FULL PAPER *Virology*

Improvement of a Rapid Diagnosis Kit to Detect Either Influenza A or B Virus Infections

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ABSTRACT. To improve the sensitivity of a kit, ESPLINE® INFLUENZA A&B for rapid diagnosis of influenza by detecting influenza A or B virus specific nucleoproteins (NP), the ESPLINE® INFLUENZA A&B-N was developed by using newly established monoclonal antibodies (MAbs) to the respective NP molecule. MAbs FVA2–11 and FrB1–03 recognize the epitope on the amino acid region 59–130aa of the NP molecule of influenza A virus, and that on the region 72–191aa of the NP of influenza B virus, respectively. The new kit detected influenza A and B virus antigens with a detection limit of $10^{2.0}$–$10^{2.7}$ pfu/test, which is 4–1000 times higher than that of the original kit. Importantly, this kit detected each of influenza A viruses of the known hemagglutinin (HA) subtypes (H1-H15) including the H5N1 viruses recently isolated from human and avian sources in Asia. The kit also detected all of the 15 representative influenza B virus strains tested. The ESPLINE® INFLUENZA A&B-N is thus a rapid and highly sensitive and specific kit for the diagnosis of either influenza A or B virus infections.

KEY WORDS: influenza, monoclonal antibody, rapid diagnosis, sensitivity.

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Influenza A viruses infect humans and other mammals and birds, whereas influenza B viruses exclusively infect humans with an exception of infections of seals [12]. All of the influenza A virus hemagglutinin (HA) subtypes H1-H15 [16] and possibly H16 [4] are circulating in aquatic birds, especially in migratory ducks in nature. It has been shown that pigs are susceptible to influenza A viruses of each of the known HA subtypes and the generation of genetic reassortants in the cells lining the upper respiratory tract of pigs upon concurrent infection with influenza virus strains of avian and mammalian origin is an indication that avian viruses of any HA subtype could contribute genes in the production of reassortants [9]. It is, therefore, evident that each virus of the known HA and neuraminidase (NA) subtypes has the potential to provide genes to the virus which may cause future pandemics. In addition, direct transmission of H5N1 avian influenza A viruses to humans in 1997 and during 2003–2005, and H9N2 in 1999 in Asia, as well as another one caused by H7N7 influenza virus in the Netherlands, signaled the necessity to have information on the epidemiology of avian influenza worldwide [3, 10, 13].

The rapid and specific detection of influenza viruses is of significant importance in influenza monitoring and control programs as well as in patient management. The rapid tests for the detection of influenza viruses can easily be used in health care offices or small laboratories that lack complex diagnostic capabilities [1, 2]. In addition, differentiation of influenza A and B viruses may provide health care takers with valuable information regarding possible treatment and prophylaxis. Early detection of infection is of cardinal importance since anti-influenza virus medications are most effective when they are given in the first two days of the onset of symptoms [7]. The influenza virus NP antigen detection kit, ESPLINE® INFLUENZA A&B, has been shown to be specific and widely used for the rapid diagnosis of influenza A and B viruses. To improve its sensitivity, newly established monoclonal antibodies (MAbs) were used to develop the ESPLINE® INFLUENZA A&B-N. In the present study, we evaluated the new kit for the detection of influenza A and B viruses and demonstrated that the sensitivity had improved by 4–1000 times higher than that of the original one.

MATERIALS AND METHODS

Cells: Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% bovine serum.

Viruses: In the present study, 37 human, 19 equine, 8 swine, one seal, and 43 avian influenza A virus strains, and 15 influenza B virus strains were used.

