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Rapid identification of H5 avian influenza virus in chicken throat swab specimens using microfluidic real-time RT-PCR†

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Avian influenza virus H5 is a widespread virus among humans and animals which has caused fatally systemic diseases through poultry-to-person transmission in the past few years. Reverse transcription polymerase chain reaction (RT-PCR) has been proved to be an effective approach for the identification and detection of avian influenza viruses. However, conventional tube RT-PCR is slow and reagent consuming and cannot meet the need for rapid and low cost detection of pathogenic bacteria and viruses. Microfluidic PCR is a burgeoning field among the techniques based on molecular analysis. In this paper, we reported a microfluidic PCR system that integrated RT-PCR and real time fluorescence detection for rapid identification of avian influenza virus H5. This microfluidic device mainly consisted of a thermal controlling unit providing actuation for the temperature cycling needed for amplification, an optical inspection system for online recording fluorescence and a microfluidic chip fabricated using polydimethylsiloxane (PDMS). In this study, influenza virus H5 from clinical chicken throat swab specimens was rapidly detected using the RT-PCR microfluidic system, which was consistent with the results of embryonated egg culture. Compared with a large-scale device, the integrated microfluidic system presented here can perform rapid nucleic acid amplification and analysis, possibly making it a crucial platform for pathogenic bacterium and virus detection in the future.

Introduction

Highly pathogenic avian influenza virus is a major challenge to global public health. A novel avian-origin influenza A (H7N9) virus has caused more than 130 human infection cases in China, including 35 deaths and brought enormous economic losses to the poultry industry.[1] Most avian influenza pandemics are associated with type A which represents a widespread virus among human and animals.[2] Among the influenza virus A, H5 and H7 subtypes are considered as highly pathogenic and responsible for enormous economic losses to the poultry industry.[3–6] Since the first human disease caused by H5N1 was reported in Hong Kong in 1997, there have been numerous cases of human death caused by avian influenza virus according to the report. This threat makes it necessary to develop a rapid, specific, and sensitive method for detecting H5.[7]

One-step reverse transcription polymerase chain reaction (RT-PCR) based on molecular biology technology is considered as an effective approach for the rapid identification and detection of avian influenza viruses.[8] Compared with conventional diagnostic methods, such as virus inoculation in embryonated eggs and enzyme-linked immunosorbent assay (ELISA) based on antigen capture, one step RT-PCR just needs a pair of specific primers and a few hours. It allows a more rapid and sensitive identification of the avian influenza virus. Previous studies have employed RT-PCR for the detection of avian influenza viruses and subtypes.[9–11]

In recent years, with the development of microfluidic technology, it has found a number of applications in biological automation.[12] The ability to make arbitrary fluidic manipulations at the nanometer scale makes it a new tool in protein crystallization, gene expression, and cell culture.[13] It allows processes to be miniaturized and integrated, compared with what was previously done at larger scale in a separate operation, which enhanced the speed and efficiency.[14] Exploiting a microfluidic chip in nucleic acid amplification has significant advantages, including shorter assay time, lower reagent consumption, rapid heating/cooling and it is easily integrated.[15] These unique advantages have the potential to meet the needs

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of rapid and accurate detection of the influenza virus when the epidemic breaks out, such as H7N9 in China in 2013.

Although there are many publications on microfluidic PCR devices, only few researchers have integrated a compact fluorescence detection unit in their studies. The compact real-time PCR systems reported by Higgins[16] need tens of microliters of the reaction mixture to perform the PCR. Xiang's[17] on-chip real-time PCR used a miniature thermal cycler to detect *E. coli*; however, the real-time detection was done by using a desktop fluorescent microscope. Qiu's[18] PCR system utilized a commercial thermal cycler and a fluorescent reader, the cost was as expensive as that of a conventional real time PCR system.

In this paper, we provided an integrated microfluidic PCR system combined with online fluorescence detection for identification of influenza virus H5. The system allows rapid temperature cycling with high accuracy (*i.e.* the average heating and cooling rate of PCR reagents are 7.2 °C s^{-1} and 5 °C s^{-1} respectively) and the fluorescence detection module permits continuous monitoring of the PCR reaction in the microfluidic chip over thermal cycles, real-time image/signal data processing and gets instant results after PCR completion. In addition, the microfluidic PCR chip was made using PDMS for simple fabrication, low cost and disposability. Therefore, the system presented in this work has great potential as an inexpensive, rapid and accurate method for the detection of the virus. In order to verify its feasibility and sensitivity, the system is tested with clinical chicken throat swabs from influenza virus H5 infected chickens for 2, 3 and 4 days. We also set up an embryonated egg culture as a comparative study, which is the gold standard for avian influenza virus detection.

