Letter to the Editor

Lateral Flow Immunoassay for Quantitative Detection of Ractopamine in Swine Urine*

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A strip reader based lateral flow immunoassay (LFIA) was established for the rapid and quantitative detection of ractopamine (RAC) in swine urine. The ratio of the optical densities (ODs) of the test line (A_T) to that of the control line (A_C) was used to effectively minimize interference among strips and sample variations. The linear range for the quantitative detection of RAC was 0.2 ng/mL to 3.5 ng/mL with a median inhibitory concentration (IC_{50}) of 0.59±0.06 ng/mL. The limit of detection (LOD) of the LFIA was 0.13 ng/mL. The intra-assay recovery rates were 92.97%, 97.25%, and 107.41%, whereas the inter-assay rates were 80.07%, 108.17%, and 93.7%, respectively.

Ractopamine (RAC) misused in livestock production can increase the potential toxicological risks to humans. Many countries in the world, including China and most European countries, have forbidden the use of RAC as feed additives[1]. Various confirmation methods, including high-performance liquid chromatography[2], liquid chromatography plus mass spectrometry[3], have been used to monitor the illicit use of RAC, but these methods are unsuitable for routine screening because of the high cost of instruments, extensive clean-up procedures and the operation by trained people. Lateral flow immunoassay (LFIA) has the advantages of both chromatographic separation and immunoassay specificity. It is considered as an effective field test and has been widely used for quantitative detection of RAC residue in swine urine[4-5].

In the present study, we established a portable strip reader-based LFIA for the rapid quantitative detection of RAC residue in swine urine. The detection parameters, including interference from the urine matrix, the sample volume and the interpretation time, were optimized by analysing the dynamic curves of A_T, A_C, and the A_T/A_C ratio against incubation time. The specificity, reproducibility, and accuracy of the established LFIA were evaluated. The reliability of the new method was further compared with a commercial ELISA kit by analysing 48 real swine urine samples.

The RAC quantitative strip was produced as described previously[6] with some modification. Briefly, the colloidal gold probe was prepared by adding 1 mL anti-RAC mAb (15 μg/mL) to 10 mL colloidal gold solution. After being blocked with 1 mL of 10% BSA and centrifuged at 4500 g for 30 minutes, the colloidal gold probe was resuspended with 1 mL of PBS containing 5% sucrose, 2% fructose, 1% PEG 20 000, 1% BSA and 0.4% Tween-20[7], and then sprayed onto a treated conjugate pad at a density of 2.5 μL/cm. The BSA-RAC (0.3 mg/mL) and goat anti–mouse IgG (1 mg/mL) were dispensed onto the nitrocellulose (NC) membrane as test line (T) and control line (C) at a density of 0.8 μL/cm. The assembled strips were cut into pieces (4 mm) and then sealed in a plastic bag with desiccant gel.

The LFIA assay for RAC was based on a competitive immunoassay. The changes in ODs on both lines can indirectly reflect the dynamic process of antigens (on NC membrane) and colloidal gold probe (on conjugate pad) interaction[8]. In the present study, dynamic analysis was conducted for elaborating the effect of the interpretation time, loading sample volume, ionic strength and pH of urine on the stability and sensitivity of the quantitative LFIA. The dynamic curve was constructed as follows. After the sample was incubated for 1 min, the strip was scanned by using a commercial HG8 strip reader, which was provided by Shanghai Huguo Science Instrument Co., Ltd. The A_T and A_C were recorded every 30 seconds for 45 min.

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The immunoreaction between the colloidal gold probe and the BSA-RAC on the test line, as well as the donkey anti-mouse IgG on the control line were indicated by a curve by plotting the ODs against the incubation time.

