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Development of an Immunochromatographic Kit for Rapid Diagnosis of H5 Avian Influenza Virus Infection

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Running title: Rapid Diagnosis kit for H5 influenza virus infection

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Abstract: Highly pathogenic avian influenza (HPAI) caused by the H5N1 subtype has given rise to serious damage in poultry industries in Asia. The virus has expanded its geographical range to Europe and Africa, posing a great risk to human health as well. For the control of avian influenza, a rapid diagnosis by detecting the causative virus and identifying its subtype is essential. In the present study, a rapid diagnosis kit combining immunochromatography with enzyme immunoassay which detects the H5 HA antigen of influenza A virus was developed using newly established anti-H5 HA monoclonal antibodies. The present kit specifically detected all of the H5 influenza viruses tested, and did not react with the other HA subtypes. H5 HA antigens were detected from swabs and tissue homogenates of chickens infected with HPAI virus strain A/chicken/Yamaguchi/7/04 (H5N1) from 2 days post inoculation. The kit showed enough sensitivity and specificity for the rapid diagnosis of HPAI.

Key word: Influenza A virus, H5 HA antigen, Diagnosis kit

Abbreviations: EID₅₀, 50% egg infectious dose; HA, hemagglutinin; HPAI, highly pathogenic avian influenza; HPAIV, HPAI virus; MAbs, monoclonal antibodies; MEM, Minimum essential medium; NASBA, Nucleic Acid Sequence Based Amplification; NP, nucleoprotein; p.i., post inoculation; RT-LAMP, Reverse Transcription - Loop-mediated Isothermal Amplification; RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction; WHO, World Health Organization.

Influenza A virus infects birds and mammals including humans, and is classified into subtypes H1~H16 and N1~N9 based on the antigenic specificity of hemagglutinin (HA) and neuraminidase, respectively (10, 16, 20). H5N1 HPAI virus (HPAIV) infections have spread widely in domestic poultry and feral water birds in 60 countries in East Asia, the Middle East, Europe, and Africa. In May 1997, the first fatal case of human infection with H5N1 HPAIV was reported in Hong Kong. Avian-to-human transmission of H5N1 virus occurred in 18 human cases, with 6 fatalities (6, 8). From the end of 2003 to July 2007, a total of 318 human cases of avian influenza A virus infection including 192 deaths were reported to WHO from Asia (Azerbaijan, Cambodia, China, Indonesia, Iraq, Lao, Thailand, Turkey, and Vietnam), and Africa (Djibouti, Nigeria, and Egypt). Most of the human cases were caused by direct transmission of the virus from chickens. Although a few cases of human-to-human transmission among family members have been suggested, no case of infection between wife and husband has been found (3, 17). There is now great concern that H5N1 HPAIV may change to a new pandemic influenza virus strain.

Therefore, a rapid diagnosis kit to detect infections of the H5 strain is essential for the control of influenza. Rapid diagnosis kits are available to detect influenza virus specific nucleoprotein (NP) of which antigenicity is common among the A or B type influenza viruses, but do not identify the HA subtype of influenza A virus (1, 4). Although gene amplification systems to detect the HA gene of H5 influenza virus have been developed, these methods require a high level of skill (7, 18).

In the present study, we developed a novel diagnosis kit to detect specifically

H5 HA antigen, and evaluated its usefulness for rapid diagnosis of H5 influenza virus infection.

Materials and Methods

Viruses. In the present study, 28 strains of influenza virus were used (Table 1). These viruses were propagated in 10-day-old embryonated chicken eggs. Chickens experimentally infected with A/chicken/Yamaguchi/7/04 (H5N1) were used for the detection of H5 HA antigen with the kit.

Development of a kit to detect H5 HA antigen. Hybridoma cell lines producing MAbs against the HA of A/duck/Pennsylvania/10218/84 (H5N2) were established and examined for reactivity with a series of H5 influenza virus strains by the immunofluorescent antibody test (Soda et al., submitted to Vaccine). Anti-mouse immunoglobulin antibodies and anti-H5 HA MAbs were immobilized onto a nitrocellulose membrane for the control and test judgment regions as capture antibodies, respectively. A reagent pad containing colloidal-gold-labeled anti-H5 HA MAbs was located between the judgment regions and the sample-dropping region. The immunochromatographic test was performed by adding 100 µl of test solution to the sample-dropping region. The membrane was observed visually after a 15-min incubation period at room temperature. A single colored line appearing in the control judgment region indicates the absence of the H5 HA antigen. The concurrent presence of colored lines in both the control and test judgment regions indicates the presence of the H5 HA antigen (Fig. 1).

