

# Dual-Label Chemiluminescence Strategy for Multiplexed Immunoassay of 20 Fluoroquinolones, 15 $\beta$ -Lactams, 15 Sulfonamides, and CAP in Milk

Xiaoqi Tao<sup>1,2</sup> · Jia Wang<sup>1</sup> · Yuejie Xie<sup>1</sup> · Xiaowei Zuo<sup>1</sup> · Fang Mo<sup>1</sup> · Song Zhou<sup>1</sup> · Hongjun Li<sup>1,2</sup>

Received: 26 October 2016 / Accepted: 9 March 2017 / Published online: 18 March 2017  
© Springer Science+Business Media New York 2017

**Abstract** In this paper, a novel dual-label time-resolved chemiluminescent multiplexed immunoassay (DLTRC-MIA) based on the distinction of the kinetic characteristics of horseradish peroxidase (HRP) and alkaline phosphatase (ALP) with approximate estimation approach for simultaneous determination of 20 fluoroquinolones (FQs), 15  $\beta$ -lactams, 15 sulfonamides (SAs), and chloramphenicol (CAP) in milk was developed. The strategy integrated a single-chain variable fragment–alkaline phosphatase fusion protein (scFv-ALP), a recombinant penicillin-binding protein (PBP) 2<sup>\*</sup>, a monoclonal antibody (MAb), and a polyclonal antibody (PAb) in one immunoassay and in a single well together to fulfill the simultaneous detection of 51 low-molecular weight contaminants (20 FQs, 15  $\beta$ -lactams, 15 SAs, and CAP). The limits of detection for FQs,  $\beta$ -lactams, SAs, and CAP range from 0.29  $\mu\text{g L}^{-1}$  for ciprofloxacin (CIP) to 81.6  $\mu\text{g L}^{-1}$  for trovafloxacin (TRO), 0.27  $\mu\text{g L}^{-1}$  for ceftiofur (CEF) to 44.1  $\mu\text{g L}^{-1}$  for cephalexin (CEL), 0.089  $\mu\text{g L}^{-1}$  for sulfadimethoxine (SDM) to 2.7  $\mu\text{g L}^{-1}$  for sulfadiazine (SDZ), and 0.028  $\mu\text{g L}^{-1}$  for CAP, respectively. The results demonstrated that the detection limits of DLTRC-MIA meet the requirement of detection levels for 51 drug residues in milk, suitable for high-throughput screening of low-molecular weight contaminants.

**Keywords** Chemiluminescence · Multiplexed immunoassay · Fluoroquinolones ·  $\beta$ -Lactams · Sulfonamides · Chloramphenicol

## Introduction

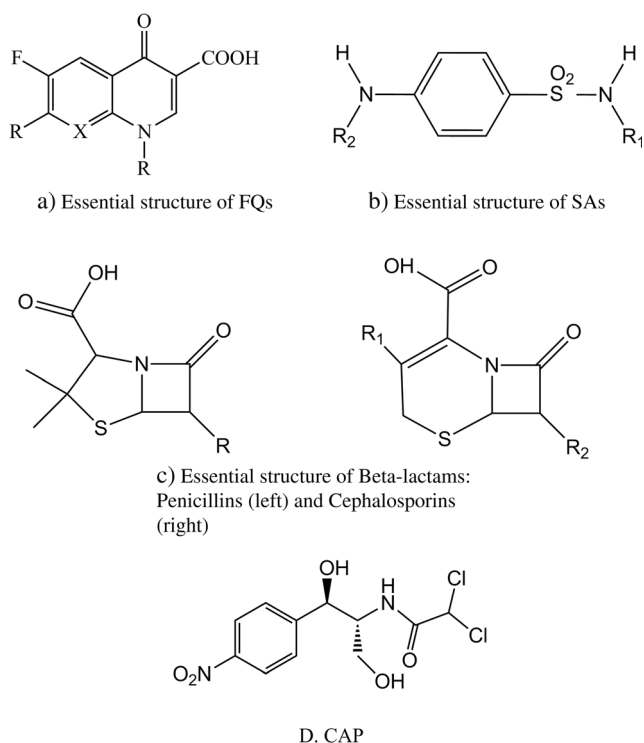
Fluoroquinolones (FQs, Fig. 1a) and sulfonamides (SAs, Fig. 1b) are two families of synthetic antibiotics that have broad-spectrum antimicrobial activity and used extensively to treat and prevent various infections in animals and humans and to promote growth in low dosage animal husbandry (Chen et al. 2014b; Yu et al. 2015).  $\beta$ -Lactams (Fig. 1c) are a broad class of antibiotics containing  $\beta$ -lactam nucleus in their molecular structure and have been widely used for the treatment of diseases in livestock animals, such as bovine mastitis, pneumonia, and so on (Goto et al. 2005). Chloramphenicol (CAP, Fig. 1d) is a very effective broad-spectrum antibiotic which was widely used in both human and veterinary practice for prevention and treatment of many bacterial infections (Shi et al. 2007). Residues of these drugs may enter the food chain either by illegal use or by non-compliance of producers with existing animal treatment protocols (withdrawal times). The presence of these chemical residues in milk constitutes a potential hazard for humans due to allergic and toxic reactions and/or to increase resistance of pathogenic bacterial towards antibiotics (Jiang et al. 2013; Mitchell et al. 1998; Shi et al. 2007).

Many countries have established regulatory rules for using these antimicrobial agents in food animals in order to protect consumers from exposure to harmful residues. In Europe, the Commission approved maximum administrative residue limits (MRLs), ranging from 10 to 100  $\mu\text{g L}^{-1}$  for FQs, 4–125  $\mu\text{g L}^{-1}$  for several  $\beta$ -lactams, 25–100  $\mu\text{g L}^{-1}$  for SAs in milk (EU Regulation 470/2009; Commission Regulation

✉ Xiaoqi Tao  
77179000@qq.com

<sup>1</sup> College of Food Science, Southwest University, NO. 2 Tiansheng Road, Beibei District, Chongqing 400715, China

<sup>2</sup> Chongqing Engineering Research Center for Regional Foods, Chongqing 400715, China



**Fig. 1** The essential structures of FQs,  $\beta$ -lactams, SAs, and CAP

37/2010). The minimum required performance limit (MRPL) for CAP is  $0.3 \mu\text{g L}^{-1}$  (Commission Regulation 37/2010; Stolker and Brinkman 2005). Therefore, it is of great importance to develop sensitive and reliable methods for FQs,  $\beta$ -lactams, SAs, and CAP detection in milk samples for food safety and quality control.

A number of analytical instrumental methods have been developed over the years to monitor the presence of FQs,  $\beta$ -lactams, SAs, and CAP in milk (Turnipseed et al. 2008; Karageorgou et al. 2013; Liu et al. 2016; Chen et al. 2016). However, the demand for rapid screening techniques cannot be fulfilled alone by these instrumental analytical methods. The immunoassay-based methods (Wang et al. 2016; Zeng et al. 2013; Wang et al. 2013; Tao et al. 2014) have been gradually employed to assess milk quality and safety because of its simplicity, selectivity, and cost-effectiveness. The most commonly used immunoassay format is enzyme-linked immunosorbent assay (ELISA), but it is normally a single-plex method, i.e., one assay for one analyte and several independent detecting runs for multiple targets for detection of FQs,  $\beta$ -lactams, SAs, and CAP (Wang et al. 2016; Zeng et al. 2013; Wang et al. 2013; Tao et al. 2014). Hence, the current trend of immunoassay is developing multi-analyte immunoassays. To date, the multi-analyte immunoassays for veterinary drugs are mainly based on generic (class-selective or broad-specific) antibodies or on the combinations of specific antibodies (multi-antibody strategy). The former mode is more popular, and these assays are more favorable to determine the gross quantity of all veterinary drugs that have similar structures,

but they can only detect one class of veterinary drug simultaneously (Wang et al. 2013). In the latter strategy, assays are performed in spatial resolved mode with different antigens or antibodies immobilized on planar supports (planar array) (Zhu et al. 2011; Jiang et al. 2013), on microsphere (Charlemroj et al. 2013), on beads (Guo et al. 2013), and on nitrocellulose membrane such as lateral-flow immunoassay (LFIA) (Li et al. 2015). Among the above two strategies, the latter which could simultaneously detect more than one class of veterinary drug is recommended. However, the operation is more time consuming, especially the coloration procedure of ELISA. Chemiluminescence with the advantages of high sensitivity, fast emission of light, and instant termination of the chemiluminescence reaction by removal of substrate (Dodeigne et al. 2000) could solve the time-consuming problem. In our previous study (Zhang et al. 2014), a sensitive dual-label time-resolved reverse competitive chemiluminescent immunoassay, based on the distinction of the chemiluminescent kinetic characteristics of horseradish peroxidase (HRP) and alkaline phosphatase (ALP), was developed for simultaneous detection of CAP and clenbuterol (CLE) in milk. This strategy could enhance the sensitivity of the immunoassay for CAP and CLE and reduce the assay duration. However, the method in our previous study is only a primary model, which could not detect multi-analyte and not take full advantage of the strategy of the dual-label time-resolved.

