On-site Screening of Avian Influenza Virus (AIV)

by Polystyrene Microfluidic Chip Immunoassay

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ABSTRACT

In this study, AIV (AIV) Ag of A type with viral nucleoprotein (NP), which covers all AIV subtypes, including high-pathogenic AIV, was demonstrated by the novel format of polystyrene microfluidic chip ELISA. In micro-channels of chip instead of conventional 96-well microtiter plates, a double-antibody sandwich ELISA (DAS-ELISA) was developed, in which rabbit Ab, mouse nuclear protein (NP) mAb and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were used as coating, primary and secondary antibodies, respectively. Recombine Ag of AIV was prepared as positive control to ensure the on-site test safety. With TMB as substrate, AIV Ag could distinctly be judged by comparing colors of sample detection windows with that of the negative control in micro-channels, a preliminary result was obtained in accordance with conventional ELISA while total analytical time were within 1 h. The on-chip DAS-ELISA was well applicable to rapid and real-time screening of AIV. The sensitivity could be further improved by optimization experiments.

Key Words: AIV, DAS-ELISA, Microfluidic Chip, Immunoassay

INTRODUCTION

Avian Influenza Virus (AIV) is a rapidly spread erosolized bird disease and rapidly infects flocks or birds when outbreak occurs. Infection can range from asymptomatic to severe, depending on the virulence of the virus and the susceptibility of the avian host. Up to now, embryonating chicken eggs (ECE), hemagglutination-inhibition (HI) and neuraminidase- inhibition tests has been the common tests to identify each specific subtype of AIV in the clinical diagnosis although it is a labor-intensive and time-consuming procedure[1]. Although somewhat effective, most of the current methods or technologies are subject to low sensitivity and high amount-consuming issues, moreover, expensive microscopy equipments and necessary sophisticated imaging operation were not facile for those experiments under indoor conditions, and that has been the largest obstacle to their widespread uses, let alone on-spot performance to generate at a high throughput. To reduce the time required for target detection, a minimal amount of sample manipulation is essential. Therefore, a novel feasibility, more automatic, integrated and miniature, was to be obtained by combining the current dot ELISA with the microfluidic chip, demonstrating more competitive and attractive potential whether in parallel batching process on a single substrate, or on the aspects of increasing analytical throughputs. Special types of microfluidic and nanofluidic structures are applied in so-called micro total analysis systems (μ-TAS) [2] and lab-on-a-chip systems [3], in miniaturized drug delivery systems as well as in areas of tissue engineering [4]. These are predominantly passive microcomponents, e.g. simple microdepressions that are frequently applied in conventional microtiter plates as reservoir areas or miniaturized sample chambers, so-called wells. Miniaturized analysis systems are additionally equipped with capillary microchannel structures, mainly in the form of inlet or supply channels or as reaction or separation sections. Microchannels with integrated microcomponents may take over either mixing or filter functions. Via smallest pores, a precisely adjusted sample transfer into and from microfluidic systems can be achieved

[5].A typical lab-on-a-chip application would involve the integration of multiple sample preparation and analytical functionalities, such as mixing, reactions, extraction, separation and detection. The combination of microchip analytical power and antigen-antibody reaction specificity could overcome some of the drawbacks of conventional immunoassay and keep the potential development. A portable immuno-microchip system consisting of low-cost biocompatible-polystyrene chips, a mini-vacuum pump and a base unit for LED light source as a miniaturized optical readout was devised. The surfaces of the micro-channels were processed with plasma to be more hydrophilic and were characterized by kinetic coating with a CCD scanning method

2 EXPERIMENTAL

2.1 Chemicals and Reagents

The preparation techniques on antigen, McAb and PcAb of AIV were supplied by the laboratory of Pro. Wang in Yangzhou University. 3,3′,5,5′- Tetramethylbenzydine (TMB) was obtained from *Sigma* (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was supplied by Huamei Biochemicals (Beijing, China). The analytes were prepared in 10mM phosphate-buffered saline (PBS: 136 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4,1.5 mM KH_2PO_4, pH 7.2), respectively. All reagents were of analytical grade.

