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Rapid and amplification-free detection of fish pathogens by utilizing a molecular beacon-based microfluidic system ☆

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ABSTRACT

Nervous necrosis virus (NNV) and iridovirus are highly infectious pathogens that can cause lethal diseases in various species of fish. These infectious diseases have no effective treatments and the mortality rate is over 80%, which could cause dramatic economic losses in the aquaculture industry. Conventional diagnostic methods of NNV or iridovirus infected fishes, such as virus culture, enzyme-linked immunosorbent assays and nucleic acid assays usually require time-consuming and complex procedures performed by specialized technicians with delicate laboratory facilities. Rapid, simple, accurate and on-site detection of NNV and iridovirus infections would enable timely preventive measures such as immediate sacrifice of infected fishes, and is therefore critically needed for the aquaculture industry. In this study, a microfluidic-based assay that employ magnetic beads conjugated with viral deoxyribonucleic acid (DNA) capturing probes and fluorescent DNA molecular beacons were developed to rapidly detect NNV and iridovirus. Importantly, this new assay was realized in an integrated microfluidic system with a custom-made control system. With this approach, direct and automated NNV and iridovirus detection from infected fishes can be achieved in less than 30 min. Therefore, this molecular-beacon based microfluidic system presents a potentially promising tool for rapid diagnosis of fish pathogens in the field in the future.

1. Introduction

There are more than 50 different species of groupers inhabiting around Taiwan and grouper farming represents a 7.7-billion industry in Taiwan (Kuo et al., 2011). For this industry, viral infection poses a significant threat and causes heavy economic losses. In particular, nervous necrosis virus (NNV) and iridovirus are two strains of highly contagious virus that cause high mortality rates among grouper larvae and juveniles (Harikrishnan et al., 2010). Furthermore, because there are no effective treatments for diseases caused by NNV and iridovirus, fishes infected with NNV or iridovirus are immediately sacrificed in order to prevent further transmission (Kuo et al., 2012a, 2012b). Simple, rapid and accurate methods for detecting NNV and iridovirus directly from fishes are therefore crucial for monitoring the infected fishes and minimizing economic losses. Up to date, conventional methods for detecting these viruses, including histopathology (Tanaka et al., 2004), virology (Fukuda et al., 1996; Chou et al., 1998), immunohistochemistry (Shi et al., 2003) or molecular diagnosis (Kurita et al., 1998; Dalla Valle et al., 2005) have been employed. Unfortunately, these methods suffer from several drawbacks, such as complex and time-consuming procedures and requirements of large amount of samples/reagents, bulky instruments and well-trained technicians, which have significantly limited their effectiveness as simple, rapid and accurate detection methods.

Micro-total-analysis-systems (μ-TAS), also called integrated microfluidic systems or lab-on-a-chip (LOC), are systems that can perform complex processes, such as sample pretreatment, transportation, mixing, separation, reaction and detection within a single miniaturized chip, and have recently been actively explored

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Nomenclature

ANOVA	analysis of variance
BHQ1	Black Hole Quencher-1
DEV	dengue virus type 2
DNA	deoxyribonucleic acid
EV	enterovirus type 71
FRET	fluorescence resonance energy transfer
H1	influenza A/H1N1 virus
H3	influenza A/H3N2 virus
IB	influenza B virus
LOC	lab-on-a-chip
MCP	major capsid protein
NNV	nervous necrosis virus
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S/*N* ratio	signal-to-noise ratio
VIB	*Vibrio* sp.
a.u.	arbitrary unit
ddH_2O	double-distilled water
FAM	6-carboxyfluorescein
μ-TAS	micro-total-analysis-systems

in the research community. Such μ-TAS integrates various microfluidic modules, such as microvalves, micropumps, micromixers, microfilters, microheaters, microsensors, and microreactors and achieves chemical and biomedical analysis with lower sample or reagent consumption, shorter reaction times and higher throughput (Hessel et al., 2005; Thorsen et al., 2002). Furthermore, these systems could perform the entire process in an automatic format. Therefore, μ-TAS may offer a robust and effective platform for detection of fish viruses that may replace traditional complicated diagnostics and potentially save considerable analysis time and costs.

