# MULTIPLE PATHOGEN DETECTION FOR POULTRY BY ULTILIZING INTEGRATED MICROFLUIDIC SYSTEM

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## ABSTRACT

Avian influenza virus (AIV) and Newcastle disease virus (NDV) are highly contagious and fatal diseases affecting most species of poultry. The mortality rate of poultry infected by NDV is almost 100% and is over 80% for high pathogenic AIV. High fatality rate may cause significant economic loss of agricultural products. Furthermore, high pathogenic AIV may cause viral transmission from avian to human and genetic mutation to infect from person to person, therefore leading to human pandemics. In this study, a new approach for using magnetic beads-conjugated molecular beacons to rapidly diagnose AIV was demonstrated. The diagnosis process can be automatically performed on a microfluidic system within 30 minutes. The developed system may provide a useful platform for multiple pathogen detection for poultry.

## KEYWORDS

microfluidics, avian influenza virus (AIV), Newcastle disease virus (NDV), molecular beacon

## INTRODUCTION

Avian Influenza viruses (AIV) belong to the Orthomyxoviridae family and can be classified into two categories, including highly pathogenic AIV which produces severe fatal infection and low-pathogenic AIV [1]. Newcastle disease virus (NDV) is one of the paramyxoviruses which is a contagious and lethal disease infecting most specious of birds [2]. High fatality rate could cause significant economic loss of agricultural products. Furthermore, highly pathogenic AIV may cause viral transmission from avian to human and cause genetic mutation to infect from person to person, finally lead to human pandemics. Besides, clinical symptoms of AIV and NDV are very similar. Therefore, to distinguish AIV and NDV is crucial for pathogen diagnosis and surveillance. Thus, a rapid and accurate detection of pathogens is of great need.

Traditional diagnostic methods such as virus culture, enzyme-linked immunosorbent assay (ELISA), reverse-transcription polymerase chain reaction (RT-PCR) may have some drawbacks. For instance, ELISA is relatively time-consuming, exhibits poor specificity and low sensitivity. Alternatively, RT-PCR is a relatively complex procedure and requires special laboratory facilities and well-trained technicians. In this study, a microfluidic system using molecular beacons was used for rapid detection of pathogens. Molecular beacons have been widely used due to their ease of synthesis, unique functionality, molecular specificity and structural tolerance to various modifications. The structure of molecular beacons can be divided into three parts. One is a stem-loop with sequences complementary to the target sequence, the others are a pair of quencher and fluorophore. When molecular beacon forms the stem-loop structure, it holds the quencher and fluorophore close to each other. Hence, the energy from the fluorophore is transferred to the quencher. Once the single stranded loop portion of the molecular beacon hybridizes to the target, the stem melts and the resulting spatial separation of the fluorophore from the quencher leads to an enhancement in fluorescence signal [3]. Therefore, it can be used for recognizing the target molecules and fast diagnosis.

## EXPERIMENTAL

The working principle of diagnostic system is schematically shown in Fig 1. Briefly, the molecular beacon, test samples and magnetic beads coated with virus specific probes to capture virus RNA were first loaded into a reaction chamber. Then, virus lysis and beacon denature were performed under 95℃ for 5 minutes. It was then followed by decreasing temperature for beacon and probes annealing to the target sequence. Finally, the fluorophore was excited by laser and the fluorescence signal was measured for virus detection.

Figure 2 shows an integrated microfluidic chip made of two polydimethylsiloxane (PDMS) layers and one glass plate, which was first fabricated by a computer-numerical-control machining process, followed by PDMS casting and oxygen plasma treatment for bonding. The length, the width and the depth of the chip were measured to be 59, 74 and 5 mm, respectively. This microfluidic chip integrating washing buffer chambers, reagent/reaction chambers, waste units, suction-type micropumps and normally-closed valves was designed and fabricated to perform the entire process for virus detection. This chip contained six identical modules for positive control, negative control and 4



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individual detections (AIV and ADV), respectively.

