ed for rapid diagnosis of influenza in experimentally infected chickens and pigs. The swab samples and tissue homogenates, 30 μl, were dropped onto the kit. After 15 min, positive samples showed double blue lines and negative samples, only a control line.

Cells. Madin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with 10% bovine serum. For the titration of viruses, the cells were cultured in Eagle's minimal essential medium without bovine serum.

Viruses and other microorganisms. A/chicken/Yamaguchi/7/04 (H5N1) (13, 14) and A/chicken/aq-Y-55/01 (H9N2) (12) were used as HPAI and LPAI viruses, respectively. A/swine/Hokkaido/2/81 (H1N1), A/swine/Miyagi/5/03 (H1N2), A/swine/Nakorn Pathom/02 (H3N2), and A/duck/Pennsylvania/10128/84 (H5N2) were used for the inoculation of miniature pigs. A/swine/Nakorn Pathom/02 (H3N2), and A/duck/Pennsylvania/10128/84 (H5N2) (19) were obtained from Dr. S. Parchariyanon, National Institute of Animal Health, Thailand, and Dr. R.G. Webster, St. Jude Children's Research Hospital, U.S.A., respectively. Other microorganisms were used to test the specificity of the kit for avian influenza-like disease. These included the avian paramyxovirus (APMV) strains NDV/duck/Mongolia/705/02 and Chicken/California/Yucaipa/56 from the repository of viruses in our laboratory, as well as avian infectious bronchitis virus strain B-42, infectious laryngotracheitis virus strain NS175, fowl poxvirus strain Sishui, *Mycoplasma gallisepticum* strain CP5PT and *Haemophilus paragallinarum* strain HK-1, which were purchased from the Japanese Association of Veterinary Biologics (Tokyo).

To further evaluate the specificity of the kit, swine viruses and bacteria were also tested. These included Aujeszky's disease virus strain Y-S81, classical swine fever virus strain GPE$^-$, transmissible gastroenteritis virus strain TO-163, porcine reproductive and respiratory syndrome virus strain 93-259, *Haemophilus parasuis* strain HA66, *Erysipelothrix rhusiopathiae* strain NF4-E1, *Salmonella choleraseuis* strain B-90, *Pasteurella multocida* strain No. 6, and *Bordetella bronchiseptica* strain Bb-11, all purchased from the Japanese Association of Veterinary Biologics.

Experimental infection of chickens and miniature pigs with influenza viruses. Four-week-old specific pathogen-free (SPF) white leghorn chickens were hatched and raised in our laboratory. The chickens were infected intranasally with 100 μl of allantoic fluid containing A/chicken/Yamaguchi/7/04 (H5N1) at a 50% egg infectious dose (EID_{50}) of $10^{4.0}$. Tracheal and cloacal swabs were collected daily from the infected chickens for up to 4 days post-inoculation (p.i.) by which time all the chickens had died. In addition, tissue samples of the trachea, lungs, colon, spleen, kidneys, and liver were collected from sacrificed or dead chickens. A separate group of chickens was infected with A/chicken/aq-Y-55/01 (H9N2) at an EID_{50} of $10^{8.8}$. Tracheal and cloacal swabs were collected daily from the infected chickens for up to 8 days p.i. since all chickens survived the infection. Some chickens were sacrificed and tissue samples of the trachea, lungs, colon, spleen, kidneys, and liver were taken daily. The tracheal and cloacal swabs were kept in transport medium containing 10,000 U/ml of penicillin and 10 mg/ml of streptomycin (10). The tissue specimens were homogenized, tracheal, and cloacal swabs were inoculated onto MDCK cell monolayers, and viral titers were calculated (18).

Two-month-old SPF miniature pigs (Sankyo Labo Service Co., Tokyo) were intranasally inoculated with 0.5 ml of allantoic fluid containing $10^{6.2}$ EID_{50} of A/swine/Hokkaido/2/81 (H1N1), $10^{8.4}$ EID_{50} of A/swine/Miyagi/5/03 (H1N2), $10^{8.0}$ EID_{50} of A/swine/Nakorn Pathom/02 (H3N2), and $10^{9.2}$ EID_{50} of A/duck/Pennsylvania/10128/84 (H5N2) for each miniature pig. Nasal swabs were collected in transport medium once a day for 14 days p.i. and inoculated onto MDCK cell monolayers. Viral titers were then calculated.

Each pig and groups of 2–4 chickens were housed in self-contained isolator units (Tokiwa Kagaku, Tokyo) with a separate negative-pressure HEPA ventilated isolator. The experimental infection studies were performed in a biosafety containment level III (BSL 3) facility in the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Evaluation of the kit on healthy chickens and pigs. The specificity of the kit was further evaluated using swab samples of healthy chickens and pigs. Tracheal and cloacal swab samples were taken from a total of 212, 24-month old chickens obtained from two different chicken farms in Hokkaido, Japan, and nasal swabs were taken from a total of 220 pigs in Okinawa, Japan. The tracheal and cloacal swabs of chickens and the nasal swabs of pigs were kept in transport medium and aliquots were concurrently tested using the kit and inoculated into 10-day old embryonated chicken eggs and MDCK cells for the isolation of viruses.

Results

Detection of Antigens of Influenza A Viruses in Swabs and Tissue Homogenates from Infected Chickens

Ten chickens were inoculated with the HPAI virus A/chicken/Yamaguchi/7/04 (H5N1). From 36 hr p.i.,

the chickens showed clinical signs such as swelling of the face, depression, ruffled feathers, and dyspnea, and viral antigens were detected in the tracheal swabs and homogenates of the liver, kidney, and colon. The chickens died within 4 days and viruses were recovered from each of the tissues examined at high titers from the dead chickens. The kit detected antigens from 1 day p.i. in the kidney homogenates and from 2 days p.i. in the tracheal swabs. All swab and homogenate samples from 3 dead chickens were positive (Table 1). Meanwhile, no clinical signs were observed in the chickens infected with the LPAI virus A/chicken/aq-Y-55/01 (H9N2). Virus was recovered from the tracheal organs of the chickens from 1 to 3 days p.i. The kit gave positive results at 1, 2, 3 and 4 days p.i. in the tracheal swabs, and from 1 to 3 days p.i. in tracheal homogenates, during which time the virus was also recovered in cell cultures (Table 2). The kit detected viral antigens in samples that had titers of at least $10^{3.4}$ $TCID_{50}$/g or ml.