As shown in Figure 1A, during the 45 min incubation time, the A_T and A_C increased sharply during the first 10 minutes, then increased slowly in the next 15 to 35 min and reached a stable value in the last 10 min (data not shown). However, the A_T/A_C ratio reached a stable phase 15 min after incubation and remained stable during the subsequent 30 min of observation time under 0 ng/mL to 1.5 ng/mL RAC-spiked concentration. Thus, the incubation for 15 min was necessary for the RAC LFIA quantitative analysis. The effects of sample volume on the sensitivity of LFIA shown in Figure 1B indicated that the A_T/A_C ratio increased by 7.93% and 7.66% at sample volumes of 85 and 100 μL, respectively. However, at a sample volume of 100 μL, the A_T/A_C ratio needed more time (20 min) to reach a stable phase. Thus, a sample volume of 85 μL was used for the following experiments. The effects of ionic strength on the consistency of A_T/A_C ratio are shown in Figure 1C. The results indicated that the A_T/A_C ratio remained relatively stable from 0.80±0.01 to 0.81±0.01 (P>0.05) under different NaCl concentrations although the values of A_T and A_C changed irregularly. To explore the effect of pH on the consistency of A_T/A_C ratio, the blank urine mixtures were adjusted respectively to final pH values of 5.0, 6.0, 7.0, 8.0, and 9.0. The stable A_T, A_C, and A_T/A_C ratio shown in Figure 1D indicated that pH markedly influenced the A_T and A_C. However, the A_T/A_C ratio remained relatively stable, ranging from 0.72±0.02 to 0.78±0.01 (P>0.05) when the pH varied from 6.0 to 9.0, whereas it declined to 0.41±0.03 at pH 5.0 (P<0.01) because the A_T decreased significantly.

