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ORIGINAL PAPER

A label-free electrochemical assay for coronavirus IBV H120 strain quantification based on equivalent substitution effect and AuNPs-assisted signal amplification

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Abstract

A label-free electrochemical strategy is proposed combining equivalent substitution effect with AuNPs-assisted signal amplification. According to the differences of S1 protein in various infectious bronchitis virus (IBV) strains, a target DNA sequence that can specifically recognize H120 RNA forming a DNA-RNA hybridized double-strand structure has been designed. Then, the residual single-stranded target DNA is hydrolyzed by S1 nuclease. Therefore, the content of target DNA becomes equal to the content of virus RNA. After equivalent coronavirus, the target DNA is separated from DNA-RNA hybridized double strand by heating, which can partly hybridize with probe 2 modified on the electrode surface and probe 1 on AuNPs' surface. Thus, AuNPs are pulled to the surface of the electrode and the abundant DNA on AuNPs' surface could adsorb a large amount of hexaammineruthenium (III) chloride (RuHex) molecules, which produce a remarkably amplified electrochemical response. The voltammetric signal of RuHex with a peak near − 0.28 V vs. Ag/AgCl is used as the signal output. The proposed method shows a detection range of $1.56e^{-9}$ to $1.56e^{-6}$ μM with the detection limit of $2.96e^{-10}$ μM for IBV H120 strain selective quantification detection, exhibiting good accuracy, stability, and simplicity, which shows a great potential for IBV detection in vaccine research and avian infectious bronchitis diagnosis.

Keywords Coronavirus · IBV H120 strain · Equivalent substitution effect · AuNPs-assisted signal amplification · Electrochemical assay

Introduction

In the last few decades, viruses are a real menace to safety. The pandemic dimension spread of coronavirus disease poses a severe threat to the health and lives of seven billion people worldwide [1]. Rapid identification of viruses should be one of the best ways to prevent disease outbreaks and is of great significance to medical healthcare [1]. IBV, one kind of coronaviruses, is a positive-sense single-stranded enveloped RNA virus with a length of 27–32 kb. One IBV strain, H120 strain, usually needs to be identified from other strains for immunoprophylaxis and vaccine production, for example, NNA strain. Both of them are composed of structural and nonstructural proteins [2, 3]. The spike (S) glycoprotein is one of the major structural proteins which can be post-translationally cleaved into S1 and S2 subunits [4]. S1 subunit determines the genotype, serotype, and phenotype of IBV, which is the most significant protein for virus identification [5]. It is known that gene sequences of H120 and NNA strains

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are highly similar [6–8]; as a result, an obstacle in immunoprophylaxis presents because of the lack of accurate identification method. Therefore, developing a rapid and sensitive method for identification and quantification of different IBV strains based on hypervariable region of S1 gene can effectively solve the problem, which plays important roles in IB early diagnosis and control, especially for vaccine production.

Up to now, a number of methods have been fabricated to diagnose acute IBV infections based on IBV virus RNA detection and antibody response. These common methods include immunofluorescence assay (IFA) [9], agar gel precipitation test (AGPT) [10], virus isolation (VI) [11], immunoperoxidase assay (IPA) [12], reverse transcriptase polymerase chain reaction (RT-PCR) [13], and enzyme-linked immunosorbent assay (ELISA) [4]. Among the above methods, the sensitivity is generally unsatisfactory and those methods are relatively expensive and laborious. Meanwhile, one of present challenges in the virus detection field is the need for further non cross-reactive, stable, and sensitive biosensors [14]. Thus, the development of ultrasensitive and fast methods to detect IBV is still a great challenge and absolutely necessary. To improve the sensitivity, the signal amplification strategy should be utilized [15, 16].

Recently, some studies have shown that nanomaterials play an essential role in nanotechnology and biomedical applications [17, 18]. Among different nanomaterials, gold nanoparticles (AuNPs) have attracted tremendous interests [19, 20], due to its characteristics including easy synthesis manipulation, precise control over the physicochemical properties, strong binding affinity for thiols, and distinct electronic properties [21]. AuNPs have been used as efficient sensors for the detection of DNA and RNA based on different sensing strategies [22, 23]. Among the electrochemical methods, it has attracted great attention due to its properties [24, 25], including convenient operability, simple instrumentation, low cost, and on-site detection [26, 27]. Thus, the development of label-free electrochemical biosensor for the assay of IBV detection based on the AuNPs amplification is very promising.