Detection of Influenza A Virus Antigens in Nasal Swabs of Miniature Pigs

As shown in Table 3, swine influenza viruses A/swine/Hokkaido/2/81 (H1N1) and A/swine/Nakorn Pathom/02 (H3N2) were isolated from 1 to 7 days p.i. and A/swine/Miyagi/5/03 (H1N2) was isolated from 1 to 8 days p.i. Viral antigens were also detected with the kit in the samples positive by virus isolation except for the nasal swab of A/swine/Hokkaido/2/81 (H1N1) on 1 day p.i. Meanwhile, the avian virus A/duck/Pennsylvania/10128/84 (H5N2) was transiently isolated 2 and 3 days p.i. with viral antigens being detected at 2 days p.i. only. No viruses or viral antigens were detected after 9

Table 1. Antigen detection in swabs and tissue homogenates of chickens infected with A/chicken/Yamaguchi/7/04 (H5N1) using the ESPLINE® INFLUENZA A&B-N kit and virus isolation

Days	Swabs		Tissue homogenates					
p.i.	Trachea	Cloaca	Trachea	Lung	Liver	Spleen	Kidney	Colon
1	−/−[a]	−/−	−/3.1	−/3.1	−/3.1	−/2.6	−/3.1	−/−
	−/−	−/−	−/3.3	−/3.1	−/3.1	−/−	−/−	−/−
	−/−	−/−	−/3.1	−/2.9	−/3.1	−/3.1	+/3.4	−/−
2	+/3.9	−/−	−/3.1	−/2.9	−/2.9	−/3.3	−/3.1	−/3.1
	−/−	−/−	−/2.9	−/3.1	+/3.9	+/3.9	+/4.1	+/3.9
	+/8.1[b]	+/8.1	+/7.9	+/7.9	+/8.0	+/7.6	+/7.6	+/7.2
3	+/8.6	+/7.9	+/8.1	+/7.6	+/8.1	+/7.6	+/7.8	+/7.6
	−/−	−/−	−/3.1	−/3.3	+/4.1	−/3.1	+/3.6	+/3.9
4	+/8.3[b]	+/7.9	+/7.8	+/7.6	+/8.1	+/7.6	+/7.6	+/6.9
	+/8.6[b]	+/8.1	+/7.8	+/7.6	+/7.6	+/7.2	+/7.6	+/6.9

[a] ESPLINE® INFLUENZA A&B-N kit/Virus titers (log_{10} $TCID_{50}$/ml for swabs and log_{10} $TCID_{50}$/g for organs) in MDCK cells.

[b] dead.

−: <1.1 log_{10} $TCID_{50}$/ml in swabs, <2.1 log_{10} $TCID_{50}$/g in organs.

Table 2. Antigen detection in swabs and tissue homogenates of chickens infected with A/chicken/aq-Y-55/01 (H9N2) using the ESPLINE® INFLUENZA A&B-N kit and virus isolation

Days	Swabs		Tissue homogenates					
p.i.	Trachea	Cloaca	Trachea	Lung	Liver	Spleen	Kidney	Colon
1	+/3.6[a]	−/−	+/3.6	−/3.1	−/3.1	−/3.3	−/3.3	−/3.1
	+/3.6	−/−	+/3.6	−/3.1	−/3.1	−/3.3	−/3.3	−/3.3
2	+/3.8	−/−	+/3.6	−/3.1	−/−	−/3.1	−/3.3	−/3.1
	+/3.8	−/−	+/3.6	−/2.6	−/2.6	−/2.6	−/3.1	−/3.1
3	+/3.4	−/−	+/3.4	−/−	−/−	−/−	−/−	−/−
	+/3.4	−/−	+/3.4	−/−	−/−	−/−	−/−	−/−
4	+/3.4	−/−	−/−	−/−	−/−	−/−	−/−	−/−
	+/3.4	−/−	−/−	−/−	−/−	−/−	−/−	−/−
5	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−

[a] ESPLINE® INFLUENZA A&B-N kit/Virus titers (log_{10} $TCID_{50}$/ml for swabs and log_{10} $TCID_{50}$/g for organs) in MDCK cells.

−: <1.1 log_{10} $TCID_{50}$/ml in swab, <2.1 log_{10} $TCID_{50}$/g in organs.

Table 3. Antigen detection in nasal swabs from infected miniature pigs using the ESPLINE® INFLUENZA A&B-N kit and virus isolation

Days p.i.	Inoculum			
	Sw/Hokkaido/2/81 (H1N1)	Sw/Miyagi/5/03 (H1N2)	Sw/Nakorn Pathom/02 (H3N2)	Dk/Pennsylvania/10128/84 (H5N2)
0	−/−[a]	−/−	−/−	−/−
1	−/3.3	+/3.7	+/4.5	−/−
2	+/4.0	+/6.5	+/5.0	+/4.7
3	+/5.7	+/5.0	+/6.5	−/1.7
4	+/5.0	+/4.7	+/4.5	−/−
5	+/5.3	+/3.5	+/4.5	−/−
6	+/6.7	+/4.3	+/4.3	−/−
7	+/2.7	+/2.5	+/3.7	−/−
8	−/−	+/2.5	−/−	−/−
9	−/−	−/−	−/−	−/−

[a] ESPLINE® INFLUENZA A&B-N kit/Virus titers (Log_{10} $TCID_{50}$/ml for swabs) in MDCK cells.
−: <1.1 Log_{10} $TCID_{50}$/ml in swabs.

days p.i. in the pigs. Interestingly, the sample of A/swine/Hokkaido/2/81 (H1N1) with $10^{3.3}$ $TCID_{50}$/ml on day 1 p.i. was not detectable, but that of A/swine/Miyagi/5/03 (H1N2) with $10^{2.5}$ $TCID_{50}$/ml on day 8 p.i. could easily be detected (Table 3).

Evaluation of the specificity of the kit showed that there was no cross reactivity with other viruses and bacteria used in this study (data not shown). In addition, all the 212 tracheal and cloacal samples taken from apparently healthy chickens from poultry farms and 220 nasal swabs of healthy pigs from farms were negative using the kit as well as virus isolation (data not shown).