The human influenza A virus strains were A/PR/8/34 (H1N1), A/New Jersey/8/76 (H1N1), A/USSR/92/77 (H1N1), A/Brazil/11/78 (H1N1), A/Chile/1/83 (H1N1), A/Taiwan/1/86 (H1N1), A/Texas/36/91 (H1N1), A/Beijing/262/95 (H1N1), A/Johannesburg/82/96 (H1N1), A/New Caledonia/20/99 (H1N1), A/Hokkaido/11/02 (H1N1), A/Singapore/1/57 (H2N2), A/Adachi/2/57 (H2N2), A/Aichi/2/68 (H3N2), A/Port Chalmers/1/73 (H3N2), A/Texas/1/77 (H3N2), A/Bangkok/1/79 (H3N2), A/Philippines/2/82 (H3N2), A/Mississippi/1/85 (H3N2), A/Leningrad/360/86

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(H3N2), A/Sichuan/2/87 (H3N2), A/England/427/88 (H3N2), A/OMS/5389/88 (H3N2), A/Shanghai/16/89 (H3N2), A/Guizhou/54/89 (H3N2), A/Shanghai/24/90 (H3N2), A/Beijing/32/92 (H3N2), A/Shandong/9/93 (H3N2), A/Kitakyushu/159/93 (H3N2), A/Johannesburg/33/94 (H3N2), A/Sydney/5/97 (H3N2), A/Panama/2007/99 (H3N2), A/Hokkaido/1/03 (H3N2), A/Bangkok/157/03 (H3N2), A/Chanthaburi/230/03 (H3N2), A/Hong Kong/156/97 (H5N1), and A/Hong Kong/483/97 (H5N1).

The equine influenza virus strains were A/equine/Miami/1/63 (H3N8), A/equine/Tokyo/2/71 (H3N8), A/equine/Kentucky/1/81 (H3N8), A/equine/Suffolk/89 (H3N8), A/equine/Alaska/1/91 (H3N8), A/equine/Kentucky/1/91 (H3N8), A/equine/Rome/5/91 (H3N8), A/equine/Taby/91 (H3N8), A/equine/Hong Kong/92 (H3N8), A/equine/Lambourn/22778/92 (H3N8), A/equine/Avesta/1/93 (H3N8), A/equine/La Plata/1/93 (H3N8), A/equine/Newmarket/1/93 (H3N8), A/equine/Newmarket/2/93 (H3N8), A/equine/Kentucky/1/94 (H3N8), A/equine/La Plata/1/95 (H3N8), A/equine/La Plata/1/96 (H3N8), A/equine/Prague/1/56 (H7N7), and A/equine/Newmarket/1/77 (H7N7).

The swine virus strains were A/swine/Iowa/15/30 (H1N1), A/swine/Niigata/1/77 (H1N1), A/swine/Miyagi/3/03 (H1N2), A/swine/Miyagi/5/03 (H1N2), A/swine/Miyagi/7/03 (H1N2), A/swine/Hong Kong/81/78 (H3N2), A/swine/Hong Kong/126/82 (H3N2), and A/swine/Hong Kong/10/98 (H9N2).

The seal virus strain was A/seal/Massachusetts/1/80 (H7N7).

The avian influenza virus strains were A/duck/Mongolia/116/02 (H1N1), A/duck/Mongolia/253/03 (H1N1), A/duck/Hokkaido/17/01 (H2N3), A/duck/Mongolia/174/03 (H2N3), A/duck/Hong Kong/347/78 (H3N1), A/duck/Hokkaido/28/03 (H3N8), A/duck/Czechoslovakia/56 (H4N6), A/duck/Mongolia/107/03 (H4N6), A/chicken/Yamaguchi/7/04 (H5N1), A/chicken/Thailand/142–5/04 (H5N1), A/chicken/Thailand/144–47/04 (H5N1), A/chicken/Thailand/144–54/04 (H5N1), A/chicken/Thailand/144–99/04 (H5N1), A/chicken/Thailand/152–1/04 (H5N1), A/chicken/Suphanburi/1/04 (H5N1), A/duck/Angthong/71/04 (H5N1), A/quail/Angthong/72/04 (H5N1), A/crow/Osaka/102/04 (H5N1), A/duck/Pennsylvania/10128/84 (H5N2), A/duck/Mongolia/54/01 (H5N2), A/turkey/Massachusetts/3740/65 (H6N2), A/duck/Hokkaido/108/03 (H6N8), A/duck/Hokkaido/98/04 (H6N8), A/duck/Hokkaido/139/04 (H6N8), A/chicken/Italy/99 (H7N1), A/turkey/England/63 (H7N3), A/chicken/Pakistan/95 (H7N3), A/chicken/Netherlands/03 (H7N7), A/duck/Mongolia/555/02 (H7N7), A/duck/Mongolia/142/03 (H7N7), A/turkey/Ontario/6118/68 (H8N4), A/turkey/Wisconsin/1/66 (H9N2), A/chicken/aq-Y-55/01 (H9N2), A/chicken/Germany/N/49 (H10N7), A/duck/Mongolia/149/03 (H10N5), A/duck/England/56 (H11N6), A/duck/Hokkaido/85/97 (H11N9), A/duck/Alberta/60/76 (H12N5), A/duck/Hokkaido/66/01 (H12N5), A/gull/Maryland/704/77 (H13N6), A/mallard/Astrakhan/263/82 (H14N5), A/duck/Australia/341/83 (H15N8), and A/duck/Hokkaido/W2/04 (H15N8).

