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Anionic polymer, poly(methyl vinyl ether–maleic anhydride)-coated beads-based capture of human influenza A and B virus

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ABSTRACT

An anionic magnetic beads-based method was developed for the capture of human influenza A and B viruses from nasal aspirates, allantoic fluid and culture medium. A polymer, poly(methyl vinyl ether–maleic anhydride) [poly(MVE-MA)], was used to endow magnetic beads with a negative charge and bioadhesive properties. After incubation with samples containing human influenza virus, the beads were separated from supernatants by applying a magnetic field. The absorption of the virus by the beads was confirmed by hemagglutinin assay, immunochromatography, Western blotting, egg infection, and cell infection. Successful capture was proved using 5 H1N1 influenza A viruses, 10 H3N2 influenza A viruses, and 6 influenza B viruses. Furthermore, the infectivity in chicken embryonated eggs and Madin–Darby canine kidney (MDCK) cells of the captured human influenza virus was similar to that of the total viral quantity of starting materials. Therefore, this method of capture using magnetic beads coated with poly(MVE-MA) can be broadly used for the recovery of infectious human influenza viruses.

1. Introduction

Influenza viruses are divided into three types (A, B, and C) based on the antigenicity of nucleoprotein (NP) and matrix protein (M1).[1] The genome of influenza A viruses encodes two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), whose antigenicity defines 16 distinct HA and 9 distinct NA subtypes.[2,3] Although there are no subtypes of influenza B virus, strains can usually be divided into two antigenically and genetically distinct lineages (Yamagata-like and Victoria-like).[4] Influenza A viruses have a broad range of hosts that include humans, birds, pigs, and horses,[2] while influenza B viruses have been isolated mainly from humans, and in rare cases from seals.[5] Among all of these types and subtypes, influenza virus A/H1N1, A/H3N2, and B have been mainly associated with human infections.[1]

Human influenza viruses cause severe upper respiratory tract infections in infants, young children, and the elderly with an estimated three to five million cases of severe illness and 250,000–500,000 deaths per year worldwide.[1] The infections cannot be reliably diagnosed based on clinical features alone.[6] Notably, distinguishing them from other respiratory diseases such as infections with adenoviruses, parainfluenza viruses, respiratory syncytial viruses (RSVs), metapneumoviruses, avian influenza viruses, and hemolytic streptococcus is difficult. Furthermore, the discrimination of viral infections from bacterial infections is important for decreasing misuse of antibiotics and minimizing the emergence of antibiotic-resistant bacteria. Several methods of detecting human influenza viruses have been developed such as reverse transcription (RT)-polymerase chain reaction (PCR), enzyme-linked immunoassays (EIAs), immunofluorescence assays, rapid antigen tests (immunochromatography), and serological tests.[7] Immunochromatography is useful for the rapid diagnostic assay of human influenza virus infections and broadly used for practical diagnosis in the clinic. However, none of these methods enables us to further isolate infectious viruses, because the diagnostic processes using inorganic reagents such as detergents and phenol–chloroform inactivate the viruses. Whenever possible, cell culturing should be performed to obtain isolates for characterization, which are needed to monitor new strains and develop vaccines. However, viral isolation is sometimes difficult due to the low quantity of viruses, suggesting that a method of concentration is needed for efficient isolation.

There are three major factors in the development of methods to concentrate viruses; recovery of infectivity, compatibility with current methods of detection, and convenience. Maintaining infectivity after treatment is particularly important because infectious viruses are needed for the development of vaccines. Several approaches to the concentration of influenza virus have been proposed, including ultracentrifugation and polyethyleneglycol (PEG)

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precipitation. Both these methods are applicable to various viruses. Ultracentrifugation is time-consuming and impractical, reduces infectivity, and can increase the false-positive rate when combined with PCR.[8] Although PEG precipitation is simple and easy to perform, the PEG inhibits PCR and partially inactivates infectious viruses.[9,10] One alternative to conventional methods is the use of magnetic beads coated with molecules, which efficiently bind a virus without reducing its infectivity. One such potential molecule is poly(methyl vinyl ether–maleic anhydride) [poly(MVE-MA)] (Fig. 1), which has anionic and bioadhesive properties.[11]

In the present study, we report that magnetic beads coated with the polymer poly(MVE-MA) are useful for the capture of human influenza viruses including 5 strains of H1N1 influenza A virus, 10 strains of H3N2 influenza A virus, and 6 strains of influenza B virus.