In order to achieve simple and rapid detection, detecting the viral nucleic acids via an amplification-free method should be employed. Molecular beacons (Tyagi and Kramer, 1996) serve this purpose well because they produce an easily detectable signal directly upon hybridization with the target nucleic acid sequence. The structure of molecular beacons includes a loop that is designed to be complementary to the target sequence and a self-complementary stem to hold with a fluorophore and a quencher in proximity. It has been widely used in chemistry, biology, biotechnology and medical sciences as bio-molecular recognition probes due to their ease of synthesis, unique functionality, molecular specificity and structural tolerance to various modifications (Tan et al., 2004; Whitcombe et al., 1999; Wong and Medrano, 2005; Mercier-Delarue et al., 2014) for diagnostic assays. Furthermore, when employed in concert with a second sequence-specific deoxyribonucleic acid (DNA) capturing probe that is conjugated to magnetic beads (Wang et al., 2011), the specificity of molecular beacon-based assays could be further improved. The magnetic probes not only enhance the specificity for detecting the target sequence, but also help purify the target viral DNA or ribonucleic acid (RNA) from debris from the fish sample (Chang et al., 2013a). Therefore, the combination of molecular beacons and sequence-specific magnetic capture probes performed on an integrated microfluidic system may present an amplification-free process that decreases the detection time.

This study therefore reports the development of an integrated microfluidic chip that contains some micro-modules, including reagent and washing buffer chambers, normally-closed valves and micropumps. The specific probe conjugated magnetic beads and molecular beacons were used to purify samples and hybridize with the target nucleic acid sequences for detection, respectively. In addition, a custom-made control system including a temperature control module, a microfluidic control module and an optical detection module was used to carry out the entire diagnosis process automatically (Chang et al., 2013b). As a demonstration, this microfluidic platform could carry out the detection of NNV and iridovirus simply and directly from fish samples.

2. Materials and methods

2.1. Viral strain

The NNV-infected, iridovirus-infected fish and *Vibrio* sp. (VIB) samples were from the Institute of Biotechnology, National Cheng Kung University, Taiwan. In this study, inactivated influenza A/H1N1 virus (H1), influenza A/H3N2 virus (H3), influenza B virus (IB), dengue virus type 2 (DEV), and enterovirus type 71 (EV), were prepared and generously provided by Dr. Chih-Peng Chang from the Department of Microbiology and Immunology, National Cheng Kung University, Taiwan.

2.2. Experimental procedures

The working principle of the integrated microfluidic system for the rapid diagnosis of NNV and iridovirus developed in this work is shown in Fig. 1. First, virus-infected fishes were grinded by a bio-vortex homogenizer (No. 1083-MC, Bio Spec Products Inc., USA). Subsequently, the 10 μL of grinded fish suspension was incubated with 5 μL of specific molecular beacons and 5 μL of specific probe-conjugated magnetic beads at 95 °C for 10 min, to lyse viral particles and denature the stem-loop structure of the molecular beacons and any secondary structure of viral DNA and RNA. Then single-stranded molecular beacons were allowed to hybridize to the complementary sequence of target viral DNA or RNA at 75 °C for 5–15 min. After that, the temperature was cooled to 55 °C for the probe-conjugated magnetic beads to anneal to the target viral sequence. The beacon–probe–target complex was collected by an external magnet that unbound beacons and debris from fish samples were washed away by using double-distilled water (ddH_2O, pH 7.0) for three times. In is study, the fluorescence donor and the quencher of the molecular beacons may contact to cause direct energy transfer and then dissipate with heat energy by FRET (fluorescence resonance energy transfer). When the molecular beacon was denatured as single-strand molecular beacon by heating treatment, the ssDNA was hybridized to the target region of the tested nucleic acids. The fluorescence donor then provided a stronger signal that resulted from the donor since the quencher was located far apart for a long distance. Finally, the fluorophore of the molecular beacon was excited by a laser (473 nm) and the intensity of fluorescence signal was measured by a custom-developed software (PCR_MFD ver 1.0, Mirle Co., Taiwan) to determine the diagnostic results. Note that, with the exception of grinding the fish sample, the whole assay was carried out in an integrated microfluidic system in a rapid and automated fashion by a developed custom-made control system (Chang et al., 2013b). Note that NNV is a RNA-virus and iridovirus is a DNA-virus. A specific NNV RNA2 probe and an iridovirus major capsid