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A simple strategy for rapid and sensitive detection of avian influenza A H7N9 virus based on intensity-modulated SPR biosensor and new generated antibody

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Abstract

In 2013 a new reassortant avian influenza A H7N9 virus emerged in China, causing human infection with high mortality. An accurate and timely diagnosis is crucial for controlling the outbreaks of the disease. We therefore propose a simple strategy for rapidly and sensitively detecting the H7N9 virus using an intensity-modulated surface plasmon resonance (IM-SPR) biosensor integrated with a new generated monoclonal antibody. The novel antibody exhibits significant specificity to recognize H7N9 virus compared with other clinical human influenza isolates (p<0.01). Experimentally, the detection limit of the proposed approach for H7N9 virus detection is estimated to be 144 copies/mL, which is a 20-fold increase in sensitivity compared with homemade target-captured ELISA using the identical antibody. For the measurement of mimic clinical specimens containing the H7N9 virus mixed with nasal mucosa from flu-like syndrome patients, the detection limit is calculated to be 402 copies/mL, which is better than conventional influenza detection assays; quantitative reverse transcription polymerase chain reaction (qRT-PCR) and rapid influenza diagnostic test (RIDT). Most importantly, the assay time took less than 10 minutes. Combined, the results of this study indicate that the proposed simple strategy demonstrates high sensitivity and time-saving in H7N9 virus detection. By incorporating a high specific recognizer, the proposed technique has the potential to be used in applications and development of other emerging or re-emerging microbe detection platforms.

Keywords: Influenza, H7N9, Intensity-modulated surface plasmon resonance, Target-captured ELISA, Rapid influenza diagnostic test

INTRODUCTION

In the past Avian influenza A H7N9 virus has been detected only in birds, however, a new reassortant of avian influenza A H7N9 virus was identified in China in March, 2013. Since then, the number of human infections with the reassortant H7N9 virus has steadily increased in the country. The reassortant H7N9 virus has low pathogenicity for avian hosts, which results in asymptomatic or mild avian diseases.[1-5] This asymptomatic infection of poultry can cause poultry-to-human silent transmission through direct contact or exposure to seemingly healthy but infected birds.[5] However, according to a recent WHO report, there was a total of 1564 laboratory-confirmed cases of human infection with the reassortant H7N9 virus, and among them, 612 ended in death[6]. Most patients who are infected with the reassortant H7N9 virus progress to pneumonia and acute respiratory distress syndrome, with a case fatality rate of approximately 40%.[7] The new reassortant avian H7N9 virus possesses amino acid changes that allow it to adapt to mammalian hosts, which raises a concern for pandemics in humans. Therefore, accurate and prompt diagnosis is an important tool for a disease control plan. Currently, the laboratory diagnostic assays for the new reassortant H7N9 virus detection recommended by WHO are either by using molecular tests such as quantitative reverse transcription polymerase chain reaction (qRT-PCR) or by attempting to grow the virus via propagating the virus in a cell culture system or embryo eggs.[8,9] However, culturing this new emerging reassortant avian H7N9 virus is time-consuming, laborious, and requires BSL-3 containment.[8,9] Although qRT-PCR is relatively time-saving, it still needs several hours. Additionally, high cost, and the need of well-trained technical staff and thermocycling steps further limit the classic technique.[10-12] Therefore, there is an urgent demand for developing a simple, cost-effective, sensitive and specific biosensing platform to rapidly diagnose infections with the new reassortant avian H7N9 virus.

Recently, PCR-free detection methods have attracted tremendous attention because they exhibit

rapid and highly sensitive detection of clinically relevant viruses.[13-16] Surface plasmon resonance (SPR) technique would be a good candidate of PCR-free detection because it is a label-free and real-time technique that is sensitive to the refractive index of the medium in direct contact with a metal film. Besides the above-mentioned advantages, SPR biosensor also shows the benefits of easy operation and setup. SPR biosensor has been widely used to study biomolecular interactions, including antigen/antibody, small molecule (such as a drug)/receptor, and nucleic acids interactions.[17-21] However, the inherent characteristics of SPR technology limit its detection sensitivity, especially in the direct detection of low concentrations of small molecules.[10] Thus, in recent years, SPR biosensor has been applied to detecting macromolecules, such as viral or bacterial pathogens, which bypasses time-consuming methods, like culturing, PCR, and enzyme-linked immunosorbent assays.[22-25] Previously, we developed a paired surface plasma wave biosensor (PSPWB) that was based on optical heterodyne interferometry for swine-origin influenza A H1N1 virus (S-OIV) and polyomavirus BK (BKV) detection.[10,11] Although the PSPWB exhibits a higher detection sensitivity than conventional setup, the PSPWB needs costly and bulky instruments just like the conventional setup. In this study, we built up an intensity-modulated SPR (IM-SPR) biosensor which was equipped with relatively inexpensive devices, a current-modulated diode laser and a data acquisition (DAQ), integrated with fast Fourier transform (FFT) analysis. Experimentally, the IM-SPR biosensor shows a comparable physical performance (stability or refractive index changing testing) to the PSPWB but is much less expensive. On the other hand, the specificity of SPR biosensor is strongly dependent on the reliability of capture antibodies. However, several reports indicate that routinely used commercial rapid tests have serious flaws in reliability because they fail to recognize their corresponding target, that is, cross-reactivity may occur leading to decrease in specificity.[26-29] Hence, in order to reduce this issue and to improve the reproducibility and specificity of the proposed IM-SPR biosensor on H7N9 virus detection, a well-characterized new monoclonal antibody was generated by our own laboratory via immunization of trimeric recombinant hemagglutinin of the H7N9 virus (Tri-rHA7), which was produced

from the baculovirus-insect protein expression system.