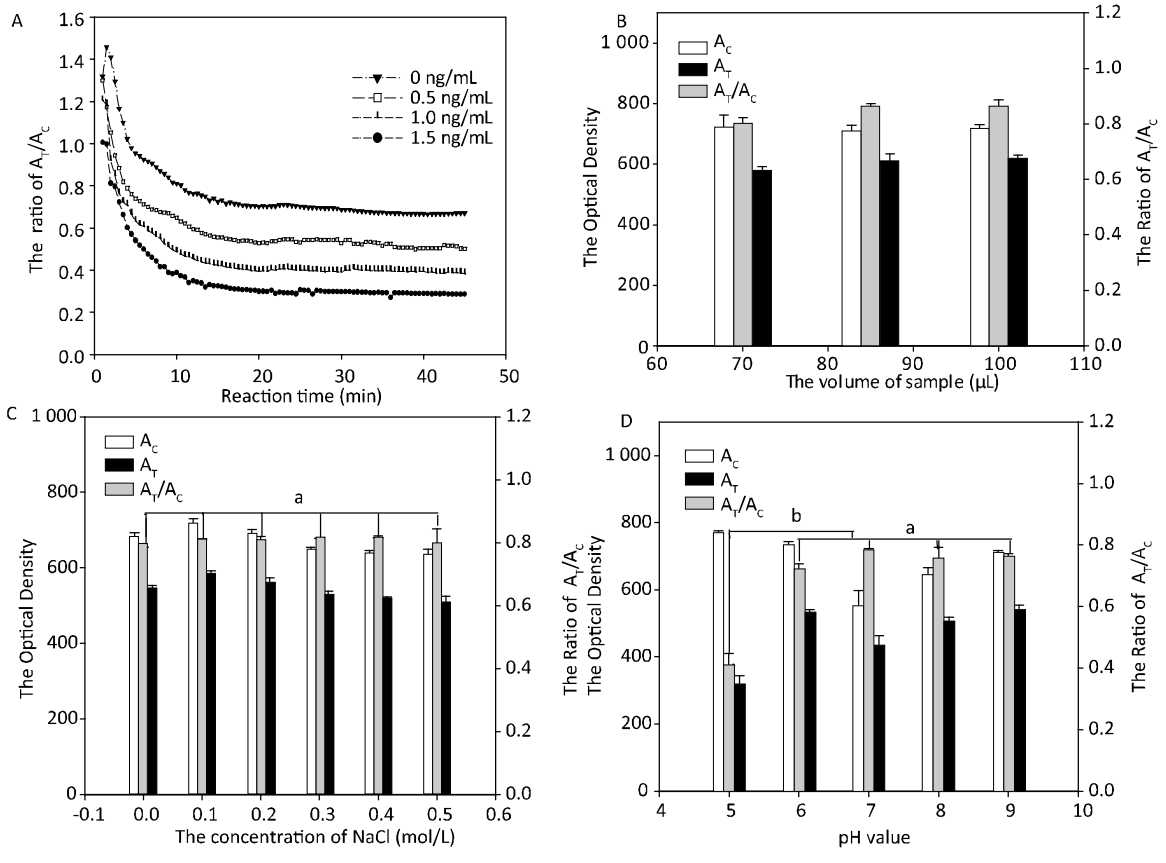

Figure 1. Optimizing the LFIA assay. (A) Immunoreaction dynamics of A_T/A_C ratio at different RAC concentrations. (B) Effect of sample volume on the A_T, A_C, and A_T/A_C ratio. (C) Effect of the ionic strength of urine samples on the A_T, A_C, and A_T/A_C ratio. (D) Effect of the pH value in urine samples on the A_T, A_C, and A_T/A_C ratio. The letter a in Figure 1C and Figure 1D indicates that the means are not significantly different (one-way ANOVA, P>0.05). The letter b in figure 1D indicates that the means are significantly different (one-way ANOVA P<0.01).

To further evaluate the interference from the urine matrix, ten RAC-free urine samples with pH values ranging from 5 to 9 were used to test the A_T/A_C ratio. The results showed that the A_T/A_C ratio of each sample varied from 0.48 to 0.90, with a variation coefficient of 16.73%. Even for samples with the same pH (pH 8.0, n=4), the A_T/A_C ratios varied from 0.60 to 0.90. The aforementioned results indicated that other unknown matrices probably existed in the urine aside from pH and ionic strength, which might interfere with the strip quantitative assay. Thus, 10 urine samples were diluted with PBS at 1:1 to 1:4. The means of A_T/A_C ratio were 0.80, 0.87, and 0.93, with a relative standard deviation of 10.74%, 7.05%, and 4.35%, which corresponded with 1:2, 1:3, and 1:4 dilution, respectively. Based on the results, 1:4 sample dilution was necessary to avoid the false positive results caused by interference from the sample matrix.

The calibration curve of the quantitative LFIA was obtained by plotting the B/B_0 against the logarithm of RAC concentration, in which B_0 and B were representing the A_T/A_C ratio of the negative sample and a series of RAC working standard solutions, respectively. The standard solutions were prepared by spiking a RAC stock solution (100 ng/mL) with the 1:4 diluted blank mixture swine to final concentration at 0, 0.1, 0.2, 0.5, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 ng/mL, respectively. The regression equation of the calibration curve was y=-0.2619 Log (x)+0.3641 with a reliable correlation coefficient (R^2=0.99) and a median inhibitory concentration (IC_{50}) of 0.59±0.06 ng/mL (n=5).

The limit of detection (LOD) of the LFIA was 0.13 ng/mL according to the calibration curve IC_{10}, whereas the LOD for real swine urine was obtained by the analysis of 20 randomly selected swine urine samples which were confirmed to be free of β-agonists through LC-MS/MS, and then diluted at 1:4 with PBS before the test. The LOD was calculated to be 0.44 ng/mL according to the mean of the measured blank signals (n=20) with three times of the standard deviations[9] and then multiplied by the dilution factor. The specificity was evaluated by the cross-reaction to other β-adrenergic compounds, including salbutamol, cimbuterol, terbutaline, clenbuterol, cimaterol, mabuterol, bambuterol, tulobuterol, clorprenaline, penbutolol, and brombutero. The results indicated that anti-RAC mAbs did not cross-react with the 11 β-agonists, and the established LFIA assay could detect the RAC in real swine urine samples specifically. To evaluate the reproducibility and accuracy of the quantitative LFIA, the 1:4 diluted urine samples with a RAC concentrations at 0.2, 0.4, and 0.8 ng/mL were used to analyse the intra-assay and inter-assay variations. Intra-assay test was performed with 5 replicates at each concentration whereas the inter-assay test was conducted for 3 days successively, three times with 3 hour intervals per day, and five replicates at each concentration. As shown in Table 1 , the average intra-assay recovery rates were 92.97%, 97.25%, and 107.41% with relative standard deviation of 4.92%, 2.72%, and 9.25% at RAC concentrations of 0.2, 0.4, and 0.8 ng/mL, respectively. The average inter-assay recovery rates were 80.07%, 108.17%, and 93.7% with relative standard deviation of 6.36%, 10.09%, and 12.74%, respectively. The fact that the variations within the intra-assay and the inter-assay recovery rates were less than 12.8% demonstrated an acceptable accuracy level for RAC LFIA quantification.