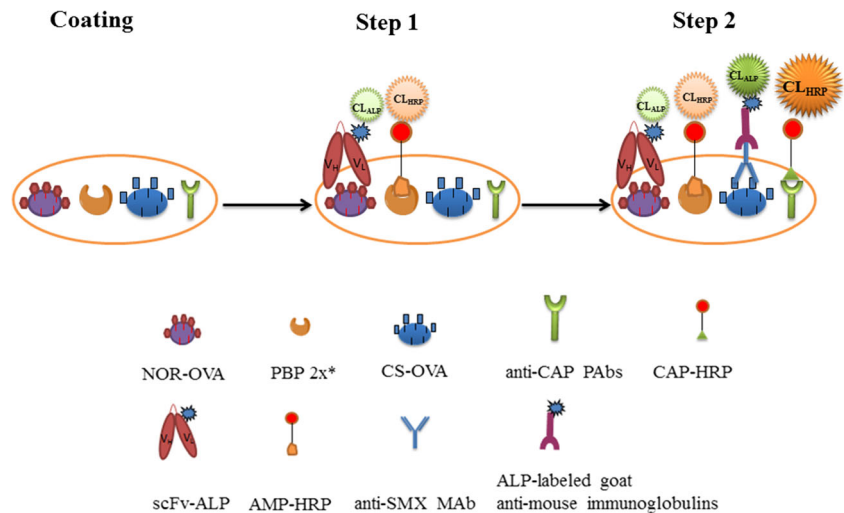
In this study, a dual-label time-resolved chemiluminescent multiplexed immunoassay (DLTRC-MIA) for simultaneous determination of 20 FQs, 15  $\beta$ -lactams, 15 SAs, and CAP in milk was developed (Fig. 2). Firstly, a single-chain variable fragment-alkaline phosphatase fusion protein (scFv-ALP), a recombinant penicillin-binding protein (PBP) 2 $\times$ \*, a monoclonal antibody (MAb), and a polyclonal antibody (PAb) were adopted to fulfill the simultaneous detection of 51 veterinary drugs. In particular, (PBP) 2 $\times$ \* instead of antibody could resolve the problem that it is difficult to develop a broad spectrum antibody reacting with  $\beta$ -lactams simultaneously because of the diversity of molecular structure of these drugs. Secondly, the combination of antigen/antibody-enzyme molecules (HRP and ALP) obtained by chemical bound and genetic fusion expression can achieve good effect of signal separation. Hence, the simultaneous detection of four kinds of veterinary drugs in one well was achieved through the sequential reaction combination of four ligand-receptor couples.

## Materials and Methods

### Apparatus

- (a) Chemiluminescence reader—Veritas Microplate Luminometer (Turner BioSystems, Sunny Vale, CA, USA).

**Fig. 2** Schematic representation of DLTRC-MIA for quantitative determination of FQs,  $\beta$ -lactams, SAs, and CAP



- (b) Microtiter plate reader—Sunrise microtiter plate reader (TECAN, Groedig, Austria).  
 (c) White Opaque high binding plates for chemiluminescent measurements (chemiluminescent microtiter plate (MTP))—Costar (Cambridge, MA, USA).  
 (d) Milli-Q system—(EMD Millipore Corporation, Belleria, MA, USA).

## Reagents

- (a) Standards—Ciprofloxacin (CIP), enrofloxacin (ENR), norfloxacin (NOR), and other FQs were purchased from VETRANAL® Fluka (Sigma-Aldrich, St. Louis, MO, USA). Sulfamethazine (SMZ), sulfamethoxazole (SMX), sulfadiazine (SDZ), sulfamonomethoxine (SMM), and other SAs were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Penicillin G, ampicillin (AMP), amoxicillin, cloxacillin and other  $\beta$ -lactams, CAP (99%), florfenicol (FF, 99% purity), thiamphenicol (TAP, 97.6% purity) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ractopamine (RAC), ivermectin (IVE), and gentamicin (GEN) were purchased from Shanghai Caienu Technology Co. Ltd. (Shanghai, China). Standard stock solutions of FQs were prepared by dissolving an appropriate amount of each standard in 0.03 M sodium hydroxide. The CAP, FF, RAC, SMX, SMZ, and other SA stock solutions were prepared in methanol; penicillin and gentamicin were prepared in PBS; TAP was prepared in dimethylformamide. All stock solutions ( $2 \text{ mg mL}^{-1}$ ) were stored at  $-20^\circ\text{C}$ , and working standards were prepared from the stock solution by 3-fold serial dilution in 0.02 M PB (buffer d). Working standard (cocktail standard of NOR, AMP, SMX, and CAP) in the range of  $0.24\text{--}58.32 \mu\text{g L}^{-1}$  (NOR),  $0.1\text{--}24.3 \mu\text{g L}^{-1}$  (AMP),  $0.067\text{--}16.2 \mu\text{g L}^{-1}$  (SMX), and  $0.017\text{--}4.05 \mu\text{g L}^{-1}$  (CAP) was prepared from the  $2 \text{ mg mL}^{-1}$  stock solution by serial dilution in 0.02 M PB. Hence, the seven corresponding concentrations of NOR, AMP, SMX, and CAP in the cocktail were standard 1 (0, 0, 0, and 0), standard 2 (0.24, 0.1, 0.067, and 0.017), standard 3 (0.72, 0.3, 0.2, and 0.05), standard 4 (2.16, 0.9, 0.6, and 0.15), standard 5 (6.48, 2.7, 1.8, and 0.45), standard 6 (19.44, 8.1, 5.4, and 1.35), and standard 7 (58.32, 24.3, 16.2, and 4.05), respectively.
- (b) Analytical grade reagents—ovalbumin (OVA),  $-(3\text{-dimethylaminopropyl})\text{-3-ethylcarbodiimide hydrochloride}$  (EDC), dimethylformamide (DMF), and  $N$ -hydroxysuccinimide (NHS) were purchased from Sigma (St. Louis, MO, USA). All other chemicals and organic solvents were of reagent grade and were from Beijing Chemical Co. (Beijing, China).
- (c) The chemiluminescence Super Signal substrate solution was purchased from Pierce (Rockford, IL, USA). The Visiglo Plus™ ALP Chemiluminescent Substrate was obtained from Invitrogen (NY, USA).
- (d) The single-chain variable fragment–alkaline phosphatase fusion protein (scFv-ALP) and NOR-OVA were obtained in our previous study (Yu et al. 2015). PBP 2 $\times$ \* and AMP-EDC-HRP were prepared in previous study (Zeng et al. 2013). The coating antigen 4-(4-aminophenylsulfonamido) benzoic acid-OVA (CS-OVA) and the production of MAb 4D11 were from previous study (Wang et al. 2013). The anti-CAP PAb (rabbit source) and CAP-HRP were from our previous study (Tao et al. 2014). ALP-labeled goat anti-mouse immunoglobulins were from Sigma (St. Louis, MO, USA).

## Buffers

- Coating buffer (CB, pH 9.6)—0.05 M carbonate buffer, made with 1.59 g Na<sub>2</sub>CO<sub>3</sub> and 2.93 g NaHCO<sub>3</sub> in 1 L of purified water.
- Blocking buffer—0.01 M PBS containing 0.5% casein.
- Washing solution (PBST)—0.01 M PBS containing 0.05% Tween-20.
- 0.02 M phosphate buffer (PB; pH 7.2)—containing 1.1 g NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 5.16 g Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O in 1 L purified water.
- Phosphate-buffered saline (PBS; pH 7.4)—0.01 M PBS was prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 0.24 g KH<sub>2</sub>PO<sub>4</sub>, and 3.63 g Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O in 1 L purified water.
- Carrez A: 0.36 M K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O; Carrez B: 1.04 M ZnSO<sub>4</sub>·7H<sub>2</sub>O.

## The Procedure of DLTRC-MIA for FQs, $\beta$ -Lactams, SAs, and CAP

In brief, 96-well chemiluminescent microtiter plates (MTP) were coated overnight at 4 °C with 100- $\mu$ L mixture of NOR-OVA (2.0  $\mu$ g mL<sup>-1</sup>), PBP 2 $\times$ \* (2.0  $\mu$ g mL<sup>-1</sup>), CS-OVA (2.0  $\mu$ g mL<sup>-1</sup>), and anti-CAP PABs (1.5  $\mu$ g mL<sup>-1</sup>), dissolved in coating buffer. The plates were washed with PBST three times and blocked with 200  $\mu$ L/well of blocking buffer, then incubated at 37 °C for 1 h.