In this study, we used reverse genetics to generate attenuated reassorted influenza A H7N9 virus (H7N9-RG virus), which is currently used as a vaccine candidate and can be handled in a BSL-2 laboratory. In addition, the surface antigenicity of the generated H7N9-RG virus was verified similar to new emerging reassortant avian H7N9 influenza A virus. Thus the H7N9-RG virus was used as target virus in place of the new reassortant avian influenza A H7N9 virus in all the experiments including IM-SPR, ELISA, RIDT, and qRT-PCR for safety consideration. The sensitivity of the IM-SPR biosensor and of other conventional influenza detection assays such as qRT-PCR and rapid influenza diagnostic test (RIDT) were compared and evaluated. Experimentally, the detection limit of the proposed IM-SPR biosensor for the H7N9-RG virus detection in mimic solution (nasal mucosa from Flu-like illness donors as the diluent) is estimated to be 402 copies/mL, which is slightly better than that of qRT-PCR at 977 copies/mL. It is noteworthy to mention that the assay time of the proposed platform is within 10 minutes, and it is easy for SPR biosensor to be turned into a portable device. We believe that the sensitive and time-saving approach has the potential to be applied for clinical H7N9 diagnosis.

EXPERIMENTAL SECTION

Materials.

Immobilization buffer (10 mM sodium acetate, pH 5.0), regeneration solution and an amine coupling kit containing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 1.0 M ethanolamine-HCl, pH 8.5 (ETH) were purchased from Bio-Rad (Sundbyberg, Sweden). 11-mercaptoundecanoic acid (MUA), and 6-Mercapto-1-hexanol (MCH) and Biotin Protein Labeling Kit (1:1000 dilution) were obtained from Sigma Aldrich. HRP-Conjugated Streptavidin (1:2000 dilution) and RIDT were bought from Thermo Fisher and Panion & BF Biotech Inc., respectively. The working and washing buffer were PBS, comprised of 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate

buffer solution (pH 7.4 at 25 °C). PBST stands for 0.1% Tween-20 in PBS. All of the chemicals were used without further purification.

Ethics statement.

Throat swabs were used to collect influenza clinical isolates from influenza infected patients. Written informed consent forms were provided and signed by the study participants, and the study procedures were conducted in agreement with ethics committee regulations. The study was approved by the Institutional Ethics Committee of the Kaohsiung Medical University, Taiwan. Regarding monoclonal antibody generation, we followed procedures according to the Institutional Animal Care and Use Committee (IACUC) guidelines. The protocols of Animal use and handle were estimated and approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University. When immunized mice showed the highest titers of anti-sera against HA7 proteins, they were considered to have reached the end point of this experiment. The mice were euthanized humanely to ameliorate suffering, following the IACUC guidelines.

H7N9-RG strain generation.

Influenza A reassortant viruses were generated from plasmids by reverse genetics methods.[30,31] The HA and NA genes of the A/Shanghai/2/2013 (H7N9) virus were amplified by PCR from pHA7 and pNA9 (HA7 and NA9 expressing plasmids were purchased from Sino Biological Inc.). The genes were cloned into a reverse genetics vector pHW2000, which contains the pol I–pol II transcription system for the synthesis of vRNA and mRNA. Reverse genetic plasmids that encode the HA7 and NA9 genes, as well as plasmids that contain the six internal genes from A/PR/8/1934(H1N1), were transfected into 293T cells using Lipofectamine 2000 (Life Tech, Grand Island, NY, USA). Viruses from transfected cells were collected and subsequently propagated in MDCK cells. The function and morphology of the generated H7N9-RG virus was confirmed using the hemagglutination assay (HA) and transmission electron microscope (TEM),

respectively. The testing titers of the H7N9-RG viruses were quantified by qRT-PCR. The detailed protocol for the H7N9-RG virus detection and quantification appears in the WHO avian influenza H7N9 detection guideline[32,33].

Anti-HA7 monoclonal antibody preparation.

The Bac-to-Bac® Baculovirus Protein Expression System (Invitrogen, Cat. No. 10359-016) was used to produce the trimeric recombinant HA7 (Tri-rHA7) proteins of the A/Shanghai/02/2013(H7N9) strain (Genbank Accession No. KF021597). The detailed protocol for generation of the trimeric protein is referenced in a previous publication.[34] With regard to the generation of monoclonal antibodies against HA7(H7-mAb), we used BALB/c mice intraperitoneally immunized with 2 μg of Tri-rHA7 proteins mixed with complete Freund's adjuvant (Sigma Aldrich, Cat. No. F5881). Then BALB/c mice received twice intraperitoneal boosters using the same dose of Tri-rHA7 proteins emulsified with incomplete Freund's adjuvant (Sigma Aldrich, Cat. No. F5506). After a final boost, the mice splenocytes were collected and subjected to fusion with NS-1 cells (myeloma cells), as described previously.[35,36] After serial dilution, several clones that produced specific individual antibodies were selected.[35,36]

Specific epitopes in HA7 protein recognized by H7-mAb.

The epitopes recognized by H7-mAb on hemagglutinin were determined by using phage display and mutant escape assays described previously.[37-40] Phage-displayed library (7-mer peptides) was purchased from New England Biolabs (NEB), Inc. (Cat. No. E8102L) and the procedures of biopanning were conducted following the NEB manufacture's protocol. The detailed protocols of phage display and viral mutant escape assays are described in the supplement. The mAb recognized epitopes were directly mapped to hemagglutinin crystal structure of the A/Shanghai/02/2013 influenza A (H7N9) strain (RCSB Protein Data Bank No. 4LCX). The three-dimensional visualization of HA7 was shown using Accelrys DS Viewer 5.0.