2. Results

To examine the capacity of magnetic beads coated with the anionic polymer poly(MVE-MA) to capture viruses, hemagglutination assays, immunochromatography, egg inoculations, and cell infections were performed. Viral capture was performed as described in Section 4 (Fig. 2). Immunochromatography showed that the influenza A virus NP was efficiently concentrated in the beads fraction (BD) after incubation compared to the sample before incubation with the beads (BF) using cell culture medium (CM), allantoic fluid (AF), and nasal aspirate (NAS) (Fig. 3). In contrast, the influenza A virus NP was undetectable in the supernatant fraction (SP). These results suggest this method of capture to be applicable to CM, AF, and NAS.

Next, we examined the efficiency with which the influenza virus was concentrated by conducting hemagglutination assays. Results showed that the hemagglutination titer was concentrated by the anionic magnetic beads from A/PR8/34 (H1N1)-infected AF at levels from 2.3 $\log_2$ to 9.6 $\log_2$, whereas it was under the detectable limit in SP from infected and uninfected AF and BD from uninfected AF (Figs. 4 and 5 and Supplemental Table 1). Furthermore, the hemagglutination titer recovered with anionic magnetic beads (BD) was similar to that in samples containing the same quantity of start material as BD (TL). These results suggest that influenza virus is efficiently concentrated by the anionic magnetic beads.

Second, we examined whether this method is applicable to other influenza viruses including influenza A viruses (H1N1 and H3N2) and influenza B viruses (Yamagata-like and Victoria-like).

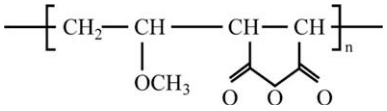

Figure 1. The structure of poly(methyl vinyl ether–maleic anhydride) [poly(MVE-MA)].

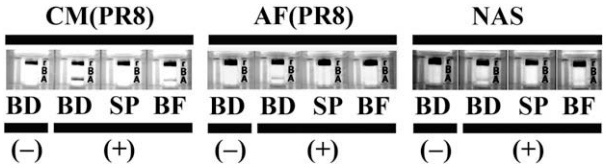

Figure 3. Detection of nucleoprotein (NP) in influenza A virus absorbed on poly(MVE-MA)-coated magnetic beads from cell culture medium (CM), allantoic fluid (AF), and nasal aspirates (NAS). AF and CM of Madin–Darby canine kidney (MDCK) cells infected with influenza A virus (A/PR8/34) (+) and left uninfected (−) as well as influenza A virus-infected and uninfected NAS from patients were diluted with phosphate-buffered saline (PBS) and subjected to incubation with poly(MVE-MA)-coated magnetic beads. Esprine Influenza A and B-N (Fujirebio Inc., Tokyo, Japan) was used for detection of influenza virus nucleoprotein (NP) by immunochromatography. Results regarding the presence of influenza A virus (A) and influenza B virus (B) were interpreted on the basis of the presence and absence of a line, with positive control, which is included for internal kit positive control (r). Samples were divided into categories; a beads fraction (BD), the sample before incubation with the beads (BF), supernatant after the incubation (SP), and total sample containing the same quantity of starting material (CM, AF, or NAS) as BD (TL), which were solubilized with lysis buffer and subjected to immunochromatography.