## Step 1

After washing as described above, 100  $\mu$ L/well of standard solution (cocktail of NOR, AMP, SMX, and CAP) in 0.02 M PB or sample solution was added, followed by 50  $\mu$ L/well mixture of scFv-ALP (2.0  $\mu$ g mL<sup>-1</sup>) and AMP-HRP (2.0  $\mu$ g mL<sup>-1</sup>) dissolved in PBST. The competitive reaction was allowed to take place for 30 min at room temperature (RT). After washing five times, the chemiluminescence signal was measured with a chemiluminescence reader at 2 min after automatic addition of 100  $\mu$ L/well Visiglo Plus™ ALP Chemiluminescent Substrate in injector A, and the results were expressed in relative light units (RLU) and marked as RLU<sub>1A</sub>. At 2.5 min, the HRP activity was revealed by automatically adding 100  $\mu$ L/well of a freshly prepared substrate mixture of Super Signal substrate solution in injector B. The signal was measured using a chemiluminescence reader at 4 min, and the results were expressed as RLU<sub>1B</sub>. After that, the chemiluminescence substrates were removed and washed with PBST three times to halt the chemiluminescence reaction.

## Step 2

Then, 50  $\mu$ L/well of ALP-labeled goat anti-mouse immunoglobulins (1/5000 dilution in PBST) were added, followed by 50  $\mu$ L/well mixture of CAP-HRP (1.5  $\mu$ g mL<sup>-1</sup>), and anti-SMX MAb (1.5  $\mu$ g mL<sup>-1</sup>) dissolved in PBST. The competitive reaction was allowed to take place for 15 min at RT. After washing five times, the chemiluminescence signal was measured with a chemiluminescence reader at 2 min after automatic addition of 100  $\mu$ L/well Visiglo Plus™ AP Chemiluminescent Substrate in injector A, and the results were expressed as RLU<sub>2A</sub>. At 2.5 min, the HRP activity was revealed by automatically adding 100  $\mu$ L/well of a freshly prepared substrate mixture of Super Signal substrate solution in injector B. The signal was measured using a chemiluminescence reader at 4 min, and the results were expressed as RLU<sub>2B</sub>.

## Data Analysis

Standards and samples were run in quadruplicate wells, and mean chemiluminescence intensity values were divided by RLU<sub>max</sub> (chemiluminescence intensity in the absence of analyte). The ratio is defined as B/B<sub>0</sub>. Standard curves were obtained by plotting B/B<sub>0</sub> against the logarithm of analyte concentration and fitted to a four-parameter logistic equation using Origin (version 8.0, Microcal, Northampton, MA, USA) software packages

$$y = \left\{ (A-D) / \left[ 1 + \left( x/C \right)^B \right] \right\} + D$$

where A is the asymptotic maximum 1, B is the curve slope at the inflection point, C is the x value at the inflection point (corresponding to the analyte concentration that reduces RLU<sub>max</sub> to 50%), and D is the asymptotic minimum (RLU<sub>background signal</sub>/RLU<sub>max</sub>).

## Cross-reactivity

Each of the IC<sub>50</sub> values of the four different types of analytes was determined in the DLTRC-MIA described above. The ability of the four types receptors (scFv, PBP2 $\times$ \*, MAb, and PAb) to recognize other analogs was investigated by evaluating the percentage of cross-reactivity (% CR) (Table 1). The % CR values were calculated with the following equation:

$$\%CR = 100 \times IC_{50,(\text{haptent})} / IC_{50,(\text{analogs})}$$

Furthermore, to help define the specificity of the antibodies, the conventional veterinary drugs, RAC, IVE, GEN, FF, and TAP were also tested.

**Table 1** The IC<sub>50</sub> and cross-reactivity (% CR) to FQs (1 to 20), β-lactams (21 to 35), SAs (36–50), and CAP (51)

Analogue	IC <sub>50</sub> (μg L <sup>-1</sup> )	% CR	Analogue	IC <sub>50</sub> (μg L <sup>-1</sup> )	% CR		
1	CIP	0.50	138.0	29	CEF	0.72	294.44
2	ENR	0.68	101.47	30	CEM	4.17	50.84
3	NOR	0.69	100	31	CEQ	1.99	106.53
4	PEF	0.74	93.24	32	CEZ	6.64	31.93
5	DAN	0.98	70.41	33	CER	27.30	7.76
6	LEV	1.02	67.65	34	CEP	4.37	48.51
7	OFL	1.10	62.72	35	CET	8.17	25.95
8	AMI	0.93	74.19	36	SMX	1.75	100
9	ENO	0.95	72.63	37	SCP	5.27	33.21
10	ORB	1.14	60.53	38	SDM	0.26	673.08
11	FLER	1.08	63.89	39	SDT	0.24	729.12
12	SPA	1.23	56.10	40	SDZ	9.89	17.69
13	MAR	1.13	61.06	41	SIM	1.42	123.24
14	LOM	1.20	57.50	42	SMD	0.95	184.21
15	FLU	2.85	24.21	43	SMM	1.34	131.58
16	PAZ	2.78	24.82	44	SMP	0.67	261.20
17	PRU	19.11	3.61	45	SMR	1.33	56.32
18	SAR	35.20	1.96	46	SMZ	2.60	67.31
19	DIF	35.75	1.93	47	acetyl-SMZ	5.04	34.72
20	TRO	140.82	0.49	48	SPY	1.41	124.11
21	PEN	0.97	218.56	49	SNT	0.34	514.70
22	AMP	2.12	100	50	SQX	2.12	82.55
23	AMO	1.86	113.97	51	CAP	0.14	100
24	CLO	20.50	10.34	52	RAC	>1000	<0.1
25	DIC	4.17	50.84	52	IVE	>1000	<0.1
26	OXA	2.72	77.94	54	GEN	>1000	<0.1
27	NAF	1.49	142.28	55	FF	>1000	<0.1
28	CEL	117.83	1.80	56	TAP	>1000	<0.1

FQs: *CIP* ciprofloxacin, *DAN* danofloxacin, *DIF* difloxacin, *ENO* enoxacin, *ENR* enrofloxacin, *FLER* fleroxacin, *AMI* amifloxacin, *FLU* flumequine, *LEV* levofloxacin, *LOM* lomefloxacin hydrochloride, *MAR* marbofloxacin, *NOR* norfloxacin, *OFL* ofloxacin, *ORB* orbifloxacin, *PAZ* pazufloxacin, *PEF* pefloxacin-d 5, *PRU* prulifloxacin, *SAR* sarafloxacin, *SPA* sparfloxacin, *TRO* trovafloxacin

β-lactams: *PEN* penicillin G, *AMP* ampicillin, *AMO* amoxicillin, *CLO* cloxacillin, *DIC* dicloxacillin, *OXA* oxacillin, *NAF* nafcillin, *CEL* cephalixin, *CEF* ceftiofur, *CEM* cefalonium, *CEQ* cequinome, *CEZ* cefazolin, *CER* cefoperazone, *CEP* cephalirin, *CET* cefacetile

SAs: *SMX* sulfamethoxazole, *SCP* sulfachloropyridazine, *SDM* sulfadimethoxine, *SDT* sulfadimethoxypyrimidine, *SDZ* sulfadiazine, *SIM* sulfisomidine, *SMD* sulfamethoxydiazine, *SMM* sulfamonomethoxine, *SMP* sulfamethoxy-pyridazine, *SMR* sulfamerazine, *SMZ* sulfamethazine, *acetyl-SMZ* N<sup>4</sup>-acetyl-sulfamethazine, *SPY* sulfapyridine, *SNT* sulfanitran, *SQX* sulfaquinoxaline

*RAC* ractopamine, *IVE* ivermectin, *GEN* gentamicin, *FF* florfenicol, *TAP* thiamphenicol

## Sample Preparation

For extraction of FQs, β-lactams, SAs, and CAP from milk, 500 μL Carrez A and 500 μL Carrez B were added to 10 mL milk and mixed thoroughly. Then, the mixture was centrifuged for 10 min at 4000g in 4 °C. Two aliquots of aqueous supernatant (2.2 mL) were transferred to two new tubes. The pH of one aliquot was adjusted to 11 with 1 M NaOH and mixed with

8.0 mL ethyl acetate thoroughly for 10 min in a new tube; the pH of another one was adjusted to 7.0 and mixed with 8.0 mL ethyl acetate thoroughly for 10 min in a new tube. Each mixture was centrifuged at 4000g for 10 min, and 4 mL of organic supernatant was transferred to a new tube and dried by nitrogen at 60 °C. The residue was dissolved in 0.5 mL of 0.02 M PB. Then, the two redissolutions were combined to one sample solution (1 mL) and could be used for determination.

## Recovery and Precision

Standard solutions (AMP/NOR/SMX/CAP) were added into the blank milk samples, known to be free of FQs,  $\beta$ -lactams, SAs, and CAP, to yield different concentrations (Table 2). All samples were analyzed 10 times in duplicate to assess accuracy and precision.

