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Multi-colored immunochromatography using nanobeads for rapid and sensitive typing of seasonal influenza viruses

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A B S T R A C T

Immunochromatography (IC) is an antigen-detection assay that plays an important role in the rapid diagnosis of influenza viruses because of its rapid turnaround and ease of use. Despite the usefulness of IC, the limit of detection of common IC kits is as high as 10^3–10^4 plaque forming units (pfu) per reaction, resulting in their limited sensitivities. Early diagnosis within 24 h would provide more appropriate timing of treatment.

In this study, a multi-colored NanoAct™ bead IC was established to detect seasonal influenza viruses. This method has approximately 10-fold higher sensitivity than that of colloidal gold or colored latex bead IC assays, and does not require specific instruments. More notably, NanoAct™ bead IC can distinguish influenza A and B viruses from clinical samples with a straightforward readout composed of colored lines. Our results will provide new strategies for the diagnosis, treatment, and a chance to survey of influenza viruses in developing countries and in the field research.

1. Introduction

Influenza is a highly contagious respiratory disease of humans, caused by the negative-strand RNA viruses, Influenza A virus (IAV), and Influenza B virus (IBV) of the family Orthomyxoviridae (Wright et al., 2007). Seasonal outbreaks of influenza caused by IAV and IBV present global health problems involving morbidity, mortality, and economic losses. IAVs are classified into subtypes based on two viral surface proteins, hemagglutinin (HA) and neuraminidase (NA). There are 16 HA and 9 NA subtypes. Influenza viruses with almost all possible combinations of HA and NA subtypes have been isolated from aquatic birds, poultry, and other avian species. In contrast to IAVs, IBVs are not classified into subtypes according to HA or NA, but consist of only two phylogenetic and antigenic lineages: the B/Victoria/2/87-like (Victoria) lineage and the B/Yamagata/16/88-like (Yamagata) lineage (Kanegae et al., 1990; Rota et al., 1990; Yamashita et al., 1988). IAV has the potential to become pandemic through reassortment among avian and human IAVs, whereas IBV does not (Webster, 1997).

The threat of highly pathogenic avian influenza (HPAI)-H5N1 occurred in 1997 (Claas et al., 1998; de Jong et al., 1997; Subbarao et al., 1998). The World Health Organization (WHO) reported that the HPAI-H5N1 virus had infected 620 individuals causing 367 deaths (~59% mortality) as of February 15, 2013 (World Health Organization, 2013a). A recent pandemic of Influenza virus A/H1N1pdm occurred in 2009 following triple-reassortment with swine influenza virus and Eurasian-lineage swine influenza virus (Dawood et al., 2009). Influenza virus A/H1N1pdm caused more than 18,849 deaths in more than 214 countries (World Health Organization, 2010b). The WHO announced that the pandemic had transitioned into a post-pandemic phase on September 10, 2010 (World Health Organization, 2010a).

Recently, swine influenza viruses were isolated from humans (Shu et al., 2012). In 2011 and 2012, an outbreak of Influenza virus A/H3N2 (H3N2v) occurred in 12 states of the United States of America. This resulted in 309 human cases, including 16 hospitalizations

Abbreviations: IC, immunochromatography; IAV, Influenza A virus; IBV, Influenza B virus; HA, hemagglutinin; NA, neuraminidase; HPAI, highly pathogenic avian influenza; NA-IC, multi-colored NanoAct® beads; CG-IC, colloidal gold-immunochromatography (Prorast™-Flu); CL-IC, colored latex-immunochromatography (QuickNavi™-Flu); FLIC, fluorescent immunochromatography; qRT-PCR, quantitative real-time PCR; LOD, limit of detection.

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and one death (Centers for Disease Control and Prevention, 2012). H3N2v infection has been associated with exposure to swine at agricultural fairs, and is characterized by the matrix segment of the viral genome of Influenza virus A/H1N1pdm (Bowman et al., 2012). These reports indicated that variant swine influenza viruses have the potential to spread between humans.

A recently discovered H7N9 influenza virus has caused 135 human infections and 44 deaths in China since February 18, 2013 (Chen et al., 2013; Kageyama et al., 2013; World Health Organization, 2013b). Despite its high pathogenicity in humans, H7N9 virus has low pathogenicity in avian hosts (Chen et al., 2013; Liu et al., 2013). Infection of mice with H7N9 lead to weight loss without fatality (Belser et al., 2013; Mok et al., 2013; Watanabe et al., 2013). These results indicate that H7N9 is likely to spread in birds or other animals without symptoms. H7N9 genome consists of an *HA* gene that was derived from the Eurasian wild bird H7N3 virus, an *NA* gene that was derived from the Eurasian wild bird H2N9 or H11N9 virus, and six other viral gene segments derived from the poultry H9N2 virus (Lam et al., 2013; Liu et al., 2013). The HA protein of H7N9 virus isolated from humans has a significantly higher affinity for α-2,6-linked sialic acid analogs than that of avian H7 virus (Watanabe et al., 2013; Xiong et al., 2013; Zhou et al., 2013). In addition, the human H7N9 virus retains the ability to interact with α-2,3-linked sialic acid analogs. Thus, this virus may have the potential to infect both human and avian hosts. Epidemiological investigation suggests the evidence of transmission of the human H7N9 virus from human to human within a family cluster (Zhang et al., 2013). However, the transmission was limited and non-sustainable, as 43 other close contacts of the first and second patients were serologically negative for the H7N9 virus. One of the H7N9 virus strains, A/Anhui/1/2013 (H7N9), has been reported to be transmitted through respiratory droplets from infected ferrets to contact ferrets with limited efficiency (Belser et al., 2013; Richard et al., 2013; Watanabe et al., 2013; Zhang et al., 2013). These results suggest that H7N9 viruses have the potential to produce pandemics. Collectively, these reports indicate the possibility of the occurrence of a pandemic caused by new animal influenza viruses such as HPAI-H5N1, H3N2v, and H7N9. If deleterious mutation of these viruses were to confer efficient human-to-human transmission, they might pose a serious threat to human health and the global economy.