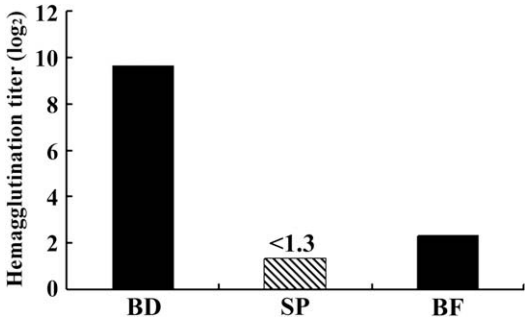

Figure 4. Concentration of hemagglutination titers by poly(MVE-MA)-coated magnetic beads. Influenza A virus (A/PR8/34) in infected AF was diluted with PBS and captured by poly(MVE-MA)-coated magnetic beads. The resultant beads fraction (BD), supernatant after incubation (SP), and sample before incubation with the beads (BF) were subjected to a hemagglutination assay. The results of the assay are shown as $\log_2$ values.

Immunochromatography for NP showed that not only A/H1N1 but also A/H3N2, B/Yamagata-like, and B/Victoria-like could be recovered in BD by the viral capture method from CM and AF (Fig. 6). These tendencies were also confirmed by hemagglutination assays (Tables 1 and 2). Meanwhile, there were differences in reactivity among the strains of each virus, possibly due to slight conformational differences in viral components.

Third, proteins recovered by anionic magnetic beads from influenza virus-infected CM were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) transferred onto polyvinylidene difluoride (PVDF) membranes and stained with Coomassie brilliant blue (CBB) and anti-influenza virus NP. The results showed that BD but not SP in influenza virus (A/PR8/34)-in-

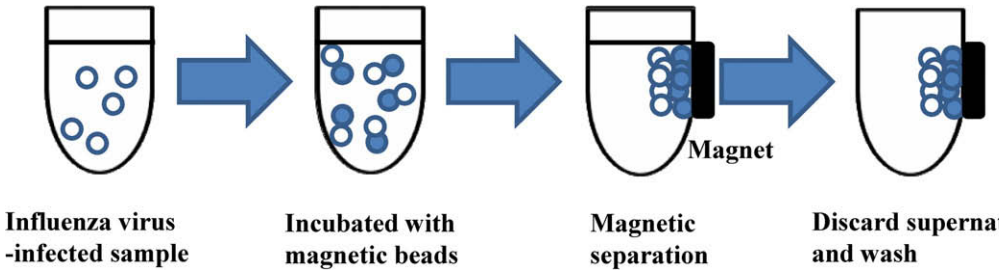

Figure 2. The procedure for capturing influenza virus using poly(MVE-MA)-coated magnetic beads.

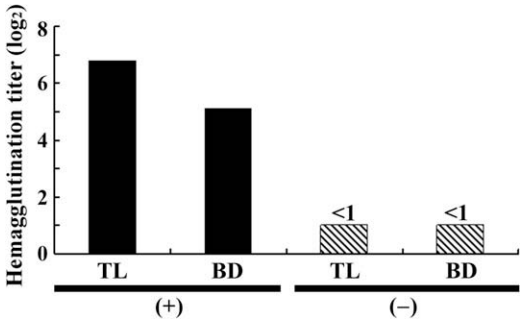

Figure 5. Efficient recovery of hemagglutination titers by poly(MVE-MA)-coated magnetic beads. Influenza A virus (A/PR8/34)-infected AF (+) and uninfected AF (−) were diluted with PBS and captured by poly(MVE-MA)-coated magnetic beads. The resultant beads fraction (BD) and sample containing the same quantity of AF as BD (TL) were subjected to a hemagglutination assay. The results of the assay are shown as $\log_2$ values.

fected CM showed major bands of 56, 63, and 66 kDa (Fig. 7). The size of 56 and 63 kDa was consistent with that calculated from the deduced amino acid sequence of influenza virus (A/PR8/34) NP and HA0, respectively.[12] The 56-kDa band was also close to the deduced size of the cleaved form of HA, viz. HA1.[12] Thus, the 56-kDa band was probably a mix of NP and HA1. On the other hand, there was a non-specific band (66 kDa) in the total sample fraction (TL) and BD of influenza virus-infected (+) and uninfected (−) CM. This band is possibly due to albumin in CM, as deduced from its size.[13] These results suggest that the anionic magnetic beads bind mainly to influenza virus but also non-specifically to other components of CM. No bands for influenza virus-derived proteins were detected in non-infected samples besides the 66-kDa band. Taken together, these results showed the presence of influenza virus-derived proteins such as NP, HA1, and HA0 in the fraction recovered with the anionic magnetic beads. However, the non-specific binding of a serum-derived protein, albumin, in CM was also observed.

