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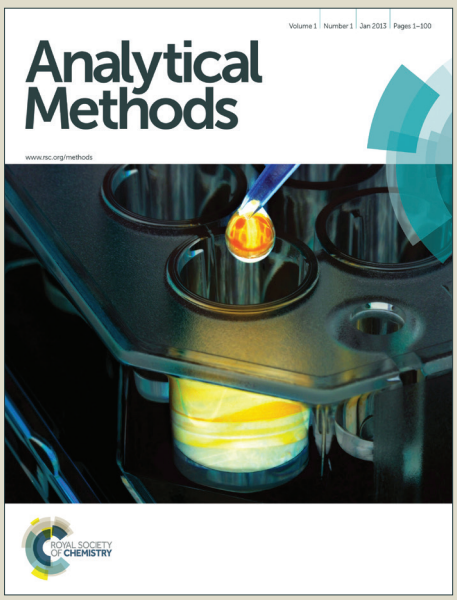

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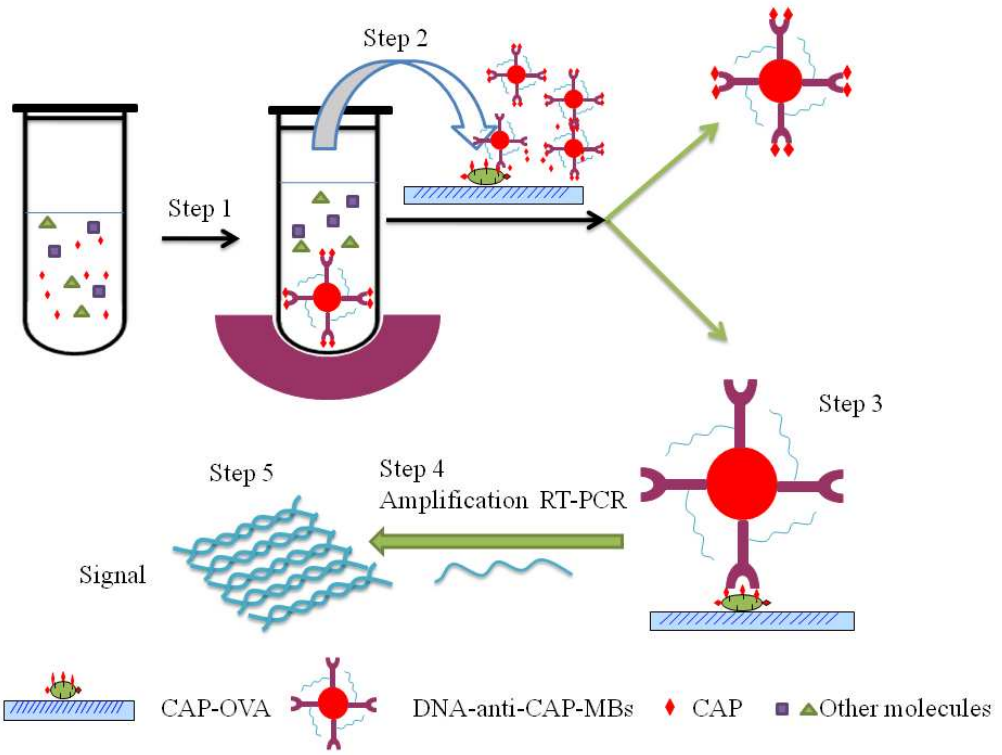
Graphic Abstract



The real-time immuno-quantitative PCR (RT-IPCR) schematic illustration of the determination of CAP in milk based on magnetic beads capturing

Development of a Highly Sensitive Real Time Immuno-PCR for the Measurement of Chloramphenicol in Milk Based on Magnetic Beads Capturing

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Abstract

Chloramphenicol (CAP) is a forbidden antibiotic that enters the food chain by illegal use for food-producing animals, potentially posing aplastic anaemia in human. Immuno-PCR has the potential to address the need of meeting the strict limits by detecting trace levels of CAP present in animal derived food. A real-time immuno-quantitative PCR (RT-IPCR) assay for quantification of CAP based on simple and quick immunomagnetic beads recovery of CAP in milk was developed. The immunomagnetic beads were obtained by linking the reporter DNA to anti-CAP monoclonal antibody with N-Succinimidyl S-Acetylthioacetate (SATA) and succini-midyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), and immobilizing the anti-CAP monoclonal antibody/DNA-label conjugate on the magnetic beads with 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (SNHS). The RT-IPCR assay leads to the sensitive detection and quantification of CAP from 0.001-0.11 μg L^{-1} (R^2=0.9986) with 50% inhibition concentration (IC_{50}) values of 0.008 μg L^{-1}. The RT-IPCR approach discussed here is presented as a model system that could easily be adapted for small molecule detection in a variety of food using a simple immunomagetic beads recovery.

Keywords

Chloramphenicol (CAP); Real time immuno-PCR; Magnetic beads

Introduction

Immuno-PCR was the combination of antibody with PCR emerges by covalently linking a reporter DNA sequence to an antibody.[1] Later, immunodetection was combined with real-time PCR and used for quantification of vascular endothelial grow factor.[2] Although the assays showed high sensitivity for large molecules (for example virus or protein) detection, they required two sets of wells (for immunodetection and PCR), and therefore were not suitable for high throughput screening and were fraught with high risk of contamination. The method was further modified in such a way that both protein detection and real-time PCR were performed in the same well of the TopYield strip.[3] Due to the high sensitivity, the real time quantitative immuno-PCR (RT-IPCR) was widely used in food safety, especially for pathogens and other macromolecules detection.[4,5] However, there were almost no reports about small molecules detection using RT-IPCR. Hence, the establishment of RT-IPCR methods will greatly improved detection sensitivity for small molecules. However, the detection of small molecules based on competitive immunoassay, including the RT-IPCR, often encountered matrix interferences that are consistently present in a sample and may lead to increased false positive or false negative rates, low or high bias or poorer precision. Fortunately, magnetic beads (MBs) have potentially larger surface area combination with surface modification group such as carboxyl, amino group which may provide a high density of antibody or protein and convenient connection of MBs and the protein, and target antigen can be directly captured, easily separated and analyzed by immunoassay.[6,7] When MBs were introduced to RT-IPCR,

matrix interferences would be removed.

