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Research paper

Detection of two different influenza A viruses using a nitrocellulose membrane and a magnetic biosensor

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ABSTRACT

Here we describe a new analytical method for the detection of two influenza A viruses by nitrocellulose membrane and magnetic sensors that employ a special frequency mixing technique. The combination of the nitrocellulose membrane and magnetic bead detection permits a rapid assay procedure and excludes two steps (the development of color and the stop reaction) required for usual immunochemical detection methods such as ELISA. Quantitative virus detection was performed using magnetic beads conjugated with secondary antibody. The results were compared with conventional assay methods and with a dot-blot assay with fluorescence compound (FITC). Under optimum conditions, our new assay procedure is capable of detecting picograms of virus per well. This new method combining the nitrocellulose membrane and magnetic bead detection reduces analytical time and allows stable and repeatable analyses of samples in point-of-care applications.

1. Introduction

Influenza A virus belongs to the RNA virus family *Orthomyxoviridae* that infects mammals, including human beings. It is generally transmitted through the air by coughs, sneezes, and direct contact with the contaminated materials such as body fluids (Subbarao and Katz, 2000). In order to prevent further infections and provide the proper treatment, rapid detection of infection is critical. However, one of the many problems faced by clinicians and the concerned public is proper methods of virus detection. Usually, a few diagnostic methods are widely accepted and used for the clinical diagnosis of patients (Dwyer et al., 2006). Although the RT-PCR method is a very accurate method, it requires expensive reagents, good facilities, and skillful operators (Amano and Cheng, 2005).

Rapid antigen detection methods such as immunofluorescence or enzyme immunoassay are simple and based on the specific binding between antigen and antibody (de Boer et al., 1990; Remarque et al., 1998). The labeling compounds typically used in these assays are the following: enzymes such as horseradish peroxidase and alkaline phosphatase, fluorophores, chemiluminescent molecules (Acridinum esters), radioisotopes like ^{125}I, ^{3}H and ^{57}Co, and nano- or microsized magnetic beads. Among these, enzymes have been used extensively due to their good sensitivity. The drawbacks of these enzymes are as follows: 1) several incubation and washing steps are needed. As a result the method is labour intensive. 2) The enzyme and the substrates are subject of several deactivation reactions and require well defined storage conditions. Dot-blot assay employing fluorescence compounds such as FITC is another alternative method. The method is simple and fast. The major drawback is quenching and bleaching of the fluorescence tag. As a result the method is not very robust.

Accordingly, other methods not requiring enzymes and chemicals have been suggested as alternatives. Paramagnetic

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or superparamagnetic beads are becoming more widely accepted materials because they can act as the labeling compound as well as can separate targeting materials from the sample. The research of labeling magnetic beads on biomaterials to create analytical signals has been widely developed (Haun et al., 2010; Janssen et al., 2008). Several studies have demonstrated the feasibility of GMR, TMR, Spin vale sensor, and SQUID as biosensors. Although they are analytical instruments with excellent abilities, they have several deficiencies with respect to practical aspects. First, they cannot cover the range of densities necessary for analysis because the surface of the sensor is so limited. Furthermore, the sensing element is difficult to prepare. Due to these shortcomings, a new analytical instrument to detect the magnetic beads was suggested (Krause et al., 2007; Meyer et al., 2007a,b, 2007c); in order to measure the sample, an ABICAP® column including ABICAP filter® was employed. Although there was a good correlation between the amount of the sample and that of the magnetic beads, the usage of the column and filter limited the measurement of the many samples. The standard ELISA can handle 96 samples or more at once.

Enzyme linked immunoflow assay (ELIFA), which is principally the same method as ELISA, uses a nitrocellulose membrane. Generally, the membrane is widely used for a variety of detection methods because of its ability to tightly bind bio-material such as proteins, DNA, or cells (Paffard et al., 1996; Shields et al., 1991). This adaptation of the membrane provides a reduction in assay time and flexibility of the sample substrate.

In this study, magnetic beads were used as a labeling material (Fig. 1). The detection and quantification of the magnetic beads was performed by a magnetic biosensor based on frequency mixing detection. The combination of the nitrocellulose membrane and the magnetic bead detector resulted in elimination of two analytical steps and hence reduction of assay time. This method also makes it possible to prepare the samples with a usual 96-well microplate that is the commonly accepted preparation platform for immunoassays.