Experimental section

Materials and apparatus

AL2000 DNA marker and 1 kb DNA Ladder marker were obtained from Nanjing Zhongding Biotechnology Co. Ltd. Prime Script II 1st Strand cDNA Synthesis Kit, SYBR Premix Ex Taq II (Tli RNaseH Plus) Bulk, pMD19-T Vector Cloning Kit, and EcoRI were purchased from Dalian Bao Biological Engineering Co. Ltd. The AxyPrep DNA Gel Extraction Kit was obtained from Axygen (USA). Chloroauric acid, sodium citrate, and tris(2-carboxyethyl)-phosphine (TCEP) were purchased from Sigma-Aldrich Chemical Co. Ltd. (USA). Other reagents in the method were of analytical grade. All solutions in the study were prepared with ultrapure water, which was obtained from Milli Q water purification system (USA).

The DNA immobilization buffers are 10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, and 10 μM TCEP (pH 7.4). The reaction buffer is phosphate-buffered saline (10 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.4) with 140 mM NaCl and 5 mM $MgCl_2$. DNA oligonucleotides (HPLC purified) were synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). All the electrochemical measurements were performed on CHI 660D electrochemical workstation (Shanghai, China). DNA sequences used in this electrochemical assay were shown in Table 1.

IBV RNA extraction and S1 gene amplification

The AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit was used to extract viral RNA. The Prime Script II 1st Strand cDNA Synthesis Kit and Roche's PCR enzyme FastStart Universal SYBR Green Master (ROX) were for RNA inversion and S1 gene amplification, respectively. The S1 gene amplification reaction system (total volume 25 μL) contained 12.5 μL of 2 × PCR Master Mix, 1 μL of cDNA products, 9.5 μL of ddH_2O, and 1 μL of 10 pmol of S1 forward and S1 reverse primer (Table S1). Amplification procedures were as follows: 95 °C for 5 min, then 33 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, and finally 10 min at 72 °C. The products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Construction of standard plasmid

After purification, PCR product was connected with pMD19-T. Then, it was transformed into susceptible *E. coli* by conventional method and coated on plates with ampicillin and LB. White colonies were selected and inoculated in LB medium containing ampicillin. Finally, the plasmids were extracted for sequencing and identification by enzyme digestion. The concentration of standard plasmids was measured by ultraviolet spectrophotometer and copy number was calculated through the equation: copies/mL = plasmid concentration × $6.02 \times 10^{23} \times 10^{-9}$/(plasmid length × 660). After

Table 1 List of DNA sequences used in this experiment

Name	Sequence (5′-3′)
SH-H120-Probe1	TTT TTT TCA GGT GAG TTA
H120-Probe 2-SH	GAT CAT AAT ATA TAT ATA T
H120-Target	ATT ATG ATC TAA CTC ACC TGA

determination of copy number, a series of standard plasmids at different copies were prepared for real-time fluorescence quantitative PCR to obtain the standard linear curve, which was used to calculate the concentration of real virus samples.

Preparation of probe 1-functionalized AuNPs

The synthesis of AuNPs was according to our previous report [28]. Then, 100 μL of AuNPs colloid was incubated with 40 μL of 10 μM probe 1 and 360 μL of PBS buffer. The mixed solution was oscillated slowly in 37 °C for 12 h. After that, 2 M NaCl was added slowly at room temperature for 4 times. The final concentration of NaCl was 0.3 M. The salt was used to block the negative charges on DNA strands to allow high density loading and rearrangement of DNA on AuNPs' surface. After incubation at room temperature for another 8 h, the solution was centrifuged at 12,000 rpm for 20 min. Then, the solution was purified through three times of centrifugation at 12,000 rpm for 20 min. Finally, the AuNPs were resuspended in 20 mM Tris-HCl (pH 8.0) solution and stored at 4 °C. The characterizations of probe 1 DNA-modified AuNPs were investigated by ultraviolet visible absorption spectroscopy (Fig. S1).