Chloramphenicol (CAP), a broad spectrum antibiotic, is frequently employed in animal production for its excellent antibacterial and pharmacokinetic properties. However, in humans it leads to hematotoxic side effects,[8] in particular CAP-induced aplastic anaemia for which a dosage-effect relationship has not yet been established, leading to a prohibition of CAP for the treatment of food-producing animals in China, USA and EU.[9,10] Moreover, milk is a daily food to improve nutrition , so it is of great significance to detect trace CAP residues in milk. Although a variety of analytical methods to detect and qualify CAP in food matrices existed, such as liquid chromatography (LC) with an iron trap detector,[11] LC-mass spectrometry (LC-MS) or LC-MS/MS,[12-15] enzyme linked immunoassay (ELISA),[16,17] surface plasmon resonance-based biosensor[18], chemiluminescent immunoassay based on gold nanoparticles and magnetic beads[19] and piezoelectric immunosensor,[20] there was still a need for more sensitive methods to detect trace CAP in milk. In our previous studies, the chemiluminescent immunoassay based on gold nanoparticles and magnetic beads[19] and the direct competitive chemiluminescent ELISA (CL-ELISA)[21] represented the most sensitive methods for detecting CAP in milk. However, the process of extracting CAP with ethyl acetate and drying by nitrogen for overcoming the matrix interference was a time-consuming work and harmful to human health and environment.

In this study, the MBs were used to separate the resultant immunocomplex and remove the matrix interference. Moreover, the application of MBs also could improve the sensitivity of RT-IPCR by enriching CAP and magnifying signal with covalent

binding several DNA-anti-CAP monoclonal antibodies (DNA-anti-CAP) on MBs. The scheme of the competitive RT-IPCR based on immunomagnetic beads was depicted in Figure 1, and it was applied to CAP analyte in milk. The coating antigen CAP-OVA was immobilized on the surface of polypropylene PCR-plate. The CAP-OVA competed with CAP enriched by DNA-anti-CAP-MBs to bind the anti-CAP on MBs. Next, CAP-DNA-anti-CAP-MBs complex, not binding with the CAP-OVA, was discarded. Finally, the CAP-OVA-anti-CAP-DNA-MBs complex was formed on the PCR wells and the DNA was amplified by the real time-PCR. The fluorescence signals responses decreased linearly with the increase in the concentrations of CAP since a competitive immunoassay mode was employed, that is, the Ct values were positively correlated with the concentrations of the CAP in the standard or milk sample. Here we tested the suitability of RT-IPCR based on magnetic beads capturing for detection of trace CAP in milk and defined the conditions for simplified detection of CAP by RT-IPCR.

Experimental

Apparatus

The real-time PCR was performed with Bio-Rad /MJ Research Opticon 2 (CA, USA). Chemiluminescence was measured with Veritas Microplate Luminometer (Turner BioSystems, Sunny Vale, CA, USA). The colorimetric-ELISA was made by Sunrise microtiter plate reader (TECAN, Groedig, Austria). The polypropylene PCR-plate (AB-0600) was purchased from Thermo Scientific (MA, USA). Transparent 96-well

microtiter ELISA plates for colorimetric assay and 96-well chemiluminescent white opaque MTP were purchased from Costar (Cambridge, MA, USA). Monodisperse magnetic polystyrene microspheres with amine as the surface functional group [100-200 nm, 1% (w/v)] and magnetic rack were provided by Tianjin Baseline Chromtech Research Centre (Tianjin, China). All buffers were prepared using Milli-Q H_2O system (18 MΩ/cm) (EMD Millipore Corporation, Belleria, MA, USA).

Reagents

(a) Standards.—CAP (99% purity, Sigma-Aldrich, St. Louis, MO, USA); florfenicol (FF, 99% purity), florfenicol amine (FFA, 97.6%) and thiamphenicol (TAP, 97.6% purity) were obtained from Schering-Plough Corp (Kenilworth, NJ, USA); sulfadiazine (SUL), ciprofloxacin (CIP), penicillin (PEN) were purchased from Shanghai Caienfu Technology Co. Ltd. (Shanghai, China).

(b) Analytical grade regents.—1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (SNHS), 2-(N-morpholino) ethanesulfonic acid (MES, pH 4.7), N-Succinimidyl S-Acetylthioacetate (SATA) and succini- midyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) were purchased from Thermo (MA, USA). All other chemicals and organic solvents were of reagent grade and were from Beijing Chemical Co. (Beijing, China).

(b) The purified anti-CAP and coating antigen CAP-OVA were from our previous study. [19,22]

(c) The amino modified DNA sequence 5'-TGCCCTGCGT TTATCTGCTC TCGCATGTCGCAAGCCTCATAGTTTAGGAACATTACATTGACGCAGG-3' and

the primers (Forward 5'-CCCTGCGTTTATCTGCTCTC-3'; Reverse 5'-CCTGCGTCAATG TAATGTTC-3') were synthesized by Sangon-Biotech (Shanghai, China).

