A biochemiluminescent assay for rapid diagnosis of influenza

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Abstract
A biochemiluminescent assay of influenza diagnosis is presented. The assay diagnoses influenza based on detection of the influenza viral neuraminidase activity. An instrument designed for the assay is also reported. This assay solves the problem that current influenza virus diagnosis assays are susceptible to virus mutation. A luciferase-based complex is synthesized as biochemiluminescent substrate. The substrate is cleaved to free luciferin with presence of influenza neuraminidase in specimen. Luciferase is oxidized to oxyluciferin with luciferin as catalyzer resulting in luminescence, which is proportional to the neuraminidase activity and measured by instrument. The instrument uses a photomultiplier tube as sensor, with 24 test channels. Fine optical arrangements enable the instrument with high sensitivity and accuracy. A total of 389 clinical specimens were collected to evaluate the performance of the assay in clinical settings. This assay had a sensitivity and specificity of 95.92% (95% confidence interval 91.38–98.12%) and 97.93% (95% confidence interval 95.26–99.11%), respectively, compared to the colloidal gold assay. As a biochemiluminescence assay, this assay is advantageous in sensitivity and specificity. It does not require any washing or separation steps, which makes the instrument simple in design and easy to operate or maintenance. The assay is suitable for the rapid diagnosis of influenza virus in point-of-care settings.

Keywords  Biochemiluminescence · Influenza · Luciferin · Luciferase · Neuraminidase · Instrument

Introduction
Influenza is caused by influenza virus infection. Influenza A, B, and C are 3 types of viruses responsible for illness. Infection produced by the influenza viruses A and B range from mild respiratory illness like chills, headache, persistent cough, fatigue, to fatal pneumonia, myocarditis and heart failure [1].

The benefit of influenza treatment is greatest when antiviral therapy is started within 24 h of symptom onset, so a reliable diagnostic method enabled rapid detection of influenza is essential [2, 3]. At present, viral culture, antigen detection and polymerase chain reaction (PCR) are the main conventional methods for influenza diagnosis. However, viral culture and PCR is tedious and time-consuming, and antigen detection has less sensitivity [4]. A comparison of several antigen detection assays to PCR revealed that sensitivities of immunoassays was only 60–80% for seasonal influenza virus stains [5–11].

Neuraminidase (NA) is one of the essential enzymes of influenza virus, which help progeny influenza virus cleave sialic acid and release from the host cell [2]. Therefore, NA plays an important role in virus replication and transmission [12, 13]. All type A and B influenza viruses carry NA, so NA can be an ideal marker for diagnosing influenza [14, 15]. Literature studies have shown that N-acetylneuraminic acid (NeuAc) can be recognized by NA. In addition, 4,7-di-Omethyl-NeuAc has been revealed as a specific receptor of NA for influenza A and B with excellent specificity. In this paper, the assay, homogeneous biochemiluminescent assay (HBA) for rapid diagnosis of influenza by detecting viral NA activity is introduced [16, 17].

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Material and methods

Substrate synthesis

In this assay, the team used a luciferase-based biochemiluminescent substrate, luciferin-4,7-di-O-methyl-neuraminic acid [18]. In the presence of influenza virus in a reaction, the substrate is cleaved to free luciferin, which becomes an active substrate of firefly luciferase. In the presence of firefly luciferase, the free luciferase is oxidized to oxyluciferin, resulting in a stable luminescent signal that can be detected by instruments [19, 20].

The process of substrate synthesis has been reported previously [16]. The synthesis of luciferin-4,7-di-O-methyl-neuraminic acid is based on NeuAc and D-luciferin. The 4,7-di-O-methyl-2,8,9-tri-O-acetyl NeuAc methyl ester was achieved with optimized reaction conditions. Then the 2-chloro-4,7-di-O-methyl-8,9-di-acetyl NeuAc methyl ester was prepared by chlorination reaction. Finally, the D-luciferin had been linked to 2-chloro-4,7-di-O-methyl-8,9-di-acetyl NeuAc methyl ester by Williamson ether synthesis, followed by removing the protection groups. The product was purified by high performance liquid chromatography (HPLC) with its yield above 98%.

The test kit of the HBA contains a sample buffer and a master mixture including the NA substrate, luciferin-N-acetyl-neuraminic acid, and luciferase. Innovative reagent production technology makes the reagent as a freeze-dried bead, this allows for room temperature shipment and a longer shelf life.

Clinical samples preparation and detection

In the clinical study, a total of 389 clinical samples were collected between 26 January 2019 to 07 April 2019 at ZhuJiang Hospital of Southern Medical University, Guangzhou, China. Nasopharyngeal swabs were used to collect samples. The colloidal gold reagents were from Guangzhou Wondfo Biotech Co., Ltd., (registration number: 20153400613, production batch number: W05980202). PCR reagents and instrument were from Shanghai ZJ Bio-Tech Co., Ltd. (registration number: 20173404656, production batch number: P 20190601).

To perform a clinical sample testing, the swab was placed into a sample buffer for elution. The team added 250 uL sample buffer to the reagent tube to dissolve the reagent beads, and then incubated for 15 min in the instrument. The instrument measures the luminescent signal and prints the results automatically. For samples that were not in the sample buffer, the team diluted the samples 1:1 with the sample buffer and then tested as described above.

Data analyses

Sensitivity and specificity were used to determine the diagnostic yield among different assays. 95% confidence intervals were calculated according to the Wilson score method. Kappa was used to evaluate the consistency of the assays.

