Gold(III) enhanced chemiluminescence immunoassay for detection of antibody against ApxIV of *Actinobacillus pleuropneumoniae*†‡

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We report, for the first time, a chemiluminescence immunoassay (CLIA) method based on $AuCl_4^-$-enhanced luminol chemiluminescence (CL) reaction for the highly sensitive detection of ApxIV antibody of *Actinobacillus pleuropneumoniae* (APP). The $AuCl_4^-$, which was the dissolution product of the gold nanoparticle–rabbit anti-pig IgG conjugate, served as an analyte in the CL reaction for the indirect measurement of antibody against ApxIV. The optimal condition of gold dissolution was composed of a 5.0×10^{-2} M HCl, 1.5×10^{-2} M NaCl, and 2.5×10^{-4} M Br_2 solution. Under the optimal conditions, a good correlation between the relative CL photon counting and the dilution coefficient of serum was obtained in the dilution range of 1:160–1:40 000. Based on the analysis of clinical samples, the results indicated that CLIA had remarkable advantages in terms of reliability and practical use compared with indirect hemagglutination (IHA) and enzyme-linked immunosorbent assays (ELISA). The proposed method provided a new tool for the indirect determination of antibody against ApxIV in pig serum samples and showed great potential for numerous applications in immunoassays.

Introduction

Actinobacillus pleuropneumoniae (APP) is the etiological agent of porcine contagious pleuropneumonia characterized by fibrinous, hemorrhagic, and necrotizing pleuropneumonia in pigs of all ages. The respiratory infectious disease occurs worldwide and has resulted in great economic losses to the swine industry.[1] Several virulence factors seem to be involved in the pathogenicity of APP including capsular polysaccharides, lipopolysaccharides, outer membrane proteins and exotoxins. In order to prevent outbreaks of APP, a simple, rapid and sensitive method for the detection of the virulence factors in infected pigs would be undoubtedly beneficial. APP isolated from healthy carrier or sick pigs needs a selective media and subsequently is identified by biochemical and physiological methods (*e.g.* polymerase chain reaction), and immunological methods such as enzyme-linked immunosorbent assay (ELISA), indirect hemagglutination assay (IHA), and complement fixation (CF) test.[2–8] Nonetheless, cross-reactions between strains of the various APP serotypes and other bacterial species have remained problematic. APP has hampered attempts to produce species-specific diagnostic tests which include all serotypes.[9–10]

The Apx toxins (ApxI, ApxII, ApxIII and ApxIV), members of the RTX toxin family, are primary virulence factors of APP.[11–13] However, ApxI, ApxII and ApxIII are secreted by different serotypes of APP and other related bacteria, in contrast, ApxIV is specific to the species of APP regardless of the serotypes and inducible only *in vivo*, so it is optimal for developing a species-specific assay.[14,15] Several methods for the detection of ApxIV have been developed based on the polymerase chain reaction, ELISA and IHA.[16–18] However, these methods have some drawbacks, such as tedious procedures, subjective results, and insufficient accuracy.[19] Hence, a convenient, rapid, and sensitive diagnostic test, such as a chemiluminescence immunoassay (CLIA), would be extremely valuable for the diagnosis of APP in clinical field and field applications.

CLIA has some advantages, such as high sensitivity and selectivity, simple instrumentation, wide linear response range and low background signals for the emission of chemiluminescence (CL) coming from the chemical reaction.[20] Therefore, CLIA has been exploited with a wide range of applications in different fields, such as biotechnology, pharmacology, molecular biology, routine clinical analysis and clinical research applications.[21–23] CLIA is generally achieved by employing labels, such as enzymes (glucose oxidase labels, horseradish peroxidase labels and alkaline phosphatase labels) and CL reagents (luminol, isoluminol and their derivatives).[24–26] But enzyme labels are unstable and prone to interference from inhibitors present in the sample, and the method of CL reagents labelling is very complex and could cause protein denaturation. Therefore, the biomedical applications of immunoassay were limited.[27] In recent years, metal-based labels, especially gold nanoparticles, have been extensively studied to overcome the problems because of their good biocompatibility and stability.[28–35] Each gold nanoparticle contains thousands of gold atoms (*e.g.*, 1.1×10^5 gold atoms are