Immunochromatography (IC), an antigen-detection assay by using specific antibody that can be completed within 15 min, is an useful and rapid method for clinical diagnosis and surveillance of influenza (Sakai-Tagawa et al., 2010; Sakurai and Shibasaki, 2012; Sasaki et al., 2012). The specificity of commonly available ICs is >90%, whereas the overall sensitivity is approximately 60% (Cruz et al., 2006). Improvement in the sensitivity of IC would increase the value of this technique in the diagnosis and typing of influenza viruses. Here, we describe the newly developed IC assay that utilizes the detection antibody conjugated with multi-colored NanoAct® beads (NA-IC) to type influenza virus. NA-IC method permit to detect and distinguish strains of IAV and IBV in over 200 clinical samples which were confirmed to contain influenza viruses with culture and a quantitative real-time PCR (qRT-PCR) in the 2012 and 2013 seasons. The sensitivity of NA-IC method demonstrated approximately 10-fold higher than that of classical IC methods without any specific instrument.

2. Materials and methods

2.1. Cells and virus strains

Madin–Darby canine kidney cells (MDCK cells; American Type Culture Collection, VA, USA) were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin solution.

All influenza virus strains (family Orthomyxoviridae, genera Influenza virus A and Influenza virus B, species Influenza virus A and Influenza virus B, respectively) used in this study are listed in Tables 2–4. These viruses were described previously (Sakurai et al., 2011, 2013). All viruses were grown in MDCK or 10-day-old embryonated chicken eggs.

2.2. Establishment of immunochromatography with colored cellulose nanoparticles for typing influenza viruses

NanoAct™ (Asahi Kasei Fibers Corporation, Miyazaki, Japan, http://www.asahi-kasei.co.jp/fibers/en/index.html) is a cellulose nanoparticle product, which has an average diameter of 330 nm, colored in red, blue, or green. Compared with other labels for IC, NanoAct™ has advantages regarding higher visibility and multi-color labeling (Table 1). The surface size of NanoAct™ is larger than that of colloidal gold (CG) in the same volume, and the color is more intense than that of latex beads because the cellulose can be easily and strongly modified by color chemicals. In addition, unlike other high sensitivity IC methods such as FLIC (Sakurai et al., 2013) or silver amplification (Wada et al., 2011), NanoAct™ signal can easily detect under visible light condition without any specific instrument such as a chemiluminescence and a fluorescent scanner. Prorast™-Flu (Mitsubishi Chemical Medience Corporation, Tokyo, Japan, http://www.medience.co.jp/english/index.html; CG-IC) is a commercialized IC for typing Influenza A and B viruses with a colloidal gold (CG)-conjugated antibody. And QuickNavi™-Flu colored latex (CL) IC assay (Otsuka Pharmaceutical Company Limited; Tokyo, Japan, http://www.otsuka.co.jp/en/; CL-IC) is also for typing Influenza A and B viruses with color latex bead-conjugated antibody. Immunochromatography with NA-IC was established by replacing colloidal gold with NanoAct™ beads conjugated with the antibody used in CG-IC (Fig. 1).

For detecting IAV, immobilized mouse monoclonal anti-IAV NP clone 56-1 (subclass IgG2a) or conjugated clone A43-6 (subclass IgG2a) antibodies were used. For the detection of IBV, immobilized mouse monoclonal anti-IBV NP clone 47-23 (subclass IgG2b) or conjugated clone B58-17 (subclass IgG2a) antibodies were used. The anti-NP antibodies against IAV and IBV were conjugated with different-colored NanoAct™ beads. NanoAct™ beads were diluted in 1 mM Tris–HCl (pH 8.0) at a final concentration of 0.1% (w/w). Anti-IAV NP antibody A43-6 or anti-IBV NP antibody B58-17 (1 mL, 100 μg/mL) was incubated with 10 mL of the NanoAct™ beads solution at 25 °C for 3 h. The mixture was blocked with Block Ace (DS Pharma Biomedical Company Limited, Osaka, Japan, http://www.dspbio.co.jp/index.html) at a final concentration of 1% for 2 h. The solution was centrifuged (13,000 × *g*, 5 min, 4 °C) and the pellet was washed 3 times with 50 mM Tris–HCl (pH 8.0)/0.05% Triton X-100. The washed pellet was resuspended in suspension buffer (50 mM Tris–HCl (pH 7.2), 0.05% Triton X-100, 0.1% BSA, 5% saccharose, and 0.095% sodium azide) at a final concentration 0.1% (w/w).

2.3. Isolation of samples from patients

Collection of all human samples and clinical trials were performed under the approvals of IRB in all facilities and hospitals (Supplementary Table 1). The clinical protocol was accepted by Japanese Pharmaceuticals and Medical Devices Agency (PMDA) and the clinical trials were performed under the regulation of PMDA.

Samples were collected from nasal swabs and self-blow nasal discharge specimens of patients who had a diagnosis of influenza-like respiratory disease based on signs and symptoms such as fever. Samples for IC were suspended in 500 μL of IC dilution buffer