The influenza B virus strains were B/Lee/40, B/Hong Kong/8/73, B/Singapore/222/79, B/Norway/1/84, B/Ann Arbort/1/86, B/Beijing/1/87, B/Victoria/2/87, B/Yamagata/16/88, B/Panama/45/90, B/Harbin/7/94, B/Shandong/7/97, B/Yamanashi/166/98, B/Hokkaido/26/99, B/Chanthaburi/218/03, and B/Bangkok/227/03.

These viruses were prepared from the repository of viruses in our laboratory and propagated in MDCK cells or 10-day-old embryonated chicken eggs.

Expression of panels of NP fragments of influenza A and B viruses: The recombinant NP of influenza A virus (A/NP) and its truncated fragments A/NP 1–159aa, A/NP 162–327aa, A/NP 327–498aa, and A/NP 59–130aa, and that of the influenza B virus (B/NP) and the truncated fragments B/NP 1–200aa, B/NP 190–330aa, B/NP 320–560aa, and B/NP 72–191aa were prepared. These truncated proteins were used for the identification of their respective epitopes. The NPs and the fragments were prepared from the NP genes of A/New Calidonia/20/99 (H1N1) and B/Yamanashi/166/98, respectively. The NP gene segments were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) and digested by proper endonucleases and ligated into the pW6A expression vector [5] produced from pGEX-2T (Amersham Biosciences). The resulting constructs were transformed into *Escherichia coli* competent cells BL21(DE3) (Novagen) for the expression of panels of NPs. The expressed NPs were purified by a DEAE Sepharose Fast Flow System (Amersham Biosciences) according to the instructions of the manufacturer. The flow-through was applied onto a 50%, 30%, and 15% sucrose gradient and centrifuged at 100,000 × g for 15 hr. The fractions were then analysed by SDS-PAGE. The fraction containing the target protein was dialyzed against 5% sucrose solution for 20 hr. The dialyzed fraction was then applied onto 50% and 15% sucrose gradient and centrifuged as above and the resulting purified protein fraction was used as antigen in this study.

Production of MAbs against NPs of influenza A and B viruses: The MAbs against the NPs of influenza A and B viruses were produced as previously described [8]. Briefly, spleen cell donor BALB/c mice were immunized with the respective recombinant NPs of influenza virus strains A/New Caledonia/20/99 (H1N1) or B/Yamanashi/166/98. The recombinant NPs of the respective viruses were used for the screening of the MAbs-producing hybridoma cells by ELISA. The hybridoma cells producing the MAbs were cloned by limiting dilution and the MAbs were purified from the supernatant fluids of the hybridoma cell cultures.

Establishment of ESPLINE® INFLUENZA A&B-N kit: The immunochromatography and enzyme immunoassay kit was established for the rapid simultaneous detection of influenza A and B viruses. In the assay system, MAbs against the NP of influenza A or B viruses were divided into two parts, one for the capture line on the nitrocellulose membrane and the other for labeling with the alkaline phosphatase. The IgG Fc fragments of the MAbs FVA2–11 and FrB1–03 for influenza A and B viruses, respectively, were

removed and only the IgG Fab fragments were used in subsequent experiments. The IgG Fab fragments of the MAbs against the NP of either influenza A or B viruses were labeled with alkaline phosphatase. The anti-alkaline phosphatase antibodies were fixed at the reference line. The substrate BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) that migrated with the activation buffer reacted with the alkaline phosphatase on the nitrocellulose membrane. When a specimen containing the corresponding viral antigen was dropped onto the kit, a sandwich complex was formed at the judgement line and reacted with the substrate. The newly developed ESPLINE® INFLUENZA A&B-N kit indicated influenza A or B positive results when blue lines were formed on the influenza A or B judgement lines, as well as on the control line. The development of the color on the control line only was indicative of negative results for influenza A or B virus antigens.