## Analysis of Field Milk Samples

Forty whole cow milk samples with packages were purchased from retail outlets in Chongqing. Each sample was divided into 7 portions in brown polystyrene bottle, which would be analyzed by developed DLTRC-MIA, conventional ELISA kits, and LC-MS/MS (Chen et al. 2016; Zhang et al. 2008). All of the samples were stored at  $-20\text{ }^{\circ}\text{C}$  until use.

## Results and Discussion

### The Principle of DLTRC-MIA

The procedure of DLTRC-MIA for FQs,  $\beta$ -lactams, SAs, and CAP based on time-resolved chemiluminescence (CL)

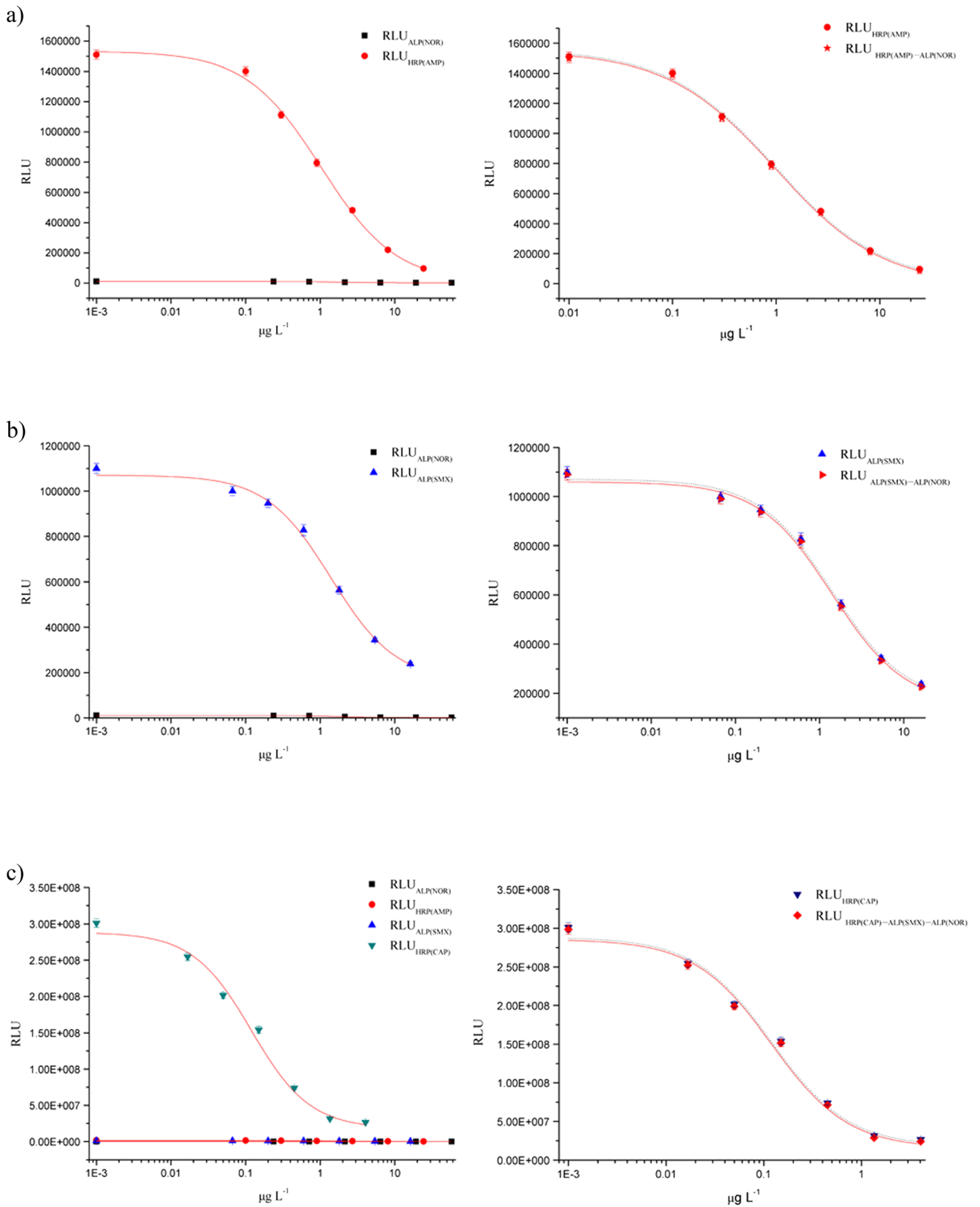
strategy is illustrated in Fig. 2. ALP and HRP were adopted as the signal probes to form the conjugates scFv-ALP, AMP-HRP ( $\beta$ -lactams), ALP-labeled goat anti-mouse immunoglobulins (SAs), and CAP-HRP (CAP), respectively. With a competitive immunoassay format, the ALP-tagged and HRP-tagged immunocomplexes were formed in the well of the MTP.

In step 1, the AP-tagged and HRP-tagged immunocomplexes (NOR-OVA—scFv-ALP and PBP 2 $\times$ \*—AMP-HRP) were formed in the well of the MTP. The two CL signals were successively triggered by adding the two different CL substrates (ALP and HRP CL solutions were prepared in different injections in the automatic luminometer).

As observed in our previous study (Zhang et al. 2014), the CL signal of ALP-tagged immunocomplex (NOR-OVA—scFv-ALP) reached the maximum value at 2 min after injection of ALP CL substrate in injector A (0 min) and showed a steady plateau in the whole measured period. The CL signal of the HRP-tagged immunocomplex (PBP 2 $\times$ \*—AMP-HRP) reached the maximum value at 4 min after injection of HRP CL substrate in injector B (at 2.5 min) and showed a steady plateau in the whole measured period (data not shown). The signal for AMP was collected at 4 min after adding HRP CL substrate (injector B) into the wells at 2.5 min.

**Table 2** A comparative analysis of the developed DLTRC-MIA with other immunochemical methods and commercial ELISA kits for FQs,  $\beta$ -lactams, SAs, and CAP detection in milk

Manufacturer	LOD ( $\mu\text{g L}^{-1}$ )	Number of detection	Refer to
		FQs	
Developed DLTRC-CIA	0.4	20	Reported here
FQs ELISA kits (R-Biopharm)	6.0 (shrimp)	10	<a href="http://www.r-biopharm.com/products/food-feed-analysis/residues/antibiotics/chinolone/item/ridascreen-chinolonequinolones">http://www.r-biopharm.com/products/food-feed-analysis/residues/antibiotics/chinolone/item/ridascreen-chinolonequinolones</a>
Fluorescence polarization immunoassay	3.5	19	Chen et al. 2014a
		$\beta$ -lactams	
Developed DLTRC-CIA	0.8	15	Reported here
$\beta$ -lactams ELISA kits (R-Biopharm)	0.2	10	<a href="http://www.bioon.com.cn/reagent/show_product.asp?id=934563">http://www.bioon.com.cn/reagent/show_product.asp?id=934563</a>
ELISA based on receptor	1.22	15	Zeng et al. 2013
		SAs	
Developed DLTRC-CIA	0.6	15	Reported here
SAs ELISA kits (R-Biopharm)	3.5	18	<a href="http://www.r-biopharm.com/products/food-feed-analysis/residues/antibiotics/sulfonamide/item/ridascreen-sulfonamide">http://www.r-biopharm.com/products/food-feed-analysis/residues/antibiotics/sulfonamide/item/ridascreen-sulfonamide</a>
ELISA	10.2	15	Wang et al. 2013
		CAP	
Developed DLTRC-CIA	0.028	1	Reported here
CAP ELISA kits (R-Biopharm)	0.025	1	<a href="http://www.xygen.com/pdfs/mycotoxins/antibiotics/Glucuronid-Cap.pdf">http://www.xygen.com/pdfs/mycotoxins/antibiotics/Glucuronid-Cap.pdf</a>
CL-ELISA	0.73	1	<a href="http://www.docin.com/p-339996838.html">http://www.docin.com/p-339996838.html</a> <a href="http://www.docin.com/p-339996838.html">http://www.docin.com/p-339996838.html</a> Tao et al. 2014



**Fig. 3** Gross standard inhibition curves and revised standard inhibition curves in two detection steps

Then, the ALP and HRP chemiluminescence substrates were removed and washed with PBST three times to halt the chemiluminescence reaction.