Finally, we examined the infectivity of the fraction recovered with the magnetic beads using eggs and cells. Assays using Madin–Darby canine kidney (MDCK) cells showed that the 50% tissue culture infective dose ($TCID_{50}$) value obtained with beads (BD) was similar to that in the total sample containing the same quantity of starting material as BD (TL) (Fig. 8). Infectivity recovered with the anionic magnetic beads from aliquots of AF using eggs showed that BD from influenza A virus (A/PR8/34)-infected AF exhibited a 2/3 infection rate in embryonated eggs, which was the same rate as TL (Table 3). Taken together, results supported that infectious

Table 1
Hemagglutination titer of influenza A viruses (H1N1 and H3N2) recovered with poly(MVE-MA)-coated magnetic beads from infected CM

	Hemagglutination titer ($\log_2$)	
	TL	BD
PR8	5	5
FK1	7.81	6.13
TK6	10.81	7.13
YN2	9.81	6.13
NI102	8.81	6.13
GZ54	5.81	6.13

PR8: A/PR8/34 (H1N1); FK1: A/Fukuoka/1/70 (H3N2); TK6: A/Tokyo/6/73 (H3N2); YN2: A/Yamanashi/2/77 (H3N2); NI102: A/Niigata/102/81 (H3N2); GZ54: A/Guizhou/54/89 (H3N2); BD: beads fraction; TL: total sample containing the same quantiy of CM as BD.

Table 2
Hemagglutination titer of influenza viruses (A/H1N1, A/H3N2, B/Yamagata-like, and B/Victoria-like) recovered with poly(MVE-MA)-coated magnetic beads from infected AF

	Hemagglutination titer (log2)	
	TL	BD
A/H1N1		
FM1	8.8	7.1
BK10	7.8	5.1
YG120	5.8	6.1
YG32	3.8	7.1
A/H3N2		
AI2	12.8	8.1
FKC29	6.8	5.1
KK159	10.8	7.1
WH539	6.8	7.1
SD5	6.8	6.1
B/Yamagata-like		
YN166	6.8	6.1
JB5	7.8	7.1
SH261	6.8	5.1
B/Victoria-like		
GF21	7.8	6.1
TK942	7.8	6.1
SHD7	7.8	6.1

FM1: A/FM/1/47; BK10:A/Bangkok/10/83; YG120: A/Yamagata/120/86; YG32: A/Yamagata/32/89; AI2: A/Aichi/2/68; FKC29: A/Fukuoka/C29/85; KK159: A/Kitakyushu/159/93; WH539: A/Wuhan/359/95; SD5: A/Sydney/5/97; YN166: B/Yamanashi/166/98; JB5: B/Johannesburg/5/99; SH261: B/Shanghai/361/02; GF21: B/Gifu/2/73; TK942: B/Tokyo/942/96; SHD7: B/Shangdong/7/97; BD: beads fraction; TL: total sample containing the same quantity of AF as BD.