(d) SYBR® Premix Ex Taq_{TM} II was purchased from Takara (Dalian, China).

Buffers

(a) 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.

(b) Coating buffer (CB, pH 9.6).—0.05 M carbonate buffer, made with 1.59 g Na_2CO_3 and 2.93g $NaHCO_3$ in 1L of purified water.

(c) Blocking buffer.—0.01 M PBS containing 0.5% casein.

(d) Washing solution (PBST).—0.01 M PBS containing 0.05% Tween-20.

(e) 0.02 M phosphate buffer (pH 7.2).—containing 1.1 g $NaH_2PO_4 \cdot 2H_2O$, 5.16 g $Na_2HPO_4 \cdot 12H_2O$ in 1 L purified water.

(f) Imidazole–HCl buffer (pH 7.0).—0.01M Imidazole–HCl buffer was prepared by dissolving dissolve 0.681 g of imidazole in 800 mL of distilled water, adjust to pH 7.0 with 4 N HCl and dilute with distilled water to 1000 mL.

Conjugation of reporter DNA to detection antibodies

A DNA-anti-CAP label conjugate was prepared (Figure 2). A 67 bases long DNA-label with amino-modification at the 5'-end was introduced with protected

sulfhydryls and linked covalently to anti-CAP using the heterobifunctional cross-linking agent SMCC (Product No. 22360), follwing the instructions of SATA (Product No. 26102) and SMCC.

The DNA-anti-CAP label conjugate was immobilized on the MBs (Figure 2). Briefly, a suspension of MBs (100μL), in a 1.5 μL Eppendorf tube (EP tube), were separated from the solution on a magnetic rack. The MBs were washed three times with 0.01 M imidazol-HCl buffer (pH 7.0; 3×200 μL) and then suspended to a final volume of 100 μL in the same buffer solution. The 990 μL of DNA-anti-CAP (2 μg mL^{-1}) was incubated with 10 μL of a pre-mixed solution of EDC (4 mg mL^{-1}) and SNHS (11 mg mL^{-1}) for 30 min at 37 °C to activate the carboxylate groups on the DNA-anti-CAP. The 100 μL of resulting EDC-SNHS cross-linked DNA-anti-CAP was added to the 100 μL of MB suspension and the resultant suspensions were allowed to stand overnight at 25 °C for the immobilization of the activated DNA-anti-CAP to the surface of the MBs. Finally, the resultant DNA-anti-CAP-MBs were separated from the solution magnetically and resuspended in 1 % BSA solution (100 μL) for 2 h to eliminate the risk of unspecific binding, then washed with the above buffer solution (2×100 μL) and resuspended in 100 μL of 0.02 M PBS.

Immunomagnetic beads recovery of CAP in direct competitive immunoassay

Different concentrations of CAP, constituting an array of standard solutions, were individually solution-captured using anti-CAP. The direct immune-capture of CAP was attempted (Figure 1). The CAP capture was done using immunomagnetic beads

containing anti-CAP in direct immunoassay. In brief, dilutions of CAP prepared with 0.02 M PBS (100 μL of each standard) or milk samples (100 μL) were added with 100 μL of DNA-anti-CAP-MBs. CAP capture was performed by gently shaking the tubes for 15 min at room temperature (22 °C) by the shaker (30 rpm/min) to avoid settling of the beads. Then, the magnetic beads were allowed to form a pellet using a magnetic rack and the solution containing unbound molecules was aspirated by pipette. The beads were further washed with 200 μL of PBS for three times and resuspended with 100 μL of 0.02 M PBS. The polypropylene PCR-plate (AB-0600) was added with 50 μL/well of 0.8% glutaraldehyde solution, processed at 37 °C for 5h and washed by ultrapure water (300 μL×3). Plates were coated overnight at 4 °C with 100 μL of CAP-OVA dissolved in buffer a (1.5 μg mL^{-1}). The plates were washed with 260 μL/well buffer c manually three times, blocked with 200 μL/well of buffer b and incubated at 37 °C for 1 hour. After the plates were washed as described above, then 100 μL/well of resultant immunomagnetic beads with DNA-anti-CAP-CAP complex were added. The competitive reaction was allowed to take place with delicate shaking at 25 °C for 30 min. The wells were washed three times with buffer c to remove the unbound DNA-anti-CAP-MBs and the complex (CAP-OVA-anti-CAP-DNA-MBs) was formed in the well.

RT-IPCR for detection and quantification of CAP

After optimizing real time PCR efficiency using 3-fold diluted reporter DNA tethered to detection antibodies, the development of a standard curve was done using similarly

diluted CAP working stocks prepared in 0.02 M PBS. Each standard solution was subjected to immunomagnetic beads recovery using the direct immunoassay format mentioned above. The immuno-PCR assay was performed using the MJ Research Opticon 2 (now Bio-Rad) real-time PCR detection system and the PCR signals generated were correlated to the initial CAP concentrations. Briefly, an optimum primer concentration of 0.4 μM was used (Forward, 5'-CCCTGCGTTTATCTGCTCTC-3'; Reverse 5'-CCTGCGTCAATG TAATGTTC-3') to amplify a 65-bp reporter DNA in a 25 μL reaction mix containing 12.5 μL of SYBR® Premix Ex Taq_{TM} Ⅱ (2×), 2 μL of template DNA, 1 μL of forward primer, 1 μL of reverse primer and 8.5 μL of ddH_2O. The optimized real-time PCR cycle parameters included a 95 °C initial denaturation step for 30 s, followed by 30 cycles of denaturation (95 °C for 2 s), annealing (55 °C for 30 s) and extension (72 °C for 30 s). Fluorescence measurements were taken after each annealing step. Melting curve analysis performed from 75 °C to 95 °C to detect potential nonspecific products was done with signal acquisition at every 0.2 °C melting rates for 1s hold time.

