in spiked urine samples with PEAA at the concentrations of 1,
299 5, 10 and 20 ng mL^{-1} and parallel with the strips test. The spiked urine samples were
300 diluted 1: 10 and then analyzed by using the test strip with the scanner.

301 The results of accuracy and precision to the strip was showed in Table 2, the
302 average recoveries ranged from 92% to 102%, the coefficients of variation (CV)
303 ranged from 8.70% to 10.75% for intra-assay and 12.63% to 17.65 for inter-assay .
304 The results indicated that recoveries within 25% of theoretical values and
305 coefficients of variation below 20% were acceptable for screening detection of
306 PEAA residues in swine urine.

307 The values showed in figure were the average of six repeated tests. The
308 correlation coefficient (R^2) for the test strip analysis and the LC-MS/MS analysis of

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309 PEAA in swine urine samples was 0.9789, indicating an acceptable agreement
310 between the two methods for the detection of PEAA (Fig. 6). The slope of the
311 correlation was 0.9219, indicating that the quantitative results of PEAA by
312 LC-MS/MS were little greater than the detected results by the test strip method (Fig.
313 6). The results suggested that the test strip method based colloidal gold based lateral
314 flow immunoassay was reliable for the PEAA detection in swine urine samples,
315 meanwhile, the method offered advantages of sample preparation and high
316 throughput.
317

318 **4. Conclusions**

319 In the present study, we established a colloidal gold-based lateral-flow immunoassay
320 for the rapid detection of PEAA in swine urine by using a monoclonal antibody
321 produced with the immunogen PEAA-BSA conjugate. The assay could be
322 accomplished within 10 min without the need for any sample preparation. By
323 scanning the relative optical density (ROD) of the test lines, this test strip format
324 assay could be quantitatively analyzed in accordance with the mathematical model of
325 RPNA (Qian and Bau, 2004).[45] Similar to the competitive ELISA, the IC_{50},
326 sensitivity, specificity, LOD, LOQ, accuracy and precision of PEAA test strip were
327 easy calculated and analyzed. In the present study, the IC_{50}, LOD and LOQ of the
328 test strip under an optical density scanner were calculated to be 0.52± 0.11, 0.188
329 and 0.263 ng mL^{-1}, respectively. As compared to the previous reports on the
330 detection of PEAA using ELISA, the IC_{50} of PEAA in this assay was at the similar

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331 level.[9-12] Results from visual evaluation of the lateral-flow tests of spiked swine
332 urine samples were shown that the cut-off value of PEAA was 2.7 ng mL^{-1}. The
333 developed assay was shown excellent specificity for the PEAA measurements,
334 because the monoclonal antibody was highly specific for PEAA and PEAA-NH_2,
335 with negligible cross-reactivity with other β-adrenergic agonists. Acceptable
336 recovery rates of 92-102% and the intra- and inter-assay coefficients of variation
337 (CV) of 8.70-17.65% were achieved. Results with test strips and LC-MS/MS
338 analysis for detection of PEAA in spiked swine urine samples proved the reliability
339 of the immunoassay. In conclusion, the described a colloidal gold-based lateral-flow
340 immunoassay format could be used for rapid and cost-effective screening PEAA
341 residues in swine urine samples.