Preparation of the SPR chip.

In this study, all the bare Ag/Au (35/10 nm) chips were made from the Thin Film Technology Center at the National Central University (Taoyuan, Taiwan). In addition, the gold surface was cleaned by use of the UV/Ozone Cleaner (BioForce Nanosciences, Ames, IA), before surface functionalization with self-assembled monolayers (SAMs). The capture antibody, H7-mAb, at a concentration of 10 μg/mL was covalently immobilized to the reaction spot of the SPR chip through a mixed SAMs of MUA and MCH with a molar mixing ratio of 1 : 9 via the amine-coupling protocol, which is described in detail in our previous study.[10,11]

Measurement of the H7N9-RG virus using the IM-SPR biosensor.

The optical setup of the IM-SPR biosensor is illustrated in Figure 1. A diode laser emitting at a wavelength of 635 nm, and its output power was modulated at a frequency of 1 kHz. Afterward, the beam passed through a polarizer that was oriented along the TM direction, and then, it was split into two parallel beams by using a lateral displacement beam splitter. Subsequently, the two beams were incident onto a Kretschmann SPR device, and the reflected beams ultimately were detected by two photodetectors as the signal and reference. A DAQ device was employed for simultaneously measuring the SPR signals from the reference spot and reaction spot for the detection of the H7N9-RG virus or other human clinical influenza isolates. All of the analytes flowed through the SPR chip to interact with the immobilized H7-mAb, while PBS solution flows through the reference spot.

Establishment of the homemade target-captured ELISA.

The H7-mAb was used to establish a target-captured ELISA assay. The detailed protocol of the

target-captured ELISA was described previously.[41] Briefly, 96-well round-bottom microtiter plates (Nunc, Roskilde, Demark) were coated with 2 μg purified H7-mAb in 100 μL carbonate buffer (73 mM sodium bicarbonate and 30 mM sodium carbonate) and incubated at 37°C for 1h or at 4°C overnight. The plates were washed three times with PBST and blocked with PBST that contained 5% bovine serum albumin (BSA) at 37°C for 1h. After washing three times with PBST, the H7-mAbs coated 96-well plates could be used for the H7N9-RG virus detection. The standard Tri-rHA7 proteins and qRT-PCR quantified H7N9-RG viruses were both used as target for the target-captured ELISA. The diluted and quantified H7N9-RG viruses in PBS or mimic samples (the flu-like syndrome patent's nasal mucosa) were added onto 96-well plates and then incubated at 37°C for 2h. After washing five times with PBST, biotin labeled polyclonal antibodies (Figure S-1 in the Supporting Information depicts the specificity.) were added into each well and incubated for 1h at 37°C. Then, 100-μL HRP-conjugated streptavidin was added into each well and incubated at 37°C for 1h. After washing five times with PBST, color development was performed by the addition of 100 μL of freshly prepared TMB (solutiontetramethyl benzidine) solution (ThermoFisher); the absorbance at 450 nm was read with an ELISA reader (Tecan, Switzerland).

Measurement of the H7N9-RG virus using commercial RIDT.

The commercial RIDT was used to detect the H7N9-RG virus as well. The measurement was conducted according to the manufacturer's protocol. The analytes were mixed with diluted and extraction buffer and then added to the reactive strip at room temperature for 10 min. The reactive bands of the influenza A and B viruses as well as the control were observed.

Measurement of the influenza A H7N9-RG virus using WHO guided qRT-PCR.

For the detection and quantification of H7N9 virus qRT-PCR was used. The protocol described by

WHO H7N9 detection guideline was followed and the primers and probes used are listed in (http://www.who.int/influenza/gisrs laboratory/collaborating centres/list/en/). Briefly, 1.0 μL of 10 μM HA7 and RnaseP (as quality control) primers, 0.5 μL of the corresponding probes, 5 μL of extracted viral RNA, 12.5 μL of 2× One Step RT-PCR buffer, 1 μL of Taq Polymerase, 1 μL of Script RT Enzyme Mix and an appropriate volume of Rnase-free distilled water (dH_2O) were mixed in a total 25 μL reaction volume. The thermal cycles were performed on an ABI 7500 real-time PCR system (Applied Biosystems, Foster city, CA) under the following conditions: 30 min at 50°C for reverse transcription; 5 min at 95°C, then 40 cycles at 95°C for 15s and 55°C for 45s for real-time quantitative PCR[33]. The PCR results determined if the quality controls were detected.

RESULTS AND DISCUSSION

Generation of the H7N9-RG virus.

The new reassortant avian influenza A H7N9 virus has been classified as a biosafety level 3 (BSL-3) pathogen, which must be handled in a BSL-3 laboratory. Thereby, in this study we use the H7N9-RG virus as the target virus in all the experiments including IM-SPR, ELISA, RIDT, and qRT-PCR because this virus could be handled in a BSL-2 laboratory. The plasmids that contain HA7 and NA9 full-length sequences of A/Shanghai/02/2013(H7N9) were co-transfected with six other plasmids that contain internal gene fragments of A/PR/8/34 (H1N1) into 293T, to generate the H7N9-RG strain. The results from the transmission electron microscopy (TEM) indicated that the H7N9-RG strain showed a similar structure and morphology with the clinical human H7N9 influenza isolate (Figure S-2A). Immunogold staining using commercial and our generated anti-HA7 monoclonal antibody indicated that HA7 protein was expressed on H7N9-RG viruses (Figure S-2B). Functional evaluation indicated that H7N9-RG could infect MDCK cells (Figure S-2C) and has a hemagglutination capability to aggregate turkey RBCs (Figure S-2D). The viral

qualification of H7N9-RG viruses was determined by using H7N9 quantitative RT-PCR (qRT-PCR)(Figure S-2E). Combined, this data indicates that our generated H7N9-RG virus contains functional HA7 proteins on the envelope and displays similar antigenicity as the new emerging reassortant avian H7N9 influenza A virus.