Instrumental. Microfluidic chip working system (integrated with one-time plastic chips, a mini vacuum pump and a mini-enzyme labelling apparatus, developed by CAIQ and Kionix Co.). Incucell series incubator shaker (Co., Germany) and Varian Cary 1E UV–vis spectrophotometer (Varian Instruments, USA) were used. The controlled ELISA experiment was carried out on GENios (TECAN Co., Austria).

2.2 On-chip measurement procedure

Each channel of the 8-channel microfuidic plastic chip was coated with 100μL AIV PcAb in 0.01mg/mL carbonate solution (pH 9.6) at room temperature when flowing from the channel surface by a mini vacuum pump. After washing in PBST for one time, each channel was blocked with 5% skimmed milk solution in 0.01mol/L PBS for two times at RT to prevent nonspecific adsorption of protein during subsequent steps. Then the channels were rinsed two times with PBST, and antibodies were immobilized on the surface using the same way as that mentioned above. Different concentration of antigen of AIV solution then flowed through the channels and competed with the MCAb of AIV washed again, 100μL of 1:2000 diluted HRP-labeled goat anti-rabbit IgG was added and combined with the AIV antibodies. After the same washing procedure, 5μL of phosphate buffer containing TMB and H_2O_2 was used as the substrate solution. The enzyme reaction was stooped with 3μL termination agent after 5min, then the absorbance value was read by the mini-DAD detector at 450nm. Enclosing multiple layers of substrate without compromising microstructures with high precision layer-to-layer alignment is a very challenge problem for polymeric material (Fig.1).

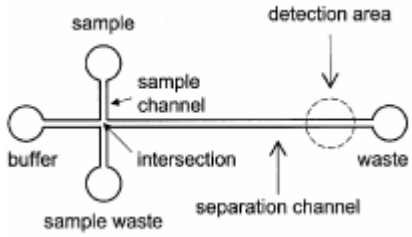

Fig.1 Setup and functioning principle of a lab-on-a-chip systems

3 RESULTS AND DISCUSSIONS

3.1 Characterization of microfludic chip on microchannels

The polystyrene (PS) chip, build-up with a substrate and a cover plate, was manufactured with precision injection molding and bonded by solvent. The microchannel structure, which was fabricated on the base plate, was designed with straight and flexure structure. Cleaning and hydrophilization of the channels were performed with oxygen plasma for 5min so as to do protein adsorption easier. Details about the characterization of the microfluidic immunoassay chip channels with rapid 3D were reported previously, and the scan results of the substrate shown that the depth of the eight microchannels

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was about 100μL, which meet the specification of our design. In order to explore the microdynamics of the channel, analysis of kinetic coating of proteins onto microchannels of PS chip has been studied, which indicated that the reaction on the channel surface was different from that of the ELISA plate

3.2 Rapid qualitative determination of AIV with immuno-chip

The most common format to perform a complete-assay for small molecular measurement is enzyme –linked immunosorbent assay (ELISA), which is conducted in plastic microtiter plate. The miniaturized chip system we proposed based on solution flowing in the channels by means of pressure. Specific binding results of the target analytes and the antibodies with their increase of specific surface area, which is a great benefit to the immunoreaction. The adsorption and desorption of protein molecular on the channel surface is different from that of ELISA plate well, which lead to the acceleration of reaction speed and the reduction of reaction time. For example, the plate was coated overnight at 4℃ and blocked for 2h at 37℃ in ELISA process, and the coating solution flowed through the channel only need one minute and blocking for 2 minutes in chip system. All kinds of time-consuming incubation procedure are being leaved out in experiment operating. The blank value represents the signal generated from substrate in the absence of target AIV. The stop solution color of blank was deeper than that of analytes, and the color was close to that of blank when the concentration of target was more lower due to the sandwich immunoreaction mechanism. Within a whole assay time of 30min only, AIV could be qualitative measurement by visual observation, while ELISA needs about one day to obtain the similar results.In order to achieve a good sensitivity and a low detection limit, the concentration of coating antigen, polyclonal antibodies and HRP-conjugated goat anti-rabbit IgG have to be optimized carefully. The target solution was introduced into the sampling hole and was pushed forward in the channels by where three tiny polystyrene valves open and close alternately.