Discussion

There are various rapid and highly sensitive diagnostic kits for human influenza including the ESPLINE® INFLUENZA A&B-N (2, 6, 8), but none have been tested on animals. We, here, have evaluated the use of the ESPLINE® INFLUENZA A&B-N kit to detect influenza A viruses in experimentally infected animals for the first time. The kit detected viral antigens in tracheal and cloacal swabs and tissue homogenates of dead chickens infected with the HPAI virus A/chicken/Yamaguchi/7/04 (H5N1). It also detected viral antigens in tracheal swabs and tissues of chickens infected with the LPAI virus A/chicken/aq-Y-55/01 (H9N2) from 1 to 4 days p.i. These results are consistent with those of virus isolation in MDCK cells. All samples in which viral titers were more than $10^{3.4}$ $TCID_{50}$/g or ml, tested positive with this kit. This detection limit for animal specimens is consistent with that for *in vitro* samples determined previously ($10^{3.5}$–$10^{4.2}$ pfu/ml) by Bai et al. (2). The HPAI and LPAI viruses showed different replicative forms. The HPAI virus induced a systemic infection and titers of swabs and organs in dead chickens were higher than the limit of detection for this kit (Table 1). On the other hand, replication of the LPAI virus was restricted to areas like respiratory organs and the titers of tracheal swabs and tracheal tissues were enough to detect viral antigens (Table 2). It is evident that tracheal samples are most suitable for the diagnosis of HPAI and LPAI using this kit.

Viral antigens were also detected in the nasal swabs of all the pigs infected with A/swine/Miyagi/5/03 (H1N2) and A/swine/Nakorn Pathom/02 (H3N2) from 1 to 7 days p.i., and with A/swine/Hokkaido/2/81 (H1N1) from 2 to 7 days p.i. As shown in Table 3, these results were largely in agreement with those obtained by virus isolation, except for A/swine/Hokkaido/2/81 (H1N1) in which antigens could not be detected 1 day p.i. with the kit despite a titer of $10^{3.3}$ $TCID_{50}$/ml. Interestingly, the titer of $10^{3.3}$ $TCID_{50}$/ml was not detectable 1 day p.i. even though a lower titer, $10^{2.5}$ $TCID_{50}$/ml, could be detected 8 days p.i. This result could be attributed to the increased accumulation of the viral nucleoproteins, the targets of the kit, on the nasal mucosa relative to the progression of the disease. Additionally, infectivity titers of virus dropped during the late infection since virus-antibody complexes were formed due to the immune response.

As shown previously (10), pigs were susceptible to avian influenza virus strain A/duck/Pennsylvania/10128/84 (H5N2) and viral antigens were detected on day 2 p.i. in the nasal swab using this kit although the period of virus shedding was short and the viral titer was low as compared with swine isolates. The surveillance of influenza viruses in pigs and identification of avian influenza viruses in pig populations will contribute to the prediction of new pandemic viruses. The

present results indicate that this antigen detection kit will be a useful tool for the surveillance of influenza viruses in pig populations.

RT-PCR assays have been used to detect influenza viruses in throat and nasal specimens collected from humans, pigs, and horses (3, 6, 7, 15–17). They potentially have high sensitivity, but require a high level of skill and complex laboratory infrastructure and take several hours to perform. They do not necessarily fall into the category of tests that are rapid and easy to perform with a low level of expertise. It takes only 15 min to obtain results with this detection kit and interpretation of the results does not require expertise. The kit would enable early detection of infections of influenza viruses, and the implementation of appropriate control measures in time to abate epidemics. The kit is, thus, recommended as a tool for screening flocks even in live-bird markets and nature. Nevertheless, virus isolation is the standard method for the diagnosis of influenza as it provides for the characterization of viruses.

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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.

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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

(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).

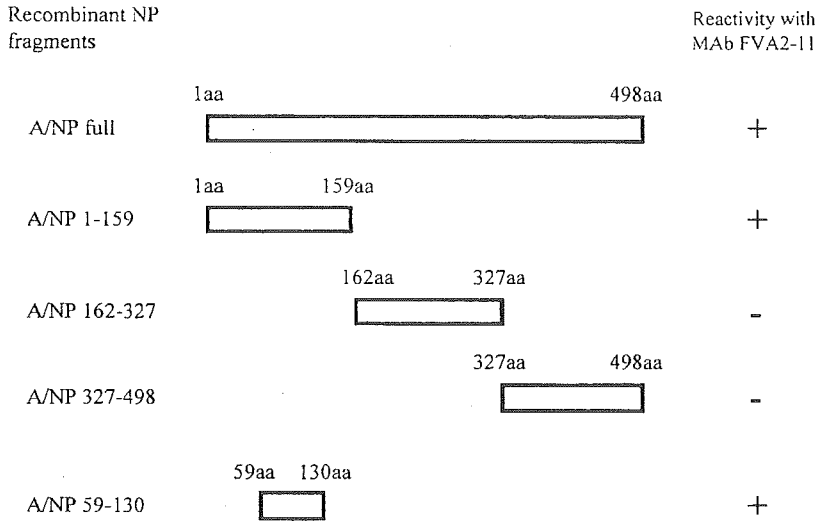

Fig. 1. Determination of the epitope of the FVA2–11 MAb on the NP of influenza A viruses. The Western blotting analysis was used for the determination of the epitope. The A/NP full, A/NP 1–159aa, A/NP 162–327aa, A/NP 327–498aa, and A/NP 59–130aa indicate the lengths of the recombinant NP fragments of 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.

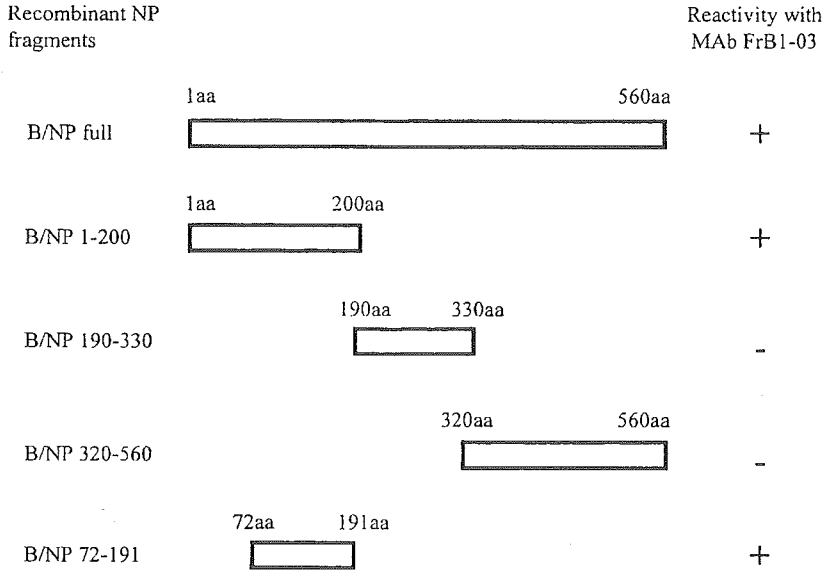

Fig. 2. Determination of the epitope of the FrB1–03 MAb on the NP of influenza B viruses. The B/NP full, B/NP 1–200aa, B/NP 190–330aa, B/NP 320–560aa, and B/NP 72–191aa indicate the lengths of the recombinant NP fragments of the influenza B virus used in this study. The MAb FrB1–03 reacted with B/NP 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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Editor-Communicated Paper

