Supplementary materials

Materials and Methods

Buffers

The following buffers were used:

(a) Coating buffer (CB)—coating buffer, 0.05 M carbonate/bicarbonate buffer, pH 9.6

(b) Blocking buffer—0.01 M sodium phosphate-buffered saline (PBS) with 0.5% casein, pH 7.4

(c) Phosphate-buffered saline (PBS; pH 7.4)—0.01M PBS was prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 0.24 g KH_2PO_4, and 3.63 g $Na_2HPO_4 \cdot 12H_2O$ in 1 L purified water

(d) PBST was prepared by 0.01 M PBS with 0.05% Tween-20

(e) 0.2 M sodium phosphate, pH 7.2 (PB)

(f) Carrez A: 0.36 M $K_4Fe(CN)_6 \cdot 3H_2O$; Carrez B 1.04 M $ZnSO_4 \cdot 7H_2O$

Reagents

(a) Standards.—The CAP, FF and FFA stock solutions were prepared in methanol; CLE, SAL, RAC and sulfadiazine were prepared in ethanol; ciprofloxacin and penicillin were prepared in purified water; TAP was prepared in dimethylfomamide. The stock solution (2 mg mL^{-1}) was stored at -20 °C, and working standards were prepared from the stock solution by serial dilution in 0.02 M PB. Working standard (cocktail of CAP and CLE) in the range of 0.0033–2.43 μg L^{-1} (CAP) and 0.0074–5.4 μg L^{-1} (CLE) (Scheme A), 0.0033–2.43 μg L^{-1} (CAP) and 0.0667–16.2 μg

L^{-1} (CLE) (Scheme B) were prepared from the 2 mg mL^{-1} stock solution by serial dilution in 0.02 M PB.

Preparation of Coating Antigen and Synthesis of HRP-conjugated CAP

The synthesis of coating antigens and preparation of HRP-conjugated CAP were according to the previously reported procedure with minor revision. Briefly, the CAP-succinate (3.1 mg), NHS (5.5 mg), and DCC (4.6 mg) were mixed and stirred in 0.5 mL dimethylformamide (DMF) for 6 hours at room temperature. The horseradish peroxidase (HRP, 4.3 mg) in 1.5 mL of 0.2 M $NaHCO_3$ was then added to the reaction mixture with stirring at room temperature for 16 hours. The reaction solutions were then dialyzed against PBS for three days and mixed with 2 mL of 50% glycerol and stored at -20 °C.

CLE (10 mg) was dissolved in 1.33 mL of distilled water (7.5 mg mL^{-1}), and the pH was adjusted to 2.5 by adding 1 N HCl. To this CLE solution, $NaNO_2$ (0.67 mL, 15 mg mL^{-1}) solution was added dropwise in the dark at 48 °C with constant stirring followed by incubation for 30 min at 48 °C to diazotise CLE. To remove unreacted nitrous acid, ammonium sulphamate (50 mg mL^{-1}) was added until no more nitrogen bubbles were given off. Diazotisation of CLE was confirmed by the formation of a deep yellow color after reaction of an aliquot of the above solution with N, N-dimethylaniline. The diazo-CLE solution was added to carrier OVA (3.33 mL; 55 mg mL^{-1} in 0.1M PBS, pH 7.5), then the final pH was adjusted to 7.5 with NaOH (1 N) followed by overnight incubation at 48 °C. CLE-OVA conjugate was dialysed against

0.01 M PBS at 48 °C for three days in the dark with frequent changes of the dialysis solution.

Strategy of the Two Immunoassays

Scheme A

HRP-CAP/CAP—anti-CAP PAb, HRP-goat anti mouse immunoglobulins—anti-CLE MAb—CLE-OVA/CLE

The subtlety of Scheme A lied in different coating concentration of CLE-OVA and anti-CAP PAb, in which $RLU_{max\text{-}CLE}$ (chemiluminescence intensity in the absence of CLE) values of HRP-goat anti mouse immunoglobulins—anti-CLE MAb—CLE-OVA/CLE (CLE) system was only 1/50 of that for HRP-CAP/CAP—anti-CAP PAb system. Although based on the same HRP-luminol chemiluminescence system, the impact of first detection for CLE chemiluminescence signal (2 million) on the latter detection for CAP signal (100 million) is negligible. After the first detection of CLE, added the CAP standard or sample solution and HRP-CAP, reacted for 15 min for CAP detection.