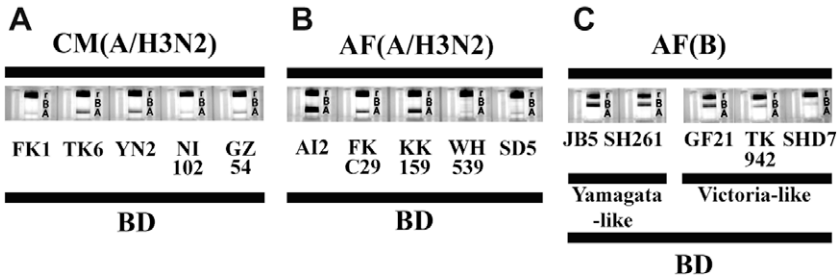

Figure 6. Detection of NP in influenza A virus (H3N2) and influenza B virus absorbed on poly(MVE-MA)-coated magnetic beads from cell culture medium (CM) and AF. Influenza A viruses (H3N2) in infected CM (A) and AF (B) and influenza B viruses (Yamagata-like and Victoria-like) in infected AF (C) were diluted with PBS and subjected to incubation with poly(MVE-MA)-coated magnetic beads. Esprine Influenza A and B-N (Fujirebio Inc., Tokyo, Japan) was used for detection of influenza NP by immunochromatography. Results regarding the presence of influenza A viruses (A) and influenza B viruses (B) were interpreted on the basis of the presence and absence of a line, with positive control, which is included for internal kit positive control (r). The beads fraction (BD) of samples was solubilized with lysis buffer and subjected to immunochromatography. Influenza A virus H3N2 strains such as FK1: A/Fukuoka/1/70, TK6: A/Tokyo/6/73, YN2: A/Yamanashi/2/77, NI102: A/Niigata/102/81, GZ54: A/Guizhou/54/89, AI2: A/Aichi/2/68, FKC29: A/Fukuoka/C29/85, KK159: A/Kitakyushu/159/93, WH539: A/Wuhan/359/95, and SD5: A/Sydney/5/97 were used. Influenza B viruses such as JB5: 5/Johannes Burg/5/99, SH261: B/Shanghai/361/02, GF21: B/Gifu/2/73, TK942: B/Tokyo/942/96, and SHD7: B/Shangdong/7/97 were also used.

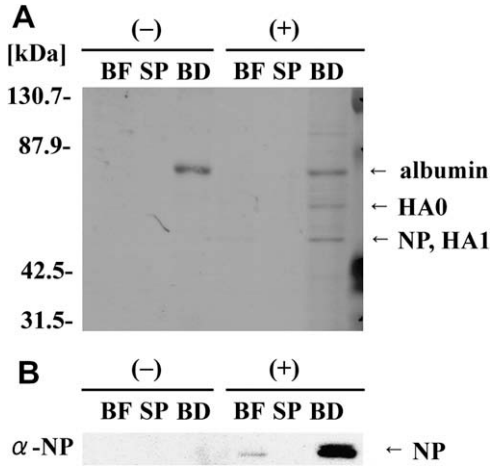

Figure 7. Detection of proteins in influenza A virus recovered with poly(MVE-MA)-coated magnetic beads using Coomassie brilliant blue (CBB) staining and Western blotting. Influenza A virus (A/PR8/34) in 500μl of diluted CM infected with the virus (+) or left uninfected (−) was captured by anionic magnetic beads. Samples were divided into three categories; a beads fraction (BD), supernatant after the incubation (SP), and sample before incubation with the beads (BF), which were solubilized with sodium dodecyl sulfate (SDS)-gel-loading buffer and separated by SDS–polyacrylamide gel electrophoresis (PAGE) and analyzed by CBB (A) and Western blotting (B) with anti-influenza A virus NP. The bands corresponding to albumin, NP, HA0, and HA1 in CBB and NP in Western blotting are shown. NP showed doublet bands by Western blotting, possibly due to cleavage of NP by caspase.[31,32] This is consistent with previous reports of NP in influenza A virus (A/PR8/34)-infected MDCK cells.[33,34]

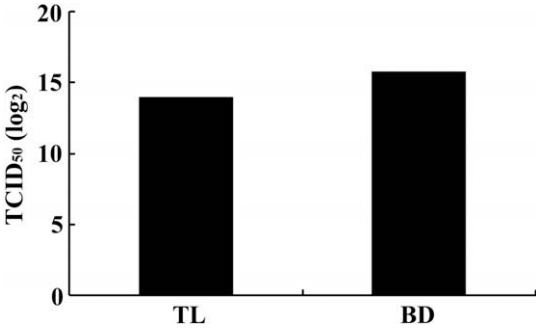

Figure 8. Efficient recovery of infectivity using poly(MVE-MA)-coated magnetic beads from CM. Influenza A virus (A/PR8/34) in CM of infected MDCK cells was captured by poly(MVE-MA)-coated magnetic beads. The beads fraction (BD) and sample containing the same quantity of CM as the BD (TL) were subjected to a $TCID_{50}$ assay as described in Section 4. Results of the $TCID_{50}$ are shown as $\log_2$ values.