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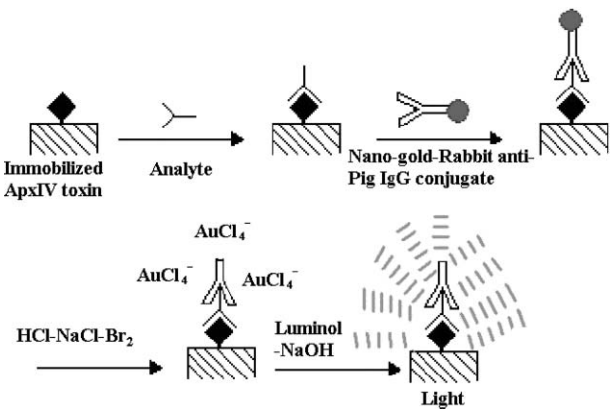
‡ The HTML version of this article has been enhanced with colour images.

theoretically contained in a 15 nm spherical gold nanoparticle), and consequently, lower detection limits can be attained by determining gold ion.[36] To the best of our knowledge, CLIA for the detection of animal-borne disease based on gold ion has not been reported, and has not yet gained the attention from chemical scientists.

In this article, we report for the first time a CLIA based on the gold ion enhanced luminol CL reaction for the detection of ApxIV antibody from APP by employing oxidative gold nanoparticles dissolution in an acidic solution. The principle of CLIA for the detection of ApxIV antibody is shown in Scheme 1. Primarily, the purified recombinant ApxIV protein is adsorbed passively on the surface of polystyrene wells. The antibody in the serum samples is captured by the recombinant ApxIV protein and then sandwiched by gold nanoparticle–rabbit anti-pig IgG conjugate. Next, the unbound gold nanoparticle–rabbit anti-pig IgG conjugate is removed. A large number of $AuCl_4^-$ ions from gold nanoparticles anchored on the surface of polystyrene wells are released in an oxidative HCl–NaCl–Br_2 solution, and then quantitatively determined by a simple and sensitive $AuCl_4^-$ enhanced luminol CL reaction. The concentration of sample could be quantified by the accompanied increment of CL photon counting, $\Delta I = I_s - I_0$, where I_s and I_0 are the CL photon counting in the presence and absence of antibody against ApxIV, respectively. The proposed method has the advantage of showing the specificity of ELISA and sensitivity of a CL assay. The optimal conditions for the gold dissolution and the optimal conditions for CL reagents were studied in detail. Under the optimal conditions, a good correlation between the relative CL photon counting and the dilution coefficient of serum was obtained in the dilution range of 1:160–1:40 000. The typical available immunoassays including IHA and ELISA were compared with CLIA for the detection of antibody against ApxIV, which showed that CLIA was a new tool for the indirect measurement of antibody against ApxIV in pig serum samples.



Scheme 1 Schematic representation of the gold(III) enhanced CLIA for the determination of ApxIV.

Experimental

Apparatus

CL measurements were performed with a BPCL ultra-weak CL analyzer (Institute of Biophysics Academic Sinica, Beijing, China). The ultraviolet and visible (UV-vis) absorption spectra were acquired on a Thermo Nicolet Corporation Model evolution 300 UV-visible spectrometer. Optical density was measured by ELISA reader (Bio-Tek Instruments Inc, USA). The pH measurements were made with a Model pHS-3C meter (Shanghai Leici Equipment Factory, China).

Reagents

Polystyrene 96-well microtiter plates were used to perform the immunoreactions. A commercially available IHA kit was obtained from Lanzhou Institute of Veterinary Medicine, Lanzhou, China. Rabbit anti-pig IgG was purchased from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Bovine serum albumin (BSA) was from German Roche Co. (Germany). $HAuCl_4{\cdot}4H_2O$ was obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Peroxidase-conjugated rabbit anti-pig IgG conjugate and orthophenylinediamine dihydrochloride substrate were obtained from Sigma Chemical Co. (USA). The recombinant ApxIV protein, standard positive and negative sera were made by ourselves.[37] The positive control serum was a polyclonal anti-sera from a pig experimentally infected with APP.[37] The negative control serum was obtained from a pig from an APP-free herd. Clinical sera were obtained from naturally infected and non-infected pigs. The luminol stock solution (1.00×10^{-2} M) was prepared by dissolving luminol (obtained from Sigma Chemical Co.) in 0.1 M NaOH solution and stored in a dark place. The buffers used were as follows: (A) coating buffer, 0.05 M carbonate–bicarbonate buffer solution, pH 9.6; (B) incubation buffer, 0.1 M sodium phosphate buffered saline (PBS), pH 7.2–7.4; (C) washing buffer, buffer B with 0.05% Tween 20; (D) dilution buffer, buffer B with 1% BSA. All other reagents were of analytical reagent grade and used as purchased without further purification. Milli-Q water was used throughout.