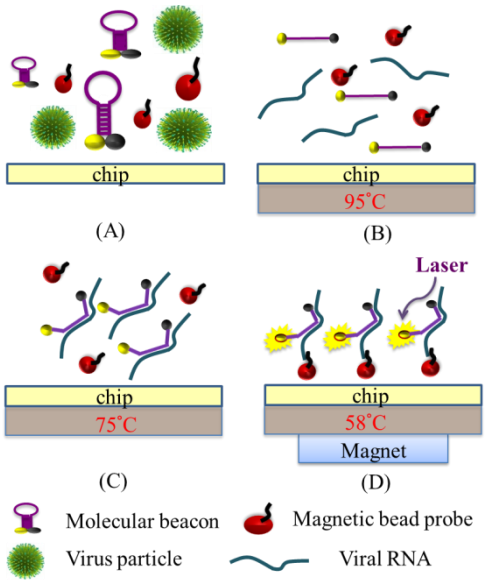



***Fig.1*** *Schematic illustrations about the experimental procedures for rapid detection of AIV.* ***(A)*** *Loading molecular beacons, virus and magnetic beads into a reaction chamber.* ***(B)*** *Increasing temperature to 95℃ for virus lysis and beacon denaturation.* ***(C)*** *Decreasing temperature to 75℃ for beacon annealing onto the target sequence.* ***(D)*** *Cooling down to 58℃ for probe annealing, washing out waste and diagnosis of fluorescence signal.*

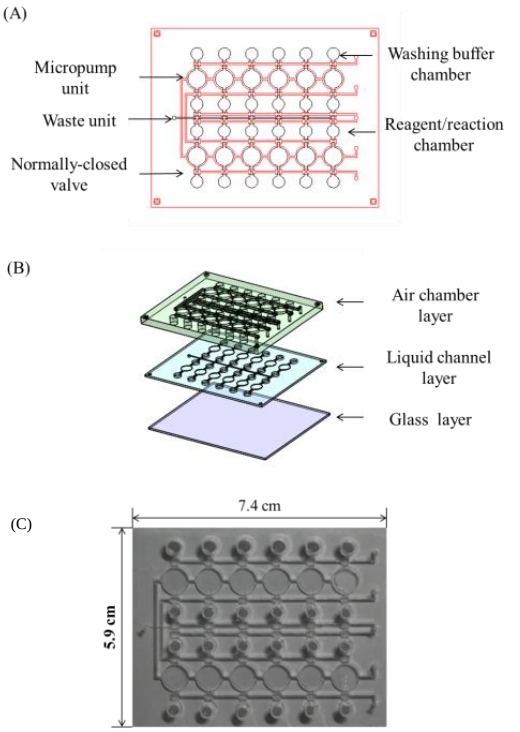
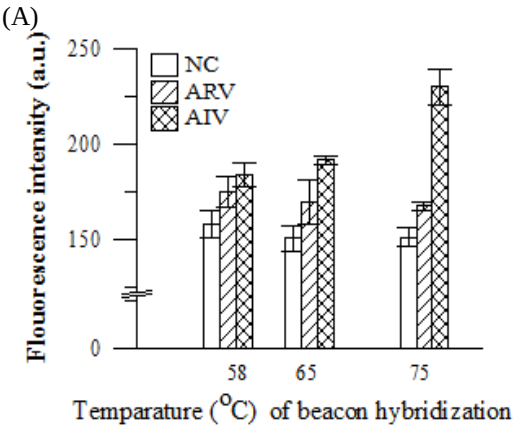