Materials and methods

Microfluidic PCR chip fabrication

The PCR microchip was designed using SolidWorks, and fabricated with PDMS by using a soft-lithography method (Fig. 1). PDMS is a very attractive polymeric material to manufacture microfluidic channels, for its favorable optical property, easy bonding and benefit for insulation. The design of microchip was patterned on a silicon wafer by contact photolithography with SU-8 photoresist (MicroChem, MA, USA) and the PDMS replicas were then fabricated by molding PDMS against the master. Specifically, Sylgard 184 (Dow Corning, MI, USA) was stirred thoroughly with curing agent in a weight ratio of 10 : 1. The mixture was poured onto the mold master and baked at 95 °C for 1.5 h. After cooling to room temperature, the PDMS sheets were peeled off from the mold and cut into an appropriate size according to the substrate glass (25 × 25 × 1 mm). The surfaces of the PDMS sheet and glass substrate were bound together after treating in oxygen plasma.

The PCR efficiency is limited by PDMS and the increase of the surface-to-volume ratio in a microscale environment. Therefore, to produce PCR-friendly inner surfaces, the PDMS surface was modified by graft polymer coating and a competing protein adjuvant bovine serum albumin, which were used to coat the micro-channel surface to decrease the surface adsorption.[19]

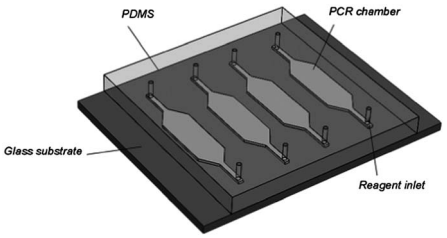

Fig. 1 Schematic illustration of the PCR microchip.

Design of the real-time PCR system

The integrated microfluidic real-time PCR system consisted mainly of a thermal cycling control unit and a fluorescent detection system. As known, there are three steps of denaturation, annealing and extension in every PCR amplification process. To achieve fast thermal cycling, the thermal cycling control unit should possess the essential functions of heating and cooling. Therefore, a sandwiched platform using a thermoelectric element (TEC; FPH1-19912-ACS1, Fulianjing Electronics, China) based on the principle of Peltier was configured as shown in Fig. 2. A 2 mm thick aluminum sheet was attached on the top of the TEC to attain spatial thermal homogeneity, and a slot was cut in the middle of the aluminum sheet to accommodate a Pt100 sensor (Hayashi Denko, Japan) that was used to acquire temperature information of the TEC surface. A fan-cooled heat sink was equipped under the thermoelectric element for air convection.

For real-time fluorescence detection, we developed a compact fluorescent detection system as shown in Fig. 3. Two blue LED (470 ± 20 nm, CREE) and a filter set (excitation wavelength: 470 ± 25 nm; emission wavelength: 525 ± 25 nm, Bd-optic. Ltd) were used for exciting the fluorescent dye. Fluorescence measurement was performed at the end of each thermal cycle and the fluorescent signal was collected by a CCD camera (ICX412, Sony) followed by real-time image/signal analysis using a built-in computer. The detection limit of the fluorescent reader is 10^{-10} mol of fluorescent molecules per liter sample.

Ethics statement

All experiments involving live H5 avian influenza viruses were performed in a BSL-3 facility in accordance to the relevant guidelines and regulations provided by the College of Veterinary Medicine at South China Agricultural University. The study protocol for the experiment was approved by the Ethics Committee of South China Agricultural University.

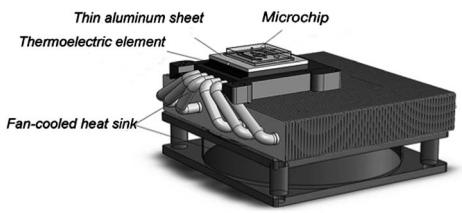

Fig. 2 A schematic depiction of the thermal cycling control unit.

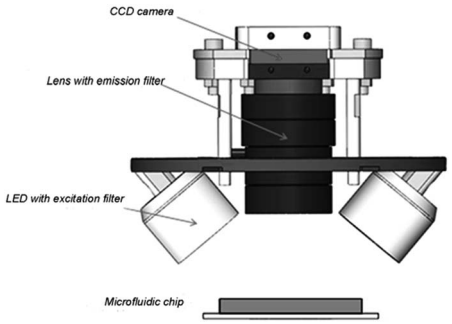

Fig. 3 A schematic illustration of the fluorescence detection system.