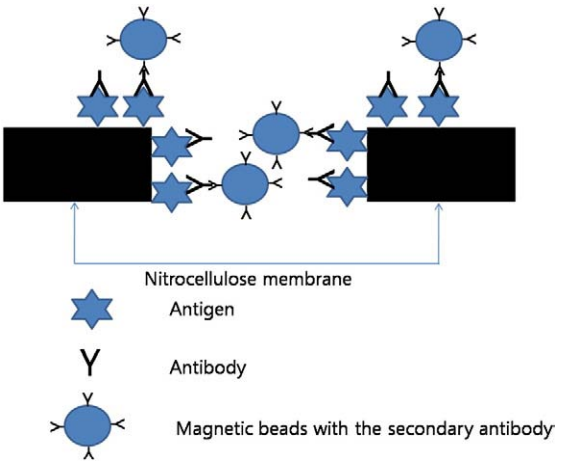

Fig. 1. Scheme showing the detection of viruses captured on a nitrocellulose membrane. The calibration curve was determined from the data of three separate measurements. The coefficient of determination is 0.96 which is close to linearity.

2. Materials and methods

2.1. Materials

Influenza A/Beijing/262/95 and A/Kiev/301-94 were purchased from Fitzgerald Industries International (MA, USA) with concentrations of 0.8 mg/ml and 1.3 mg/ml (mg of virus protein/ml), respectively. Influenza A/Beijing/262/95 and A/Kiev/301-94 are the inactivated antigens produced by inoculation with the strains to allantonic fluid of 10 day old embryonic eggs. Thus, these viruses do not have the capability of infection. For the detection of the viruses, two types of polyclonal antibodies were employed in the assay. According to the manufacturer's data sheet, both of them were produced in goat using H1N1 and H3N2 strain as immunogen. The detection antibodies were also purchased from the same company. The concentration of antibodies was 5.0 mg/ml. The antigens and antibodies were stored at −20 °C in 10 μl single-use aliquots. Before each experiment, the aliquots were diluted 50 fold with phosphate-buffered saline (PBS) containing 0.02% sodium azide. Magnetic beads (FluidMAG-ARA) were purchased from Chemicell GmbH (Berlin, Germany). The surfaces of the 100-nm beads are covered with glucuronic acid-carboxyl. 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, PBS, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), rabbit–anti-goat IgG (H + L) unconjugated, rabbit–anti-goat IgG conjugated with Horseradish Peroxidase (HRP), fluorescein isothiocyanate (FITC) antibody labeling kit, Easy titer apparatus and nitrocellulose membranes (pore size: 0.45 μm) were purchased from Thermo Fisher Science (IL, USA). Blocking buffer containing 1.0% BSA was purchased from Sigma-Aldrich (MO, USA). Just before the assay, rabbit–anti-goat conjugated with HRP was diluted in PBS (1/20,000) and FITC labeled one was also diluted in PBS (1/2000).

2.2. Preparation

Two hundred microliters of magnetic bead solution (50.0 mg/ml and ~1.8×10^{15}/g) was washed two times with 1.0 ml of MES buffer with a pH range of 5.5–6.5. After washing, the bead solution was dissolved in 250.0 μl of MES buffer containing 10.0 mg of EDC. The mixture was incubated at room temperature for 10 min. Subsequently, it was washed 2 times with 1.0 ml of MES buffer and re-dissolved in 250.0 μl of MES buffer. Subsequently, 2.0 mg of rabbit–anti-goat IgG (Fc-specific) was dissolved in 2.0 ml of ultrapure water from a Millipore unit, and 100.0 μl of the solution was mixed with the washed magnetic bead solution. The mixture was incubated for 2.0 h with gentle shaking. Finally, the bead solution was washed 3 times with phosphate-buffered saline (PBS) and re-dissolved in 30.0 ml of PBS. The bead solution prepared was diluted 1/5 in PBS before the assay. For labeling the anti-goat IgG unconjugated dissolved in PBS with FITC, 40.0 μl of borate buffer was added to 0.5 ml of 2.0 mg/ml in PBS. The protein solution was transferred to the vial of FITC reagent and incubated for 60 min at room temperature. Protein was purified using the purification resin and spin column of the labeling kit.