Determination of the sensitivity of the ESPLINE® INFLUENZA A&B-N kit: The analytical sensitivity was assessed for two human, one seal influenza A and one influenza B virus strains. Serial 10-fold dilutions of each virus were made and each of the dilutions of the samples was inoculated onto the MDCK cell monolayers for plaque assay [14] and concurrently tested by the kit. The analytical sensitivity was the lowest virus titer (pfu/test) detectable by the kit and was expressed as $\log_{10}$ pfu/test.

Western blotting analysis: Western blotting analysis was used for epitope mapping [15]. The fragments of the recombinant NPs of influenza A and B viruses were separated by 12.5% SDS-polyacrylamide gel electrophoresis. Separated proteins were then electrophoretically transferred to a nitrocellulose transfer membrane (Schleicher & Schuell Bioscience, Germany). The membrane was then blocked with 1% non-fat milk and treated with MAbs FVA2–11 and FrB1–03 against NP of influenza A and B viruses, respectively. Finally, the membrane was treated with peroxidase-labelled goat anti-mouse IgG (Dako Cytomation). Signals were detected by using 4-chloro-1-naphthol substrate (Sigma).

RESULTS

Production of MAbs: A total of 45 hybridoma clones secreting MAbs to the NP of influenza A virus and 51 clones to that of influenza B virus were obtained. Upon further screening, two highly reactive and specific IgG1 MAbs, FVA2–11 and FrB1–03 to the recombinant NPs of influenza A and B viruses, respectively, were selected for use in the diagnostic assay. After purification of the MAbs, their IgG Fab fragments were obtained by papain digestion and labeled with alkaline phosphatase.

Detection of different influenza virus strains with ESPLINE® INFLUENZA A&B-N kit: The kit reacted with each of the human influenza A virus strains tested; 11 H1N1, two H2N2, and 22 H3N2 strains, and two H5N1 strains isolated from humans in Hong Kong in 1997, and 15 influenza B virus strains. The kit also reacted with each of avian influenza virus strains of known HA (H1-H15) and NA (N1-N9) subtypes, swine influenza viruses of H1, H3, and H9 subtypes, equine influenza viruses of subtypes H3 and H7, and an H7N7 seal influenza virus. The sensitivity of the kit was assessed with A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2), A/seal/Massachusetts/1/80 (H7N7), and B/Lee/40 influenza viruses. The serially diluted samples (30 μl) were dropped onto the kit for testing. As shown in Table 1, the detection limit of the new kit was $10^{2.0}$–$10^{2.7}$ pfu/test for influenza A viruses and $10^{2.1}$ pfu/test for influenza B virus, whereas the detection limit of the original kit was $10^{3.1}$–$10^{5.7}$ pfu/test for influenza A viruses and $10^{3.1}$ pfu/test for influenza B virus. The sensitivity of the detection of influenza A and B virus antigens in the improved kit was 4–1000 times higher than that of the original one (Table 1).

Epitope mapping: To map the epitopes recognized by the MAbs on the NP molecule of influenza A virus, a series of influenza NP fragments were produced. Three NP fragments, A/NP full, A/NP 1–159aa, and A/NP 59–130aa gave positive reactions with MAb FVA2–11 in the Western blotting analysis (Fig. 1). On the other hand, no signal was detected with the fragments A/NP 162–327aa or A/NP 327–498aa. These results indicated that an epitope recognized by MAb FVA2–11 existed on the A/NP 59–130aa fragment.

