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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.

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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.

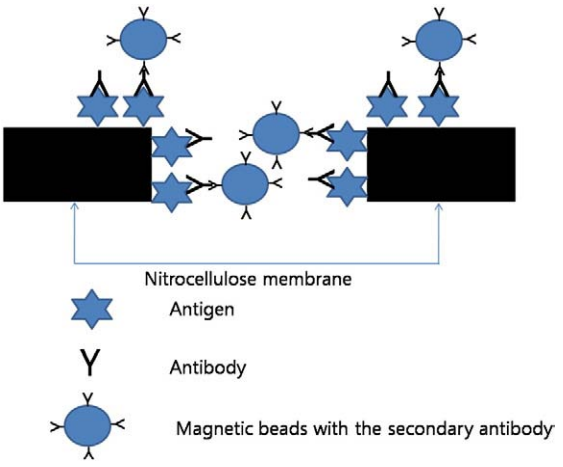

Fig. 1. Scheme showing the detection of viruses captured on a nitrocellulose membrane. The calibration curve was determined from the data of three separate measurements. The coefficient of determination is 0.96 which is close to linearity.

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.

2.3. Assays

The Easy titer apparatus was prepared as described in previous studies (Paffard et al., 1996). Two hundred microliters of diluted antigen solution was placed in the first wells and serially diluted to 210.0 pg/200 μl for H1N1 and 450.0 pg/200 μl for H3N2. After pulling the solution through the membrane for 5 min, the wells were blocked with 200.0 μl of blocking buffer. Then, 10.0 μl of antibody solutions (H1N1 and H3N2) was diluted in 10.0 ml of PBS, and 200.0 μl of this mixture was added to the wells. Subsequently, 200.0 μl of the bead solution prepared above was added to wells, which were then washed 5 times with PBS. After washing, the nitrocellulose membrane was cut into pieces for insertion in the detector. In case of the conventional ELIFA, 200.0 μl of HRP solution was added, and in case of fluorimetric dot-blot assay, 200.0 μl of the secondary antibody labeled with FITC was added instead of the bead solution. After washing with PBS 5 times, 100.0 μl of TMB solution was added to the wells with HRP. This solution was pulled through the membrane for 5 min. The solution was then collected in a clean microplate and 100.0 μl of 1 N HCl solution was added. The absorbance of each well was measured using a microplate reader for the ELIFA (iMARK, Bio-RAD, USA). For the dot-blot assay (DBA), the membrane was placed on the microplate, and the fluorescence intensity was measured using a fluorescence reader (Infinite M200; Tecan Trading AG, Switzerland).

2.4. Measurement of magnetic beads

In order to measure the amount of magnetic beads bound to the antibody, a magnetic biosensor consisting of a measurement head with a 9-mm bore and a touch-panel magnetic reader was employed. The detection principle is based on a two-frequency magnetic excitation and the detection of a sum frequency component that is selectively generated by the magnetic nanoparticles due to their nonlinear magnetization characteristics (Krause et al., 2007).

The magnetic measurement head comprises two excitation coils wound on a common coil former and a differential pickup coil consisting of two identical coils placed next to each other but wound in opposite orientations. The outer low frequency coil generates a magnetic field at $f_2 = 61$ Hz with an amplitude of approximately 4 mT which periodically drives the magnetic particles close to saturation. The middle high frequency coil generates an excitation field of about 0.5 mT at $f_1 = 61$ kHz. The excitation coil can be moved with respect to the pickup coil using a fine thread, thus allowing precise balancing of the measurement head when no sample is present. The parameters of the coils are listed in Table 1.