Highly Pathogenic H5N1 Influenza Virus Causes Coagulopathy in Chickens

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Abstract: **Severe hemorrhage at multiple organs is frequently observed in chickens infected with highly pathogenic avian influenza (HPAI) A viruses. In this study we examined whether HPAI virus infection leads to coagulation disorder in chickens. Pathological examinations showed that the fibrin thrombi were formed in arterioles at the lung, associated with the viral antigens in endothelial cells of chickens infected intravenously with HPAI virus. Hematological analyses of peripheral blood collected from the chickens revealed that coagulopathy was initiated at early stage of infection when viral antigens were detected only in the endothelial cells and monocytes/macrophages. Furthermore, gene expression of the tissue factor, the main initiator of blood coagulation, was upregulated in the spleen, lung, and brain of HPAI virus-infected chickens. These results suggest that dysfunction of endothelial cells and monocytes/macrophages upon HPAI virus infection may induce hemostasis abnormalities represented by the excessive blood coagulation and consumptive coagulopathy in chickens.**

Key words: **H5N1, Avian influenza virus, Blood coagulation**

Influenza A viruses are found in a variety of birds and mammals. Viruses of 16 hemagglutinin (HA) and 9 neuraminidase subtypes have been identified in aquatic birds, the natural reservoir of influenza A viruses (7, 14, 25, 39). These viruses occasionally transmit to other animals and cause disease. Some viruses of particular HA subtypes (H5 and H7) are known to cause highly pathogenic avian influenza (HPAI) in chickens. In comparison with avirulent influenza viruses that induce only local infection in the respiratory or gastrointestinal tracts with subclinical or mild disease (28), HPAI viruses induce lethal systemic infection in chickens. The different pathogenicity of these viruses for chickens is based on the susceptibility of HA to proteolytic cleavage (11, 26). The HPAI viruses have multiple basic amino acids at the cleavage site of HA, which is susceptible for proteolytic activation with ubiquitous endoproteases such as furin (12, 29). Therefore, HPAI viruses are able to infect cells of a broad range of the tissues in chickens, leading to systemic infection with viremia. On the other hand, HA of avirulent influenza viruses has only a single arginine at the cleavage site, which is activated only by trypsin-like proteases at restricted organs, resulting in local infection.

In systemic infection by HPAI viruses, chickens generally show severe depletion, hemorrhages, edema, cutaneous ischemia, and neurological signs and finally

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die within a few days. Particularly, hemorrhages are observed at multiple organs, for example, lungs, leg shanks and intestines (17, 30, 32). Such severe large hemorrhages imply that HPAI viruses induce blood coagulation disorder to chickens, since blood coagulation plays an important part for the cessation of blood loss from a damaged vessel.

When blood vessel is injured, bleeding initiates the platelet aggregation and the blood coagulation to form the clots. Upon the aggregating of the platelets, the subendothelial coagulation factor named tissue factor initiates extrinsic coagulation cascade by activation of the coagulation factor VII, followed by the activation of the coagulation factors IX, X, VIII, and V, leading to thrombin and fibrin formation, and finally solid clots are generated (16). Tissue factor is constitutively present on the cell membrane of fibroblasts and pericytes in and around blood vessels (18, 20). By contrast, monocytes and endothelial cells contain very little tissue factor activity in normal conditions. But proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-6 induce the expression of tissue factor on those cells (9).

The high frequency of the severe hemorrhage in HPAI virus-infected chickens implies that the blood coagulation disorder is induced. In this study, to examine whether the blood coagulation system is dysregulated upon HPAI virus infection, and if so, to elucidate the possible mechanism to cause the blood coagulation disorder and its implication with the pathogenesis of HPAI virus infection, we compared chickens infected with virulent and avirulent H5N1 viruses pathologically and hematologically.

Materials and Methods

Viruses. Two recombinant influenza viruses, HK156/836 and HK911 were generated by reverse genetics as described previously (33), using avirulent strain A/duck/Hong Kong/836/80 (H3N1) (HK836) as a helper virus. HK156/836 contains HA gene of highly pathogenic A/Hong Kong/156/97 (H5N1) (HK156) and all other genes from HK836. HK911 has the modified HK156 HA gene to express avirulent type of HA and all other genes from HK836 helper virus. Viruses were propagated in the allantoic cavity of 10-day-old embryonated hen's eggs. Virus stocks were stored at −80 C until use. Fifty percent egg infectious dose (EID_{50}) was determined by inoculating serial dilutions of the viruses into eggs, followed by hemagglutination test, using the method of Reed and Muench (24). HK156/836 virus was handled in a biosafety level 3 containment.

Experimental infection of chickens. Six-week-old female specific pathogen-free white leghorn chickens (Nisseiken, Yamanashi, Japan) were infected intravenously with 10^7 EID_{50} of HK156/836 or HK911. After infection, their clinical signs were monitored and blood samples were collected every 6 hr post infection (p.i.) from both chicken groups. The blood samples were treated with sodium citrate (final 0.2%) to prevent clotting and supplied for the examinations of virus titers, thrombocyte counts, and prothrombin times. The virus titers in the blood samples were examined by EID_{50}. All experimental animal studies were undertaken in accordance with the guideline on the Care and Management of Experimental Animals (Japan).

Histopathology and immunohistochemistry. HK156/836-infected chickens were sacrificed at 12 hr post inoculation (p.i.) (2 chickens) and 24 hr p.i. (4 chickens). The comb, lung, bursa, kidney, brain, liver, heart, and spleen were taken from those chickens. Samples from dead chickens were also collected at 18 hr p.i. (1 chicken) and 24 hr p.i. (2 chickens). Collected tissue samples were fixed with 10% formalin solution and embedded in paraffin and sectioned at 4 μm for histological and immunohistochemical examinations. The sections for immunohistological examination were placed on poly-L-lysine coated glass slides. They were deparaffinized, digested by trypsin, and endogenous peroxidase activity was quenched with 3% H_2O_2 in distilled water. After blocking of nonspecific reaction with normal goat serum for 30 min at 37 C, the sections were incubated for 24 hr at 4 C with rabbit anti-strain 499 (H5N3) hyperimmune serum at a 1:2,000 dilution (27). After 5-min wash with phosphate buffered saline (PBS), they were incubated with biotinylated goat anti-IgG antibody (DAKO, Glostrup, Denmark) for 1 hr at 37 C, followed by 5-min wash with PBS and 1 hr incubation at 37 C with peroxidase-conjugated streptavidin (DAKO). Specific reaction was visualized with diaminobenzidine and hydrogen peroxidase.