The ALP CL signal for NOR ( $RLU_{1A}$ ) was collected at 2 min without any other material interference. When the chemiluminescence reader collected the CL signal at 4 min, there were two chemiluminescent systems—ALP CL system for NOR and HRP CL system for AMP, and the CL value ( $RLU_{1B}$ ) was the sum of  $RLU_{1A}$  and HRP CL for AMP ( $RLU_{HRP-AMP}$ ). But the maximum  $RLU_{1A}$  ( $RLU_{max-NOR}$ , corresponding that the concentration of NOR is  $0 \mu\text{g L}^{-1}$ ) was 1/137 of  $RLU_{1B-max}$  (corresponding that the concentration of AMP is  $0 \mu\text{g L}^{-1}$ ) and 1/9 of  $RLU_{1B-min}$  (corresponding that the concentration of AMP is  $24.3 \mu\text{g L}^{-1}$ ). When the  $RLU_{max-NOR}$  was taken as the background, the HRP CL for AMP ( $RLU_{HRP-AMP}$ ) was obtained by subtracting  $RLU_{max-NOR}$  from  $RLU_{1B}$ . Standard curves were obtained by plotting  $RLU_{1B}$  and  $RLU_{HRP-AMP}$  against the logarithm of AMP concentration (Fig. 3a). When determining the background effects, interferences are quantified by comparing the inhibition curves generated from  $RLU_{1B}$  and  $RLU_{HRP-AMP}$ . The curves generated from  $RLU_{1B}$  and  $RLU_{HRP-AMP}$  were almost superposable, indicating that the background effect ( $RLU_{1A}$ ) was not insignificant and negligible (Fig. 3a). Hence,  $RLU_{1B}$  was used to represent  $RLU_{HRP-AMP}$ .

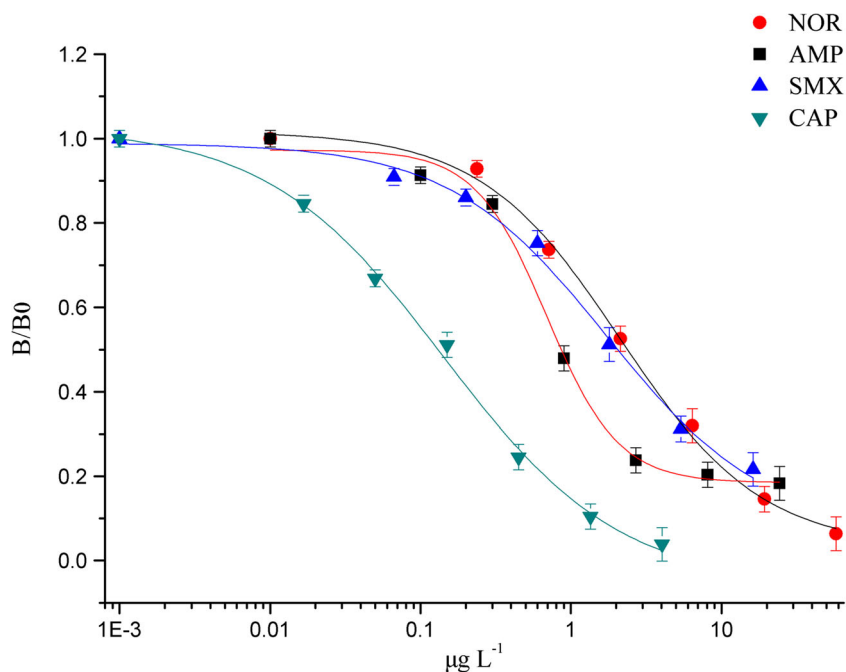
In step 2, the ALP-tagged and HRP-tagged immunocomplexes (ALP-labeled goat anti-mouse immunoglobulins—anti-SMX MAb—CS-OVA and anti-CAP—CAP-HRP) were newly formed in the well of the MTP. Then, there were four immunocomplexes: NOR-OVA—scFv-ALP and PBP 2X\*—AMP-HRP formed in step 1, and ALP-labeled goat anti-

mouse immunoglobulins—anti-SMX MAb—CS-OVA and anti-CAP PAb—CAP-HRP formed in step 2. The two CL signals were successively triggered by adding the two different CL substrates (ALP and HRP CL solutions were prepared in different injections in the automatic luminometer).

The CL signal of ALP-tagged immunocomplex (NOR-OVA—scFv-ALP and ALP-labeled goat anti-mouse immunoglobulins—anti-SMX MAb—CS-OVA) reached the maximum value at 2 min after injection of ALP CL substrate in injector A (0 min). The CL signal of the HRP-tagged immunocomplex (PBP 2X\*—AMP-HRP and anti-CAP PAb—CAP-HRP) reached the maximum value at 4 min after injection of HRP CL substrate in injector B (at 2.5 min). Then, the signal for NOR and SMX was detected at 2 min after adding ALP CL substrate (injector A) into the well. The signal for AMP and CAP was collected at 4 min after adding HRP CL substrate (injector B) into the wells at 2.5 min.

When the chemiluminescence reader collected the CL signal at 2 min, the CL value ( $RLU_{2A}$ ) was the sum of  $RLU_{1A}$  (ALP CL signal for NOR) and ALP CL for SMX ( $RLU_{ALP-SMX}$ ). But the  $RLU_{max-NOR}$  was 1/100 of  $RLU_{2B-max}$  (corresponding that the concentration of SMX is  $0 \mu\text{g L}^{-1}$ ) and 1/21 of  $RLU_{2B-min}$  (corresponding that the concentration of SMX is  $16.2 \mu\text{g L}^{-1}$ ). When the  $RLU_{max-NOR}$  was taken as the background, the ALP CL for SMX ( $RLU_{ALP-SMX}$ ) was obtained by subtracting  $RLU_{max-NOR}$  from  $RLU_{2A}$ . Standard curves were obtained by plotting  $RLU_{ALP-SMX}$  against the logarithm of SMX concentration (Fig. 3b). When determining the background effects, interferences are quantified by comparing the inhibition curves generated from  $RLU_{2A}$  and  $RLU_{ALP-SMX}$ . The curves generated from  $RLU_{2A}$  and  $RLU_{ALP-SMX}$  were

**Fig. 4** Normalized standard curves for AMP, NOR, SMX, and CAP of DLTRC-MIA





almost superposable, indicating that the background effect ( $RLU_{1A}$ ) was not insignificant and negligible (Fig. 3b). Hence,  $RLU_{2A}$  was used to represent  $RLU_{ALP-SMX}$ .

When the chemiluminescence reader collected the CL signal at 4 min, there were two chemiluminescent systems and four CL signals—ALP CL system for NOR and SMX and HRP CL system for AMP and CAP. The CL value ( $RLU_{2B}$ ) was the sum of  $RLU_{2A}$  (ALP CL signal for NOR and SMX) and  $RLU_{HRP-AMP}$  (HRP CL for AMP). When the  $RLU_{2A}$  and  $RLU_{HRP-AMP}$  ( $RLU_{1B}$ ) were taken as the background, the HRP CL for CAP ( $RLU_{HRP-CAP}$ ) was obtained by subtracting  $RLU_{max-2A}$  and  $RLU_{max-1B}$  from  $RLU_{2B}$ . When determining the background effects, interferences are quantified by comparing the inhibition curves generated from  $RLU_{2B}$  and  $RLU_{HRP-CAP}$ . The curves generated from  $RLU_{2B}$  and  $RLU_{HRP-CAP}$  were almost superposable, indicating that the background effect was not insignificant and negligible (Fig. 3c). Hence,  $RLU_{2B}$  was used to represent  $RLU_{HRP-CAP}$ .