Fig. 2 depicts a schematic diagram of the electronics for frequency generation and synchronous readout of the measured sum frequency component. Two digital oscillators (direct digital synthesis chips AD9834 from Analog Devices) that are synchronized by a common quartz reference serve as frequency generators for the two excitation fields. Two more digital oscillators with adjustable phases supply the reference frequencies for the two demodulation stages. In our case, the reference oscillator DDS2 is set to f_1 and DDS4 is set to $2 \cdot f_2$. Thus, the demodulation chain synchronously detects the frequency mixing component at $f_1 + 2 \cdot f_2 = 61{,}122$ Hz. The main advantage of this principle is that magnetic response signals from paramagnetic or diamagnetic materials are very effectively suppressed because their magnetic response in the lower millitesla range is linear. Thus, they do not generate frequency mixing components. In contrast, the magnetic particles give rise to frequency mixing components due to their strongly nonlinear magnetization curve in that field range.

Table 1
Parameters of the coils of the magnetic measurement head.

	Inner pickup coil 1	Middle high frequency excitation coil 2	Outer low frequency driving coil 3
Diameter D_i [mm]	10.8	14.7	21.5
Width W_i [mm]	2×8	24	24
Height H_i [mm]	0.8	0.7	6
Wire diameter d_i [mm]	0.15	0.18	0.2
No. of windings N_i	2×264	468	2400
Resistance R_i [Ω]	17	16	89
Inductance L_i [mH]	1.1	1.9	76

The magnetic moment sensitivity limit is given by the Johnson noise of the detection coil. According to the formula given in Krause et al. (2007), the noise equivalent magnetic moment was calculated to 1.0×10^{-13} Am$^2/\sqrt{\text{Hz}}$.

The experiments were performed in duplicate or triplicate. The signal of negative reference was subtracted from that of the samples and the calibration curves were established.

3. Results

3.1. Principle of detection

An assay described in Sections 2.2–2.4 was performed to detect two viruses. In this study, 100-nm magnetic beads were used as labeling materials instead of enzyme–substrate reactions such as IgG-HRP and TMB or IgG-FITC. Fig. 3 shows the comparison between conventional ELIFA and the magnetic methods presented in this research. The use of magnetic beads can eliminate two steps (addition of substrate and stopping the reaction by acid), resulting in reduction of the assay time and provides a much better robustness.

The nitrocellulose and magnetic bead-based immunoassay for multiple detection is presented in Fig. 1. The assay involves antigen adsorption to the membrane, formation of the antigen–antibody complex and magnetic detection of the complex after addition of magnetic beads coupled to secondary antibodies. Recognition of the complex yields distinct signals reflecting the concentration of the corresponding beads in the complex. The intensity of the signals from the detector is directly related to the amount of magnetic beads bound in the complex. The magnetic beads remaining on the surface of the nitrocellulose membrane can be measured, they reflect the amount of antibody–antigen complex. Thus, this alternative ELIFA system is suitable for magnetic biosensors due to the binding capability and flexibility of the nitrocellulose membrane. In addition, the samples can be stored after the measurement; this is not possible with the usual ELISA system employing enzyme and substrate.