Calibration curve and quantitative real time-PCR analysis

The increase in fluorescence signals after each PCR cycle during reporter DNA amplification was recorded automatically by the instrument. The cycle number where the fluorescence signal crosses a manually established threshold showing linear signal increase was labeled as the 'cycle threshold (Ct)' value. Signals generated from the

real time PCR assay were positively correlated to the initial CAP concentrations using the obtained Ct values from each standard. The Ct values were plotted against the logarithmic concentrations of the CAP standards for the calibration curve. Each analysis (CAP calibration curve standards and test samples) was performed in triplicate replication. The individual replications included the entire process of CAP extraction, magnetic bead recovery, and ultimately PCR analysis (i.e., not simply PCR reactions in triplicate). Data analysis was done using a simple linear regression analysis of the Ct values against log concentrations and plots of the amplification curves were drawn using Origin (version 8.0, Microcal, Northampton, MA, USA) software packages.

Cross-reactivity (CR)

Three compounds structurally related and three another structurally unrelated to CAP were used as the competitor and determined as the RT-IPCR assay described above. CR was calculated as follows:

$$CR\ \% = IC_{50,\ CAP} / IC_{50,\ cross\text{-}reactant} \times 100\ \%$$

Recovery and precision

Standard solution were added into the blank milk samples, known to be free of CAP to yield at 0.0004, 0.0008, and 0.0016 μg L^{-1}, respectively. Each sample was measured 10 times in duplicate and in three consecutive days to assess accuracy and precision.

Analysis of field milk samples

Twenty milk samples were collected from retail outlets in Beijing. Each sample was divided into three portions, which would be analyzed by the established RT-IPCR, CL-ELISA established in our previous study[21] and traditional ELISA kits (R-Biopharm).

Results and discussion

Optimization of RT-IPCR

Several physicochemical factors influencing immunoassay performance, including the effect of additives Tween-20, ionic strength and pH values, optimum incubation time, and optimum incubation temperature, were investigated in RT-IPCR. The 50% inhibition concentration (IC_{50}) in the standard curves were evaluated under different conditions, the lower IC_{50} indicated higher sensitivity. Checkerboard procedure was performed to select the optimal concentrations of DNA-anti-CAP conjugation with immunomagnetic beads for the competitive immunoassay. The coating antigen CAP-OVA were diluted to 6.0, 3.0, 1.5, 0.75 and 0.375 μg mL^{-1}. In conclusion, such conditions as 2.0 μg mL^{-1} per 100 μL magnetic beads of DNA-anti-CAP, 1.5 μg mL^{-1} of coating antigen CAP-OVA, 30 min of competition time and the use of 0.02 M PB as reaction buffer produced a lower IC_{50} than any other evaluated conditions. The parameters of RT-IPCR were optimized according the previous report (Lind et al., 2005) with minor revision. Melting curve analysis performed from 75 °C to 95 °C to detect potential nonspecific products was done with signal acquisition at every 0.2 °C

melting rates for 1s hold time (Supplementary Figure 1).

Performance of RT-IPCR

Under the optimal conditions, the fluorescence signals responses decreased linearly with the increase in the concentrations of CAP since a competitive immunoassay mode was employed, that is, the Ct values were positively correlated with the concentrations of the CAP. The developed RT-IPCR detected CAP in the range of 0.001-0.11 μg L^{-1} (R^2=0.9986), with IC_{50} values of 0.008 μg L^{-1}, respectively (Figure 3). The sensitivity represented by IC_{50} of the developed RT-IPCR for CAP was about 100 times sensitive greater compared to the biotin-streptavidin amplified enzyme-linked immunosorbent assay[17] (BA-ELISA) and more than 10-fold better than that of the commercial CAP ELISA kits (WDWK Biotech: 0.084 μg L^{-1}; R-Biopharm: 0.082 μg L^{-1}).

Moreover, the sensitivity of the developed RT-IPCR was about 1.7 times and 2.2 times greater than direct competitive CL-ELISA (IC_{50} =0.0136 μg L^{-1}) and indirect competitive CL-ELISA (IC_{50} =0.0172 μg L^{-1}), and even more sensitive compared to the chemiluminescent immunoassay based on gold nanoparticles and magnetic beads, which represents higher sensitivity in most cases, reported in our previous study (Table 1).[19, 21, 22] The sensitivity of this method was increased in virtue of the high sensitivity of the real time quantitative PCR and the application of MBs with a larger surface area enriching CAP by covalent binding more DNA-anti-CAP monoclonal antibody (anti-CAP) to MBs.

TAP, FF, FFA, structurally related with CAP, were selected for cross-reactivity (CR) experiments to evaluate the specificity of anti-CAP in the developed RT-IPCR. No significant CR of anti-CAP to other amphenicols was observed (Table 2). Furthermore, to help define the specificity of the anti-CAP and developed RT-IPCR, structurally unrelated drugs including SUL, CIP and PEN were also tested. No CR was observed. The CAP molecule can be described as having three main parts: the core nitrophenyl moiety, the propanediol, and dicloroacetamido groups (Table 2). The aromatic ring and the dichloroacetamido group accounted for a large portion of the immunological reactivity of the hapten. The antibody showed no or negligible CR towards other related compounds where the immunologically important nitrophenyl (TAP and FF, FFA) were lacking. In addition, the antibody showed higher CR (120%) with CAP succinate, which contained the critical immunological nitrophenyl and dichloroacetamido group.