Counting of thrombocytes in blood samples. The blood samples treated with sodium citrate were smeared on slide glasses and fixed with methanol. The smear samples were stained using anti-chicken thrombocyte monoclonal antibody (HUKT) (13). The numbers of thrombocytes and red blood cells were counted in 20–30 different microscopic fields for each sample and thrombocyte counts are represented as a ratio to red blood cells per ml.

Prothrombin time assay. Prothrombin time of blood samples were measured using a fibrometer (Cobas Fibro, Roche, Basel, Switzerland) and the reagent for the detection of prothrombin time containing 0.5 mg/ml rabbit thromboplastin and 3.0 mg/ml calcium lactate (ThrombocheckPT, Daiichikagaku, Tokyo) according to

manufacturer's protocol. Briefly, after incubation at 37 C for 3 min, 0.1 ml of blood sample was mixed with 0.2 ml of ThrombocheckPT reagent. The time to fibrin-strand formation was monitored with fibrometer.

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Chickens infected with HK156/836 or HK911 were sacrificed at 15 hr p.i. and spleens, lungs, and brains were collected. Mock-infected allantoic fluids of the chicken embryo were inoculated into control chickens. Total RNA was extracted from those tissues by Isogen reagents (Nippon Gene, Tokyo) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 600 ng of total RNA using oligo(dT) as a mRNA-specific primer and Moloney murine leukemia virus reverse transcriptase (SuperScript III, Invitrogen, Calif., U.S.A.). cDNA was amplified in PCR thermal cycler using PCR master mix, 2X (Promega, Wis., U.S.A.) with primers specific for chicken tissue factor (GenBank accession no. LOC429084) sense: 5'-GCACTCCGGAATGTAAAG-GAGACCTATACAGCTC-3', antisense: 5'-GCACCGT-GCTTTCTTGACC-3' and GAPDH (22) sense: 5'-GTCTTCACCACCATGGAGAAGGC-3', antisense: 5'-CCAAAGTTGTCATGGATGACCTTGG-3'. The amplification profile for tissue factor involved denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec, and extension at 72 C for 40 sec. And that for GAPDH involved denaturation at 94 C for 30 sec, annealing at 56 C for 30 sec, and extension at 72 C for 30 sec. The reaction cycles of PCR were performed in the range that demonstrated a linear correlation between the amount of cDNA and the yield of PCR products (28 cycles: tissue factor mRNA in lung and brain, 29 cycles: tissue factor mRNA in spleen, and 25 cycles: GAPDH mRNA in lung, brain, and spleen). The PCR products were separated by electrophoresis in 1.5% agarose gels and visualized by ethidium-bromide staining and inspection under UV light. The PCR products were found to be of the expected size, i.e., 430 base pairs for chicken tissue factor and 205 base pairs for chicken GAPDH.

Results

Pathogenicity of Recombinant H5N1 Viruses for Chickens

We prepared two recombinant viruses HK156/836 and HK911 that have different cleavability of HA. HK156/836 has HA from a highly pathogenic strain HK156. HK911 has a modified form of HK156 HA and lacks multiple basic amino acids at the cleavage site (33). All other genes of both recombinant viruses are provided from HK836 strain.

To study pathogenicity of these two viruses, chickens were infected intravenously with 10^7 EID_{50} of HK156/836 or HK911. Chickens infected with HK156/836 showed severe depression peracutely, while HK911-infected chickens did not. Figure 1A shows the survival rates of infected chickens at each time point. One chicken infected with HK156/836 died at 12 hr p.i., and 71% of chickens in this group died within 24 hr p.i. On the other hand, chickens infected with HK911 showed no clinical signs and survived until sacrificed (7 days p.i.). Then, we determined viral titers in the peripheral blood (Fig. 1B). HK156/836 replicated efficiently within 12 hr p.i. and high titers of the virus were detected during the experimental period, while HK911 in the blood was almost undetectable (<10 EID_{50}). These results indicate that HA cleavability is the only factor controlling virus pathogenicity in this experimental system, since these viruses have identical background genes. Thus, these two recombinant viruses with different HA cleavability are a useful model to directly compare the pathogenic events including host responses between virulent and avirulent avian influenza virus infections.

Pathology and Immunohistochemistry of the Chickens Infected with HK156/836

To examine the target organs and the pathologic changes in HK156/836 infection, spleens, lungs, hearts, combs, brains, livers, and kidneys were collected from infected chickens at 12, 18, and 24 hr p.i. and analyzed pathologically and immunohistochemically. At 12 hr p.i., histological changes were not remarkable anywhere except ecchymotic hemorrhage in muscle fascicles of breast in a chicken. Viral antigens were detected in endothelial cells in almost all the organs tested as well as in parenchymal cells in the spleen (Table 1). Notably, these antigen-positive parenchymal cells in the spleen were monocytes/macrophages. In addition, monocytes/macrophages in the peripheral blood were also antigen-positive at 12 hr p.i. At 18 and 24 hr p.i., viral antigens were detected in the parenchymal cells in brains, combs, lungs, and hearts in addition to spleens of the birds. The remarkable degenerative changes were observed in the organs; spleen and bursa had necrosis of lymphocytes with nuclear fragmentation and pyknosis, brain showed the formation of multiple glial nodules with degenerative necrosis of neurons, combs had necrosis with edema and hemorrhages, bursa indicated hemorrhages, and hepatocytes had mild to moderate focal necrosis. Interestingly, fibrin thrombi, consisting of thrombocytes, fibrin, red blood cells, and heterophils, were found in arterioles at lungs in two chickens (No. 7 and 9) (Fig. 2), indicating that blood coagulation was