Preparation of gold nanoparticles and gold nanoparticle–rabbit anti-pig IgG conjugate

Gold nanoparticles were synthesized using citrate to reduce $HAuCl_4$ following the procedure of Yu *et al.*[38] All glassware was soaked in highly concentrated HNO_3–HCl solution, then rinsed with water and dried before use. Briefly, 100 mL of 0.01% $HAuCl_4{\cdot}4H_2O$ solution was heated to boiling, and then 2 mL of 1% sodium citrate solution was added while stirring. After the color changed from light yellow to brilliant red, the solution was boiled for another 5 min to complete the reduction of the $HAuCl_4$, and then cooled to room temperature and stored at 4 °C. The synthesized gold nanoparticles were used for the preparation of gold nanoparticle–rabbit anti-pig IgG conjugate.

Gold nanoparticle–rabbit anti-pig IgG conjugate was prepared according to the method of Yu *et al.*, with some modifications.[38] The rabbit anti-pig IgG (10% more than the minimum amount, which was determined using a flocculation test) was added to 50 mL gold nanoparticles suspension (pH 9.0 adjusted with 0.1 M K_2CO_3), then incubated at room temperature for 2 h. The conjugate was centrifuged at 15 000*g* for 1 h, and the soft sediment was resuspended in buffer B and stored at −20 °C.

Recombinant ApxIV protein immobilization

The recombinant ApxIV protein (1.93 mg mL^{-1}) was diluted 650 times using buffer A and 100 μL dispensed in each well of the 96-well polystyrene microplate plates, which were incubated overnight at 4 °C. After washing with buffer C, a second coating was done by using 400 μL buffer D and incubated for 0.5 h at 37 °C. The solution was discarded, and the wells were washed with buffer C, dried under vacuum and stored at 4 °C.

Procedures for the immunoassay

A series of dilutions of pig sera in buffer D (100 μL) were injected into the wells and incubated for 1 h at 37 °C. The wells were washed six times with buffer C, and 100 μL gold nanoparticle-rabbit anti-pig IgG was added to each well. The wells were incubated with gentle shaking for 1 h at 37 °C. Finally, the wells were washed with buffer C and water three times.

Standard procedures for the CL detection

After the wells were thoroughly washed, gold nanoparticles were dissolved to form $AuCl_4^-$ with 100 μL per well of HCl–NaCl–Br_2 solution for 20 min to ensure that the gold nanoparticles were dissolved completely. Then, the mixture was placed in the oven at 60 °C for 30 min to completely remove bromine. Then 90 μL of the solution was injected into glass tubes containing luminol and NaOH solution, and the CL photon counting was measured for 30 s by the BPCL ultra-weak CL analyzer.

IHA

A commercially available IHA kit was obtained from Lanzhou Institute of Veterinary Medicine (Lanzhou, China) and was performed on the basis of the method described previously.[39,40] A total of 50 μL of an 8 times dilution of each serum specimen (optimal dilution) was mixed with 25 μL of either antigen-coated test cells or uncoated control cells in the wells of a U-bottom microtiter tray. Plates were incubated at 25 °C for 1 h. Hemagglutination was read on a scale of from 0 to ++++ (where + is the intensity of reaction). Positive and negative control sera were tested each time that the test was performed.

ApxIV-ELISA

The non-competitive indirect ELISA for the detection of antibody against ApxIV in sera of pigs was developed as described previously.[37] Briefly, the purified recombinant ApxIV protein was bound to 96-well polystyrene microplate plates in a final concentration of 2.97 μg mL^{-1}. Serum samples were diluted 40 times (optimal dilution) using dilution buffer, the antibody in the serum samples was captured by the recombinant ApxIV protein and then sandwiched by peroxidase-conjugated rabbit anti-pig IgG conjugate. Next, the unbound peroxidase-conjugated rabbit anti-pig IgG conjugate was removed. 100 μL of freshly prepared 37 mg orthophenylinediamine dihydrochloride substrate in 10 mL of 0.1 M citric acid phosphate buffer pH 5.0 and 10 μL of 30% H_2O_2 were added to the wells. The plate was incubated at room temperature for 15 min. The reaction was then stopped with 50 μL of 2 M sulfuric acid and the optical density was measured at 490 nm by ELISA reader.