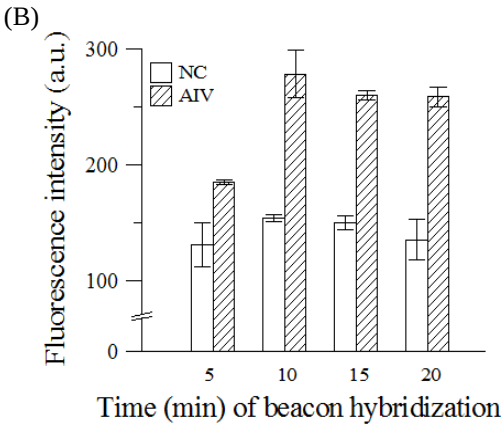

***Fig. 2 (A)*** *Top view,* ***(B)*** *exploded view, and* ***(C)*** *a photograph of the beacon-based integrated microfluidic chip consisted of normally-closed valves, washing buffer chambers, reaction/sample chambers, waste units, and suction-type micropumps*

## RESULTS AND DISCUSSION

The optimization of the beacon concentration, hybridization temperature and reaction time were first explored for the molecular beacon assay and the results are shown in Fig. 3. The fluorescence excitation signal showed that 5 mmole of AIV beacon can be used to capture AIV RNA by hybridization at 75 ℃ for 10 minutes. Note that the entire process from sample loading to final detection of fluorescent results only takes 30 min. Therefore, by using the microfludic system, the entire diagnosis process can be very simple and rapid.

Figure 4 shows the sensitivity test result for exploring the detection limit of the molecular beacon using plasmid carrying AIV specific fragment. The results show that molecular beacon can recognize AIV as low as 10 copies. The microfludic assay using molecular beacons may provide a promising tool for detection of pathogens in poultry.



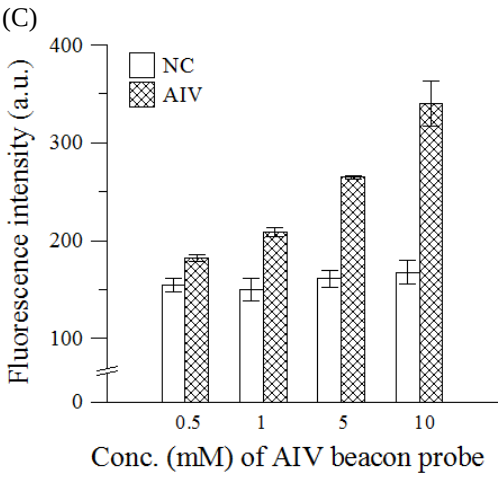



***Fig. 3*** *Optimization tests of different parameters for the molecular beacon assay.* ***(A)*** *Temperature,* ***(B)*** *hybridization time and* ***(C)*** *the concentration of beacons hybridized with target sequence. NC indicated as negative control used by distilled water. AIV was avian influenza virus as positive control. ARV was the avian reovirus as internal control to test the specificity of the beacon.*

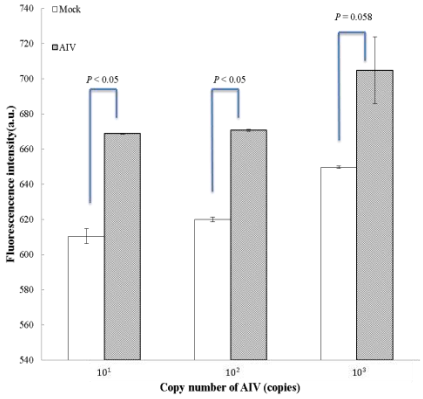



***Fig. 4*** *Sensitivity test results for the detection of AIV. A: Plasmid carrying AIV specific fragment, Mock: Plasmid only (without AIV specific fragment), NC: Negative control by using distilled water. The result indicated that molecular beacon can recognize 10 copies of AIV. P<0.05 indicated a significant difference between AIV and Mock by using student's t test.*

## CONCLUSION

An integrated microfluidic system was developed to carry out the detection of AIV. The entire process including sample pre-treatment and virus detection can be automatically performed in a short period of time (30 minute). The sensitivity of the assay has been verified by using virus particle samples, which is as low as 10 copies of AIV. The proposed microfluidic system is promising for rapid detection of aquaculture pathogens.

## ACKNOWLEDGEMENTS

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