Electrode treatment

Pretreatment of gold electrode was according to our previous report [29]. The electrode was electrochemically activated in 0.5 M H_2SO_4. Probe 2 of 0.5 μM was incubated with gold electrode for 12 h at room temperature. Then, the modified electrode was immersed into an aqueous solution of 1 mM mercaptohexanol (MCH) for 60 min to inhibit nonspecific DNA adsorption [30].

Electrochemical measurements

The target DNA was incubated with viral RNA at 90 °C for 5 min and dropped to room temperature slowly. After that, the reaction mixture was incubated with 1 μL of 80 U/μL nuclease at 37 °C for 30 min. Then, the reaction mixture was placed at 90 °C for 15 min and annealed at 4 °C. Then, the final reaction mixture was incubated with probe 2-modified electrode. Finally, probe 1-functionalized AuNPs was incubated with the modified electrode for 2 h. In this work, a three-electrode cell was used with an Ag/AgCl as the reference electrode, a platinum wire as the counter electrode, and a gold electrode as the working electrode. Two electrochemical techniques, linear sweep voltammetry and chronocoulometry, were conducted in the experiment. A Tris-HCl solution (pH 7.4) of 10 mM containing 50 μM RuHex and 10 mM PBS buffer was used. The peak current at − 0.28 V was recorded and used to quantify the concentration of IBV H120 strain.

Results and discussion

Working principle

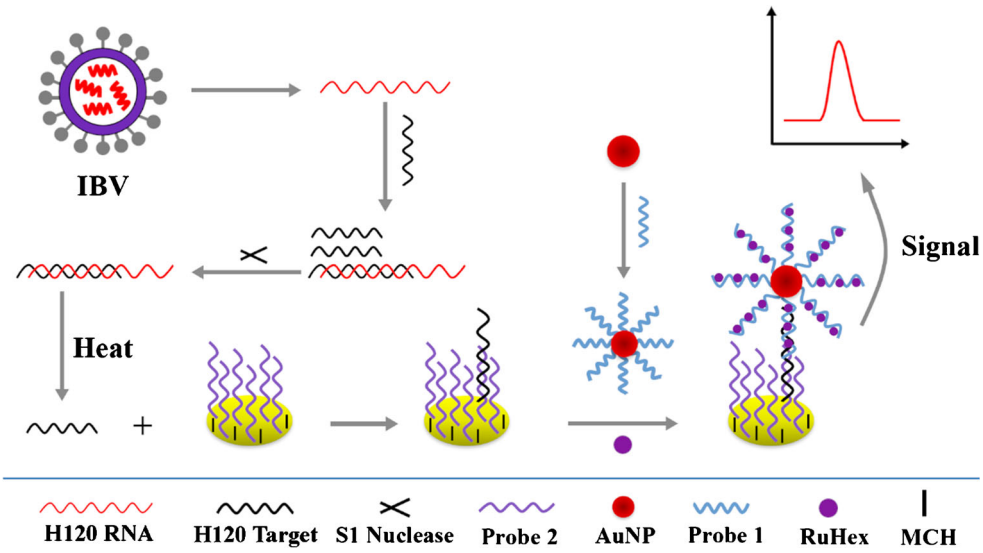
The detailed working principle is illustrated in Scheme 1. Herein, a label-free electrochemical assay based on equivalent substitution effect and AuNPs-assisted signal amplification is developed for identification and quantification detection of IBV H120 strain. We firstly design the H120 target DNA which can recognize RNA of H120 strain specifically, and the residual single-stranded H120 target DNA can be hydrolyzed by S1 nuclease. Then, the H120 target DNA is separated from the DNA-RNA hybridized double strand by heating. Thus, the concentration of virus RNA of H120 strain is equivalently substituted by the target DNA. The target DNA can partly hybridize with the probe 2 modified on the electrode surface and probe 1 on the surface of AuNPs. Due to an electrostatic interaction, positively charged signal molecules, hexaammineruthenium (III) chloride (RuHex), can be adsorbed onto the probe 1 modified on AuNPs which is pulled on the electrode surface through the target DNA, causing an intense electrochemical response. By analyzing the electrochemical response, a sensitive identification and quantification assay for IBV H120 strain is thus established and the method proposed in this work has a great potential for IBV detection in medical research and early IB diagnosis.