Moreover, in order to exclude this possibility of binding between DNA reporters with CAP, the direct competitive immunoassay was performed (Supplementary materials). The results showed that DNA reporters did not bind with CAP-OVA.

Matrix Effect

To apply a new method in real sample analysis, a matrix effect is an important issue to be considered, especially in animal tissues due to the complicated matrix. In this study, the developed RT-IPCR was used to determine CAP in milk sample. Immunomagnetic bead recovery of CAP in milk was used to concentrate CAP and eliminate the matrix

interference. When determining the matrix effects, interferences are quantified by comparing a standard inhibition curve with a standard curve generated in the milk matrix known to be free of CAP. The two curves for each concentration of CAP are superposable, indicating that immunomagnetic bead recovery of CAP in milk could effectively eliminate the matrix interference (Figure 3).

Limit of detection (LOD)

The limit of detection (LOD) was calculated as the mean of the measured content of blank different samples (n = 20) plus three standard deviations (mean + 3SD). The each of 20 blank milk samples was obtained by 20 different animals and analyzed according to the developed RT-IPCR. The LOD was 0.0008 μg L^{-1}. The good performance of developed RT-IPCR was good enough to screen the trace CAP residues in milk.

Comparison of the developed RT-IPCR based on immunomagnetic bead recovery of CAP with other immunoassays

The analytical comparison of various immunoassays for CAP detection is important to analyze their suitability for screening the residue in field samples. To date, the developed RT-IPCR is most sensitive reported immunoassay for detection CAP in milk. In our previous study, the direct competitive CL-ELISA (IC_{50} =0.0136 μg L^{-1}), indirect competitive CL-ELISA (IC_{50} =0.0172 μg L^{-1}) and the chemiluminescent immunoassay based on gold nanoparticles and magnetic beads (IC_{50} =0.017 μg L^{-1})

for measurement CAP represented the sensitive immunoassays.[19, 21, 22] Furthermore, the developed RT-IPCR is even more sensitive than the above methods in our previous study (Table 1). In this study, the sample pretreatment is very simple, only need to be incubated with the immunomagnetic beads for 15 min, enriching CAP and eliminating the matrix interference, suitable for analyzing a large amount of milk samples. Meanwhile, there is no organic solvent in the process of extracting CAP from milk, such as ethyl acetate which will be harmful to human health and the environment. Furthermore, there is no requirement of nitrogen blowing instrument when extracting CAP from milk (Table 1). In summary, the developed RT-IPCR was superior to other previously reported immunoassay for determination of CAP in milk, due to the higher sensitivity and simpler pretreatment.

Application in real samples

Precision and recovery

To confirm that the assay performed well around the LOD, the blank milk samples were fortified at 0.0004 (1/2 LOD), 0.0008 (LOD), and 0.0016 (2 LOD) μg L^{-1} with CAP prior to analysis. All samples fortified at 0.0008, and 0.0016 μg L^{-1} resulted in positive readings. 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 CAP in the milk fortified at concentrations greater than or equal to the LOD were at least 87.5 %, with coefficients of variation less than 15 %. The recovery of CAP from milk samples fortified at a concentration of 1/2 LOD was highly

variable (percent recoveries ranged from 75.0 to 155.0 %) (Table 3). Hence, the developed RT-IPCR could detect the presence of CAP above the LOD (0.0008 μg L^{-1}) and will eliminate the possibility of false-positive and false-negative results.

Analysis of CAP and CLE in field milk samples

To evaluate the determination capability of the developed RT-IPCR in milk samples, 20 field samples were analyzed by the developed RT-IPCR, direct competitive CL-ELISA established in our previous study[21] and traditional ELISA kits (R-Biopharm) (Table 4). The results demonstrated that the developed RT-IPCR could screen CAP in the incurred samples as the CL-ELISA and traditional ELISA kits did. Thereafter, the developed RT-IPCR was reliable for screening of trace CAP residues in milk samples.

Conclusion

In conclusion, we have successfully developed a sensitive and rapid RT-IPCR assay for quantitation of trace CAP directly from milk sample after simple incubation with immunomagnetic beads. The RT-IPCR method described here offers rapid recovery with sensitive detection and quantification of CAP using highly specific antibody and has several advantages of sensitivity, specificity and accuracy over enzyme catalytic immunoassays such as traditional ELISA and CL-ELISA. Due to the ban action for CAP content in animal derived food, the use of rapid and reliable sensitive methods able to quantify more trace levels CAP are in high demand and the methodology

outlined in this study can fulfiill such demands.

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Captions of Figures and Tables

Figure 1 The real-time immuno-quantitative PCR (RT-IPCR) schematic illustration of the determination of CAP in milk based on magnetic beads capturing

Figure 2 Diagram of DNA-anti-CAP- MBs coupling

Figure 3 Inhibition curves of CAP in 0.02 M PBS buffer (■) and milk extract (●)

Table 1 Comparison of the developed RT-IPCR based on immunomagnetic beads with other immunoassays for detection of CAP in milk

Table 2 CR of CAP in RT-IPCR with some structurally related and unrelated compounds

Table 3 Recovery and variation of spiked CAP in milk

Table 4 Determination of field milk samples from retail outlets in Beijing by RT-IPCR, CL-ELISA and commercial ELISA kit