342

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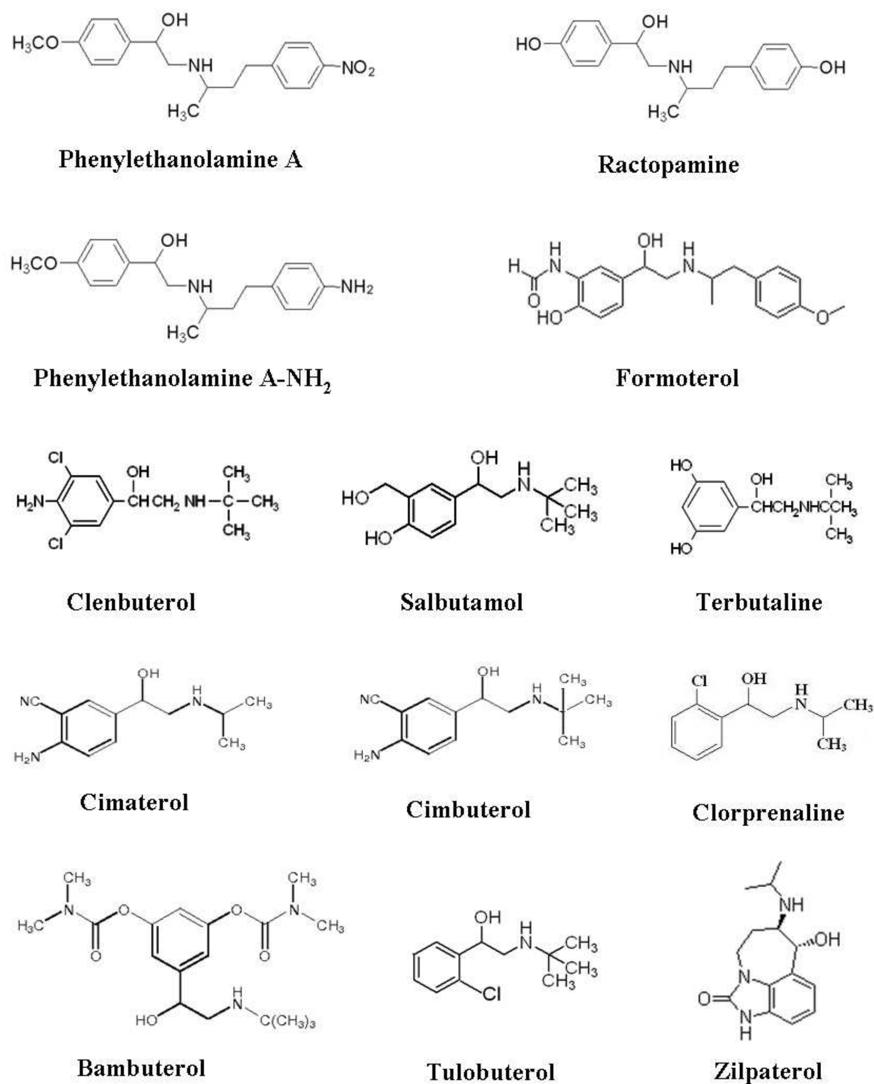

Fig.1 Chemical structures of PEAA, PEAA-NH_2 and other β - adrenergic agonists used in this study.

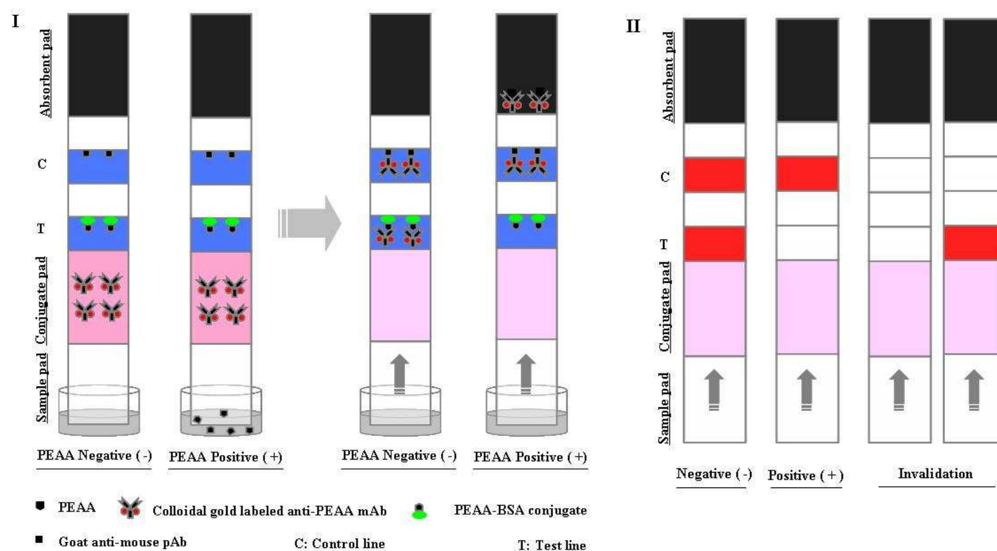

Fig. 2 A schematic diagram of the colloidal gold-based immunochromatographic assay for detection of PEAA. **I:** Model of samples tested. **II:** Model of visual result.