Generation of the H7-mAb against hemagglutinin of the H7N9 virus.

A series of H7-mAbs were generated via the immunization of Tri-rHA7 proteins, which were produced by the baculovirus-insect protein expression system (Figure S-3A). The H7-mAb 1-5 displayed a higher binding capability and could be used to detect the HA7 protein in an H7N9-RG virus-coated ELISA assay and immunoblotting assay, respectively (Figure S-3A&B). The isotype of H7-mAb 1-5 was determined, and the results indicated that this H7-mAb was an IgG1 isotype and had the κ light chain. Further, the epitopes recognized by this H7-mAb were mapped using phage display (Table S1) and mutant escape assays (Table S2). We found that the epitopes were "IERS" at amino acid site 78, 79, 80 and 84 on the HA7 lateral regions of the globular head structure (Figure S-3C,D&E). The data from protein sequence alignment of most HA7 and other HA subtypes was collected and indicated that H7-mAb 1-5 specifically recognized conserved epitopes on HA of recent circulating H7N9 isolates(Table S3). Later, we evaluated the specificity of mAb 1-5 against H7N9 virus with virus-coated ELISA using different human influenza viruses. Meanwhile, we found that anti-HA7 mAb 1-5 displayed viral neutralization capabilities against H7N9-RG virus infection using hemagglutination inhibition(HAI) (Figure S-3F) and microneutralization(MN)(Table S2) assays. In addition, our generated anti-HA7 mAb 1-5 displayed higher detection capabilities compared with two commercially available anti-HA7 mAbs under immunoblotting and H7N9 virus-coated ELISA evaluation(Figure S 4A&B). Recent reports indicate that limited specific rapid serological assays are available for avian influenza A H7N9 virus detection.[9,42] Hemagglutinin of H7N9 influenza virus is a proper target for developing the diagnostic or therapeutic H7-mAbs for avian H7N9 influenza infection.[9,43] A recent study used DNA immunization to induce specific monoclonal antibodies against HA of H7N9 virus

and were subjected to set up an immunochromatographic assay system.[44] In this study, we used the baculovirus-insect system to generate Tri-rHA7 to induce H7-mAb. Because of its existing post-modification and correct folding ability in the newly generated proteins, the baculovirus-insect expression system has been proven to be a better method to produce functional or therapeutic antibodies against influenza virus infection.[45] In addition, the recombinant trimeric HAs, which displayed similar structure with the native form of HA on the influenza envelope, could induce higher titers of type-specific antibodies.[34,36] Here, we used a similar strategy to generate H7-mAb for H7N9 virus detection.

Stability testing of the IM-SPR biosensor.

The stability testing of the proposed IM-SPR biosensor was conducted by tridistilled water at a fixed incident angle of incidence. As shown in Figure 2, the result was expressed as the percentage error of the system, which is calculated by $\frac{R_{\text{each point}} - R_{\text{mean}}}{R_{\text{mean}}} \times 100\%$, in which the response R is obtained by subtracting the signal of the reference spot from that of the reaction spot. In addition, the relative standard deviations (RSD) of the system stabilities while spanning 30 minutes was 0.13%.

Most commercial SPR instruments are operated in the angular interrogation mode, which measures analytes concentration by tracking the resonance angle shifts by use of a CCD. Gold-based SPR is the most frequently used because it provides a higher angular sensitivity than other plasmonic materials such as silver.[46,47] In contrast, the IM-SPR biosensor measures the output intensity at a fixed incident angle; it belongs to an intensity interrogation mode that silver-based SPR has a higher intensity sensitivity than gold.[48,49] Unfortunately, silver possesses fairly poor chemical stability, making it highly susceptible to oxidation. Bimetallic silver–gold structure is a good choice for silver protection, and exhibits better sensitivity performance compared to that of gold on intensity measurement.[50-52] In spite of the higher sensitivity and simpler optical setup, background noise is usually a significant issue for conventional

intensity interrogation-based SPR biosensor since a CW light source is used. By contrast, the IM-SPR biosensor is equipped with a current-modulated diode laser as the light source to produce an output power with a frequency. As a result, the reflected light from a Kretschmann SPR device incident on a detector is an AC signal. In addition, a DAQ device integrated with FFT analysis was used to extract the AC signal. When a signal is AC-coupled, the DC and low-frequency background noise can be filtered out from the input signal, improving the signal stability.[53] It is important for SPR biosensors because the performance of a SPR biosensor is strongly dominated by the system stability, which is highly dependent on the light source stability and detection noise.[54] To conclude, the IM-SPR biosensor shows more efficient, inexpensive, compact, and controllable abilities than the PSPWB which uses a complicated heterodyne interferometry and high-priced instruments.[10]

Evaluation of the specificity for H7N9 virus detection.

Here, the specificity of the new generated antibody for H7N9 virus was evaluated by the IM-SPR biosensor and homemade target-captured ELISA. The work was conducted with the influenza isolates including A/California/7/2009-like (H1N1), A/Texas/50/2012-like (H3N2) and B/Massachusetts/2/2012-like isolates and the H7N9-RG viruses. As shown in Figure 3A (IM-SPR biosensor) and Figure 3B (homemade target-captured ELISA), a significant difference in binding capacity was observed between the H7N9-RG viruses and other influenza virus strains. The results demonstrate that the new generated H7-mAb indeed has high specificity in detecting the H7N9-RG virus. In addition, it has been verified above that the generated H7N9-RG virus shows the same surface properties to new emerging reassortant avian H7N9 influenza A virus. Thus, we believe that the H7-mAb will exhibit a high specificity to the wild-type H7N9 virus as well.