A series of truncations of NP fragments of influenza B virus were also prepared. MAb FrB1-03 reacted with B/NP full, B/NP 1–200aa, and B/NP 72–191aa, but not with B/NP 190–330aa and B/NP 320–560aa fragments of influenza B virus (Fig. 2). These results indicated the presence of an epitope recognized by MAb FrB1-03 on the B/NP 72–191aa fragment.

Table 1. Sensitivity of ESPLINE® INFLUENZA A&B and ESPLINE® INFLUENZA A&B-N kits

Kits	Detection limits for these viruses ($\log_{10}$ pfu/test)			
	PR/8/34 (H1N1)	Aichi/2/68 (H3N2)	Seal/Mass/80 (H7N7)[a)]	B/Lee/40
ESPLINE® INFLUENZA A&B	3.9	3.1	5.7	3.1
ESPLINE® INFLUENZA A&B-N	2.0	2.5	2.7	2.1

a) A/seal/Massachusetts/1/80 (H7N7).

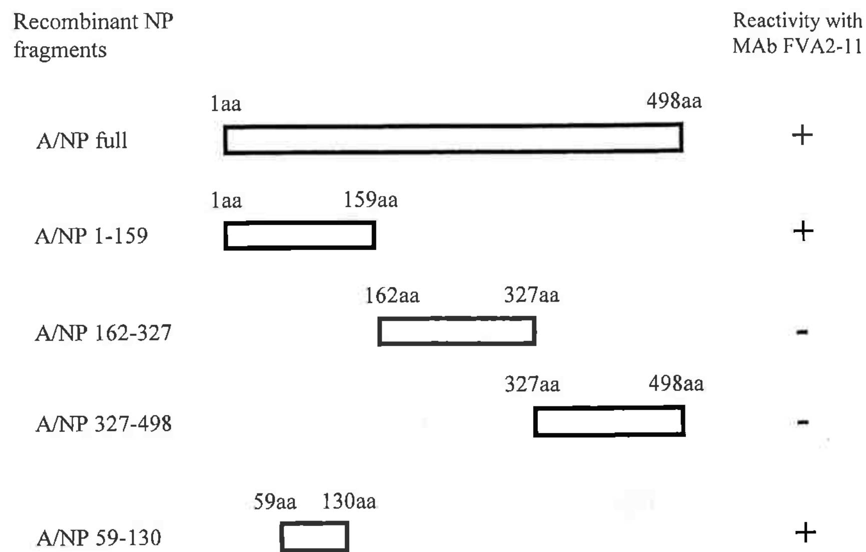

Fig. 1. Determination of the epitope of the FVA2–11 MAb on the NP of influenza A viruses. The Western blotting analysis was used for the determination of the epitope. The A/NP full, A/NP 1–159aa, A/NP 162–327aa, A/NP 327–498aa, and A/NP 59–130aa indicate the lengths of the recombinant NP fragments of the influenza A virus used in this study. The three NP fragments, A/NP full, A/NP 1–159aa, and A/NP 59–130aa gave positive reactions with the MAb FVA2–11. On the other hand, no signal was found with the fragments A/NP 162–327aa or A/NP 327–498aa. The results indicated that the fragment A/NP 59–130 had an epitope recognized by the MAb FVA2–11.