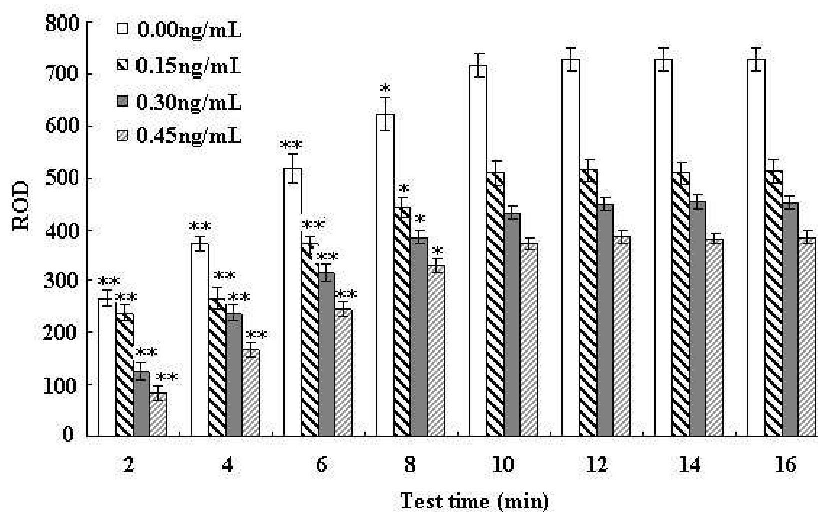

Fig. 3 The mean relative optical density (ROD) of the PEAA at 0, 0.15, 0.30 and 0.45 ng mL^{-1} standard in different time with test strips (n = 6, at 25 $^{\circ}C$).

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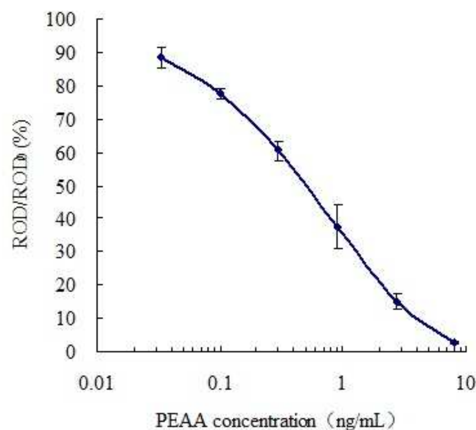

Fig. 4 Standard curves for PEAA quantitation with test strips and strip reader (n = 10, at 25 °C). ROD represents the mean relative optical density of PEAA standards and ROD_0 is the mean relative optical density at 0 ng mL^{-1}. The concentration of PEAA standards solution are 0.033, 0.1, 0.3, 0.9, 2.7 and 8.1 ng mL^{-1}, respectively.

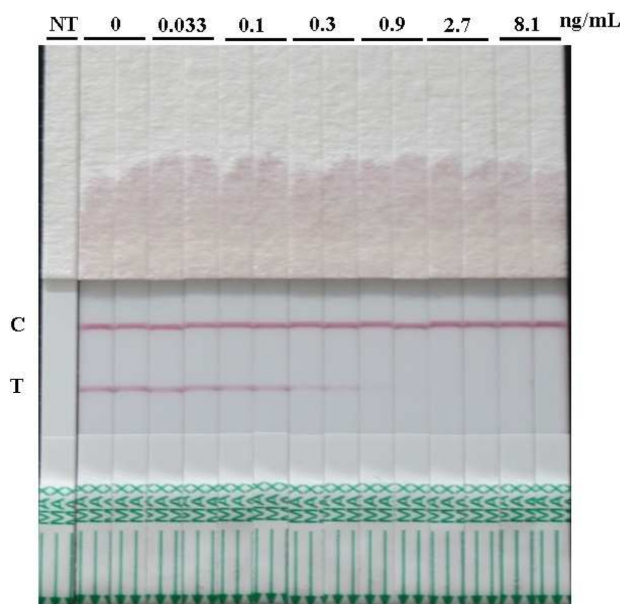

Fig. 5 Colloidal gold-based lateral-flow immunoassay for detection of PEAA in spiked swine urine samples (at 25 °C). Upper line is the control line (C), bottom line is the PEAA test line (T), respectively. NT is the un-test strip. The urine samples were spiked with PEAA at 0, 0.033, 0.1, 0.3, 0.9, 2.7 and 8.1 ng mL^{-1}, respectively.

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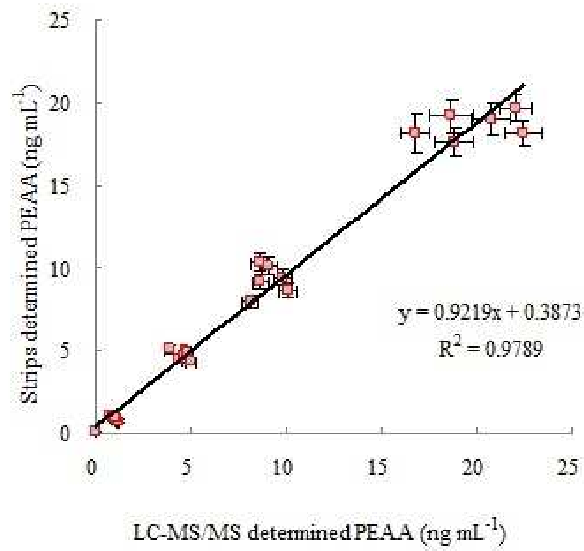

Fig.6 Correlation between test strips (n= 6) and LC-MS/MS (n= 6) for the detection of PEAA in spiked swine urine samples.

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