Results and discussion

Characterization of gold nanoparticles and gold nanoparticle–rabbit anti-pig IgG conjugate

Fig. 1 shows the UV-vis absorption spectra of gold nanoparticles and gold nanoparticle–rabbit anti-pig IgG conjugate. The gold nanoparticles exhibited a sharp plasmon absorption band with maximum absorbance at wavelength ~520 nm due to the surface plasmon resonance, and the average size of as-prepared gold nanoparticles was 15 nm (curve a).[36] After addition of the rabbit anti-pig IgG, the surface plasmon band was red-shifted (curve b) due to the interaction of the rabbit anti-pig IgG with gold nanoparticles, which indicated the gold nanoparticle–rabbit anti-pig IgG conjugate was formed.

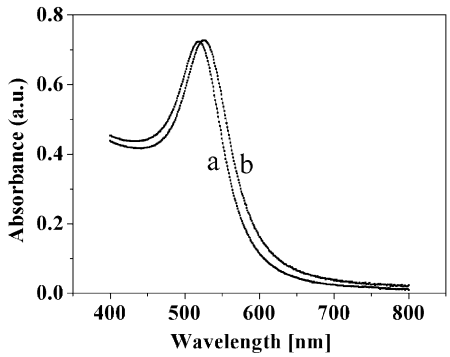


Fig. 1 UV-vis absorption spectra of (a) gold nanoparticles and (b) gold nanoparticle–rabbit anti-pig IgG conjugate.

Optimization of the CL conditions

In this paper, some experimental parameters were studied with two aims: (1) to improve immunoassay sensitivity and repeatability and (2) to study immunoassay performance under the optimal conditions. These experiments were carried out using the proposed method described above.

In this system, a large number of $AuCl_4^-$ ions from gold nanoparticles are released in an oxidative solution. The CLIA is based on the catalytic effect of $AuCl_4^-$ on the luminol CL reaction, and the possible CL mechanism of the enhancement of $AuCl_4^-$ for the luminol system can be illustrated in the following steps:[41]

$$AuCl_4^- + \text{luminol} \rightarrow AuCl_2^- + \text{3-aminophthalate}^*$$

$$\text{3-aminophthalate}^* \rightarrow \text{3-aminophthalate} + h\nu$$

$AuCl_4^-$ would accelerate the electron transfer of luminol to yield 3-aminophthalate excited state, accompanied by production $AuCl_2^-$. The state of excited 3-aminophthalate returns to the state of ground with increased CL phenomena. Therefore, the primary step is to dissolve the gold nanoparticles from the gold nanoparticle–rabbit anti-pig IgG conjugate to $AuCl_4^-$. This was achieved with the use of HCl–NaCl–Br_2 solution, which proved to be more efficient than other solutions, such as HNO_3–HCl solution, NaCl–Br_2 solution, HCl–NaCl solution, and so on. Clearly, the dissolution of gold nanoparticles in HCl–NaCl–Br_2 solution can be affected by several factors including HCl, NaCl and Br_2 concentrations. The effect of the dissolving reagent

concentration on the CL photon counting was investigated. The optimal conditions for the gold dissolution were in a 5.0×10^{-2} M HCl–1.5×10^{-2} M NaCl–2.5×10^{-4} M Br_2 solution.

CLIA for the determination of antibody against ApxIV is based on the catalytic effect of $AuCl_4^-$ on the luminol–NaOH system (initiating the CLIA emission). In order to obtain reproducible and sensitive data, optimum concentrations of NaOH and luminol on the CL photon counting were studied. The optimal conditions for initiating the CLIA emission were in a 1.0×10^{-1} M NaOH–1.0×10^{-6} M luminol solution.

Detection of different concentrations of gold nanoparticles

As noted above, the conditions for sensitive CLIA were optimized, and then the measurement of gold nanoparticles concentration was carried out. Under the above optimized experimental conditions, the relationship between the relative CL photon counting and the concentration of gold nanoparticles was investigated (Fig. 2). The calibration graph showed a linear correlation in the range of 4.9×10^{-12}–6.1×10^{-10} M, with a regression equation, $\Delta I = 1.8 \times 10^4 + (3.5 \times 10^{14} C)$ (C is the concentration of gold nanoparticles), $r = 0.997$.

