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Arch-shaped multiple-target sensing for rapid diagnosis and identification of emerging infectious pathogens

Bonhan Koo[a], Ki Ho Hong[b], Choong Eun Jin[a], Ji Yeun Kim[c], Sung-Han Kim[c,*], Yong Shin[a,*]

[a] Department of Convergence Medicine, Asan Medical Center, University of Ulsan College of Medicine, Biomedical Engineering Research Center, Asan Institute of Life Sciences, Asan Medical Center, 88 Olympicro-43gil, Songpa-gu, Seoul, Republic of Korea
[b] Department of Laboratory Medicine, Seoul Medical Center, Seoul, Republic of Korea
[c] Department of Infectious Diseases, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

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ABSTRACT

Rapid identification of emerging infectious pathogens is crucial for preventing public health threats. Various pathogen detection techniques have been introduced; however, most techniques are time-consuming and lack multiple-target detection specificity. Although multiple-target detection techniques can distinguish emerging infectious pathogens from related pathogens, direct amplification methods have not been widely examined. Here, we present a novel arch-shaped multiple-target sensor capable of rapid pathogen identification using direct amplification in clinical samples. In this study, an arch-shaped amplification containing primer sequences was designed to rapidly amplify multiple targets. Further, the sensing platform allowed for sensitive and specific detection of human coronavirus, Middle East respiratory syndrome, Zika virus, and Ebola virus down to several copies. This platform also simultaneously distinguished between Middle East respiratory syndrome and human coronavirus in clinical specimens within 20 min. This arch-shaped multiple-target sensing assay can provide rapid, sensitive, and accurate diagnoses of emerging infectious diseases in clinical applications.

1. Introduction

An increasing number of pathogens that cause unexpected illnesses and epidemics among humans and animals has led to the loss of life and economic problems (Sands et al., 2016; Allegranzi et al., 2011). Recently, emerging infectious diseases, such as Middle East respiratory syndrome-coronavirus (MERS-CoV) (Zaki et al., 2012), Ebola virus (EBOV) (Biava et al., 2018), and Zika virus (ZIKV) (Munoz-Jordan, 2017) outbreaks, have revealed that disease control systems require more effective and coordinated responses, including vaccine development, diagnostic tools, and therapeutics (Sands et al., 2016). According to the World Health Organization (WHO), an outbreak of MERS-CoV resulted in 1782 infections and 634 deaths in June 2016 (WHO, 2016). In 2015, MERS-CoV infiltrated South Korea, resulting in 186 infections and 39 deaths with high mortality rates of over 30% (WHO, 2015a; Rao and Nyquist, 2014). For more effective prevention of emerging infectious disease threats, rapid diagnostics are needed to identify new pathogens for which vaccines and effective therapeutics are not available.

Rapid diagnostics to identify and diagnose pathogens have been performed using nucleic acid-based detection techniques, such as end-point polymerase chain reaction (PCR) and real-time RT-PCR, as gold standard methods (Zumla et al., 2015; WHO, 2015b; CDC, 2016). While these methods are relatively sensitive and specific for pathogen detection, they have several limitations, including the long time to acquire results, high technicality, and costly equipment such as a thermal cycler (Yang and Rothman, 2004; Barken et al., 2007; Mori and Notomi, 2009; Huang et al., 2006). Additionally, multiplex detection techniques, which detect two or more targets simultaneously in a single reaction, are required to distinguish the target pathogen from similar family members and offers reduced time and reagent costs (Brinkmann et al., 2017; Chung et al., 2013). Multiplex detection techniques are critical for rapid diagnostics; however, self-inhibition and false-positive results caused by primer dimerization (from a primer-target template mismatch or primer itself) can reduce detection sensitivity and specificity in clinical applications. To overcome the limitations of multiplex detection, a solid-phase DNA amplification technique was developed by grafting both forward and reverse primers. However, the amplification efficiency of the grafting primer assay was approximately 90% lower than that of conventional PCR in solution (Adessi et al., 2000; Fedurco et al., 2006; Shendure and Ji, 2008). Thus, the solid-phase DNA amplification technique has not been widely explored for applications in

* Corresponding authors.
E-mail addresses: kimsunghanmd@hotmail.com (S.-H. Kim), shinyongno1@gmail.com (Y. Shin).