Design of instrument

The research team designed a compact and reliable instrument named Luminometer Helios 2000 to measure the luminescent signal emitted by the reaction mixture. The instrument consists of a reaction vessel, a heating apparatus, a measuring module, and a reaction vessel moving mechanism (Fig. 1). The reaction vessel has 24 channels for reagents incubation, reaction and luminescence (Fig. 1-(1)). The moving mechanism moves the vessel and brings particular channels to detection position (Fig. 1-(2)). A photomultiplier tube (PMT) is used to capture the luminescent light (Fig. 1-(3)). Optical fiber for transferring the luminescence light to PMT is located below the detection position and forms a confined space shielded from external light with reaction vessel. A metal cover for PMT protects it from vibration, moisture and external bright light, which may cause permanent damages.

The instrument has 24 channels for incubation and testing, so it is capable of carrying out measurement of luminescence quickly and effectively. The optical signals measured by the instrument indicated the quantification of influenza
virus. The results of the instrument are read as Relative Light Unit (RLU).

**Results**

In order to calculate the appropriate cut-off value, 460 clinical specimens were tested. The specimens were cultured for virus as well. The true positive rate (TPR) and false positive rate (FPR) were calculated under different cut-off values. The receiver operating characteristic (ROC) curve is shown in Fig. 2. Since the instrument focuses on clinical screening, in this study, 220 K RLU was used as the cut-off value. The TPR and FPR were 66.04% and 7.86%, respectively.

To evaluate linearity and linear range, samples of influenza virus strains (A/CA/07/2009; wild type) with concentrations of 2.83–5.06 log TCID$_{50}$/ml were tested. The correlation coefficient ($R^2$) was 0.9967 (95% confidence interval 0.9690–1.0).

To evaluate the variability, two positive samples, and one negative sample, were tested 4 times a day for 12 consecutive days. The results (Table 1) indicated the HBA could make correct diagnoses of these samples.

To evaluate the limit of detection (LOD), 5 samples of influenza virus strains A/CA/07/2009 and A/NC/37/2009 were tested 20 times. The concentrations of the samples were close to the detection limit. The results are shown in Table 2. The diagnostic accuracy of positive influenza samples should be at least 95%, so the detection limits of A/CA/07/2009 and A/NC/37/2009 were 995 TCID$_{50}$/mL and 953 TCID$_{50}$/mL, respectively.

To evaluate the performance of the assay in clinical settings, a total of 389 clinical samples were collected. These studies were approved by the medical ethics committee at ZhuJiang Hospital of Southern Medical University and conducted according to the requirements of the China Food and Drug Administration (CFDA). Nasopharyngeal swabs were used to collect samples, and the test results were recorded and saved according to relevant guidelines and regulations.

Of 389 samples, 147 were diagnosed positive and 242 diagnosed negative by colloidal gold assay. 146 were diagnosed positive and 243 negative by HBA. The results of 11 samples were inconsistent under these two assays. After retesting with PCR, six samples were consistent with the results of HBA. Five samples were consistent with the colloidal gold’s. The results are presented in Table 3. Overall, the HBA had a sensitivity and specificity of 95.92% (95% confidence interval 91.38–98.12%) and 97.93% (95% confidence interval 95.26–99.11%), respectively. The Kappa value was 0.94 (95% confidence interval 0.905–0.975).

**Table 1** Repeatability of different samples

| Positive Sample 1 | Positive Sample 2 | Negative Sample |
|-------------------|-------------------|-----------------|
| **Mean RLU ($n = 48$)** | 373 | 374 | 101 |
| SD | 82 | 228 | 34 |
| % CV | 21.91 | 31.49 | 33.59 |
| % Positive | 100 | 100 | 0 |

**Table 2** LOD at various concentrations of influenza virus strains

| Concentrations (TCID$_{50}$/mL) | A/CA/07/2009 | A/NC/39/2009 |
|-------------------------------|-------------|--------------|
| Mean (S/CO)                   | 0.98        | 1.15         |
| % CV (S/CO)                  | 13.81       | 8.36         |
| % Positive                   | 45%         | 100%         |

**Table 3** Test result of clinical study

| Results of the HBA | Positive | Negative |
|--------------------|----------|----------|
| Total              | 147      | 242      |

**Fig. 2** ROC curve of 460 clinical specimens
Discussion and conclusion

Influenza viruses are the most prevalent pathogens that cause acute respiratory tract infections. A rapid and sensitive diagnosis assay is essential to accurately elucidate any outbreak due to an influenza virus. High mutation rates and large numbers of variants make the diagnosis of influenza challenging [21]. Based on the existing research, NA is a conserved enzyme of the influenza virus, which is not susceptible to virus mutation, so it is an ideal influenza diagnostic marker [22].

As a biochemiluminescence assay, HBA is advantageous in sensitivity and specificity. In addition, the catalyst and substrate of HBA does not exist in the human specimens, and the analyte is captured in a liquid phase without using micro-particles, so HBA does not require any washing or separation steps that have obvious influence on measurement [23]. The HBA performs on simple and small instruments with a detection time less than 15 min, and clinical samples can be tested in batches because the instrument is designed with 24 channels. These advantages contribute to more timely results and higher laboratory workflow efficiency.

The HBA has advantages, but it also has some limitations such as the inability to identify subtypes of influenza viruses. For example, surveillance laboratories are more demanding and require comprehensive analysis of the virus including knowledge of the virus’s genetic makeup and antigenic type/subtype information, which are unachievable by HBA. Specifically, influenza C does not carry NA, so the HBA cannot be used for influenza C diagnosis.

The research team is applying for a registration certificate through the China Food and Drug Administration (CFDA) for the instrument and reagent. Future work is needed to expand the detection of viral pathogens by such rapid methods.

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Author contributions

XL involved in methodology, investigation, analysis, writing. JG participated in supervision, resources, review and editing, project administration.

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Data availability

All the data released to this work are available from the corresponding author.

Declarations

Conflict of interest

The authors did not receive support from any organization for the submitted work.
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