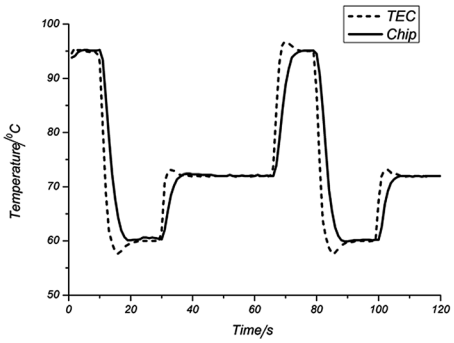

Fig. 4 The thermoelectric (TEC) temperature (dotted line) and the temperature inside of the chip (solid line) as a function of time during the PCR cycle.

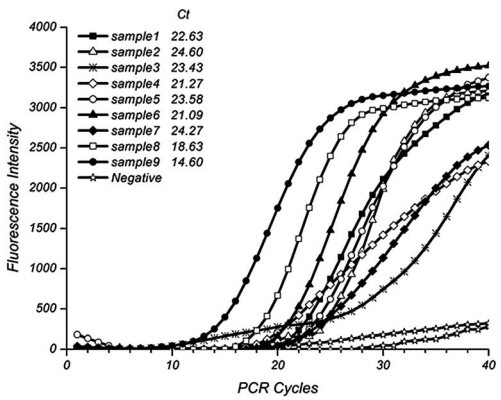

Fig. 5 One step microfluidic real time RT-PCR for detection of H5 from chicken throat swab samples on the designed system (samples 1–9 represented throat swabs from chickens infected with H5 for 2–4 days and the negative represented the samples from healthy chickens).

Sources of clinical specimens

H5N1 Avian influenza virus (A/Duck/Guangdong/383/2008) in this study was from the College of Veterinary Medicine at South China Agricultural University in China. We collected throat swabs from H5N1 infected chickens for 2, 3 and 4 days. Nine samples were stochastically chosen from inoculated chickens and two swabs were taken from healthy chickens as negative control.

RNA extraction and RT-PCR

RNA was extracted using TRIzol® Plus RNA purification kit (Invitrogen, Life Technologies, USA) according to the manufacturer's recommendations. Total RNA was extracted from 200 μl of supernatant and eluted in a final volume of 20 μl.

Reverse transcription and polymerase chain reaction (RT-PCR) were performed simultaneously in a single-step reaction using the influenza virus H5 subtype universal detection kit (Labx, YingJiuSi Sci-Tech, China). One-step RT-PCR was carried out on the integrated microfluidic system as follows: 15 min at 42 °C and 3 min at 95 °C for reverse transcription; pre-amplification was performed with 5 cycles including 10 s at 95 °C, 20 s at 55 °C and 30 s at 72 °C, and lastly with a subsequent 40 cycles at 95 °C for 5 s, 60 °C for 40 s.

Embryonated egg culture

Swab samples were washed using a phosphate buffer and extracted the supernatant after centrifugation to inoculate10 day-old embryonated specific-pathogen-free (SPF) chicken eggs (0.1 ml per egg) and propagated. After incubation at 37 °C for 48 hours, the allantoic fluid was harvested and tested by haemagglutination (HA) assay. These experiments were performed in an Animal Biosafety Level 3 (ABSL-3) laboratory at the College of Veterinary Medicine at South China Agricultural University in China.

Results and discussion

Rapid thermal cycling

Since the accuracy and sensitivity of the reactor's thermal control are very important for the efficiency and specificity of PCR, we developed a scheme to monitor the temperature variation inside the PCR chamber and TEC during PCR reaction. A temperature sensor was attached closely to the bottom inside the chip, which was running synchronously with TEC under custom-written procedure. Fig. 4 depicted the real time recording of the temperature inside the PCR chamber and TEC during heating and cooling of amplification. As the curves showed, both of them were synchronized at the point of

Table 1 The results of microfluidic RT-PCR and traditional embryonated egg culture

Method	Negative	Negative	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9
Embryonated culture	–	–	+	+	+	+	+	+	+	+	+
Microfluidic RT-PCR (Ct)	0	0	22.63	24.60	23.43	21.27	23.58	21.09	24.27	18.63	14.60

temperature variation during each cycle and the time delay between the inner temperature and the heated surface was less than 4 s. This result showed the advantage of the microfluidic chip compared to traditional tubular PCR because of the good thermal conductivity of the chip. The average cooling rate from 95 °C to 60 °C was 5 °C s^{-1} and the heating time from room temperature to 95 °C was 10 s at a heating rate of 7.2 °C s^{-1}. The significant heating and cooling rate resulted directly in a shortening of the reaction time and enhanced indirectly the efficiency of PCR amplification for enzyme activity increasing.