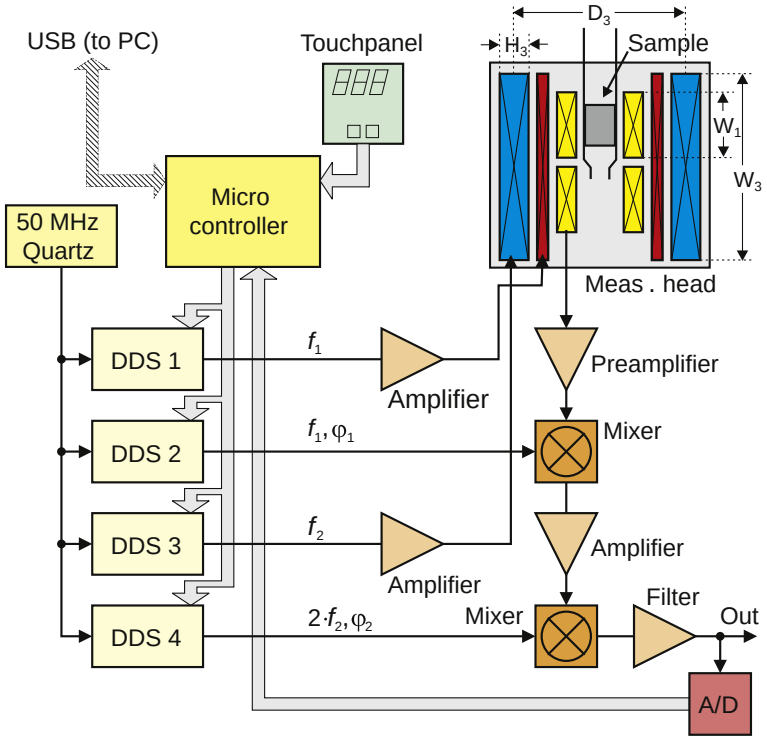

Fig. 2. Schematic of the readout electronics. The measurement head consists of two excitation coils and of a differential pickup coil with the sample in one compartment. Driven by a common quartz clock, four direct digital synthesis (DDS) chips yield the two excitation frequencies and the two demodulation frequencies. The values of the frequencies and phases are programmed by the user by remote PC connection or by touch panel via the microcontroller. After the two-stage demodulation, the output signal is filtered, digitized and processed.

3.2. Analysis

To establish the standard calibration curve for the 100-nm magnetic beads covered with the secondary antibody, serial dilutions (20.0–4.0 μg/200 μl) were prepared in PBS. The calibration curve of the system is shown in Fig. 4. The results showed a strong correlation between the concentrations of magnetic beads and the signals from the detector. The coefficient of determination (R^2) of the linear approximation was evaluated as 0.97. Average and standard deviation (SD) values from three measurements of the blank (without beads) were −0.1 and 4.07, respectively. By the linear fit, an offset of −437 mV was determined for zero particle concentration. The amount of magnetic beads based on the

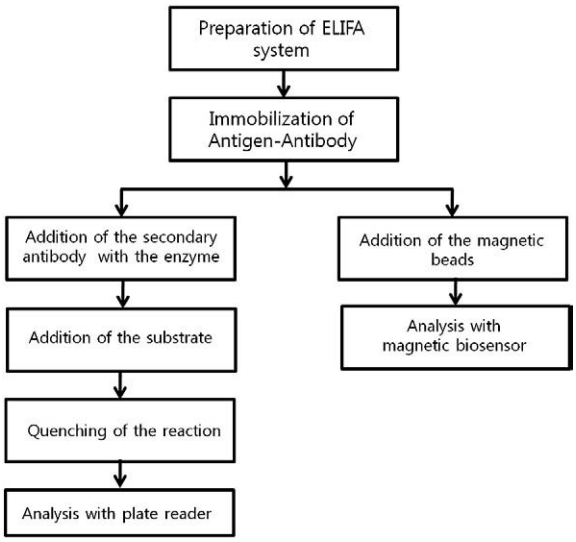

Fig. 3. Comparison of a standard ELIFA with optical detection and the new approach employing a nitrocellulose membrane and magnetic sensors.

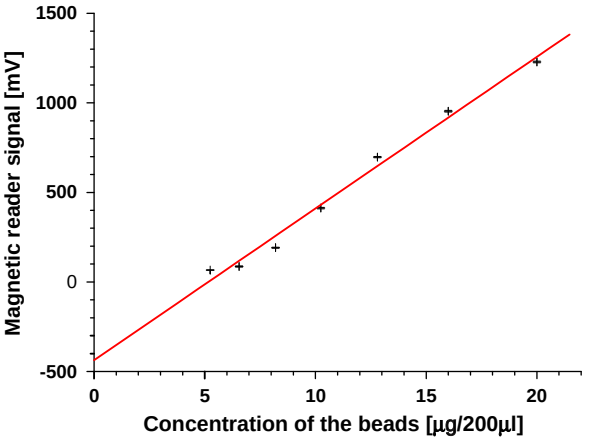

Fig. 4. Calibration curve of the new measurement system using different concentrations of magnetic beads on the surface of the nitrocellulose membrane. The 20.0 µg was serially diluted to 4.0 µg in 200 µl of PBS.

magnetic reader signals at 2 SDs below the mean absorbance of the zero point was employed to calculate the detection limit (Chen et al., 2004). The calculated detection limit based on the results of the experiments shown in Fig. 4 was found to be 3.12 µg per 200 µl.

