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Highly sensitive sandwich-type SPR based detection of whole H5Nx viruses using a pair of aptamers

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ABSTRACT

In this research, we report highly sensitive and specific sandwich-type SPR-based biosensor for the detection H5Nx whole viruses. A few of aptamers, for the first time, were successfully screened and characterized for whole avian influenza (AI) viruses, H5Nx, by using Multi-GO-SELEX method. The affinities of the aptamers developed in this study were ranged from 8×10^4 to 1×10^4 EID50/ml, and the aptamers IF22, IF23 were found to be specific to H5N1 and H5N8, respectively. In addition, some flexible aptamers IF20, IF15, and IF10 were found to bind to the H5N1 and H5N2, H5N1 and H5N8, or H5N1, H5N2, and H5N8, respectively. Moreover, aptamers IF10 and IF22 were found to bind H5N1 virus simultaneously and confirmed to bind the different site of the same H5N1 whole virus. Therefore, this pair of aptamers, IF10 and IF22, were successfully applied to develop the sandwich-type SPR-based biosensor assay which is rapid, accurate for the detection of AI whole virus from H5N1-infected feces samples. The minimum detectible concentration of H5N1 whole virus was found to be 200 EID50/ml with this sandwich-type detection using the aptamer pair obtained in this study. In addition, the sensitivity of this biosensor was successfully enhanced by using the signal amplification with the secondary aptamer conjugated with gold nanoparticles.

1. Introduction

Highly pathogenic disease caused by avian influenza A H5Nx viruses, threat to the poultry industry and human worldwide seriously. The highly pathogenic avian influenza (AI) strain H5Nx emerged in Southern China in the 1990 s and the first large-scale epizootic took place in the winter of 2004 in the East and Southeast Asia. The virus persisted in the region until the winter in August 2006–2007 and it was spread over 60 countries (Li, et al. 2004; Alexander, et al. 2007). In addition, all of the previous outbreaks have been costly and difficult to be controlled in the agricultural sector. For highly pathogenic disease, the most important control measures are rapid culling of all infected or exposed birds, proper disposal of carcasses, the quarantining and rigorous disinfection of farms, and the implementation of strict sanitary, or “biodefense”, measures. Restrictions on the movement of live poultry, both within and between countries, are another important control measures.

Highly pathogenic viruses have been known to survive for long periods in the environment, especially when temperatures are low such as highly pathogenic H5N1 virus can survive in bird feces for more than a month at low temperature. The H5N1 subtype of highly pathogenic avian virus (HPAIV) initially identified during 1996 in China, infected 18 humans with 6 deaths during 1997 in Hong Kong. This virus was highly pathogenic in chickens and humans and posed a crucial threat to public health. In addition, it has been known that H5N1 virus caused more than 60% human mortality, and as of April 2016, H5N1 virus caused of 449 deaths and more than 850 cases-confirmed human infections. Direct or indirect contact with diseased poultry is the primary route of HPAIV infections in humans. Despite of efforts which have been done to prevent of HPAIV spread by whether vaccination or culling of infected birds, several H5 influenza subtypes have already been prevalent in Asia, Europe, and Africa. Therefore, the prevention and rapid detection of H5Nx, especially for highly pathogenic

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viruses H5N1, are required to control the outbreak of AI H5Nx.

The aptamers are selected from randomized nucleic acid libraries which has diversity of $10^{12} \sim 10^{14}$ and typical short fragment of single-stranded DNA, RNA or peptide via rounds of affinity capture and amplification in SELEX process (systematic evolution of ligands by exponential enrichment) (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Upon the needs of capturing molecules, hundreds of aptamers were developed to various types of target such as antibiotics, amino acids, toxins, and small molecules or relatively bigger molecules including peptides, proteins, cells, and viruses (Ikebukuro et al., 2005; Lee et al., 2011; Niazi et al., 2008; Nitsche et al., 2007; Adler et al., 2008). Aptamers display a high affinity and specificity to their target molecules, which can be acquired through selection strategies, with impressive dissociation constants (Kd) ranging from picomolar to nanomolar levels between aptamer and its target. Compare to conventional capturing probe, namely antibody, the main advantage of aptamers is their generation using in vitro selection process, whereas the production of antibodies uses biological systems (Song et al., 2012). By isolation of aptamers in vitro condition, various aptamers can be produced to variety of targets without any roadblock of immunogenicity or toxicity. Aptamers are in general thought to be more stable than antibodies, thereby having a longer shelf life. Aptamers are also more sustainable at high temperatures such that they can be regenerated easily after denaturation. Also, chemical modifications or labeling on aptamers are easier and their binding property is not affected by these labeling or modification step. Overall, compared with traditional ligands including antibodies, peptides, and small molecules, the aptamers exhibit advantages such as low cost, low immunogenicity and toxicity, small size to enable solid tumor penetration, and high affinity to bind to the target. Therefore, the aptamers could be the ideal candidates to use in therapeutic, analytical, medical diagnosis, and biosensor applications (Herr et al., 2006; Wang et al., 2011; Yu et al., 2011).