Conventional PCR reaction is carried out in a plastic tube through a metal plate to achieve the temperature for nucleic acid amplification. The heat transfer speed is extensively limited because of the thick metal plate and cone shape of the plastic tube, which makes it not suitable for fast thermal cycle. The appearance of microfluidic technology just meets the demands of rapid amplification on the equipment. Firstly, compared with conventional PCR, the microfluidic chip has a large surface area, making the transfer between the TEC and the liquid faster and obviously shortening the total reaction time. Secondly, the microfluidic chip consumes fewer reagents and samples, which significantly reduces the cost. In addition, the microfluidic chip has the flexibility to integrate other experimental steps on one chip, such as nucleic acid extraction and product analysis, which may enable molecular diagnostics at the point of care testing.

H5 detection by microfluidic real-time RT-PCR

In order to evaluation the performance of this microfluidic platform for RT-PCR, we explored it as a tool for the detection of avian influenza virus H5. In addition, as the clinical samples were so valuable we had optimized our system using synthetic plasmid many times before the H5 experiment. And the results proved that the optical detection system and the thermal cycling control were running well under the custom-written procedure, which gave us more confidence for the next experiment with clinical samples.

We stochastically chose nine throat swab samples from chicken infected with H5 for 2–4 days and two samples from healthy chickens. RNA was extracted and one step microfluidic real time RT-PCR was performed using our system. The lowest initial concentration of positive sample is 0.02 ng μl^{-1} (RNA). The RT-PCR kit used in this study was a qualitative detection one, so we identified the positive sample or not by the cycle threshold (Ct) value in the PCR amplification curve. According to the instructions given in the real-time PCR kit, if the Ct value was less than 30, the sample was determined to be indeed positive and if the Ct value ranged between 30 and 40, the sample needed to be re-tested. These criteria have also been widely accepted and successfully applied in the field of avian influenza virus detection by real-time PCR. As shown in Fig. 5, the Ct values of the nine positive samples were all less than 30 and there were no Ct value for the two negative samples. Additionally, in this detection, the amplification of a specific fragment from H5 genome on this system just needed 65 minutes and saved nearly 1/3 of the time compared to conventional tube PCR, which was due to its rapid heating and cooling. These results imply that our microfluidic real-time PCR system has the ability to perform the amplification for rapid avian influenza virus detection.

Specificity of microfluidic RT-PCR

In order to verify the accuracy of the results obtained with the designed system, we set up an embryonated egg culture which is the gold standard for avian influenza virus identification as a comparative study . The results of the egg culture method for the same samples were consistent with those obtained using the one step microfluidic RT-PCR with the designed system (Table 1).

Although embryonated eggs have been used for the identification of influenza viruses as the gold standard, the method is time consuming, as it may require a week or more, and laborious, posing difficulties especially when a new major epidemic outbreak, like for example H7N9 in China in 2013. Other diagnostic tests employed virus culture or measurement of a rise in antibody based on immunoassays. These established techniques are complex on operation and the sensitivity of virus isolation is depended on the presence of infectious particles and some viruses are difficult to isolate.[20] Microfluidic real time PCR offers several advantages over the traditional methods, as it is rapid, specific, sensitive and reproducible. The platform we presented here is not just used for qualitative identification avian influenza virus, but also possesses the capacity to detect and measure minute amounts of nucleic acids in a wide range of samples from numerous sources. It may be used as a research tool for analysis of the different expression of genes, risk assessment of cancer recurrence, and identification of transgenes in foods.

Conclusions

The integrated microfluidic PCR system presented here has been shown to be a new method for the detection of the avian influenza virus. The result demonstrates that H5 could be successfully detected from clinical chicken throat swab specimens within 65 min using this microfluidic platform. According to the design of the platform, the compact optical detection system saves space, making the device smaller in size and more portable for use. Additionally, the rapid heating/cooling rate directly resulted in the shortening of the reaction time and enhanced the efficiency of the PCR amplification indirectly for enzyme activity increasing. Consequently, this integrated microfluidic RT-PCR system presented here may lead to a powerful tool for rapid diagnosis of the avian influenza virus and it would be possible to integrate sample pretreatment units on the chip, which is one of the directions of our future research.

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