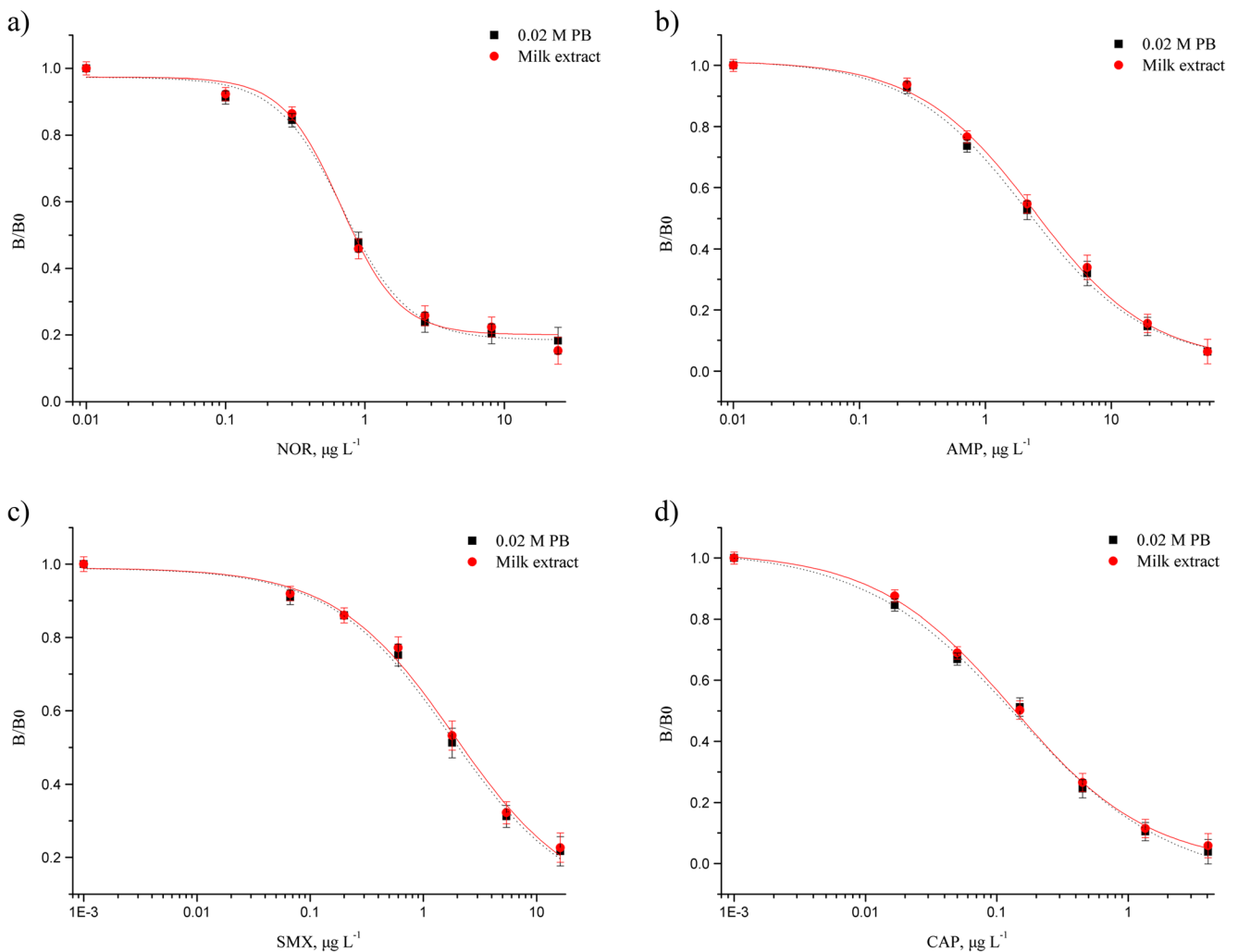
## Performance of DLTRC-MIA in Standard Solutions

### Sensitivity

The sensitivity of the DLTRC-MIA for NOR, AMP, SMX, and CAP, which were represented by 50% inhibition concentration ( $IC_{50}$ ) values, were 0.69, 2.12, 1.75, and 0.14  $\mu\text{g L}^{-1}$ , respectively (Fig. 4). The linear working range determined as the concentrations causing 20–80% inhibition of chemiluminescence intensity was 0.54–11.7, 0.34–5.99, 0.33–15.5, and 0.02–0.67  $\mu\text{g L}^{-1}$  for NOR, AMP, SMX, and CAP, respectively.

### Cross-reactivity

The CR values for 20 FQs, 15  $\beta$ -lactams, 15 SAs, and CAP were all consistent with our previous studies (Zeng et al. 2013; Yu et al. 2015; Wang et al. 2013; Tao et al. 2014) (Table 1). No significant CR of four types of receptors (scFv, PBP2 $\times^*$ ,



**Fig. 5** Inhibition curves of NOR, AMP, SMX, and CAP in buffer and milk extract

MAB, and PAB) to RAC, IVE, GEN, FF, and TAP was observed.

### Comparison with Other Immunochemical Methods

The analytical comparison of other immunochemical methods for FQs,  $\beta$ -lactams, SAs, and CAP detection is important to analyze their suitability for screening the residue in field samples (Table 2). The limits of detection (LODs) of the DLTRC-MIA for FQs and SAs were lower than those of other immunochemical methods and R-Biopharm ELISA kits. The LOD for the DLTRC-MIA for  $\beta$ -lactams was lower than that of other immunochemical methods, higher than that of R-Biopharm ELISA kits. The LOD for the DLTRC-MIA for CAP was lower than that of other immunochemical methods, equal to that of R-Biopharm ELISA kits. Moreover, the DLTRC-MIA could simultaneously detect FQs,  $\beta$ -lactams, SAs, and CAP including 51 drugs, which were much better than previous reports and R-Biopharm ELISA kits (Table 2).

### Matrix Effect

When determining the matrix effects, interferences are quantified by comparing a standard inhibition curve in buffer with that generated in the milk extract matrix known to be free of FQs,  $\beta$ -lactams, SAs, and CAP. Extracting with ethyl acetate,

drying by nitrogen, and dissolving in the same buffer of the standard inhibition curve were adopted to overcome matrix interference. The four group curves for both of NOR, AMP, SMX, and CAP are superposable, indicating that the matrix effect is not significant (Fig. 5). Then, the samples can be analyzed using the standard inhibition curve instead of the matrix curve.

### Limit of Detection

The LOD was calculated as the mean of the measured content of blank different milk samples ( $n = 20$ ) plus three standard deviations (mean + 3SD). The 20 blank milk samples, obtained by 20 different animals, extracted and analyzed according to the developed DLTRC-MIA, giving a mean and SD concentration of  $0.22 \pm 0.03 \mu\text{g L}^{-1}$  (NOR),  $0.53 \pm 0.04 \mu\text{g L}^{-1}$  (AMP),  $0.24 \pm 0.08 \mu\text{g L}^{-1}$  (SMX), and  $0.013 \pm 0.004 \mu\text{g L}^{-1}$  (CAP). The highest observed blanks were 0.4, 0.8, 0.6, and  $0.028 \mu\text{g L}^{-1}$  for NOR, AMP, SMX, and CAP in milk samples, respectively. The LODs for NOR, AMP, SMX, and CAP in milk samples were set at the level of the highest observed blank (0.4, 0.8, 0.6, and  $0.028 \mu\text{g L}^{-1}$ ) to avoid false positive, because it was greater than the mean observed concentration plus 3SD ( $0.31 \mu\text{g L}^{-1}$  for NOR,  $0.65 \mu\text{g L}^{-1}$  for AMP,  $0.48 \mu\text{g L}^{-1}$  for SMX, and  $0.025 \mu\text{g L}^{-1}$  for CAP).

**Table 3** Detection capability (CC $\beta$ ) for FQs,  $\beta$ -lactams, SAs, and CAP in milk sample

Compound	CC $\beta$ $\mu\text{g L}^{-1}$	
FQs	CIP, ENR, NOR, PEF	<1
	DAN, LEV, OFL, AMI, ENO, ORB, FLER, SPA, MAR, LOM	<2
	FLU, PAZ	<5
	PRU, SAR, DIF	<40
	TRO	<100
	$\beta$ -lactams	
$\beta$ -lactams	PEN, AMP, AMO, NAF, CEF, CEQ	<1
	DIC, OXA, CEM, CEZ, CEP, CET	<4
	CLO, CER	<20
	CEL	<100
SAs	SMX, SDM, SDT, SIM, SMD, SMM, SMP, SPY, SNT	<1
	SCP, SMR, SMZ, acetyl-SMZ, SQX	<5
	SDZ	<20
CAP	CAP	<0.1

FQs: CIP ciprofloxacin, DAN danofloxacin, DIF difloxacin, ENO enoxacin, ENR enrofloxacin, FLER fleroxacin, AMI amifloxacin, FLU flumequine, LEV levofloxacin, LOM lomefloxacin hydrochloride, MAR marbofloxacin, NOR norfloxacin, OFL ofloxacin, ORB orbifloxacin, PAZ pazufloxacin, PEF pefloxacin-d 5, PRU prulifloxacin, SAR sarafloxacin, SPA sparfloxacin, TRO trovafloxacin

$\beta$ -lactams: PEN penicillin G, AMP ampicillin, AMO amoxicillin, CLO cloxacillin, DIC dicloxacillin, OXA oxacillin, NAF nafcillin, CEL cephalixin, CEF ceftiofur, CEM cefalonium, CEQ cefquinome, CEZ cefazolin, CER cefoperazone, CEP cephalirin, CET cefacetile

SAs: SMX sulfamethoxazole, SCP sulfachloropyridazine, SDM sulfadimethoxine, SDT sulfadimethoxypyrimidine, SDZ sulfadiazine, SIM sulfisomidine, SMD sulfamethoxydiazine, SMM sulfamonomethoxine, SMP sulfamethoxypyridazine, SMR sulfamerazine, SMZ sulfamethazine, acetyl-SMZ N<sup>4</sup>-acetyl-sulfamethazine, SPY sulfapyridine, SNT sulfantran, SQX sulfaquinolaxaline