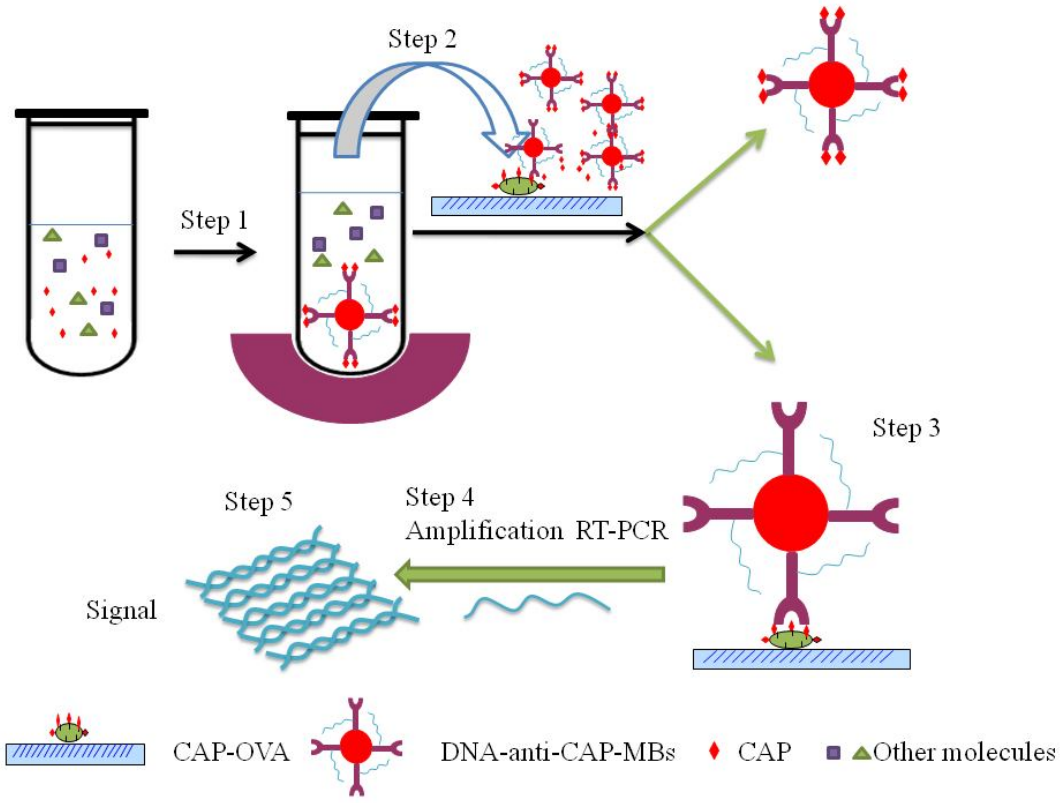


Figure 1 The real-time immuno-quantitative PCR (RT-IPCR) schematic illustration of the determination of CAP in milk based on magnetic beads capturing

Step 1 CAP in the test sample is bound to a fraction of the supplied magnetic beads. **Step 2** the beads are recovered and transferred to a pre-treated tube with surface-bound CAP. **Step 3** beads not yet bound to CAP from the test sample are sequestered to the tube surface. **Step 4** RT-PCR amplifies and measures the DNA label from the surface-sequestered beads. **Step 5** if the amplification happens faster (fewer cycles), this indicates a there were larger number of CAP-free beads in step 1 (and thus a lower sample CAP concentration) versus if amplification happens slower (more cycles), which indicates a higher number of CAP in the original sample.

