A Biochemiluminescent Assay for Rapid Diagnosis of Influenza

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Research Article

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Abstract

Background: 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.

Methods: 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 (PMT) as sensor, with 24 test channels. Fine optical arrangements enable the instrument with high sensitive and accuracy.

Results: 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.

Conclusion: As a biochemiluminscence assay, this assay is advantageous in sensitivity and specificify. It does not require any washing and 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 POC settings.

1. 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 ranges 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 hours 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 main conventional methods for influenza diagnosis. However, viral culture and PCR is tedious and time-consuming, and antigen detection is less sensitivity [4]. A comparison of several antigen detection assays to PCR revealed that sensitivities of immunoassays was only 60 to 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 diagnosis of 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 specific

receptor of NA for influenza A and B with excellent specificity. In this paper, we introduce an assay, the homogeneous biochemiluminescent assay (HBA), for rapid diagnosis of influenza by detecting viral NA activity [16,17].

2. Material And Methods

2.1 Substrate synthesis

In this assay, we use 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 chlornation reaction. Finally, the D-luciferin had been linked to 2-chloro-4,7-di-O-methyl-8,9-di-O-acetyl NeuAc methyl ester by Williamson ether synthesis, followed by removing the protection groups. The product purified by HPLC with its yield above 98%.

The test kit of the HBA contains a sample buffer and a master mixture including the NA substrate, lucifery1N-acteyl-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.

2.2 Clinical samples preparation and detection

In the clinical study, a total of 389 clinical samples were collected between 26 January 2019 to 7 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, place the swab into sample buffer for elution. Add 250uL sample buffer to reagent tube to dissolve the reagent beads, and then incubate for 15minutes in the instrument. The instrument will measure the luminescent signal and prints the results automatically. For samples that were not in sample buffer, dilute the samples 1:1 with sample buffer and then test as described above.

2.3 Data Analyses

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

the consistency of the assays.

2.4 Design of instrument

Here we designed a compact and reliable instrument to measure 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 (**Figure 1**). The reaction vessel has 24 channels for reagents incubation, reaction and luminescence. The moving mechanism moves the vessel and bring particular channel to detection position. A photomultiplier tube (PMT) is used to capture the luminescent light. 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 permanently damages.

The instrument 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).

3. Results

In order to calculate the appropriate cut-off value, 460 clinical specimens were tested, and 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 ROC curve is shown in **Figure 2**. Since the instrument focuses on clinical screening, in this study, 220K RLU was used as the cut-off value. The TPR, FPR were 66.04%, 7.86%, respectively.

To evaluate linearity and linear range, we tested samples of influenza virus strains (A/CA/07/2009; wild type) with concentrations of 2.83 to 5.06 log $TCID_{50}$ /ml. 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 that HBA could make correct diagnoses of these samples.

To evaluate the limit of detection (LOD), we tested 5 samples of influenza virus strains A/CA/07/2009 and A/NC/37/2009 for 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). We used nasopharyngeal swabs 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 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, 6 samples were consistent with the results of HBA, and 5 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)

4. 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 elucidate accurately any outbreak due to influenza virus. High mutation rate and large number of variants make the diagnosis of influenza facing many challenges [21]. Based on the existing research NA is a conserved enzyme of influenza virus, which is not susceptible to virus mutation, so it is an ideal influenza diagnostic marker [22].

As a biochemiluminscence assay, HBA is advantageous in sensitivity and specificity. In addition, the catalyzer 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 requires any washing and separation steps that have obvious influence on measurement [23]. The HBA performs on simple and small instruments with a detection time less than 15 minutes, 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 the advantages, but also it has some limitations, such as unable to identify subtypes of influenza viruses. For example, surveillance laboratories are more demanding and require comprehensive analysis of the virus includes knowledge of the virus's genetic makeup and antigenic type/subtype information, which are unachievable by HBA. Especially, influenza C does not carry NA, so the HBA cannot be used for influenza C diagnosis.

At present, the instrument and reagent is applying for registration certificate in China Food and Drug Administration (CFDA). Future work is needed to expand the detection of viral pathogens by such rapid methods.

Declarations

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Conflicts of interest

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

Availableility of data and material

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

Code availability

Not applicable.

Ethics approval

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the medical ethics committee at ZhuJiang Hospital of Southern Medical University.

Consent for publication

Patients signed informed consent regarding publishing their data.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Informed consent was obtained from legal guardians.

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Author Contributions

Xuexiang Lin: methodology, investigation, analysis, writing. Jia Gu: supervision, resources, review and editing, project administration.

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Tables

Table 1 Repeatability of different samples

	Positive Sample 1			Positive Sample 2		Negative Sample
	1	2	3	1	2	1
Mean RLU(n = 48)	373	1009	3933	724	3357	101
SD	82	198	1003	228	352	34
%CV	21.91	19.68	25.50	31.49	10.48	33.59
% Positive	100	100	100	100	100	0

Table 2 LOD at various concentrations of influenza virus strains

	A/CA/07/2009			A/NC/39/2009	
	1	2	3	1	2
Concentrations (TCID50/mL)	663	995	1,326	953	1,271
Mean(S/CO)	0.98	1.58	1.96	1.15	1.87
% CV(S/CO)	13.81	7.98	6.59	8.36	3.45
% Positive	45%	100%	100%	100%	100%

Table 3 The test result of clinical study

		Results of colloidal gold		total
		Positive	Negative	
Results of the HBA	Positive	141	5	146
	Negative	6	237	243
Total		147	242	389