**Table 4** Recovery and variation of spiked AMP, NOR, SMX, and CAP in milk

Drug	Spiked concentration $\mu\text{g L}^{-1}$	Intra-assay <sup>a</sup>		Inter-assay <sup>b</sup>	
		Measured ( $\mu\text{g L}^{-1}$ )	Recovery (%)	Measured ( $\mu\text{g L}^{-1}$ )	Recovery (%)
NOR	0.2	0.28 ± 0.11	140.0	0.25 ± 0.12	125.0
	0.4	0.34 ± 0.03	85.0	0.32 ± 0.04	80.0
	0.8	0.69 ± 0.06	86.2	0.71 ± 0.09	88.8
AMP	0.4	0.63 ± 0.20	157.5	0.68 ± 0.21	170.0
	0.8	0.72 ± 0.06	90.0	0.71 ± 0.07	88.6
	1.6	1.29 ± 0.11	80.6	1.32 ± 0.14	82.5
SMX	0.3	0.39 ± 0.15	130.0	0.42 ± 0.13	140.0
	0.6	0.51 ± 0.05	85.0	0.52 ± 0.06	86.7
	1.2	1.02 ± 0.09	85.0	1.10 ± 0.13	91.7
CAP	0.014	0.021 ± 0.003	150.0	0.020 ± 0.003	142.8
	0.028	0.024 ± 0.001	83.3	0.023 ± 0.002	77.8
	0.056	0.046 ± 0.003	80.6	0.046 ± 0.004	83.3
NOR/AMP/SMX/CAP	0.2/0.4/0.3/0.014	0.27 ± 0.09/0.61 ± 0.19/0.38 ± 0.16/0.022 ± 0.005	135.0/152.5/126.7/157.1	0.28 ± 0.11/0.59 ± 0.21/0.43 ± 0.16/0.023 ± 0.006	140.0/147.5/143.3/164.3
	0.4/0.8/0.6/0.028	0.35 ± 0.03/0.74 ± 0.05/0.53 ± 0.06/0.025 ± 0.001	87.5/92.5/88.3/89.3	0.34 ± 0.04/0.73 ± 0.05/0.52 ± 0.07/0.024 ± 0.002	85.0/91.2/86.7/85.7
	0.8/1.6/1.2/0.056	0.73 ± 0.07/1.38 ± 0.11/1.04 ± 0.10/0.047 ± 0.003	91.2/86.2/86.7/83.9	0.75 ± 0.08/1.25 ± 0.12/1.13 ± 0.13/0.049 ± 0.005	93.8/90.6/94.2/87.5

<sup>a</sup> Intra-assay variation was determined by 10 replicates on a single day<sup>b</sup> Inter-assay variation was determined by 10 replicates on 3 different days

Values of LODs inferred from the cross-reactivity data (Table 1) (Tittlemier et al. 2008) will range from approximately 0.29  $\mu\text{g L}^{-1}$  for ciprofloxacin (CIP) to 81.6  $\mu\text{g L}^{-1}$  for trovafloxacin (TRO) (FQs), 0.27  $\mu\text{g L}^{-1}$  for ceftiofur (CEF) to 44.1  $\mu\text{g L}^{-1}$  for cephalixin (CEL) ( $\beta$ -lactams), 0.089  $\mu\text{g L}^{-1}$  for sulfadimethoxine (SDM) to 2.7  $\mu\text{g L}^{-1}$  for sulfadiazine (SDZ), and 0.028  $\mu\text{g L}^{-1}$  for CAP, lower than the MRLs (FQs from 10 to 100  $\mu\text{g L}^{-1}$ ,  $\beta$ -lactams ranging from 4 to 125  $\mu\text{g L}^{-1}$ , SAs from 25 to 100  $\mu\text{g L}^{-1}$ ) or MRPL (0.3  $\mu\text{g L}^{-1}$  for CAP) in milk (EU Regulation 470/2009; Commission Regulation 37/2010); Stolker and Brinkman 2005). Hence, the sensitivity of the DLTRC-MIA in milk sample for 20 FQs, 15  $\beta$ -lactams, 15 SAs, and CAP meets the requirement of detection levels for 51 drug residues in milk.

### Decision Limit and Detection Capability

First, 20 representative blank samples (for each matrix) were extracted and analyzed using the optimized DLTRC-MIA procedure. The concentration of each blank sample was calculated, and the decision limit of each matrix was then estimated by the mean ( $n = 20$ ) plus 3 times the standard deviation, which was obtained in LOD section. Hence, the  $CC\alpha$  were 0.31  $\mu\text{g L}^{-1}$  for NOR, 0.65  $\mu\text{g L}^{-1}$  for AMP, 0.48  $\mu\text{g L}^{-1}$  for SMX, and 0.025  $\mu\text{g L}^{-1}$  for CAP, respectively. Similarly, values of  $CC\alpha$  inferred from the cross-reactivity data (Table 1) will

range from approximately 0.22  $\mu\text{g L}^{-1}$  for ciprofloxacin (CIP) to 63.2  $\mu\text{g L}^{-1}$  for trovafloxacin (TRO) (FQs), 0.22  $\mu\text{g L}^{-1}$  for ceftiofur (CEF) to 35.8  $\mu\text{g L}^{-1}$  for cephalixin (CEL) ( $\beta$ -lactams), 0.071  $\mu\text{g L}^{-1}$  for sulfadimethoxine (SDM) to 2.16  $\mu\text{g L}^{-1}$  for sulfadiazine (SDZ) (SAs), and 0.028  $\mu\text{g L}^{-1}$  for CAP, respectively.

The detection capability is defined as the lowest concentration that can be determined with an error probability of  $\beta$  ( $\leq 5\%$ ). In practice, the  $CC\beta$  was chosen as the lowest tested fortification level giving no negative result in an analysis of 20 spiked blanks; this decision should avoid the problem of false negatives. The  $CC\beta$  values obtained in this way for milk samples are listed in Table 3.

### Recovery and Precision

Some spiked milk samples were detected using the developed DLTRC-MIA to evaluate the application potential of this method. Blank milk samples were fortified with NOR and/or AMP and/or SMX and/or CAP standards at different known amounts (1/2 LOD, LOD, or 2 LOD) prior to extraction and analysis, respectively (Table 4). Each sample was evaluated 10 times in duplicate and on three consecutive days to verify the repeatability. The average intra-assay and inter-assay recoveries of NOR, AMP, SMX, and CAP in the two groups fortified at concentrations greater than (2 LOD) or equal to the LOD were at least 80.0%, with coefficients of variation (CVs) less than 15%, suggesting that the DLTRC-MIA and

**Table 5** Determination of milk samples collected from retail outlets in Chongqing by the DLTRC-CIA, conventional ELISA kits, and LC-MS/MS

Sample	DLTRC-CIA ( $\mu\text{g L}^{-1}$ )				Conventional ELISA kit (R-Biopharm) ( $\mu\text{g L}^{-1}$ )				LC-MS/MS ( $\mu\text{g L}^{-1}$ )			
	NOR	AMP	SMX	CAP	NOR	AMP	SMX	CAP	FQs	$\beta$ -lactams	SAs	CAP
S1	3.0 <sup>a</sup>	ND	ND	ND	3.5	ND	ND	ND	3.3 (PEF)	ND	ND	ND
S5	ND	0.8	ND	0.23	ND	0.9	ND	0.25	ND	40.0 (CEL)	ND	0.21
S10	1.6	ND	ND	ND	1.8	ND	ND	ND	1.3 (NOR)	ND	ND	ND
S11	ND	4.1	ND	ND	ND	3.8	ND	ND	ND	4.3 (AMP)	ND	ND
S16	ND	ND	12.5	0.60	ND	ND	13.0	0.59	ND	ND	11.8 (SMX)	0.56
S17	2.9	ND	ND	ND	3.1	ND	ND	ND	4.9 (LMO)	ND	ND	ND
S25	ND	5.8	ND	ND	ND	5.2	ND	ND	ND	5.1 (AMP)	ND	ND
S26	2.7	ND	ND	ND	3.4	ND	ND	ND	2.9 (ENR)	ND	ND	ND
S35	ND	ND	5.9	ND	ND	ND	6.0	ND	ND	ND	8.5 (SMZ)	ND
S2-S4, S6-S9, S12-S15, S18-S24, S27-S34, S36-S40					ND							

ND not detectable

<sup>a</sup> Each was determined with 3 repeats

extraction method are efficient enough for use as a screening tool. The recoveries of milk samples fortified at the concentration of 1/2 LOD with NOR, AMP, SMX, and CAP were highly variable (percent recoveries ranged from 125.0 to 170.0% and lower than for the two other fortification groups (Table 4)).

### Application in Field Milk Samples

To evaluate the determination capability of the developed DLTRC-MIA in milk samples, 40 field samples were analyzed by the developed DLTRC-MIA, conventional ELISA kits, and LC-MS/MS (Table 5). The results demonstrated that the developed DLTRC-MIA could simultaneously screen FQs,  $\beta$ -lactams, SAs, and CAP in the incurred samples. Thereafter, the developed DLTRC-MIA was reliable for the simultaneous screening of trace CAP and CLE residues in milk samples.