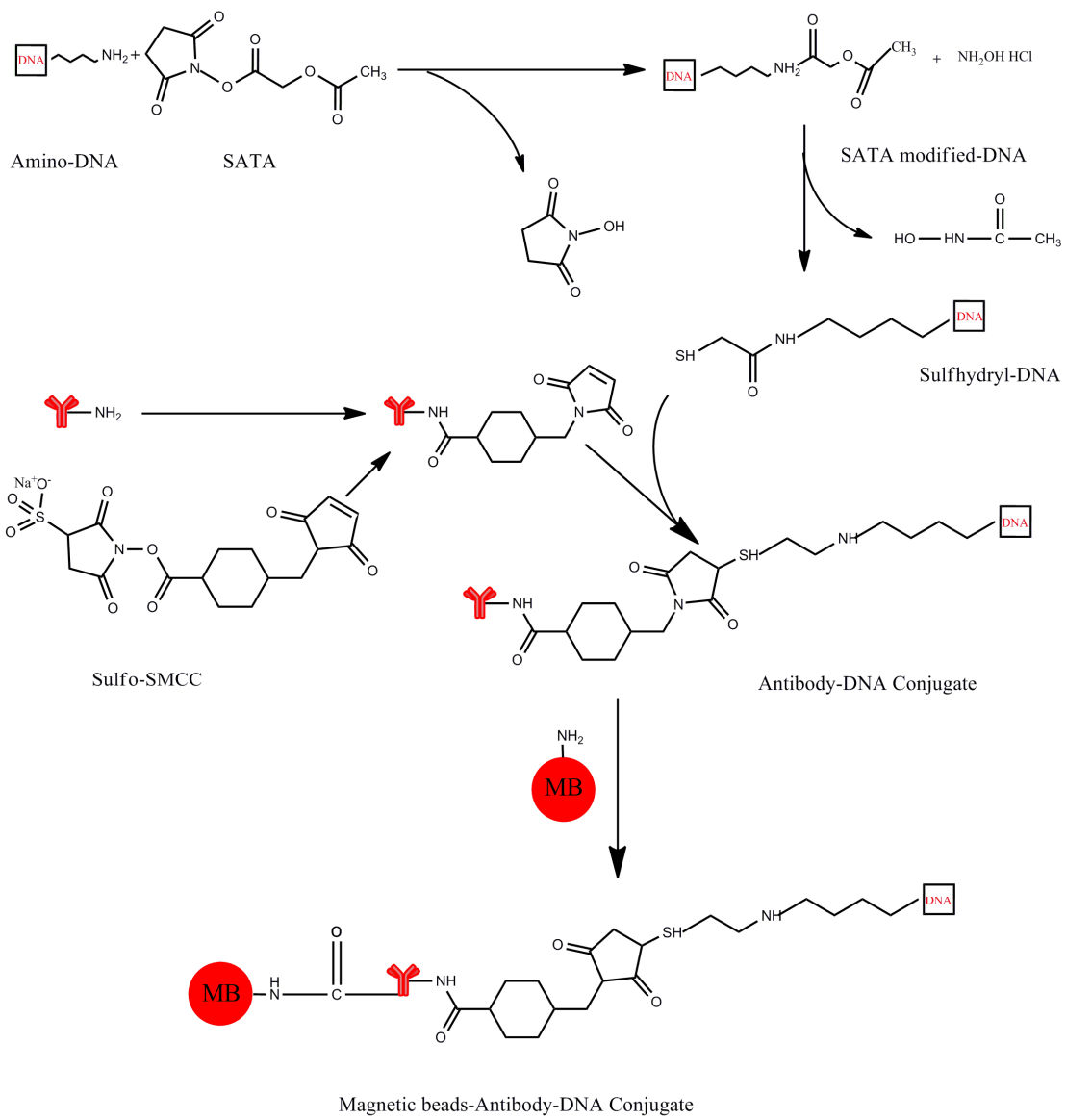


Figure 2 Diagram of DNA-anti-CAP- MBs coupling

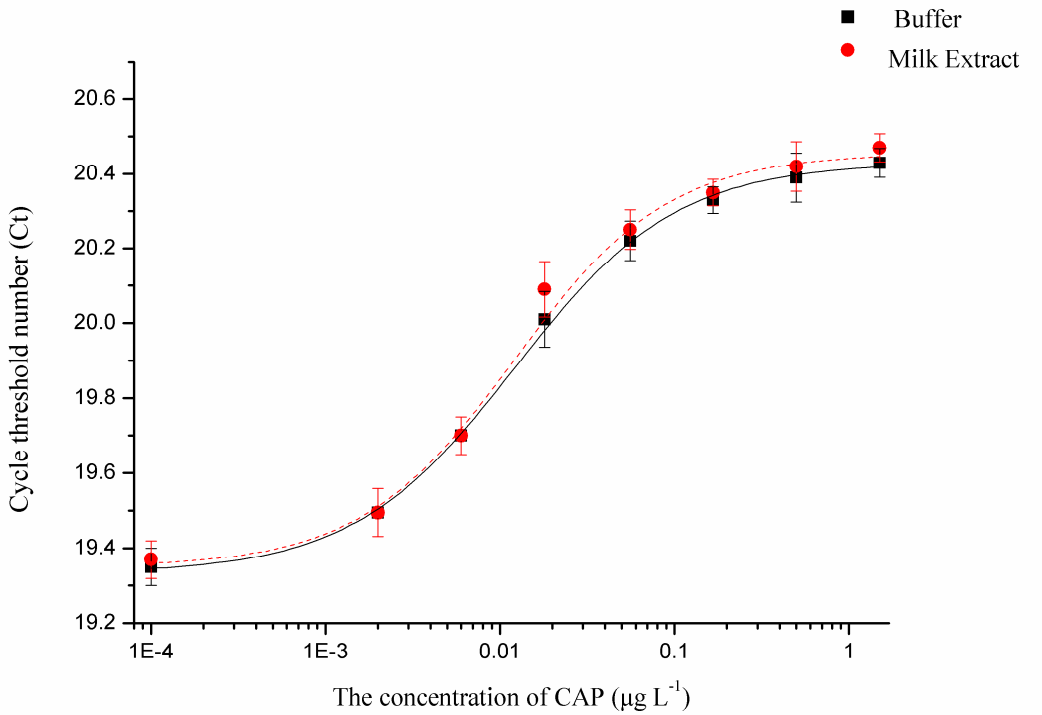
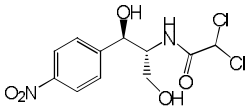
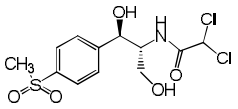
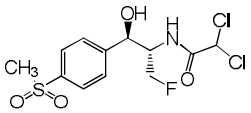
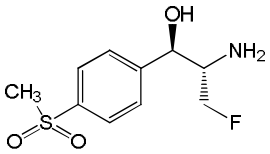
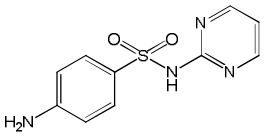
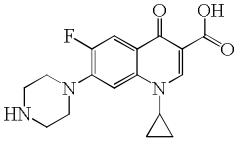
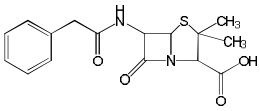


Figure 3 Inhibition curves of CAP in 0.02 M PBS buffer (■) and milk extract (●)