The use of magnetic beads as labeling substances enabled detection of concentrations of picograms per milliliter using the magnetic biosensors. Fig. 5A and B shows the calibration curves for H1N1and H3N2 influenza A viruses for all three measurement methods. To obtain a simple comparison between the methods, all data were normalized. For normalization, the values of the highest concentration and the blank were converted to 1.0 and 0.0, respectively.

For the magnetic bead measurements, after reading the low data in millivolt, the offset for zero concentration was subtracted in these measurements. The values of the coefficient of determination for each assay were 0.97 for H1N1 and 0.98 for H3N2. The calibration curves of ELIFA and fluorimetric DBA are shown in Fig. 5A and B. The correlation coefficients of the ELIFA curves were calculated as 0.90 for H1N1 and 0.96 for H3N2. The values of DBA were 0.97 for H1N1 and 0.99 for H3N2. Based on the values before the normalization, the values of the standard deviation for H1N1 measurement tests were 0.08 OD at 450 nm for ELIFA, 723.0 relative fluorescence units for DBA, and 9.45 mV for magnetic bead detection. The respective values for H3N2 were 0.02 OD, 552 relative fluorescence units, and 7.07 mV. The ratios between SD and average values of the blanks were 4.98% (ELIFA), 1.83% (DBA), and 1.67% (magnetic bead detection) for H1N1. The respective values for H3N2 were 1.69%, 1.38%, and 1.20%.

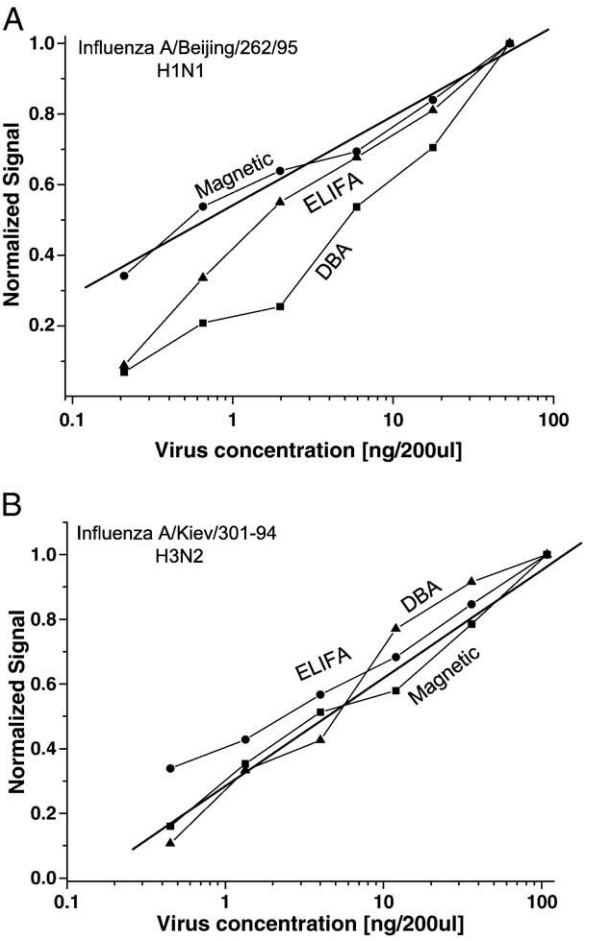

Fig. 5. Normalized calibration curves of the three techniques ELIFA, DBA and magnetic bead detection. (A) Calibration curve for inactivated H1N1 virus (A/Beijing/262/95), and (B) calibration curve for H3N2 virus (A/Kiev/301/94). The calibration curves were obtained by means of triplicate experiments.