Table 3
Capture of infectious influenza A virus (A/PR8/34) by poly(MVE-MA)-coated magnetic beads and evaluation of efficiency by infecting eggs

	Egg infection (positive/total)	Hemagglutination titer ($\log_2$)		
(+) BD	2/3	8	7	<1
(+) TL	2/3	14	9	<1
(−) BD	0/3	<1	<1	<1

Aliquots of influenza A virus (A/PR8/34)-infected CM (+) or mock-infected CM (−) were captured with poly(MVE-MA)-coated magnetic beads. The resultant beads fraction (BD) and total sample containing the same quantity of AF as BD (TL) were used to infect embryonated eggs as described in Section 4. The infection of eggs were determined by immunochromatography and hemagglutination assay of the AFs from the eggs. Rates of infection are shown as the positive number per total number. Results of the hemagglutination assay are shown as a hemagglutination titer ($\log_2$).

influenza virus was efficiently captured by the anionic magnetic beads without a decrease in infectivity.

3. Discussion

Conventional methods of concentrating viruses such as PEG precipitation and ultracentrifugation are relatively complex, reduce infectivity, and are incompatible with viral detection methods. The use of magnetic beads coated with a bioadhesive polymer or other molecule is promising. Polyethyleneimine (PEI), a water-soluble and highly positively charged polyamine, has been studied extensively as a vehicle for gene delivery.[14] Reports showed that PEI could be used to concentrate simian virus 40 (SV40),[15] herpes simplex virus type1 (HSV-1),[15] Sindbis virus,[15] vesicular stomatitis virus (VSV),[15] amphotropic murine leukemia virus (MuLV),[16] poliovirus (PV),[13] hepatitis A virus (HAV),[13] hepatitis B virus (HBV),[13] hepatitis C virus (HCV),[13] and cytomegalovirus (CMV).[17] Negatively charged lipids or proteins are proposed to be involved in the interaction.[15] Sulfonated magnetic (SO-magnetic) beads in the presence of divalent cations were also reported to efficiently concentrate CMV,[17] Sindbis virus,[17] PV,[17] and porcine parvovirus.[17] Recently, it has been shown that polycationic materials such as poly-L-arginine, protamine, and chitosan have the potential to promote absorption of biological materials.[18–21] However, the above reports did not deal with the recovery of infectivity but merely showed a reduction of infectivity after incubation and recovery of the viral genome. Furthermore, the proteins concentrated using PEI-coated beads were not viral proteins but serum proteins such as complement and IgM.[13] In the present study, we examined the recovery and concentration of infectivity and components of influenza A and B viruses using magnetic beads coated with an anionic and bioadhesive polymer, poly(MVE-MA). Poly(MVE-MA) and its derivatives are copolymers with molecular and physicochemical characteristics that permit them to be used like a bioadhesive.[11] They occur in water as an ionized free acid form and therefore are soluble.[11] The highly polar polymeric free acid is highly negatively charged in water and could be used for endowing an anionic charge after the grafting of the anionic polymer to magnetic beads. They also have large numbers of reactive groups[22] and are able to efficiently capture biomolecules including glycine,[11] phosphatidylcholine,[11] oligonucleotides,[23] enzymes,[24] and calcium.[25] Although the mechanism by which poly(MVE-MA)-coated magnetic beads bond to influenza A and B viruses remains unclear, components on the viral surface such as proteins and lipids, and sugar chains may contribute to the binding. Our preliminary studies have shown that HBV having envelope proteins could not be captured by the poly(MVE-MA)-coated anionic magnetic beads (Sakudo, unpublished result). Therefore, the binding seems not to be dependent on the presence of envelope proteins. A recent preliminary report has shown that the spatial organization of the polymer affected binding activity.[26] Moreover, interestingly, beads were aggregated after incubation with influenza A and B virus-infected samples (Supplemental Fig. 1). The aggregation may be related to the viral capture mechanism and have some contribution to the binding between influenza viruses and beads. It should be noted that this phenomena resembles the hemagglutination in chicken erythrocytes. Regretfully, as this aggregation was caused by not only influenza viruses but also RSVs (Sakudo, unpublished result), the phenomenon is not dependent on hemagglutination by HA of influenza viruses. Regardless, charge density and steric spatial organization would provide useful information on the binding mechanism. In addition, as described above, attention should be paid to non-specific binding to other components such as albumin. Such caution was also recommended in the reaction of poly(MVE-MA) with oligonucleotides.[23] Therefore, this method can be used to concentrate but not purify influenza viruses. However, modifications affecting the charge density and steric spatial organization of the polymer through surface chemistry may reduce non-specific binding.