452 Table 1 Comparison of the developed RT-IPCR based on immunomagnetic bead with other methods (immunoassays and instrumental methods) for detection of CAP

		sensitivity ($\mu g\ L^{-1}$)	Reaction or detection time (min)	Sample pretreatment time (min)	Organic solvent costs
The present study		0.008	90	15 (CAP Enrichment)	No
Chemiluminescent immunoassay based on gold nanoparticles and magnetic beads[19]	Extract method I	0.017	30	60	Ethyl acetate
	Extract method II	0.17	30	10	No
Direct CL-ELISA[21]		0.0136	15	60	Ethyl acetate
Indirect CL-ELISA[22]		0.0172	75	60	Ethyl acetate
Conventional ELISA kit (R-Biopharm)		0.080	45 (including coloration)	10	No
LC-MS/MS[23]		0.1	8	>120	ethyl acetate, ammonium Hydroxide, hexane etc
GC-MS[24]		0.1	8	>180	ethyl acetate, ammonium Hydroxide, acetonitrile, methanol

Table 2 CR of CAP in RT-IPCR with some structurally related and unrelated compounds

Compound	Structure	IC_{50} (μg L^{-1})	CR (%)
CAP		0.008	100
TAP		>1000	<0.1
FF		>1000	<0.1
FFA		>1000	<0.1
SUL		>1000	<0.1
CIP		>1000	<0.1
PEN		>1000	<0.1

458 Table 3 Recovery and variation of spiked CAP in milk

Spiked CAP	Intra-assay[a]		Inter-assay[b]	
μg L^{-1}	Measured (μg L^{-1})	Recovery (%)	Measured (μg L^{-1})	Recovery (%)
0.0004	0.00030±0.00012	75.0	0.00062±0.0008	155.0
0.0008	0.00082±0.00010	102.5	0.00086±0.0006	107.5
0.0016	0.0015±0.00018	93.75	0.0014±0.001	87.5

459 [a]Intra-assay variation was determined by 10 replicates on a single day.

460 [b]Inter-assay variation was determined by 10 replicates on 3 different days.

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Table 4 Determination of field milk samples from retail outlets in Beijing using RT-IPCR, direct competitive CL-ELISA and commercial ELISA kit

Method	RT-IPCR	CL-ELISA	ELISA kit (R-Biopharm)
Sample	μg L^{-1}	μg L^{-1}	μg L^{-1}
M2	0.074[a]±0.005	0.071±0.006	0.076±0.005
M3	0.009±0.001	0.010±0.001	ND[b]
M7	0.029±0.003	0.032±0.003	0.030±0.002
M11	0.016±0.001	0.018±0.001	ND
M15	0.090±0.006	0.086±0.007	0.083±0.007
M16	0.012±0.002	0.015±0.002	ND
M20	0.081±0.008	0.076±0.007	0.083±0.008
M1, M4-M6, M8-M10, M12-M14, M17- M19	ND		

[a] Each value was determined with 3 repeats.

[b] ND not detectable

Supplementary materials

Development of a Highly Sensitive Real Time Immuno-PCR for the Measurement of Chloramphenicol in Milk Based on Magnetic Beads Capturing

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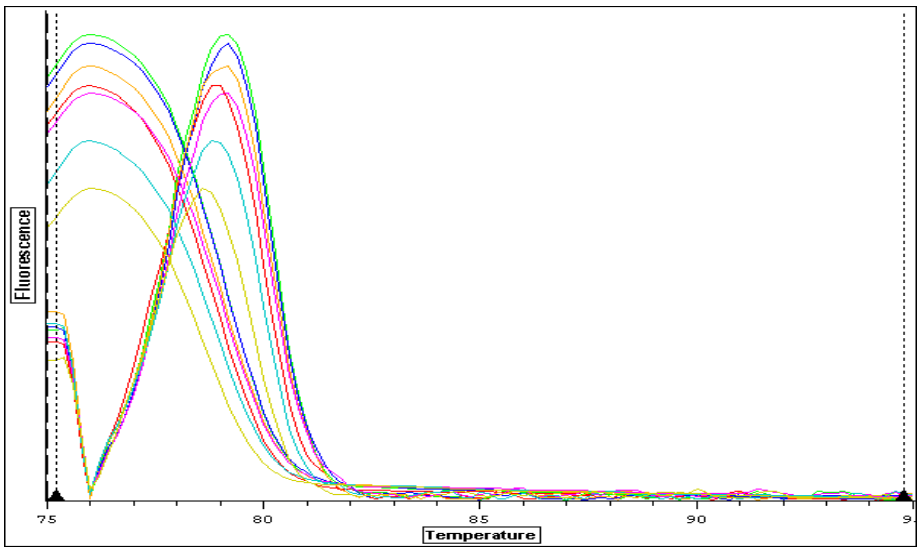

Supplementary Figure 1 Melting curve analysis of RT-IPCR for CAP

Procedure of direct competitive CL-ELISA

Plates were coated overnight at 4 °C with 100 μL of CAP-OVA dissolved in buffer a (1.5 μg/mL). The plates were washed with 260 μL/well buffer c manually three times, blocked with 200 μL/well of buffer b and incubated at 37 °C for 1 hour. After the plates were washed as described above, then 100 μL/well of mixture of DNA reporters (1.5 μg/mL) and HRP-conjugated anti-CAP MAb (1/5000 dilution) (scheme A) or 100 μL/well of HRP-conjugated anti-CAP MAb (1/5000 dilution)(scheme B) in buffer d were added, respectively. The competitive reaction was allowed to take place for 30 min at room temperature. After washing five times and finally the HRP tracer activity was revealed by adding 100 μL/well of a freshly prepared substrate mixture of SuperSignal substrate solution. The intensity of light emission was measured at 425

nm using a chemiluminesence reader immediately after the addition of the substrate and the results were expressed in relative light units (RLU). Eventually, the RLU for scheme A was equal to that for scheme B when the concentration of anti-CAP was 0, proving that the DNA reporters in scheme A did not bind with anti-CAP.