Scheme B

HRP-CAP/CAP—anti-CAP PAb, ALP-goat anti mouse immunoglobulins—anti-CLE (MAb)—CLE-OVA/CLE

The Scheme B utilized two different enzymatic systems (HRP and ALP), corresponding to the HRP-CAP/CAP—anti-CAP PAb and ALP-goat anti mouse

immunoglobulins—anti-CLE MAb—CLE-OVA/CLE system. In this strategy, the HRP chemiluminescence and ALP chemiluminescence systems did not interfere in each other. Hence the CAP and CLE could be screened in one well.

Overall, both methods could simultaneously determinate CAP and CLE. The detection sensitivity of Scheme B was slightly lower than that of Scheme A due to increased concentration of coating antigen and antibody resulting in sensitivity losses.

The reaction time of Scheme A was 30 +15 min for two step reaction times and the intensity of light emission was measured immediately after the addition of the substrate. Therefore, the total time was basically the two step reaction times. The reaction time of Scheme B was only one step reaction time 30min. Hence, the total time was much shorter than that of Scheme A.

CL-ELISA for CLE

Procedure of CL-ELISA

Plates were coated overnight at 4 °C with 100 μL CLE-OVA (1:100000 dilution) dissolved in coating buffer. The plates were washed with 260 μL/well PBST manually three times and blocked with 200 μL well^{-1} of blocking buffer, and the plates were incubated at 37 °C for 1 hour. The plates were washed as described above (conditioned ELISA plates can be stored at -4 °C for four weeks). CLE standard in 0.02 M PB or sample solution (100 μL well^{-1}) was added, followed by 50 μL/well MAb (CLE) at a dilution of 1/1500000 in 0.02 M PB. The competitive reaction was

allowed to take place for 30 min at room temperature. After washing the plates five times, 100 μL well^{-1} HRP-labeled goat anti-mouse immunoglobulins (1/5000 dilution of in PBST) were added. The reaction was allowed to take place for 30 min at room temperature. After washing the plates five times and finally the HRP activity was revealed by adding 100 μL well^{-1} freshly prepared Super Signal substrate solution. The intensity of light emission was measured using a chemiluminesence reader immediately after the addition of the substrate and the results were expressed in relative light units (RLU).

Assay Sensitivity

The IC_{10}, the sensitivity (IC_{50}) and the linear working range of the CL-ELISA for CLE, were 0.008 μg L^{-1}, 0.054 μg L^{-1} and 0.018-0.67μg L^{-1}, respectively. Meanwhile, the LOD of CLE in milk was 0.011 μg L^{-1} calculated from 20 blank milk sample values.

Legends of supplementary Tables and Figures

Supplementary Table 1 Optimized Concentrations of the Anti-CAP Polyclonal antibody and Coating Antigen CLE-OVA for the Integrated Scheme A Determination by the Checkerboard Test

Supplementary Figure 1 Gross standard inhibition curves and revised standard inhibition curves for second step in Scheme A