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pathogen diagnostics. These limitations have made it difficult to apply diagnostic methods in hospitals. Resolving these limitations will require the development of diagnostic methods that are easy-to-use, accurate, and rapid for use in hospitals.

Here, we report a novel arch-shaped multiple-target sensing platform for rapid diagnosis and identification of emerging infectious pathogens. An isothermal amplification enzyme and oligonucleotide primers were immobilized on a silicon microring resonator (SMR) for specific detection of target nucleic acids from emerging infectious pathogens, such as MERS-CoV, Zika, and Ebola. In this study, rather than applying the amplified target and probe hybridization assay, we established long (> 50 base pair, bp) oligonucleotide primers at a high concentration (5 μM) to create an arch shape on the sensor surface for solid-phase amplification, thus overcoming primer dimerization. The SMR sensor allows for sensitive, label-free, and real-time sensing (Kim et al., 2018; Koo et al., 2017), and the long primers at a high concentration greatly increased the rate and detection sensitivity of this method. For nucleic acid amplification, we used a recombinase polymerase amplification (RPA) enzyme, which requires a small instrument for isothermal amplification (Piepenburg et al., 2006). Using the arch-shaped sensing platform, we detected RNA from human coronavirus (HCoV), MERS-CoV, ZIKA, and EBOV with high sensitivity and specificity. The detection limit was 10-fold more sensitive than that of real-time reverse transcription-PCR. Furthermore, this multiple-target sensing platform rapidly (< 20 min) detected MERS-CoV in 20 clinical specimens, including MERS-CoV and HCoV infections. Therefore, the arch-shaped multiple-target sensing platform can be used to rapidly identify pathogens in various clinical applications.

2. Material and methods

2.1. Development and operation of the arch-shaped multiple-target sensor

To use the arch-shaped multiple-target sensing platform as a multiple detection system, we structured the SMR sensor as previously described (Kim et al., 2018; Koo et al., 2017). For arch-shaped amplification and detection with specific primers immobilized on the sensor, a three-step primer modification was required on the surface of the sensing chip. First, amine group modification using 3-aminopropyltriethoxysiane (APTES, Sigma-Aldrich, St. Louis, MO, USA) was performed on the sensing surface. Subsequently, the sensors were treated with oxygenated plasma and soaked in a solution of 2% APTES in a mixture of ethanol–H_2O (95:5, v/v) for 2 h, followed by thorough rinsing with ethanol and deionized (DI) water. Second, carboxyl group modification was conducted using glutaraldehyde (GAD, Sigma-Aldrich) as a linker. The sensors were cured by heating to 120 °C for 15 min. Next, the sensors were incubated with 2.5% GAD in DI water containing 5 mM sodium cyanoborohydride (Sigma-Aldrich) for 1 h, rinsed with DI water, and dried under a nitrogen stream. Third, both forward and reverse primers were immobilized to target specific primers containing the 5′ amino-modifier C12. The pretreated sensor was prepared by overnight incubation at room temperature in a 5 or 10 μM solution of forward and reverse primers containing 5 mM sodium cyanoborohydride. After incubation, unbound target specific primers were removed by washing with phosphate-buffed saline and the sensors were dried using nitrogen. The prepared sensor chip was stored at room temperature until further use. To test the target amplification and detection in the primer immobilized sensor chip, recombinase polymerase amplification-reverse transcription (RPA-RT, TwistDx, Cambridge, UK) containing 29.5 μL of rehydration buffer, 15 μL of RNase inhibitor and water, 2 μM of DTT, recombinase, polymerase enzyme, and 2.5 μL of magnesium acetate solution. To start the reaction, 10 μL of RPA-RT solution and 5 μL of target RNA were mixed. Next, 15 μL of this mixture was added to the sensor chip with an acrylic well surrounding the microring sensor area. Additionally, mineral oil was added to prevent evaporation of the mixture during amplification. The arch-shaped multiple-target sensor assay was operated at a constant temperature (43 °C). The sensor consists of four rings connected to a common input and separate dedicated output waveguides. One of the four microrings was used as a reference sensor to monitor temperature-induced drift. The 3 remaining microrings were used for sensing three targets (MERS, EBOV, ZIKV) with a vertical grating coupler. For multiple target monitoring, each dedicated output waveguide corresponding to the microring immobilized different target specific primer was measured sequentially by moving the optical fiber. The tunable laser emits light at wavelengths of 1550–1580 nm (EXFO IQS- 2600B, Quebec, Canada). To obtain a baseline as a reference, the resonance wavelength was immediately measured. The resonance wavelength shift for multiple target detection was then measured every 5 min for up to 30 min to monitor the arch-shaped amplification of MERS, ZIKV, EBOV, and HCoV RNAs in a label-free and real-time manner.