There are a few research papers that have reported the selection of avian influenza virus aptamers for the detection and inhibition of avian influenza viruses. Some research groups have been used HA proteins or peptides, such as purified influenza B virus HA proteins, HA proteins of H5 and H9 in order to select DNA or RNA aptamers (Cheng et al., 2008; Suenaga et al., 2014; Choi et al., 2011; Jeon et al., 2004; Gopinath et al., 2005, 2006b). Interestingly, whole influenza H3N2 virus has been used for RNA aptamer selection to obtain HA-specific RNA aptamer (Gopinath et al., 2006a). Very recently, Wang et al. (2013) reported the selection of influenza H5N1 aptamer from purified HA and whole virus, and then the specific aptamer was applied to develop biosensor for detection AI virus (Wang and Li, 2013). So far, there are no report that use various H5Nx whole viruses for aptamer selection and a pair of aptamers for the detection of H5Nx whole viruses.

In this research, we successfully screened out specific aptamers for H5Nx whole viruses by using Multi-GO-SELEX protocol (Nguyen et al., 2014). Among selected aptamers, a pair of aptamers was obtained and applied to SPR chip for aptamer-based sandwich type detection of the whole virus successfully.

2. Material and methods

2.1. Screening specific aptamer of avian influenza virus (AI) H5 using Multi GO-SELEX

2.1.1. General H5Nx SELEX

To prepare the denatured ssDNA, the 2 μl of 100 μM of 66mer ssDNA's random library is added into 98 μl of 1x binding buffer (BB) and the mixture is heated for 15 min at 95 °C and then slowly cooling down for 5 min on ice. After the 200 μl of 10^6 EID50/ml of counter targets (in BB) and the denatured ssDNA library are gently mixed, the mixture is incubated 30 min at RT by rotating. Then, the 100 μl of GO solution (5 mg/ml), 2 × BB (100 μl), 1 × BB (500 μl) are added in the tube and it is incubated for 2 h, RT, rotator. (Total volume 1 ml, GO 0.5 mg/ml). After that, the mixture is centrifuged at 14,680 rpm for 10 min and then the 800 μl of supernatant is removed. The ssDNA binding to GO is washed 2 times with 1xBB. The 200 μl of 10^6 EID50/ml of three main targets: H5N1, H5N2, H5N3 whole viruses and 1 × BB 700 μl are added in to the reaction tube and gently mixed, then the mixture is incubated for 2 h at RT by rotator. The reaction solution proceeds to centrifuge at 14,680 rpm for 10 min. After supernatant are collected (2 separate tube, 400 μl), the centrifugation process is repeated to remove remaining GO (2 ×). Finally, ethanol precipitation process is conducted by adding Glycogen 3 μl (5 mg/ml), NaOAc 100 μl (3 M), and Isopropanol (same volume as solution), then the solution is incubated for 2 h at −20 °C refrigerator.

2.1.2. Negative SELEX

The 20 μl (2000 ng of ssDNAs) from the 5th round sample in the H5Nx whole virus-based SELEX is added into 80 μl of 1 × binding buffer (BB) and the mixture is heated for 15 min at 95 °C and then slowly cooling down for 5 min on ice. The 500 μl of 10^6 EID50/ml of counter targets (H1N2, H2N1, H3N8, H4N6, H6, H7N8, H9N2, H10N4, H11, H12N5), others viruses (IBV, NDV), Mock allantoic fluid and feces solution (in 1 × BB) are gently mixed with 100 μl of the denatured ssDNAs of the 5th round sample and 500 μl of 2 × Binding buffer, and then the mixture was incubated for 30 min at RT in the rotating rocker. Then, the 100 μl of GO solution (5 mg/ml), 2 × BB (100 μl), 1 × BB (500 μl) are added in the tube, and it is incubated for 2 h at the room temperature in the rotator (total volume 1 ml, GO 0.5 mg/ml). After that, the mixture is centrifuged at 14,680 rpm for 10 min, and then the 800 μl of supernatant is removed. The ssDNA bound to GO was washed 2 times with 1 × BB. The 200 μl of 10^6 EID50/ml of three main target viruses, H5N1, H5N2, H5N3, and 1 × BB 700 μl are added into the reaction tube, and gently mixed, then the mixture was incubated for 2 h at RT in the rotator rocker. Further steps are followed for the general H5Nx SELEX protocol mentioned above.

2.2. Characterization of aptamer candidates using SPR

The binding affinity and the specificity of aptamers were analyzed and characterized using SPR method. The aptamers were immobilized on SPR gold chip using EDC/NHS as following previous studies (Lee, et al., 2008; Liu, et al., 2013; Ahmad Raston and Gu, 2015). Briefly, the surface of SPR was cleaned using ethanol (x3times) and distillation water (× 3 times). Then, the cleaned chip was immersed into the ethanol solution (100%) containing of 50 mM DTP for 14–18 h (overnight) at RT. Then, carboxyl functional groups on chip surface were activated with 200 mM EDC and 50 mM NHS for 30 min. Subsequently, chip was incubated with 100 μg/ml (1.9 μM) of streptavidin for 90 min on ice. The unreacted functional groups were blocked by addition of 50 mM ethanolamine solution for 30 min. Then 5′-T_{10}-biotin labeled aptamers were incubated (1 μM) for 60 min at room temperature. Finally, chip was blocked with 50 μg/ml BSA solution for 30 min and washed with DW. By using this aptamer modified gold chip, SPR (Eco Chemie, Netherlands) analysis was performed after injection of 50 μl of each main target (H5N1, H5N8, and H5N2) and mixed counter targets (H1, H2, H3, H4, H6, H7, H9, H10, H11, H12) as well. The binding reaction was performed for 30 min with a 5 min dissociation time at room temperature. To analyze the dose dependent and Kd value, 5 aptamers with high specific to AI H5 were selected from out of 18 aptamer. The dissociation constant