Performance of the IM-SPR biosensor in detecting the H7N9-RG virus.

The initial titer of the culture supernatant of H7N9-RG virus was 2.3×10^5 copies/mL and it was quantified via WHO guided qRT-PCR. 10-fold serial dilutions of the H7N9-RG virus culture supernatant in PBS solution (from 2.3×10^2 to 2.3×10^5 copies/mL) were injected into the reaction spot to interact with the immobilized H7-mAb and form an <H7-mAb/H7N9-RG> complex on the SPR sensor chip. At the same time, a 10-fold diluted culture medium in the absence of the H7N9-RG virus was treated as the control group (blank). The real-time SPR curve of the interaction is represented in Figure 4A. It can be seen that a clear nonspecific adsorption on the sensing surface after the PBS solution washing was observed in control group. This finding is not surprising because the antifouling ability of the conventional mixed SAM is inefficient. Fortunately, the SPR signals seem to differentiate these H7N9-RG virus concentrations. The standard curve of the H7N9-RG virus detection by IM-SPR biosensor over the range of 2.3×10^2 to 2.3×10^5 copies/mL is shown in Figure 4B. The SPR signal was obtained from the average of the y-axis value over the last 30 data points from Figure 4A. The results were analyzed using a sigmoidal shape by means of the 4-parameter nonlinear logistic equation; the correlation coefficient (R^2) is 0.9642, and the error bar indicates 1 SD in each measurement. Based on IUPAC definition, the theoretical limit of detection (LOD) of the IM-SPR biosensor for the H7N9-RG virus detection was estimated to be 144 copies/mL from the experimental results.

To mimic a clinical sample, the 10-fold diluted culture medium was added with nasal mucosa from Flu-like illness donors as the mimic sample. In the mock experiments, the control group (blank) was 10-fold diluted culture medium with mimic solution in the absence of H7N9-RG virus. The real-time SPR curve of interaction is represented in Figure 4C. A similar result was obtained for the standard curve of the H7N9-RG virus detection in the mimic solution over the range of 2.3×10^2 to 2.3×10^5 copies/mL, as depicted in Figure 4D. The correlation coefficient (R^2) is 0.9914, and the theoretical detection limit of the IM-SPR biosensor for H7N9-RG virus detection in the mimic solution was calculated to be 402 copies/mL.

Moreover, the regeneration of the chip surface was tested by regeneration solution (10 mM Glycine, pH 1.7). The regeneration procedure was performed after passing recombinant HA7 solutions in PBST pH 7.4 at concentrations of 10 μg/ml over the SPR chip to remove the bound recombinant HA7 from the immobilized H7-mAb. These steps were repeated three times and the sensorgram is shown in Figure S-4. The SPR signals for the specific binding were 23.7 mV, 24.0 mV, and 23.1 mV, respectively, with a coefficient of variation of 1.93%.

Detection of influenza H7N9 virus using qRT-PCR, RIDT and ELISA assays.

Next, we evaluated the detection sensitivity for the H7N9 virus using the homemade target-captured ELISA. The qRT-PCR quantified H7N9 virus culture supernatant ($2.3x10^5$/mL) under different dilutions using PBS or mimic solution. The results presented in Figure 5A using homemade target-captured ELISA demonstrate that the detectable concentration of the H7N9 virus in PBS and in mimic solution were 2.3×10^3 copies/mL (100-fold dilution) and 2.3×10^4 copies/mL (10-fold dilution), respectively.

Serial 10-fold dilutions of H7N9-RG virus culture supernatant (23 copies/mL to 2.3×10^5 copies/mL) were used to execute the detection sensitivity test of qRT-PCR. Figure 5B shows the relationship between the Ct value and the concentration of the H7N9-RG virus over the range of 23 to 2.3×10^5 copies/mL (1 to 10^4-fold dilution) by using qRT-PCR. Results indicated that the limit of detection of H7N9-RG virus in PBS and mimic diluent was 105 and 498 copies/mL, respectively. Further, the 90% confidence interval for limit of detection (LOD90) of qRT-PCR was measured using probit analysis. Results indicated that LOD90 of H7N9-RG virus detection was 251 and 724 copies/mL in PBS and mimic solution, respectively (Figure 5C&D).

Further, the RIDT was conducted. At the moment, the commercial RIDT is used for first-line clinical diagnosis of influenza A and B virus infection. Thus, we select this approach to detect the H7N9-RG virus isolate diluted in PBS and in mimic solution. The results showed that the sensitivity for detecting the H7N9

viruses was 2.3×10^4 copies/mL in either PBS buffer or mimic solution (Figure 5E).

Comparison of the diagnostic parameters of the IM-SPR biosensor with other assays for H7N9 virus detection.

Further, the diagnostic parameters of the IM-SPR biosensor were compared with homemade target-captured ELISA, commercial RIDT and WHO recommended qRT-PCR. The results indicate that the IM-SPR biosensor showed higher sensitive detection capabilities (144 copies/mL) than RIDT (2.3×10^4 copies/mL) and homemade target-captured ELISA (2.3×10^3 copies/mL) as well as qRT-PCR (105 copies/mL) when H7N9-RG viruses were in PBS (Table 1). Especially, we noted that the IM-SPR biosensor displayed slightly higher detection capabilities than qRT-PCR when the H7N9 viruses were in mimic solutions (Table 1). We think that the mimic samples could contain certain proteases or RNases that influence and reduce the H7N9 virus stabilities through the digestion of viral protein or viral RNA. The largest benefit of the IM-SPR biosensor is its time-saving nature, because it took under 10 minutes as well as a smaller amount of sample to complete whole diagnosis (Table 1). Reports have indicated that earlier detection of H7N9 virus suspected patients is the most effective measure to control and prevent further transmission of the virus.[55] A significant reduction in the mortality rate was observed when early diagnosis was accomplished on patients who were infected with avian influenza A H7N9 virus.[55,56] Taken together, we believed that the IM-SPR biosensor has a high potential to be an efficient diagnostic platform for avian influenza A H7N9 virus detection and a tool for virus control and monitoring.