Establishment and identification of standard plasmids

As shown in Fig. 1a and b, electrophoresis results indicate PCR products of S1 gene are successfully amplified. Electrophoresis results of standard plasmids are shown in Fig. 1c. After being completely cleaved by EcoRI enzyme, two bands can be seen in the electrophoretic diagram that demonstrate the successful construction of plasmid.

Determination of H120 strain concentration in real virus sample

After construction of the standard plasmid containing characteristic sequence in S1 gene of H120 strain, virus copy numbers in plasmids are measured by real-time fluorescence quantitative PCR. Amplification and melting curve of plasmid in Fig. 2a and b indicate that there is no nonspecific amplification. A linear relationship between Ct value and the logarithm of the initial copy number of the standard plasmid (Fig. 2c) is obtained, and the linear regression equation is $Y = -3.3191x + 33.0203$ ($R^2 = 0.99951$). Thus, a series of H120 standard samples at different concentrations is successfully prepared for the following electrochemical detection. Meanwhile, according to calculation, the concentration of H120 virus sample is $1.56e^{-6}$ μM (RSD = 0.67%, $n = 3$).



Scheme 1 Schematic illustration of the electrochemical method for IBV H120 strain detection

Optimization of the experimental conditions

In this work, the experimental conditions have been optimized to achieve the best signal-to-noise level. First, the annealing condition of target DNA and IBV H120 strain has been investigated. In this assay, we have adopted two kinds of annealing (Fig. S2a). One is 90 °C water bath for 5 min and then natural cooling to room temperature, and the other is metal bath cooling by means of gradient temperature (90 °C for 5 min, 70 °C for 10 min, 50 °C for 10 min, 30 °C for 10 min, 10 °C for 25 min). As shown in Fig. 3a, the former performance is better. Thus, 90 °C water bath for 5 min with natural cooling to room temperature is chosen for the following experiments.

Second, the concentration of RuHex is investigated (Fig. S2b). In this assay, we have applied 5 μM and 50 μM RuHex for the detection of IBV H120 strain. As shown in Fig. 3b, the electrochemical signal with 50 μM RuHex is better. Thus, 50 μM is chosen as the optimized concentration.

Finally, the electrolyte of this assay is studied (Fig. S2c). We have respectively used 10 mM Tris-HCl (pH 7.0) and 10 mM PBS (pH 7.0) as the electrolytes. As shown in Fig. 3c, the Tris-HCl buffer is better than PBS buffer. Therefore, the Tris-HCl buffer is chosen as the electrolyte.

Electrochemical detection of IBV H120 strain

The sensitivity of the sensing system is evaluated under the optimized experimental conditions. Firstly, we detect different concentrations of IBV H120 strain; a gradual increase of RuHex peak current corresponding with the elevated concentration of H120 strain from $1.56e^{-9}$ to $1.56e^{-6}$ μM is obtained (Fig. 4a and b). And the peak current is linear with the logarithm of IBV H120 strain. The linear range of IBV H120 strain from $1.56e^{-9}$ to $1.56e^{-6}$ μM with the detection limit at $2.96e^{-10}$ μM is obtained (S/N = 3, RSD = 1.65%, n = 3). The linear regression equation is $Y = 7.9821 + 0.6406x$ (R^2 = 0.99837), where Y is the peak current and x is the logarithm of IBV H120 strain concentration. To further evaluate the specificity of our proposed method, the chronocoulometry is used. We have detected H120 and NNA strain using H120 target DNA. As shown in Fig. 4c, NNA strain does not produce significant electrochemical signal and H120 strain could