Figures

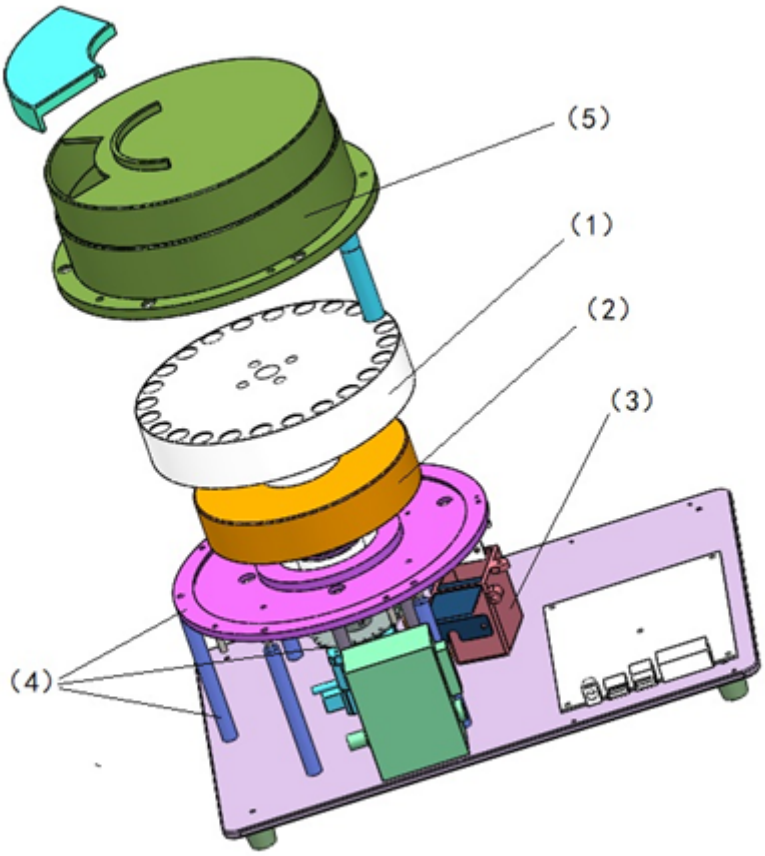

Figure 1

Instrument structure diagram (1Reaction vessel; (2) heating apparatus; (3) measuring module; (4) moving apparatus; (5) light-shielding cover

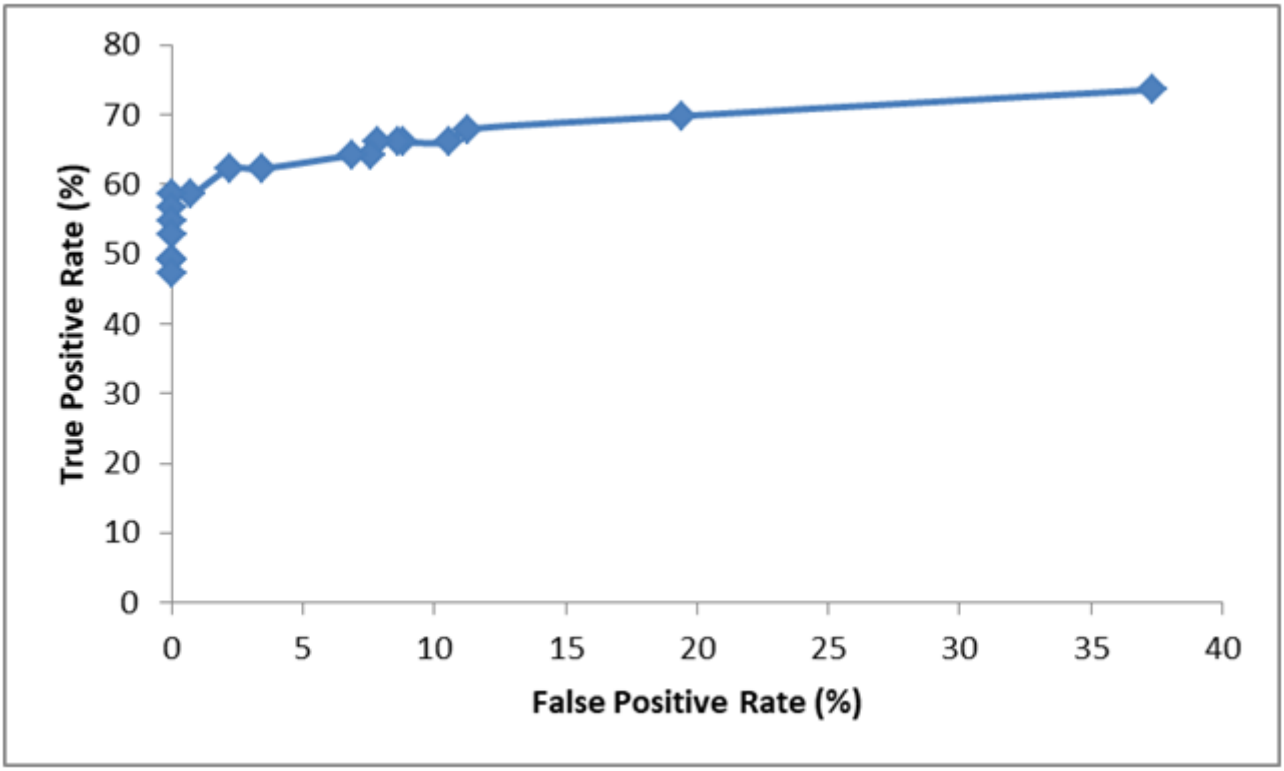

Figure 2

The ROC curve of 460 clinical specimens