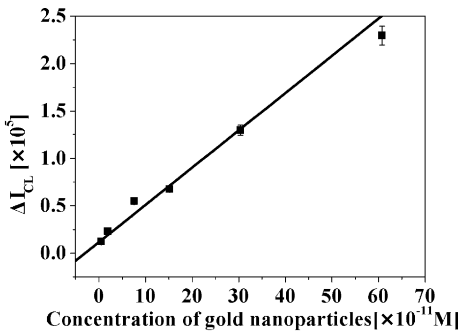


Fig. 2 The concentration of 15 nm gold nanoparticles *vs.* the relative CL photon counting. The error bars represent standard errors (SE) of the means. The data in the figure are means ± SE ($n = 6$).

Dose-response curve

CLIA based on the gold ions enhanced luminol CL reaction for the detection of ApxIV antibody has also been studied. As shown in Fig. 3, the relationship between the relative CL photon counting and different dilution coefficient of ApxIV antibody (standard positive serum) was investigated. A good correlation between the relative CL photon counting and the dilution coefficient of serum was obtained in the dilution range of 1:160–1:40 000. As the dilution coefficient of ApxIV antibody increased, the relative CL photon counting decreased between 160 times dilution and 40 000 times dilution. The results demonstrate the possibility to detect ApxIV antibody at very low concentrations.

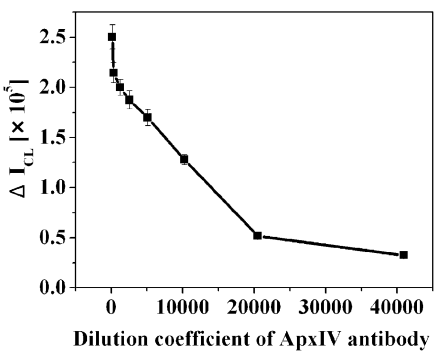


Fig. 3 The dilution coefficient of ApxIV antibody *vs.* the relative CL photon counting. The error bars represent standard errors (SE) of the means. The data in the figure are means ± SE ($n = 6$).

The optimal dilutions

The optimal dilutions were obtained by determination of the positive control serum. Titration experiments showed that a 160 times dilution of serum produced the best resolution for CLIA. However, the optimal dilution times are 8 times and 40 times for IHA and ApxIV-ELISA, respectively. Dreyfus *et al.* also studied the optimal dilution times of serum by ApxIV-ELISA, which showed that the optimal dilution of serum was 20 times.[42] The results show that CLIA is more sensitive than IHA and ApxIV-ELISA.

The limit of detection

The limit of detection (LOD) was obtained by determination of the positive control serum. According to the definition by Roda *et al.*, the LOD of CLIA and ApxIV-ELISA is given as the mean of 20 blank serum samples collected from the pigs from an APP-free herd plus three times the standard deviation (SD).[43] Therefore, the LOD was determined to be 40 000 times and 320 times dilution of serum for CLIA and ApxIV-ELISA, respectively. The LOD of IHA is the largest dilution of serum we can measure. Therefore, the LOD was determined to be 128 times dilution of serum for IHA. The LOD of CLIA was lower than ApxIV-ELISA and IHA.

Concordance between assays

ELISA and IHA have been recognized as sensitive and specific methods used in diagnosis of APP. To evaluate the diagnostic performance of the CLIA, it was necessary to compare CLIA with the reference standards (IHA and ApxIV-ELISA). The clinical serum samples ($n = 100$), collected from naturally infected pig without an appropriate reference test to classify animals into truly infected and non-infected, were analyzed by three methods. Results are summarized in Table 1. As shown in Table 1, 4 serum samples (4%) are positive with CLIA and negative with both ApxIV-ELISA and IHA; however, 1 serum

Table 1 Tabulated data of the test results of CLIA, ApxIV-ELISA and IHA used on 100 serum samples[a]

CLIA	ELISA	IHA	Total
+	−	−	4
−	−	+	8
−	+	+	1
−	+	−	2
+	+	−	3
+	−	+	1
−	−	−	42
+	+	+	39

[a] + positive; − negative.