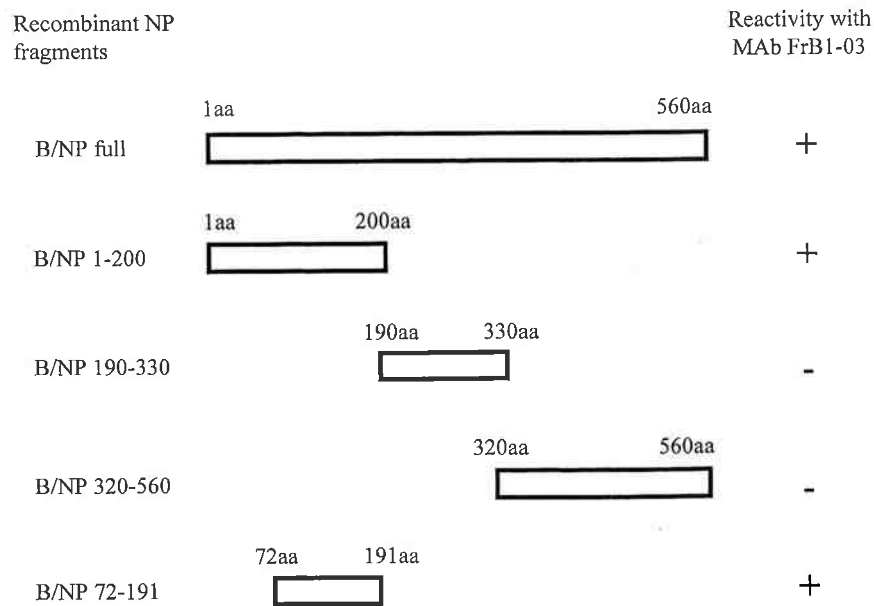

Fig. 2. Determination of the epitope of the FrB1–03 MAb on the NP of influenza B viruses. The B/NP full, B/NP 1–200aa, B/NP 190–330aa, B/NP 320–560aa, and B/NP 72–191aa indicate the lengths of the recombinant NP fragments of the influenza B virus used in this study. The MAb FrB1–03 reacted with B/NP full, B/NP 1–200aa and B/NP 72–191aa but not with the B/NP 190–330aa, and B/NP 320–560aa fragments of the influenza B virus. The results show that the B/NP 72–191aa fragment had an epitope recognized by MAb FrB1–03.

DISCUSSION

In the present study, it has been demonstrated that the ESPLINE® INFLUENZA A&B-N kit is useful for the detection of influenza A and B viruses and can differentiate between them. The reactivity of the kit was assessed on representative human and animal influenza viruses. Each of influenza viruses of human, equine, swine, seal, and avian origin was detected by the kit. The kit is of valuable importance in influenza monitoring and control programmes as it has potential for use in the early detection of influenza outbreaks in humans and animals. All tested influenza A virus strains were detected, indicating that a conserved epitope that is also recognized by MAb FVA2–11 is present in the 37 strains of human and 71 strains of animal influenza A viruses.

Using Western blotting analysis for epitope mapping (Fig. 1), the fragment of the 59–130aa domain of influenza A virus reacted with MAb FVA2–11, whereas, those of the 162–327aa and 327–498aa domains did not. The results indicated that MAb FVA2–11 recognized an epitope on the 59–130aa domain of NP of influenza A viruses. Five hydrophilic regions have been found in the 59–130aa domain by sequence analysis of 66 influenza A viruses (data not shown). The results demonstrated that the domain was highly conserved on the NP molecule in human and animal influenza A virus strains.

In addition, to the use of the newly developed MAbs FVA2–11 and FrB1–03, the labeling of the Fab fragments of the MAbs with alkaline phosphatase may have significantly contributed to the enhancement of the sensitivity of this new kit by 4–1000 times higher than that of the original one. In Table 1, the results of sensitivity were shown only for three subtypes (H1N1, H3N2 and H7N7) of influenza A viruses. Our preliminary investigations demonstrated that the sensitivities of this improved kit for all other influenza A viruses including highly pathogenic H5N1 avian influenza viruses were of the same or higher level than those of the original kit (data not shown). Additionally, no cross-reactivity of the improved kit with a panel of 50 other microorganisms that included 30 respiratory viruses and 20 bacteria was found (data not shown). Further practical study should be necessary to evaluate the usefulness of this new kit for the clinical samples of human and animal influenza.

The detection time is very short since only 15 min are required and performance of the test and interpretation of the results do not require an expert technologist. PCR-based assays offer alternative methods for the diagnosis of influenza virus infections [6, 11]. They potentially have high sensitivity and specificity, but require skill and complex laboratory infrastructure and take several hours to perform. It is concluded that the ESPLINE® INFLUENZA A&B-N is a useful, rapid, reliable, convenient, and simple test for the diagnosis of influenza A and B virus infections in both humans and animals. Clearly, the kit is a valuable addition to the tests already available for the diagnosis of influenza. However, virological examinations including virus isolation should be carried out for the evaluation of the diagnosis by the kit and further characterization of the isolated virus.

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