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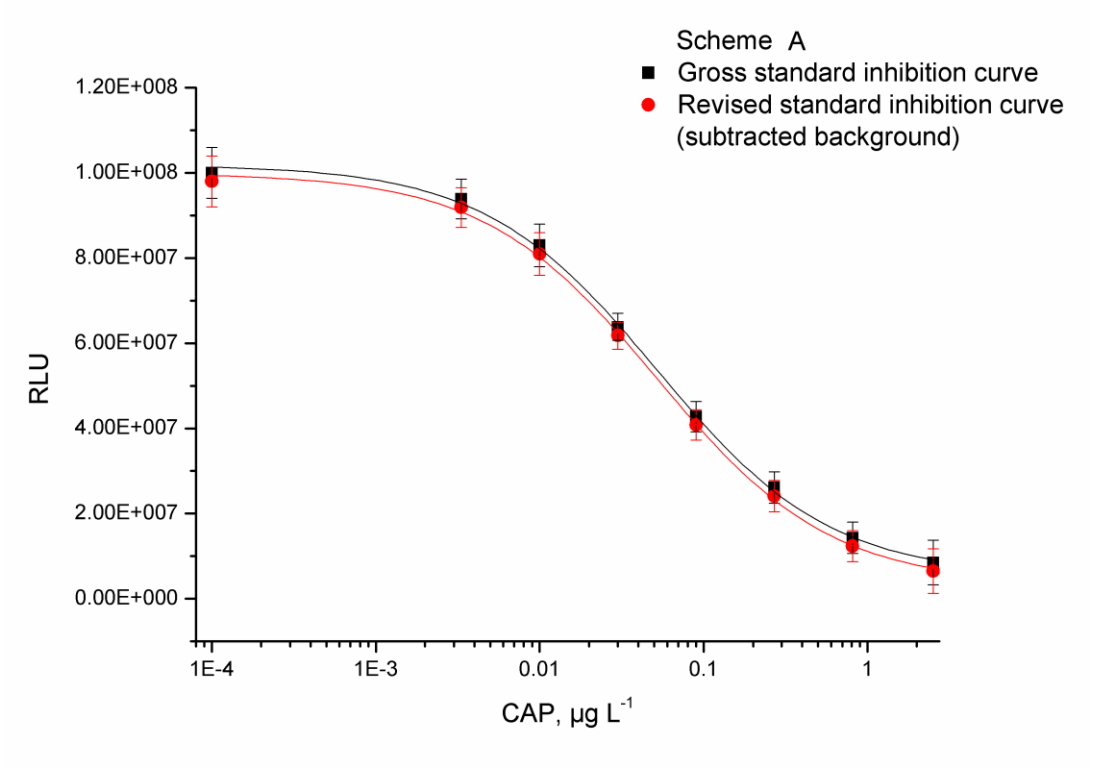

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114 Supplementary Figure 1 Gross standard inhibition curves and revised standard

115 inhibition curves for second step in Scheme A

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Supplementary Table 1 Optimized Concentrations of the Anti-CAP Polyclonal antibody and Coating Antigen CLE-OVA for the Integrated Scheme A Determination by the Checkerboard Test

Note: These values are $RLU_{max\text{-}CAP}$/ $RLU_{max\text{-}CLE}$

The standard deviations (SDs) of the values were omitted to simplify this Table

The CAP polyclonal antibody (×10000)	The coating antigen of CLE (×10000)						
	0	0.4	0.8	1.6	3.2	6.4	12.8
0	0/0	0/3.0	0/2.1	0/1.6	0/1.3	0/0.64	0/0.56
2	13.0/0	10.6/2.4	11.5/1.6	12.4/1.1	12.6/0.9	12.4/0.81	12.7/0.92
4	12.6/0	10.2/1.41	10.6/1.35	11.3/0.78	10.1/0.62	11.9/0.59	12.4/0.62
6	11.5/0	9.8/0.82	9.3/0.79	8.8/0.68	8.6/0.64	7.9/0.56	8.3/0.59
8	11.1/0	8.8/0.75	9.3/0.73	8.5/0.86	9.1/0.91	**10.0/0.20**[a]	8.1/0.53
10	8.6/0	7.9/0.69	7.6/0.79	6.9/0.83	7.2/0.88	8.4/0.93	7.2/0.56
12	7.5/0	7.2/0.61	7.3/0.62	8.1/0.78	7.8/0.81	6.9/0.89	6.1/0.41

[a] Taking $RLU_{max(CAP)}$/ $RLU_{max(CLE)}$, $IC_{50(CAP)}$ and $IC_{50(CLE)}$ into consideration, the value of 10.0/0.20 was the best for the Scheme A.