Table 2 Comparative results of the CLIA and IHA of 100 serum samples

		IHA			Performance				
		Positive	Negative	Total	Efficiency[a] (%)	Sensitivity[b] (%)	Specificity[c] (%)	FP rate[d] (%)	FN rate[e] (%)
CLIA	**Positive**	40	7	47	84.0	81.6	86.3	13.7	18.4
	Negative	9	44	53					
	Total	49	51	100					

[a] Efficiency = (TP + TN) × 100/Total. [b] Sensitivity = TP × 100/(TP + FN). [c] Specificity = TN × 100/(TN + FP). [d] False-positive rate = FP × 100/(FP + TN). [e] False-negative rate = FN × 100/(TP + FN).

Table 3 Comparative results of the CLIA and ApxIV-ELISA of 100 serum samples

		ApxIV-ELISA			Performance				
		Positive	Negative	Total	Efficiency[a] (%)	Sensitivity[b] (%)	Specificity[c] (%)	FP rate[d] (%)	FN rate[e] (%)
CLIA	**Positive**	42	5	47	92.0	93.3	90.9	9.1	6.7
	Negative	3	50	53					
	Total	45	55	100					

[a] Efficiency = (TP + TN) × 100/Total. [b] Sensitivity = TP × 100/(TP + FN). [c] Specificity = TN × 100/(TN + FP). [d] False-positive rate = FP × 100/(FP + TN). [e] False-negative rate = FN × 100/(TP + FN).

sample (1%) is negative with CLIA and positive with both ApxIV-ELISA and IHA. 2 serum samples (2%) are positive with ApxIV-ELISA and negative with both CLIA and IHA; however, 1 serum sample (1%) is negative with ApxIV-ELISA and positive with both CLIA and IHA. 8 serum samples (8%) are positive with IHA and negative with both CLIA and ApxIV-ELISA; however, 3 serum samples (3%) are negative with IHA and positive with both CLIA and ApxIV-ELISA. 39 serum samples (39%) are positive and 42 serum samples (42%) are negative with all methods. The agreement between ApxIV-ELISA and IHA is 86%. The agreement between CLIA and the other two methods (ApxIV-ELISA and IHA) is 94.2% (81/86), which is very high in a clinical diagnostic test.

Further, to evaluate the diagnostic performance of the CLIA, the comparison between CLIA and IHA and between CLIA and ApxIV-ELISA have also been studied, and are shown in Tables 2 and 3. As shown in Table 2, compared with IHA, CLIA showed good results: high efficiency (84%), high sensitivity (81.6%), high specificity (86.3%), moderate false-positive rate (13.7%) and moderate false-negative rate (18.4%). Among 47 positive sera in CLIA, 40 sera were positive in IHA, while among 53 negative sera in CLIA, 44 sera were negative in IHA. Table 3 shows the comparative results of the CLIA with ApxIV-ELISA. CLIA also showed good results here: high efficiency (92%), high sensitivity (93.3%), high specificity (90.9%), moderate false-positive rate (9.1%) and low false-negative rate (6.7%). Among 47 positive sera in CLIA, 42 sera were positive in ApxIV-ELISA, while among 53 negative sera in CLIA, 50 sera from were negative in ApxIV-ELISA. Therefore, a high percentage of agreement (84%) was found between CLIA and IHA, and a similarly high agreement (92%) was found between CLIA and ApxIV-ELISA. Compared with IHA and ApxIV-ELISA, our present CLIA provides remarkable advantages in terms of reliability and in practical uses, such as high sensitivity and specificity.

Conclusions

In summary, we provided, for the first time, a CLIA method based on $AuCl_4^-$-enhanced luminol CL reaction for the highly sensitive detection of ApxIV antibody of APP in pig serum samples that showed great potential for numerous applications in immunoassay. The agreement of CLIA and the other two methods (ApxIV-ELISA and IHA) was 94.2%, which is very high in clinical diagnostic test, and the LOD of CLIA was lower than ApxIV-ELISA and IHA. We believe that the method is extremely suitable for detecting, preventing, or controlling animal-borne disease outbreaks. From the clinical medicine point of view, the protocol can be readily extended to a large variety of animal-borne disease and zoonoses. Moreover, multiplexing detection is possible through labeling of different metal nanoparticles.

Acknowledgements

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