CONCLUSIONS

At present, the avian influenza A H7N9 virus is still circulating between birds and humans. However, there are just a few commercial diagnostic kits or platforms that are available for diagnosis of clinically

H7N9 suspected patient. Besides, most advanced laboratory methods for detection of influenza viruses are technically demanding and are not suitable for on-site use in field investigations. Thus, in this study, we developed a simple platform to rapidly and sensitively detect the H7N9 virus using an IM-SPR biosensor integrated with a new generated monoclonal antibody. Our work reveals that the biosensing platform is capable of time-efficient (~10 min) detection of H7N9-RG virus, which shows similar surface properties to avian influenza A H7N9 virus. Morover, the detection limit at 402 copies/mL in mimic solution which is much better than homemade target-captured ELISA, commercial RIDT, and even qRT-PCR. We therefore conclude that the biosensing platform would be suitable for rapid diagnosis of avian influenza A H7N9 virus infection.

ASSOCIATED CONTENT

Supporting Information

Specificity of anti-HA7 polyclonal antibody, generation of H7N9 reverse-genetics (RG) influenza virus, generation of anti-HA7 monoclonal antibody (mAb), the specificities of HA-7 mAb and polyclonal antibodies, regeneration sensorgram of SPR chip surface, two type of IM-SPR, Epitope mapping, determination, and recognition.

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Notes: The authors declare no competing financial interest.

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FIGURES:

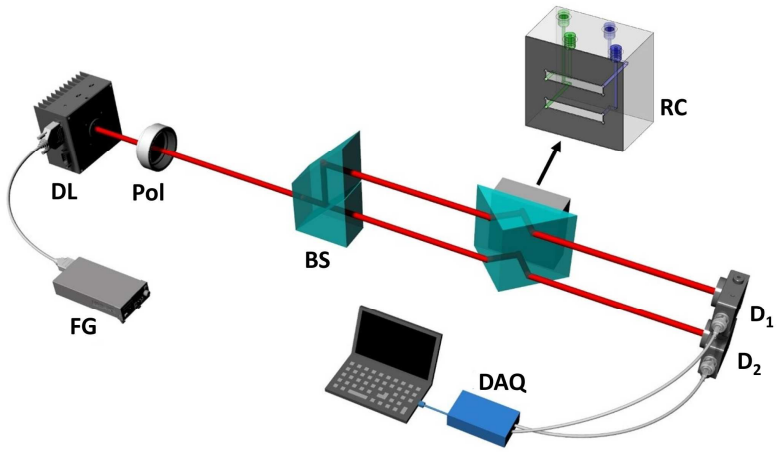


Figure 1. Experimental setup of the IM-SPR biosensor. FG, function generator; DL, diode laser; Pol, polarizer; BS, beam splitter; RC, reaction chamber; D1 and D2, photo detector; DAQ, data acquisition.

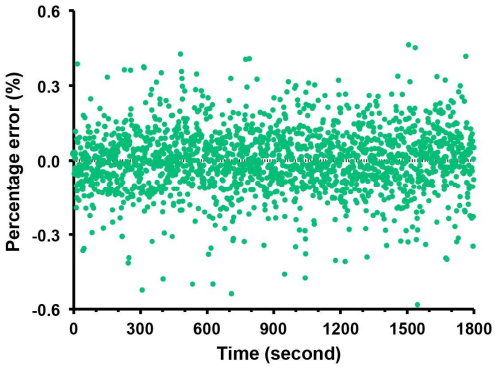


Figure 2. Stability of the IM-SPR biosensor by testing tridistilled water for the duration of 30 minutes.

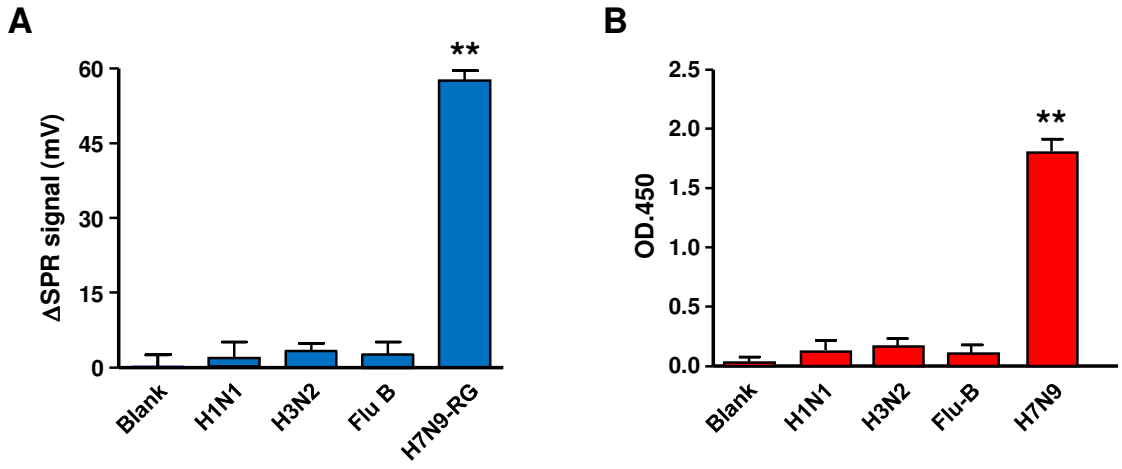


Figure 3. Evaluation of the specificity of the IM-SPR biosensor and homemade target-captured ELISA. Clinical human influenza isolates that include influenza H1N1, H3N2 and influenza B viruses as well as the H7N9 viruses were detected by (A) IM-SPR biosensor and (B) homemade target-captured ELISA. The results are from one representative experiment out of three independent experiments. "**" indicate $p<0.01$. "△SPR signal " is defined as to subtract the SPR signal of blank group from that of the other groups.