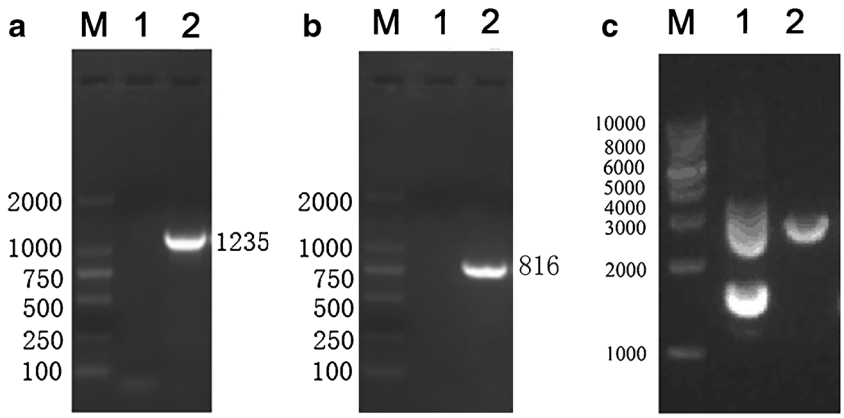


Fig. 1 Agarose gel electrophoresis diagrams. **a** Lane M: 2000 bp DNA marker, Lane 1 negative control, Lane 2: H120 S1 gene sample. **b** Lane M: 2000 bp DNA marker, Lane 1: negative control, Lane 2: NNA sample. **c** Lane M: 1 kb DNA Ladder marker, Lane 1: H120 plasmid, Lane 2: H120 plasmid is cleaved by EcoRI enzyme

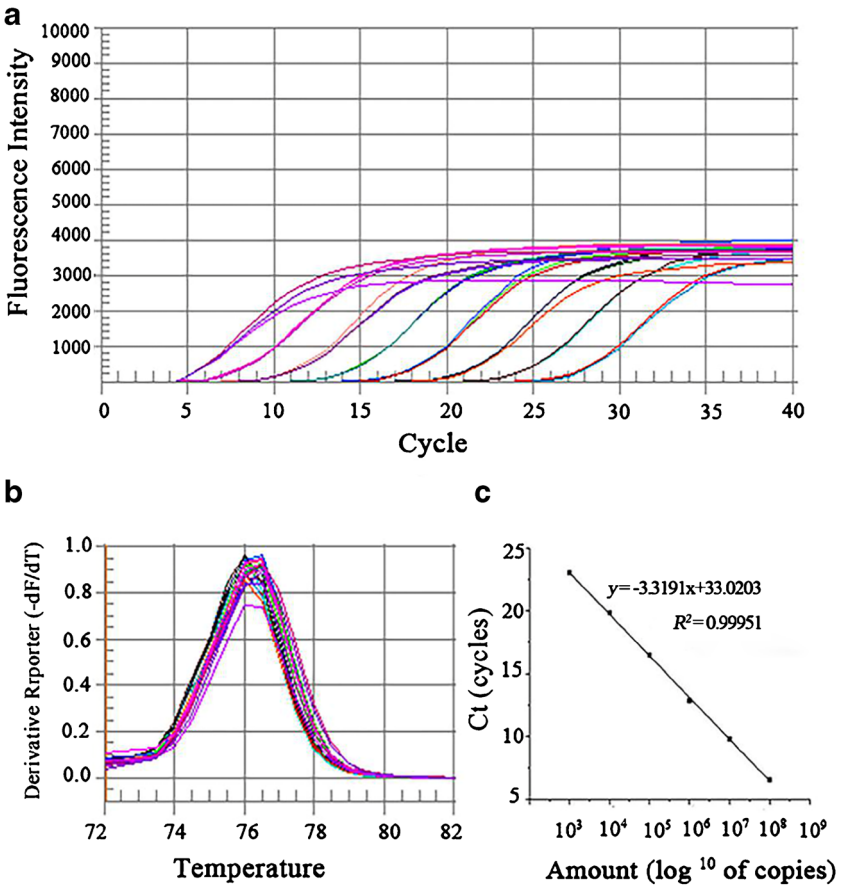


Fig. 2 Real-time fluorescence quantitative PCR plot. **a** Amplification plots. **b** Melting curves of H120 standard samples at 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 copy numbers. **c** The linear relationship between Ct value and the logarithm of the initial copy numbers

produce significant electrochemical signal, which signifies the excellent specificity of this method.

In order to evaluate the performance of the present sensor system, a comparable table for IBV analysis methods has been listed in the electronic supplementary material (Table S2). Though the construction of AuNPs is time-consuming and the introduction of the signal amplification makes the detection system a little bit complicated, it still can be seen from the table that our analysis method is more excellent than others due to the following attractive advantages. First, the IBV H120 strain RNA detection process is transformed into DNA detection which can effectively avoid RNA degradation. Second, the use of gold nanoparticles greatly increases the electron transfer efficiency of electrode surface. Third, AuNPs-assisted electrochemical signal amplification highly enhances the sensitivity of IBV detection.