2.2. Primer length and concentration for arch-shaped multiple-target sensing

We designed the Arch-RT-PCR primer based on genome sequence information for MERS, ZIKV, EBOV, and HCoV (Supplementary Table S1). The non-specific poly dT spacer sequence at the 5′ end of the primer allowed for the amplification product to warp to an arch shape in solid-phase amplification (Guo et al., 1994). As such, primers were designed that were 10, 20, and 30 bp longer than the conventional primers. Different lengths of MERS primers for detecting MERS and HCoV were immobilized to the microring on the SMR sensor. Subsequently, primers of suitable length for detecting MERS, ZIKV, EBOV, and HCoV were immobilized on the microring resonance sensors. For multiplexing experiments, we used an ultra-small dosage dispensing system with sciFLEXARRAYER SX (SCIENION AG, Monmouth Junction, NJ, USA) to avoid cross-contamination. Subsequently, droplets of other target specific primers solutions were dispensed to the microring at desired locations. Because a smaller amount of primer volume was used than in the previous method, a higher concentration of primer solution was used. Finally, the resonance wavelength shift by products of arch-shaped amplification was measured on the microring.

2.3. End-point and real-time RT-PCR assays

The utility of end-point and real-time reverse transcription PCR were compared to that of the arch-shaped multiple-target assay. We designed RT-PCR primers of standard lengths (approximately 24 bp) based on genome sequence information for MERS, ZIKV, EBOV, and HCoV (Table S1). End-point RT-PCR consist of an initial reverse transcription step at 50 °C for 30 min, followed by at 95 °C for 15 min and 45 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s and final elongation step 72 °C for 10 min. The reaction mixtures containing 25 μL of end-point RT-PCR reagent mixtures, 5x one-step RT-PCR buffer (Qiagen One-step RT-PCR kit), 0.25 mM deoxynucleotide triphosphate, 25 pmol of each primer, 1 μL of one-step RT-PCR Enzyme Mix (Qiagen), and 5 μL of RNA template. Gel electrophoresis was used to separate RT-PCR products on a 2% agarose gel containing ethidium bromide (EtBr). The gel was visualized using a GenoSens 1500 gel documentation system (Clinx Science Instruments, Shanghai, China). Real-time RT-PCR consisted of an initial reverse transcription step at 50 °C for 20 min, followed by at 95 °C for 15 min and 45 cycles at 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s, followed by a cooling step at 40 °C for 30 s. The real-time RT-PCR assay was conducted using the manufacturer's protocol (AriaMx, Agilent Technologies, Santa Clara, CA, USA). Additionally, reaction mixtures containing 20 μL of real-time RT-PCR reagent mixtures, 2x brilliant SYBR green RT-qPCR master mix, 25 pmol of each primer, and 5 μL of RNA template were prepared. The amplified products were detected by SYBR Green signals using an AriaMx Real-Time PCR System (AriaMx, Agilent Technologies).