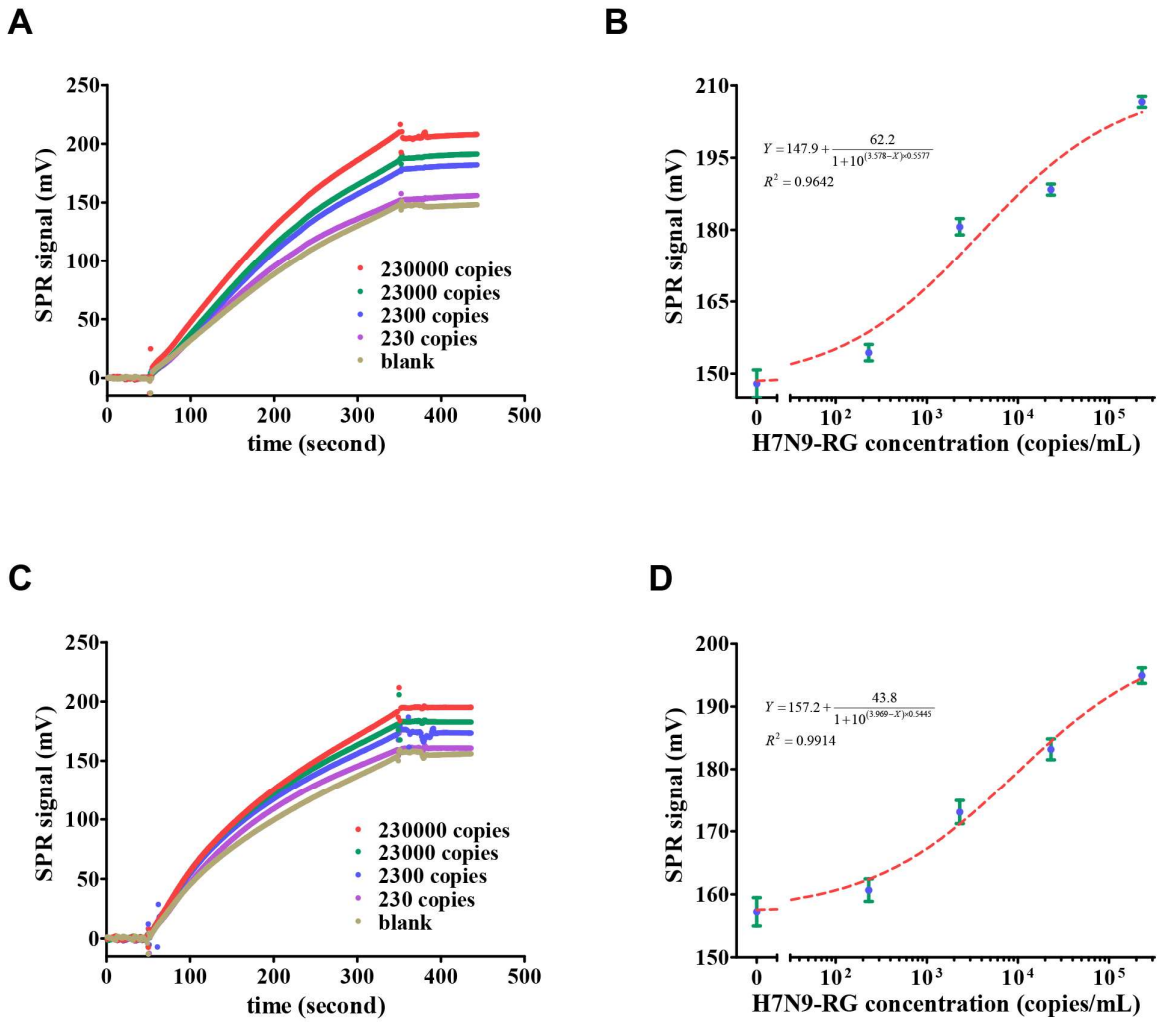


Figure 4. Detection of the H7N9 virus using the IM-SPR biosensor. Binding processes of H7-mAb interaction with different concentrations of the H7N9 virus exists in PBS and mimic diluent (A and C) over the range 2.3×10^2 - 2.3×10^5 copies/mL. Correlation between the SPR signal and the concentration of the H7N9 virus existing in PBS and mimic diluent (B and D) over the range 2.3×10^2 - 2.3×10^5 copies/mL. The error bar indicates 1 SD in each measurement. The results are from one representative experiment out of three independent experiments.