There is increasing recognition of the contribution of polymers to the design of experimental tools. Concentrating viruses using poly(MVE-MA)-coated magnetic beads is compatible with conventional methods of detecting influenza viruses such as immunochromatography, hemagglutination assay, and Western blotting. Our preliminary results have shown that the enzyme-linked immunosorbent assay (ELISA) and PCR could be compatible, too (Sakudo, unpublished result). More importantly, the influenza viruses recovered using the beads had fully preserved infectious activity in eggs and cells. The efficient capture of infectious influenza viruses from humans may enhance the early development of vaccines for newly emerged strains. Presently, the mechanisms by which viruses infect cells in the form of a non-specific poly(-MVE-MA)-virus complex, remain unknown. However, as several studies have shown that poly(MVE-MA) derivatives can be used as a vehicle for intracellular transfer,[27] viruses may be incorporated into cells in the form of the complex and infect cells. In the present study, we confirmed the broad applicability of this method using 5 H1N1 influenza A viruses, 10 H3N2 influenza A viruses, and 6 influenza B viruses. To date, we have shown its applicability using human influenza A viruses (H1N1 and H3N2) (this study), human influenza B viruses (this study), avian influenza viruses (H5N1, H5N2, and H5N3),[28] and RSVs (Sakudo, unpublished result). The method has great potential for finding emerging viruses by enhancing isolation and so contributing to global public health.

4. Experimental

4.1. Reagents

Unless otherwise specified, chemical reagents were obtained from Sigma (St. Louis, MO) or Wako Pure Chemical Industries (Osaka, Japan). Small (300 nm in diameter) monozide magnetic particles (reducing sedimentation and offering a broad surface) with a high ferrite content (allowing for face separation under a magnetic field) prepared by the grafting of poly(MVE-MA) in dimethyl sulfoxide (DMSO)/phosphate buffer 5/95 solution for 3 h at 37 °C were used.[26] These anionic beads, Viro-adembeads, are available commercially (Ademtech, Pessac, France).

4.2. Virus

A/PR8/34 (A/H1N1), A/FM/1/47 (A/H1N1), A/Bangkok/10/83 (A/H1N1), A/Yamagata/120/86 (A/H1N1), A/Yamagata/32/89 (A/H1N1), A/Fukuoka/1/70 (A/H3N2), A/Tokyo/6/73 (A/H3N2), A/Yamanashi/2/77 (A/H3N2), A/Niigata/102/81 (A/H3N2), A/Guizhou/54/89 (A/H3N2), A/Aichi/2/68 (A/H3N2), A/Fukuoka/C29/85 (A/H3N2), A/Kitakyushu/159/93 (A/H3N2), A/Wuhan/359/95 (A/H3N2), A/Sydney/5/97 (A/H3N2), B/Yamanashi/166/98 (B/Yamagata-like), B/Johannesburg/5/99 (B/Yamagata-like), B/Shanghai/361/02 (B/Yamagata-like), B/Gifu/2/73 (B/Victoria-like), B/Tokyo/942/96 (B/Victoria-like), and B/Shangdong/7/97 (B/Victoria-like) were used.