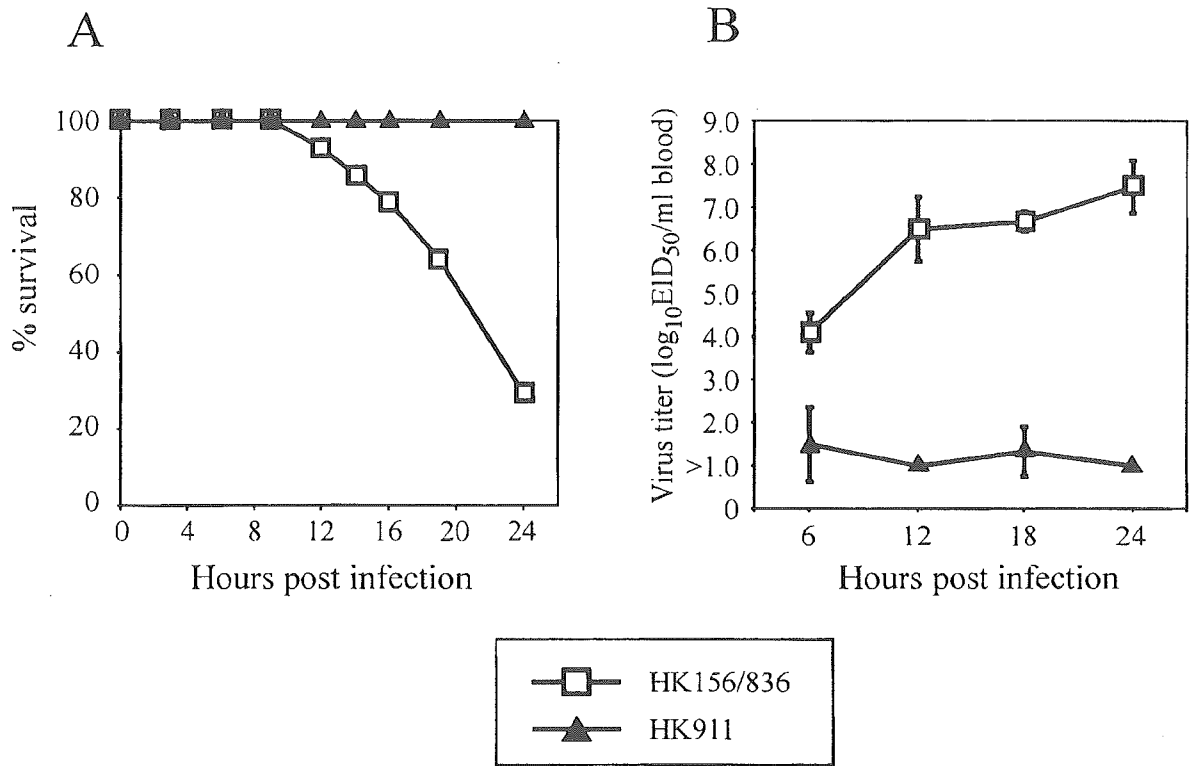

Fig. 1. Comparison of pathogenicity of HK156/836 and HK911 in chickens. (A) Survival rates of virus-infected chickens. Fourteen chickens were infected with HK156/836 intravenously and three chickens were infected with HK911. Chickens were monitored until 24 hr p.i. (B) Virus titers in the peripheral blood of chickens. Blood samples were collected every 6 hr after infection from three chickens infected with HK156/836 or HK911. Virus titers are shown as averages with standard deviations of three chickens of each group.

Table 1. Viral antigens detected in HK156/836-infected chickens in immunohistochemical analysis

Chicken No.	hr p.i.		Spleen		Lung		Heart		Comb		Brain		Liver		Kidney		Monocytes/ macrophages[a]
			E[b]	P[c]	E	P	E	P	E	P	E	P	E	P	E	P	
1	12	sacrificed	+	+	+	−	+	−	+	−	+	−	+	−	−	−	+
2	12	sacrificed	+	+	+	+	+	+	+	−	+	−	+	−	+	−	+
3	18	died	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
4	24	sacrificed	+	+	+	+	+	+	+	−	+	+	+	−	+	−	+
5	24	sacrificed	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+
6	24	sacrificed	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+
7	24	sacrificed	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+
8	24	died	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+
9	24	died	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+

[a] Monocytes/macrophages in peripheral blood samples.
[b] Endothelial cells.
[c] Parenchymal cells.

initiated in the vessels.

Coagulopathy Induced by HK156/836 Infection

We then examined the numbers of thrombocytes and the prothrombin time of the peripheral blood samples. It is known that thrombocyte is responsible for clot formation and that prothrombin time is an indicator for lack of blood coagulation factors VII, IX, X, prothrombin, or fibrinogen. We collected the peripheral blood from chickens infected with HK156/836 or HK911 every 6 hr sequentially, and counted the number of thrombocytes as described in "Materials and Methods"

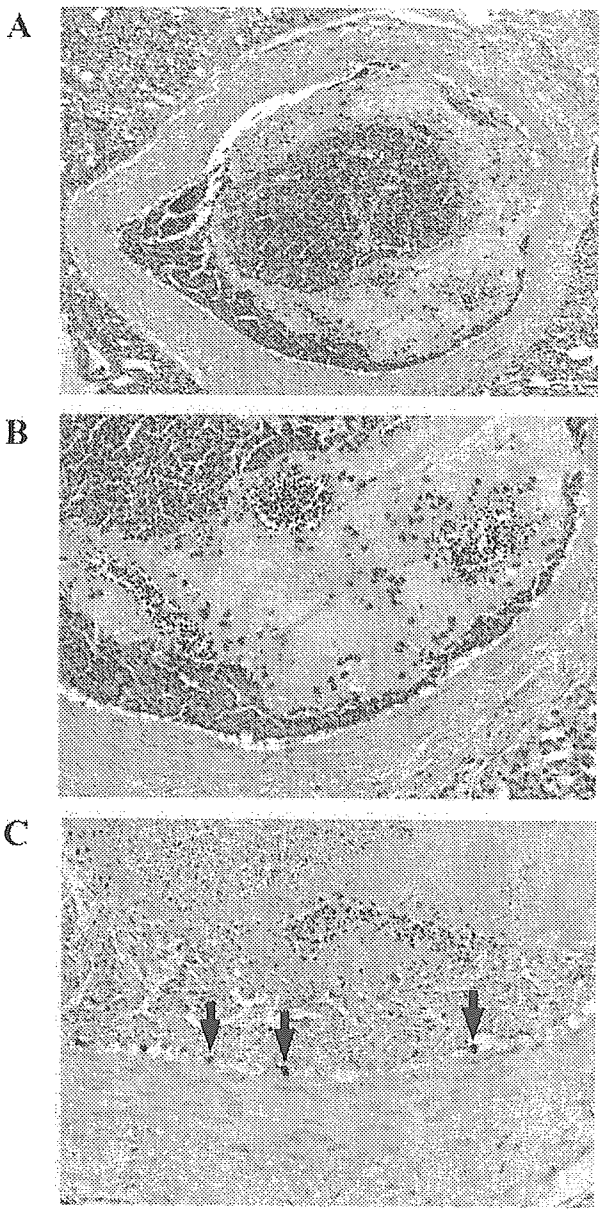

Fig. 2. Fibrin thrombus in the lung of HK156/836-infected chicken at 24 hr p.i. (A) Lower magnification photomicrograph of lung tissue in chicken with hematoxylin and eosin staining. Fibrin thrombus was found in the arteriole. (B) Higher magnification photomicrograph of fibrin thrombus with hematoxylin and eosin staining. The thrombus consists of thrombocytes, fibrin, red blood cells and heterophils. (C) Immunohistochemical analysis around the thrombus (higher magnification photomicrograph). Viral antigens were detected in the endothelial cells (arrows).