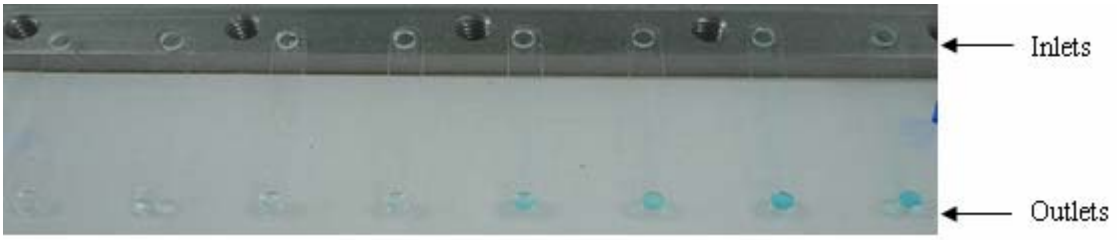

Fig.2 The color comparison of reaction by naked eyes detection limit close to 0.125HAU could be visualized rapidly.

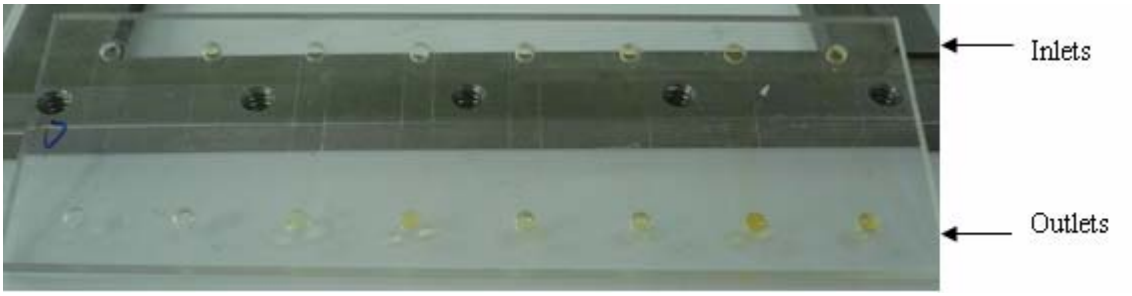

Fig.3 The determination limit of AIV with microchip
(from left to right, the color of first two channels in outlets were the blank sample, other channel contained 0.063HAU,0.125HAU, 0.25HAU, 0.50HAU, 1.00HAU, 2.00 HAU, 4.00HAU of AIV antigen, individually).

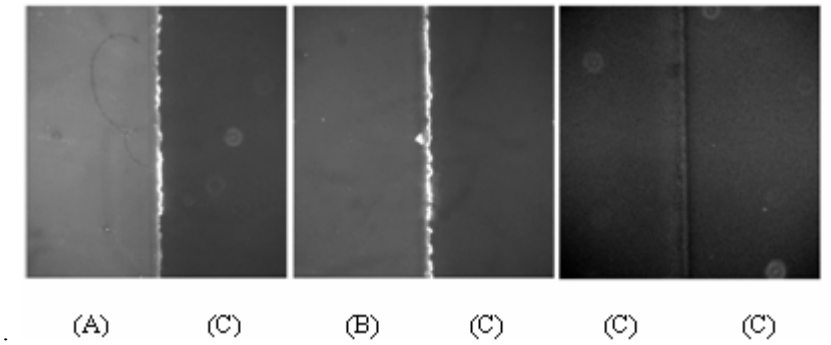

Fig. 4 Images of micro-channels coated with Proteins
A. Coated with FL-BSA B. Coated with FL-OVA C. Blank

4. CONCLUSION

The portable immuno-microchip system designed for rapid and sensitive detection was suitable for on-site application. The determination of AIV antigen and its analogs was successively performed using this system. The developed instrument has been designed and further study will focus on high-throughput measurement, which not only be fit for the qualitative and quantitative determination of small molecular, but also macro-molecular, such as protein, virus and peptide. This system has great potential for simultaneous measurement of structure analogs and batch process of complex sample, and could be applied extensively. Devices featuring a higher degree of integration and a future improved performance can be expected for the near future.

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