To further evaluate the reliability of the quantitative LFIA assay to detect RAC, the performance of RAC LFIA was compared with a commercial ELISA kit, which was provided by Wuxi Zodoboer Biotech. Co., Ltd. Totally 48 RAC-free swine urine samples were diluted at 1:4 with PBS and then spiked with RAC stock solution to final concentrations ranging from 0.1 ng/mL to 3.5 ng/mL before use. As shown in Figure 2, the two methods were highly correlated with a correlation coefficient (R^2) of 0.92 and a slope of 0.96.

Table 1. Reproducibility and Stability of LFIA in RAC-Spiked Swine Urine Samples

RAC-spiked urine (ng/mL)	Intraassay				Interassay[a]			
	Mean[b]	Recovery (%)	SD	RSD (%)	Mean[b]	Recovery (%)	SD	RSD (%)
0.2	0.19	92.97	0.04	4.92	0.16	80.07	0.06	6.36
0.4	0.39	97.25	0.02	2.72	0.43	108.12	0.06	10.09
0.8	0.86	107.41	0.06	9.25	0.75	93.70	0.09	12.74

Note. [a]The assay was completed every 3 days for 15 days. [b]The mean of five replicates at each spiked concentration.

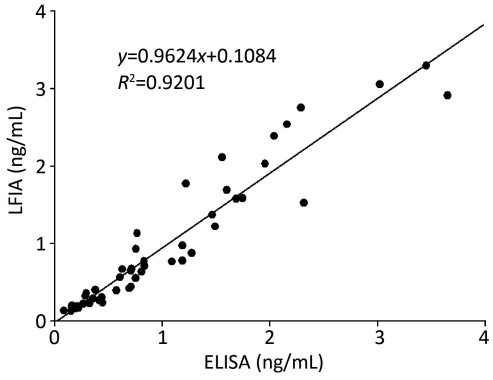

Figure 2. Correlation between results from ELISA (X-axis) and LFIA (Y-axis) analyses of RAC in 48 spiked samples.

In conclusion, to our knowledge, this is the first study on the quantitative detection of RAC residue in real swine urine based on the A_T/A_C ratio to offset the inherent heterogeneity of the strips. This quantitative approach is much more reliable and applicable than the barely effective detection of the test line. With the sensitive quantitative system based on the A_T/A_C ratio and promising advantages of LFIA resulting in good performance, the established A_T/A_C ratio-based quantitative LFIA is a useful field screening test for the detection of RAC residue in swine urine.

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REFERENCES

1. MOA Bulletins. The catalogue of drug banned to use in the feed and animal drinking water. Announcement by the Ministry of Agriculture, 2002; No. 176, China.
2. Shelver WL and Smith DJ. Determination of ractopamine in cattle and sheep urine samples using an optical biosensor analysis: comparative study with HPLC and ELISA. J Agric Food Chem, 2003; 51, 3715-21.
3. Li C, Wu YL, Yang T, et al. Simultaneous determination of clenbuterol, salbutamol and ractopamine in milk by reversed-phase liquid chromatography tandem mass spectrometry with isotope dilution. J Chromatography A, 2010; 1217, 7873-7.
4. Li X, Zhang G, Deng R, et al. Development of rapid immunoassays for the detection of ractopamine in swine urine. Food Addit Contam, 2010; 27, 1096-103.
5. Zhang MZ, Wang MZ, Chen ZL, et al. Development of a colloidal gold-based lateral-flow immunoassay for the rapid simultaneous detection of clenbuterol and ractopamine in swine urine. Anal Bioanal Chem, 2009; 395, 2591-9.
6. Hayat M. Colloidal gold: principles, methods and applications. Academic Press, New York, 1989, p. 421.
7. Chen XL, Xu HY, Lai WH, et al. A sensitive chromatographic strip test for the rapid detection of enrofloxacin in chicken muscle. Food Addit Contam, 2012; 29, 383-91.
8. Li CH, Luo W, Xu HY, et al. Development of an immuno-chromatographic assay for rapid and quantitative detection of clenbuterol in swine urine. Food Control, 2013; 34, 725-32.
9. Shen JZ, Zhang Z, Yao Y, et al. A monoclonalantibody based time resolved fluoroimmunoassay for chloramphenicol in shrimp and chicken muscle. Analytica Chimica Acta, 2006; 575, 262-6.