(Fig. 3A). We found that the number of thrombocytes significantly decreased in chickens infected with HK156/836 within 12 hr p.i. At 24 hr p.i., the number of thrombocytes dropped to approximately 18% of that collected before infection. By contrast, no appreciable decrease was observed in HK911-infected chickens. Prothrombin times of the peripheral blood were also significantly prolonged by HK156/836-infection (Fig. 3B). Prothrombin times of the sample collected at 12 and 24 hr p.i. were 5.9 and 7.8 sec longer, respectively than that of the sample collected before infection. On the other hand, HK911-infected chickens maintained baseline levels of prothrombin time during the experimental period. These results indicate that HK156/836 infection caused depletions of thrombocytes and coagulation factors in the peripheral blood, leading to severe coagulopathy in chickens.

Such acute depletions of thrombocytes and coagulation factors in the peripheral blood, as well as the formation of fibrin thrombi in vessels, implied that the acute and massive coagulation was induced in those chickens. Thus, to confirm whether HK156/836 indeed initiate blood coagulation, we examined the activation of tissue factor gene, the trigger of blood coagulation cascade, in the spleen, lung, and brain of virus-infected chickens by semiquantitative RT-PCR (Fig. 4). In most of the HK911-infected chickens, the transcripts of the tissue factor gene were under the level of detection in lungs and brains as was the case with mock-infected chickens. Although tissue factor transcripts were detected in the spleens of most of the chickens, HK911-infected chickens expressed slightly higher levels of the transcripts than mock-infected chickens. By contrast, HK156/836-infected chickens showed higher levels of the tissue factor expression in all organs tested than chickens of the other groups. These results suggest that increased level of tissue factor expression upon HPAI virus infection triggers tissue factor-mediated blood coagulation cascade, leading to consumptive coagulopathy in chickens.

Discussion

In this study, we showed that experimental infection of chickens with highly pathogenic HK156/836 caused coagulopathy in chickens, while avirulent HK911 did not. Since the only difference between these two viruses is HA cleavability, it was evident that the ability to undergo multiple replications in any organs is the critical determinant for their pathogenicity. As shown in Table 1, HK156/836 antigens were detected mainly in endothelial cells and monocytes/macrophages at the early stage of infection. Endothelial cells are thought to be one of the major targets for HPAI viruses in chickens as reported previously (3, 6, 19, 23, 30, 32). It is thus conceivable that the endothelial dysfunction by viral infection causes systemic hemorrhage manifestations in chickens.

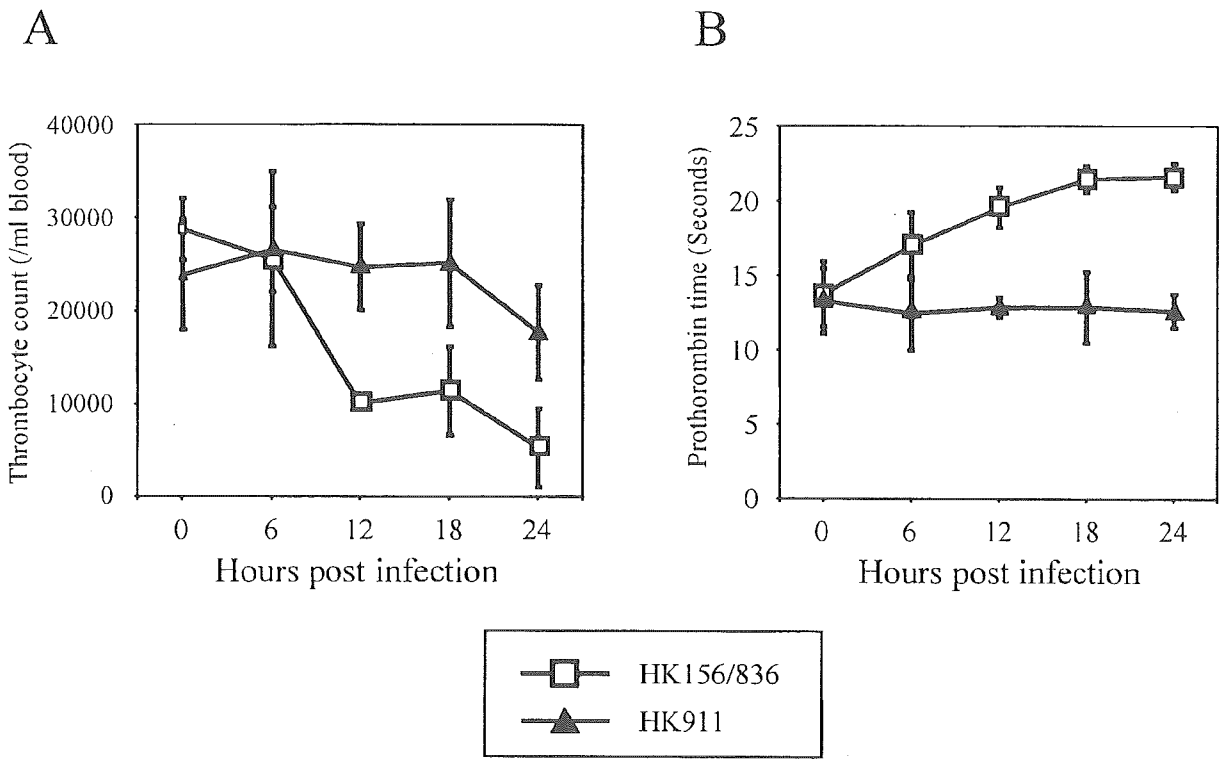

Fig. 3. Coagulopathy in chickens infected with HK156/836. (A) The number of peripheral blood thrombocytes in virus-infected chickens. Peripheral blood samples from three chickens infected with HK156/836 or HK911 were smeared on slide glasses and stained with anti-thrombocyte antibody. The number of thrombocytes was counted as described in "Materials and Methods." Averages and standard deviations of each group are shown. (B) Prothrombin time of peripheral blood in virus-infected chickens. Peripheral blood samples from three chickens infected with HK156/836 or HK911 were examined using an automated fibrometer. Averages and standard deviations of each group are shown.