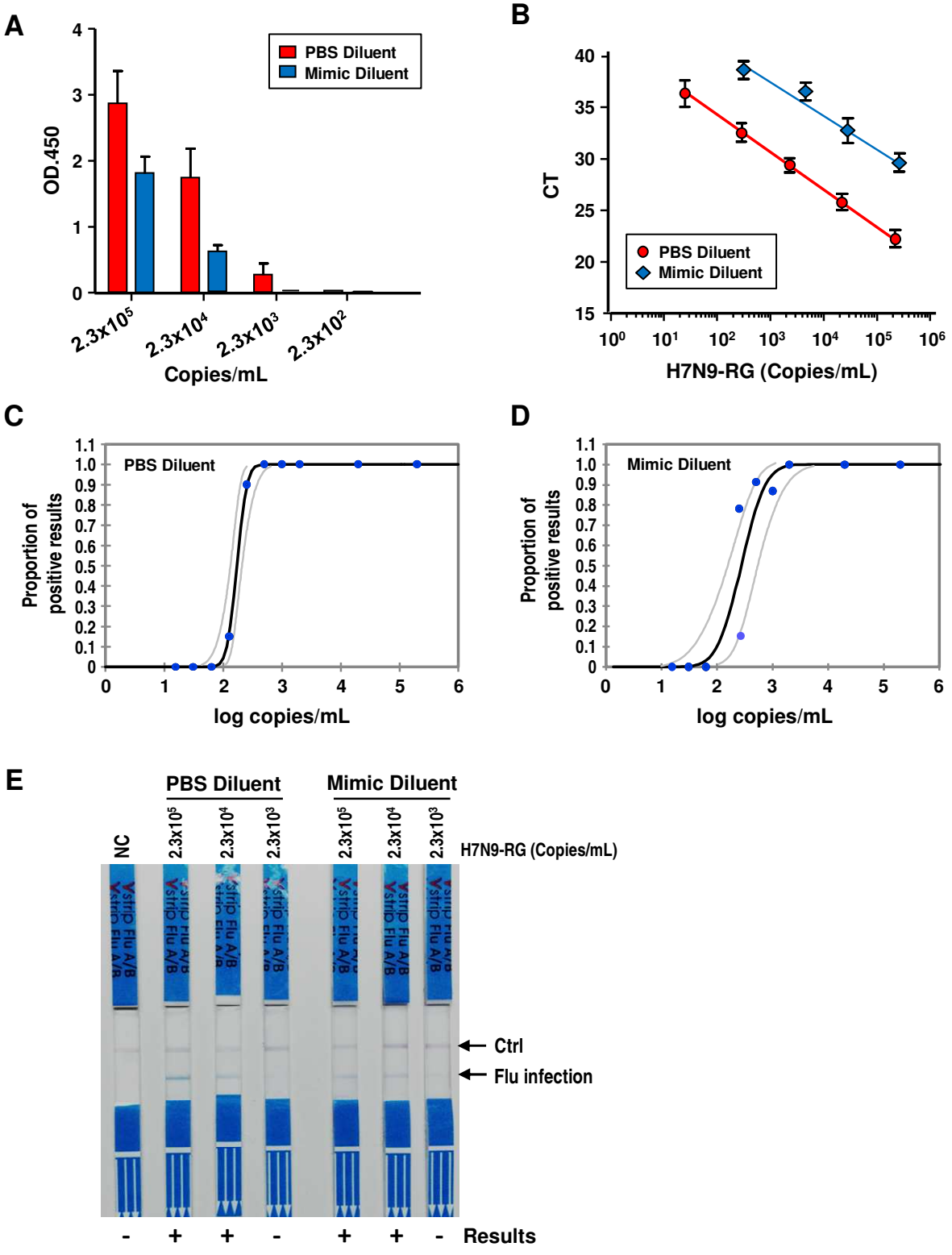


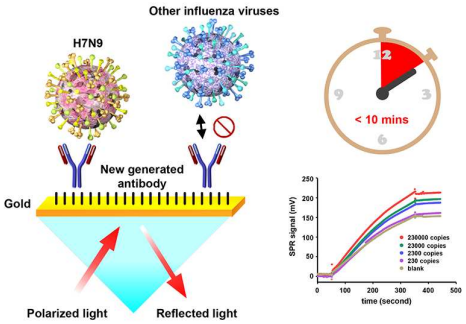
Figure 5. Detection of the H7N9 virus using qRT-PCR, homemade target-captured ELISA and RIDT. (A) Detection of the H7N9 virus (2.3×10^5 copies/mL) serial dilution in PBS and mimic solution (nasal mucosa

from Flu-like illness donors as the diluent) with target-captured ELISA was measured. (B) Detection of the serial dilution of the H7N9 virus (2.3×10^5 copies/mL) in PBS and mimic solution with qRT-PCR was measured. (C) Probit analysis to determine 90% confidence interval of limit of detection(LOD90) of H7N9 virus in PBS diluent by RT-qPCR was measured. (D) Probit analysis to determine 90% confidence interval of limit of detection(LOD90) of H7N9 virus in mimic diluent by RT-qPCR was measured. (E) Different dilutions of the H7N9 virus using PBS and mimic diluent were subjected to RIDT detection. The results are from one representative experiment out of three independent experiments.

Table 1. Method comparison for H7N9 detection.

Detection Assay	qRT-PCR	target-captured ELISA	RIDT	IM-SPR
Time cost	**2.5 h**	**5 h**	**15 mins**	**< 10 mins**
Detection target	**Nucleic acid**	**Hemagglutinin**	**Nucleoprotein**	**Hemagglutinin**
Limit of detection (in buffer)	**105 (copies/mL)**	**$2.3x10^3$ (copies/mL)**	**$2.3x10^4$ (copies/mL)**	**144 (copies/mL)**
Limit of detection (in mimic solution)	**498 (copies/mL)**	**$2.3x10^4$ (copies/mL)**	**$2.3x10^4$ (copies/mL)**	**402 (copies/mL)**

for TOC only


Other influenza viruses
H7N9
New generated antibody
Gold
Polarized light
Reflected light
< 10 mins
SPR signal (mV)
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