4.3. Collection of nasal aspirates

Clinical nasal aspirates were collected from pediatric patients at the Baba pediatric clinic between December 2006 and March 2007. The method of collection was described previously.[29] Briefly, saline was introduced into the nasal cavity, and fluid was collected using a nasal aspirator, Belvital (Melisana, Nogent-sur-Marne, France). In order to remove cell debris, the nasal fluid was filtered using a stainless steel mesh [200 grids per inch (25.4 mm)]. The nasal aspirates were subjected to immunochromatography for influenza A and B viruses as described below. Nasal aspirates similarly collected from healthy donors were also used. The research project for the development of diagnostic methods for respiratory infectious diseases was approved by the Ethics Committee of the Research Institute for Microbial Diseases in Osaka University and written informed consent was obtained from patients and healthy donors.

4.4. Capture of influenza viruses

Viral capture was performed as described previously.[28] Briefly, after two washes with binding buffer, the magnetic beads (50 μl) were further washed twice with phosphate-buffered saline (PBS). Then, 20 μl samples of AF, NAS or CM infected with influenza viruses were diluted with 500 μl of PBS and incubated with the washed magnetic beads for 20 min at room temperature. The tubes containing the beads were set under a magnetic field. Then, the beads were subjected to magnetic separation by discarding the supernatants, and washed three times with PBS. The washed beads were resuspended with PBS and subjected to a hemagglutination assay, immunochromatography, egg infection, or $TCID_{50}$ assay.

4.5. Hemagglutination assay

Samples were diluted serially two- or threefold in 25 μl of PBS in U-shaped well plates, an equal volume of 1% chicken erythrocytes in suspension was added, and then incubation was conducted at room temperature for 1 h. The agglutination pattern was read and the hemagglutination titer was defined as the reciprocal of the last dilution of sample that showed hemagglutination.

4.6. Immunochromatography

Immunochromatography for the influenza A and B virus nucleoprotein was performed using Epsrine Influenza A and B-N (Fujirebio Inc., Tokyo, Japan).

4.7. $TCID_{50}$ assay

Viral titers were determined by performing twofold serial dilutions of samples in 96-well plates containing MDCK cells. Before infection, cells were washed with PBS. Infected cells were incubated at 37 °C with 5% CO_2 and fresh trypsin was added (final concentration, 0.03%). Viral titers were read as a $\log_2$ value of $TCID_{50}$, calculated by the method of Reed & Muench.[30]

4.8. Western blotting and CBB staining

Each fraction was solubilized in an equal volume of 2× sodium dodecyl sulfate (SDS) gel-loading buffer [90 mM Tris–HCl (pH 6.8), 10% mercaptoethanol, 2% SDS, 0.02% bromophenol blue, and 20% glycerol], boiled for 5 min, and separated on a SDS–8% PAGE before being electrically transferred onto a PVDF membrane (Hybond-P; Amersham-Pharmacia Biotech, Piscataway, NJ) for 60 min at 15 V. Blots were treated with 5% skim milk for 1 h at room temperature and then incubated with a polyclonal anti-human influenza A virus NP antibody (IMG-5134A, IMGENEX Co.) in PBS containing 0.1% Tween 20 (PBS-T) and 0.5% skim milk for 1 h at room temperature. After three washes with PBS-T, the membrane was incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS-T and 0.5% skim milk for 1 h at room temperature. After three washes with PBS-T, the probed proteins were detected using an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech). The proteins transferred onto the PVDF membrane were also visualized with CBB staining.

4.9. Infection of eggs

Infectivity was determined with 100-μl inoculums using standard procedures. The inoculation was conducted in 11-day-old chicken embryonated eggs. The beads fraction (BD) and sample containing the same quantity of starting material as BD (TL) from culture medium of MDCK cells infected with influenza virus (A/PR8/34) were subjected to inoculation. AF was collected from the eggs 48 h post-inoculation and subjected to immunochromatography and a hemagglutination assay.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.11.046.

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