4. Discussion and conclusion

Influenza is an acute respiratory disease that infects many creatures. Recently, the influenza subtypes H1N1 and H5N1 occurred globally and caused many problems. Although highly accurate methods, such as RT-PCR and MDCK cell culture, are available to detect these viruses, these methods are limited in their on-site performance ability. In the case of an epidemic or pandemic disease, hospitals or government agencies should be able to handle a large amount of samples in short periods to prevent further spread of the disease. Because of the fast transmittance of influenza and the limited treatment methods available, fast detection and isolation of patients with influenza is critical. Thus, reliable screening methods that can be used on site and can handle many samples simultaneously are urgently required.

In light of this need, we propose a new detection system that combines nitrocellulose membrane and magnetic sensors that are based on frequency mixing. In this method, the reaction between antigen and antibody occurs both on and in the nitrocellulose membrane, which has a larger internal surface than that of a standard 96-well microplate. The porous characteristic of the membrane gives the assay a larger detection range. The micro or nanomagnetic beads are much bigger than usual labeling compounds, suggesting the importance of securing enough surface space for the beads. Therefore, the utilization of polymers as the solid phase has been suggested (Meyer et al., 2007a,b; Nikitin et al., 2007). These studies also suggested the advantages of utilizing a membrane: Nikitin et al. used small polyethylene POROBLAST

filters in combination with their own liquid handling system and Meyer et al. used ABICAP® polyethylene filters. Both these methods used plastic or glass columns to contain the filters and bio-materials. Although these methods are convenient when performing assays with a small number of samples, it is necessary to purchase many materials and prepare many columns. For example, to ensure compatibility with a standard 96-well microplate, 96 columns should be prepared. Although the strip-type microplate can be an alternative for analysis, the surface of the well and limited size of the sample insertion hole for the magnetic sensors limit the use of the standard microplate. In order to use a different well size of the plate, new biosensors should be designed and prepared.

In this study, nitrocellulose membranes were used as the material support substrate. There are three advantages of using the membranes instead of the solid support substrate: first, it is not necessary to change the system except for the use of magnetic beads instead of chemicals; second, the use of the membrane reduces the analytical time because of the very short incubation period; and third, there is little to no limitation on the size or shape of the sample holders of the detectors. The results from the comparison with the ELIFA and fluorimetric DBA showed that the there are no significant differences between the techniques regarding their detection capabilities, thus confirming the feasibility of the new method under experimental conditions.

The drawback of using the membrane is the non-specific binding of the beads. However, non-specific binding is a problem common to all assays using a membrane as the immobilization substrate. We also observed relatively high non-specific binding in the other methods used in this research (data not shown). It is assumed that there are two mechanisms of non-specific binding of the beads. The first is the binding between the membrane and beads. The second is the coagulation of the beads to fill up the pores of the membrane. Thus, the detection limit and range seem to be affected by the concentration of the bead solution and the pore size. Previous measurements of the different concentrations with similar virus concentrations yielded different calibration curves (data not shown). For example, we could not make a calibration curve with beads of 1.0 μm size. The previous studies also suggested that the type of beads and the pore size are important to define the detection capability of assays and that the interface chemistry should be improved to reduce non-specific binding of beads (Meyer et al., 2007a,b; Nikitin et al., 2007). To reduce non-specific binding of beads and avoid clogging of the membrane pores, we used beads of a smaller size. Further improvement of the assay could be achieved by adopting a different assay format, e.g. a double antibody sandwich assay. Although the new method suggested here was used for the detection of viruses in this study, it can be used for different immunoassays such as the detection of pathogens, DNA, etc. In addition, this new approach can promote the use of magnetic beads as a labeling compound and magnetic sensors in different detection methods.

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