Conclusions

In this work, we designed the sequence of the target DNA based on the hypervariable region in the S1 gene between different IBV strains, then, constructed the standard plasmid containing characteristic sequence of S1 gene in H120 strain,

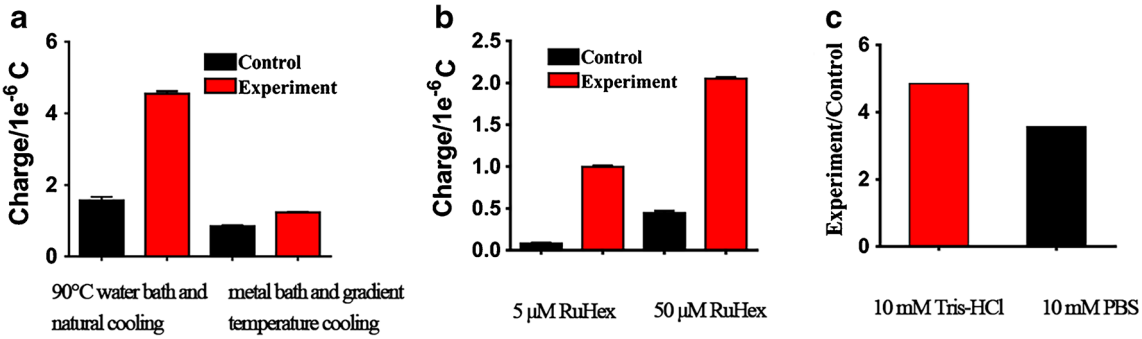


Fig. 3 Chronocoulometric-int numerical bar graph. a The annealing condition of target DNA and virus RNA. b The chronocoulometric response of RuHex. c The pH 7.0 electrolyte

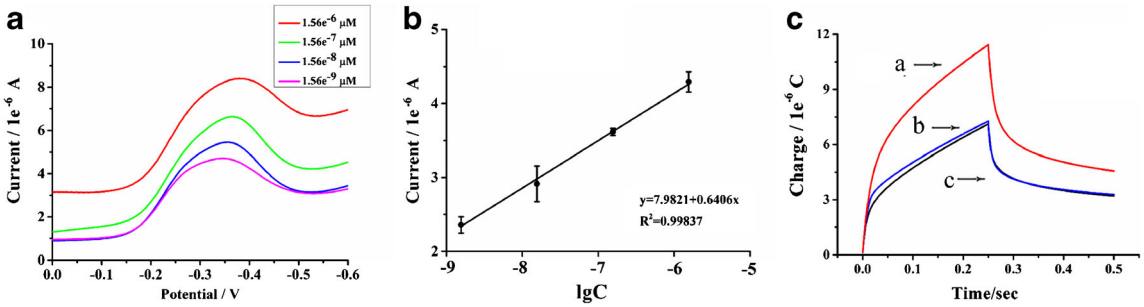


Fig. 4 Electrochemical detection of H120 RNA: **a** Linear sweep voltammetry corresponding to different concentration (1.56e^{-9} μM, 1.56e^{-8} μM, 1.56e^{-7} μM, 1.56e^{-6} μM, $n = 3$). **b** The linear relationship between the peak current and the concentration of H120 RNA. **c** Chronocoulometric response of (a) 10 μL H120 virus, (b) 10 μL NNA virus, (c) 0 μL H120 virus

and at last, proposed a label-free ultrasensitive electrochemical assay applicable for the detection of IBV H120 strain in real sample, which can distinguish between H120 and NNA. In this electrochemical method, H120 strain detection is based on equivalent substitution effect and AuNPs-assisted signal amplification with a detection range from 1.56e^{-9} to 1.56e^{-6} μM. Compared with previously reported IBV detection methods that may focus on the portable and simplified analysis, our electrochemical assay still has some advantages. For example, converting RNA detection to DNA detection effectively avoid RNA degradation. The use of gold nanoparticles and AuNPs-assisted electrochemical signal amplification greatly increases the electron transfer efficiency and the sensitivity of IBV detection. Furthermore, by analyzing the hypervariable region of S1 gene and replacing the target and probe DNA sequence by other customized sequences, the developed sensing strategy can be easily used to detect other virus. Therefore, the method might hold a great potential for further applications in virus bioanalysis, early clinical diagnosis, and biomedical research.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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