Immunohistochemistry performed in this study could not clarify whether monocytes/macrophages were infected with the viruses, because the viral antigens detected might be the viral proteins phagocytosed. However, since it has been shown that influenza A viruses could infect chicken, human, and mouse monocytes/macrophages *in vitro* (2, 4, 10, 21, 38), it is conceivable that both endothelial cells and monocytes/macrophages are the major targets for HPAI viruses in chickens.

Interestingly, fibrin thrombi were found in arterioles at the lungs in HK156/836 virus-infected chickens, suggesting that the HPAI virus infection caused disseminated intravascular coagulation (DIC). DIC is a syndrome characterized by the excessive activation of coagulation cascade up to intravascular fibrin formation, accompanied by secondary fibrinolysis and coagulopathy for the consumption of platelets, coagulation factors, and fibrinogen. DIC is observed in a wide range of disease states including sepsis, burns, polytrauma, tumors, and viral infections. Some previous reports showed fibrin thrombi in HPAI virus infection in chickens, suggesting that DIC was induced in HPAI virus infections (1, 15, 17, 37). In the present study, the coagulopathy was indeed observed in the early stage of infection (12 hr p.i.), when virus replication was restricted mainly to endothelial cells and monocytes/macrophages. Thus, these findings suggest that replication of HPAI virus in endothelial cells and/or monocytes/macrophages triggers activation of coagulation cascade leading to DIC in infected chickens.

One possible mechanism underlying DIC in HPAI virus-infected chickens is the direct destruction of the large amount of endothelial cells by viral infection, which forms many clots to cover the large damaged regions in HPAI virus-infected endothelium, leading to the consumptive coagulopathy as suggested by Kobayashi et al. (17). The other possibility is that proinflammatory cytokines produced by HPAI virus infection may be involved in developing coagulation disorder. Proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 are the inducers of tissue factor, a trig-

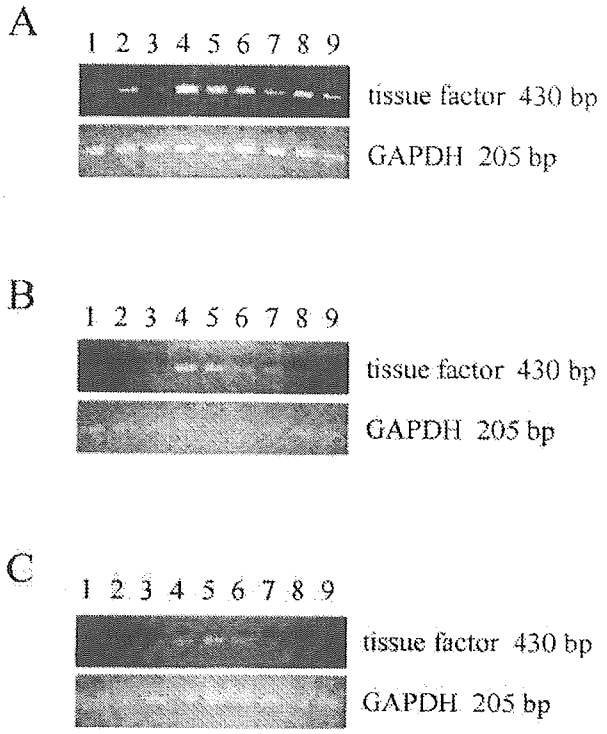

Fig. 4. Upregulation of the tissue factor mRNA in HK156/836-infected chickens. Three chickens from each group were sacrificed at 15 hr p.i. and spleens (A), lungs (B), and brains (C) were collected. Total RNA was extracted from each tissue and then tissue factor mRNA was detected by RT-PCR. RT-PCR for chicken GAPDH mRNA was also performed as a control. Lanes 1–3: mock-infected chickens, 4–6: HK156/836-infected chickens, 7–9: HK911-infected chickens.

ger of blood coagulation, on monocytes/macrophages and endothelial cells (9). It is also reported that these proinflammatory cytokines were secreted from human, rat, and mouse monocytes/macrophages infected with influenza A viruses *in vitro* (8, 10, 21). In addition, other *in vitro* studies showed that HPAI virus infection in human macrophages induced more intensive secretion of TNF-α than avirulent influenza A viruses did (4). Therefore, it is hypothesized that HPAI virus infection induces DIC by two independent mechanisms: 1) direct destruction of the endothelial cells, and 2) induction of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) from monocytes/macrophages to initiate massive coagulation.

In Hong Kong in 1997, highly pathogenic H5N1 avian influenza viruses, which were prevalent among the poultry, transmitted to humans (5, 31). Furthermore, since 2003 H5N1 HPAI viruses have been causing widespread disease not only in poultry but also in humans with many fatal cases in Asia. It was reported that some patients who progressed severe fatal influenza showed the following complications; coagulopathy, pulmonary hemorrhage, thrombocytopenia, elevation of TNF-α and so on (34–36, 40), although the H5N1 influenza virus replications were restricted in respiratory and gastrointestinal tracts in the patients (34, 36). The present results suggest a mechanism involved in pathogenesis of HPAI, which may have similarity to that of human H5N1 virus infection. Further studies are required to fully understand the pathogenesis of HPAI virus infection, which would facilitate the development of antiviral strategies against highly pathogenic influenza virus infections.

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Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals

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Summary. Outbreaks of highly pathogenic avian influenza (HPAI) have been occurring in domestic poultry in Asia since 1996. In the beginning of 2004, HPAI outbreaks were caused by H5N1 virus in two farms and a group of pet chickens in different areas of Japan. In the present study, the pathogenicity of A/chicken/Yamaguchi/7/04 (H5N1), which had been isolated from a dead chicken during the first outbreak in Japan, was assessed in chickens, quails, budgerigars, ducklings, mice, and miniature pigs by experimental infection. The virus was highly pathogenic to all the birds tested. Mice were susceptible to infection with a low mortality rate and miniature pigs were resistant to infection with the virus.

Introduction

A wide variety of species of birds and mammals are susceptible to influenza A virus infection. Viruses of all 16 hemagglutinin (HA) (H1–H16) and 9 neuraminidase (N1–N9) subtypes have been isolated from avian species [1, 6]. Aquatic birds are the natural reservoirs of influenza A viruses [12]. Influenza viruses are perpetuated in nature by continuing to circulate in migratory ducks and frozen lake water [9]. Based on the severity of the disease they cause in chickens, avian influenza viruses are divided into two groups, highly pathogenic and low pathogenic [1]. Low pathogenic avian influenza (LPAI) viruses replicate in limited tissues where host proteases such as trypsin-like enzymes are found. Highly pathogenic avian influenza (HPAI) viruses possess inserted multiple basic amino acid residues at the site of cleavage of their HAs into HA1 and HA2 by ubiquitous proteases such