### Conclusions

In this study, a novel immunoassay based on the chemiluminescent distinction of the kinetic characteristics of HRP and AP with approximate estimation approach was developed. It is the first report where two different enzymes (HRP and ALP) were used twice in one immunoassay and in a single well for detecting multiple low molecular weight chemical residues. The integrated assay was developed to detect 20 FQs, 15  $\beta$ -lactams, 15 SAs, and CAP in milk, capable of detecting multi-analyte residues, particularly in high-throughput screening. This study could also be considered as a model for custom measurement of other multiple low-molecular weight compounds of concern.

**Acknowledgments** The PAb, MAbs, PBP 2 $\times$ \*, and scFv-ALP were donated by the Department of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, China Agricultural University.

### Compliance with Ethical Standards

**Funding** We acknowledge financial support National Natural Science Foundation of China (Grant No. 31672605) (Grant No. 31671787), China Postdoctoral Science Foundation (Grant No. 2016 M590855) and Fundamental Research Funds for the Central Universities (Grant No. XDJK2015C040) (Grant No. SWU114043) (Grant No. XDJK2016C164) and Chongqing Science and Technology Commission (cstc2014pt-gc8001).

**Conflict of Interest** Xiaqi Tao declares that he has no conflict of interest. Jia Wang declares that he has no conflict of interest. Yuejie Xie declares that he has no conflict of interest. Xiaowei Zuo declares that he has no conflict of interest. Fang Mo declares that he has no conflict of interest. Song Zhou declares that he has no conflict of interest. Hongjun Li declares that he has no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** All the authors named in the manuscript are entitled to the authorship and have approved the final version of the submitted manuscript.

### References

- Charlarmroj R, Himananto O, Seepiban C, Kumpoonsiri M, Warin N, Oplatowska M, Gajanandana O, Grant IR, Karoonuthaisiri N, Elliott CT (2013) Multiplex detection of plant pathogens using a microsphere immunoassay technology. *PLoS One* 8:e62344
- Chen DM, Yu J, Tao YF, Pan YH, Xie SY, Huang LL, Peng DP, Wang X, Wang YL, Liu ZL, Yuan ZH (2016) Qualitative screening of veterinary anti-microbial agents in tissues, milk, and eggs of food-producing animals using liquid chromatography coupled with tandem mass spectrometry. *J Chromatogr B* 1017:82–88
- Chen M, Wen K, Tao XQ, Ding SY, Xie J, Yu XZ, Li JC, Xia X, Wang Y, Xie SL, Jiang HY (2014a) A novel multiplexed fluorescence polarisation immunoassay based on a recombinant bi-specific single-chain diabody for simultaneous detection of fluoroquinolones and sulfonamides in milk. *Food Addit Comtam: Part A* 31:1959–1967
- Chen M, Wen K, Tao XQ, Xie J, Wang LM, Li Y, Ding SY, Jiang HY (2014b) Cloning, expression, purification and characterization of a bispecific single-chain diabody against fluoroquinolones and sulfonamides in *Escherichia coli*. *Protein Express Purif* 100:19–25
- Dodeigne C, Thunus L, Lejeune R (2000) Chemiluminescence as diagnostic tool. *Talanta* 51:415–439
- European Commission (2009) Regulation (EC) no 470/2009 of the European Parliament and of the council of 6 may 2009 laying down community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing council regulation (EEC) no 2377/90 and amending directive 2001/82/EC of the European Parliament and of the council and regulation (EC) no 726/2004 of the European Parliament and of the council. *Off J Eur Union* L152:11–22
- European Union (2010) Commission regulation (EU) no 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Off J Eur Union* L15:1–32
- Guo YR, Tian J, Liang CZ, Zhu GN, Gui WJ (2013) Multiplex bead-array competitive immunoassay for simultaneous detection of three pesticides in vegetables. *Microchim Acta* 80:387–395
- Goto T, Ito Y, Yamada S, Matsumoto H, Oka H (2005) High throughput analysis of tetracycline and penicillin antibiotics in animal tissues using electrospray tandem mass spectrometry with selected reaction monitoring transition. *J Chromatogr A* 1100:193–199
- Jiang WX, Wang ZH, Beier RC, Jiang HY, Wu YN, Shen JZ (2013) Simultaneous determination of 13 fluoroquinolone and 22 sulfonamide residues in milk by a dual-colorimetric enzyme-linked immunosorbent assay. *Anal Chem* 85:1995–1999
- Karageorgou E, Myridakis A, Stephanou EG, Samanidou V (2013) Multiresidue LC-MS/MS analysis of cephalosporins and quinolones in milk following ultrasound-assisted matrix solid-phase dispersive extraction combined with the quick, easy, cheap, effective, rugged, and safe methodology. *J Sep Sci* 36:2020–2027
- Li XM, Wang WJ, Wang LM, Wang Q, Pei XY, Jiang HY (2015) Rapid determination of phenylethanolamine a in biological samples by enzyme-linked immunosorbent assay and lateral-flow immunoassay. *Anal Bioanal Chem* 407:7615–7624

- Liu HY, Lin SL, Fuh MR (2016) Determination of chloramphenicol, thiamphenicol and florfenicol in milk and honey using modified QuEChERS extraction coupled with polymeric monolith-based capillary liquid chromatography tandem mass spectrometry. *Talanta* 150:233–239
- Mitchell JM, Griffiths MW, McEwen SA, McNab WB, Yee AJ (1998) Antimicrobial drug residues in milk and meat: causes, concerns, prevalence, regulations, tests, and test performance. *J Food Protect* 61:742–756
- Shi XZ, Wu AB, Zheng SL, Li RX, Zhang DB (2007) Molecularly imprinted polymer microspheres for solid phase extraction of chloramphenicol residues in foods. *J Chromatogr B* 850:24–30
- Stolker AAM, Brinkman UAT (2005) Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals – a review. *J Chromatogr A* 1067:15–53
- Tao XQ, Jiang HY, Zhu JH, Wang X, Wang ZH, Niu LL, Wu XP, Shi WM, Shen JZ (2014) An ultrasensitive chemiluminescent ELISA for determination of chloramphenicol in milk, milk powder, honey, eggs and chicken muscle. *Food Agr Immunol* 25:137–148
- Tittlemier SA, Gelinat JM, Dufresne G, Haria M, Querry J, Cleroux C, Menard C, Delahaut P, Singh G, Fischer-Duran N (2008) Development of a direct competitive enzyme-linked immunosorbent assay for the detection of fluoroquinolone residues in shrimp. *Food Anal Method* 1:28–35
- Turnipseed SB, Andersen WC, Karbiwnyk CM, Madson MR, Miller KE (2008) Multi-class, multi-residue liquid chromatography/tandem mass spectrometry screening and confirmation methods for drug residues in milk. *Rapid Commun Mass Sp* 22:1467–1480
- Wang JP, Dong J, Duan CF, Zhang HC, He X, Wang GN, Zhao GX, Liu J (2016) Production and directional evolution of anti-sarafloxacin ScFv antibody for immunoassay of fluoroquinolones in milk. *J Agric Food Chem* 64:7957–7965
- Wang ZH, Beier RC, Sheng YJ, Zhang SX, Jiang WX, Wang ZP, Wang J, Shen JZ (2013) Monoclonal antibodies with group specificity toward sulfonamides: selection of hapten and antibody selectivity. *Anal Bioanal Chem* 405:4027–4037
- Yu XZ, Tao XQ, Shen JZ, Zhang SX, Cao XY, Chen M, Wang WJ, Wang ZH, Wen K (2015) A one-step chemiluminescence immunoassay for 20 fluoroquinolone residues in fish and shrimp based on a single chain Fv-alkaline phosphatase fusion protein. *Anal Methods* 7: 9032–9039
- Zeng K, Zhang J, Wang Y, Wang ZH, Zhang SX, Wu CM, Shen JZ (2013) Development of a rapid multi-residue assay for detecting  $\beta$ -lactams using penicillin binding protein 2x\*. *Biomed Environ Sci* 26:100–109
- Zhang DD, Tao XQ, Jiang HY, Wen K, Shen JZ, Cao XY (2014) Simultaneous detection of forbidden chemical residues in milk using dual-label time-resolved reverse competitive chemiluminescent immunoassay based on amine group functionalized surface. *PLoS One* 9(10):e109509
- Zhu K, Li JC, Wang ZH, Jiang HY, Beier RC, Xu F, Shen JZ, Ding SY (2011) Simultaneous detection of multiple chemical residues in milk using broad-specificity antibodies in a hybrid immunosorbent assay. *Biosens Bioelectron* 26:2716–2719
- Zhang SX, Liu ZW, Guo X, Cheng LL, Wang ZH, Shen JZ (2008) Simultaneous determination and confirmation of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine in chicken muscle by liquid chromatography tandem mass spectrometry. *J Chromatogr B* 875:399–404