Determination of specificity and sensitivity of the kit. For the evaluation of the specificity of the rapid diagnosis kit, 16 HA subtypes (H1-H16) and 11 H5 HPAIV strains of influenza virus were used. The reactivity of the kit was examined with allantoic fluid containing each virus (16~4096 hemagglutinin unit), and the appearance of colored lines in both the control and test judgment regions was determined as a positive result. The sensitivity of the kit was assessed for 4 H5 influenza viruses, A/duck/Pennsylvania/10218/84 (H5N2), A/chicken/Ibaraki/1/05 (H5N2), A/whooper swan/Mongolia/2/06 (H5N1), and A/chicken/Yamaguchi/7/04 (H5N1). Serial 4-fold dilutions of each virus were tested. In parallel, these viruses were inoculated into embryonated chicken eggs to determine the 50% egg infectious dose (EID₅₀). The sensitivity was the lowest virus titer detectable by the kit and was expressed as log₁₀ EID₅₀/test.

Experimental infection of chickens with influenza virus. Four-week-old chickens (Boris-brown) were purchased from Hokuren Central Breeding Farm (Hokkaido, Japan). The chickens were infected with 100 µl of allantoic fluid containing A/chicken/Yamaguchi/7/04 (H5N1) at 10^{5.3} EID₅₀. Tracheal and cloacal swabs were collected daily for up to 4 days post-inoculation (p.i.). These swabs were kept in 1ml of the extraction buffer provided from BL Co., Ltd. to detect the antigen, or kept in transport medium (MEM containing 10,000 U/ml Penicillin G, 10 mg/ml Streptomycin, 0.3 mg/ml Gentamycin, 250U/ml Nystatin, and 0.5% bovine serum albumin) for titration of infectivity. In addition, the trachea, kidney, and colon were collected from sacrificed or dead chickens. The 10% tissue homogenates were prepared with the transport medium as the test

samples. Samples were diluted 5-fold with the extraction buffer for the detection of the H5 HA antigens. The infectivity of the virus in the swabs and the tissue homogenates was expressed as EID₅₀/test.

Experimental infections were exclusively performed in a Biosafety Level 3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Results

Selection of the MAbs for the rapid diagnosis kit.

To develop a rapid diagnosis kit that specifically detects H5 HA antigen of influenza virus with high sensitivity, 144 combinations of 19 anti-H5 HA MAbs were tested as both capture and conjugated antibodies. Their reactivity was tested with inactivated H5 influenza virus A/chicken/yamaguchi/7/04 (H5N1). Ten combinations found to be highly sensitive were examined for reactivity with the different 5 H5 influenza viruses. The kit using Anti-H5 HA MAb 64/1 for both capture and conjugated antibodies reacted with each of the H5 strains tested at high sensitivity (data not shown).

Fig.1

Specificity and sensitivity of the rapid diagnosis kit.

The rapid diagnosis kit using anti-H5 HA MAb 64/1 was then evaluated for its specificity using H1-H16 HA subtypes and 11 H5 HPAIV strains (Table 1). The kit detected each of the H5 influenza viruses tested and did not react with any of the other HA subtypes.

Table 1

The sensitivity of the kit was, then, examined with 4 strains of H5 influenza virus. Serial 4-fold dilutions of allantoic fluids of these viruses were applied to the sample-dropping region for evaluation. As shown in Table 2, the detection limit of the kit was $10^{3.5} \sim 10^{5.5}$ EID₅₀/test for these H5 influenza viruses.

Table 2

Detection of H5 HA antigen from swabs and tissues of experimentally infected chickens.

To evaluate the applicability of the kit for the diagnosis of H5 influenza virus infections in chickens, nine chickens were experimentally infected intranasally with the HPAIV strain A/chicken/Yamaguchi/7/04 (H5N1). Two birds were sacrificed on day 1 p.i. and swab and tissue samples were collected. H5 HA antigens were not detected in these samples, because viral titers were low (Table 3). The remaining 7 birds died within 4 days, and viruses were recovered from each of the swab and tissue samples with high titers. The present rapid diagnosis kit detected H5 HA antigens in tracheal swabs and each of the tissues tested from days 2 to 4. H5 HA antigens were detected in two of the seven cloacal swabs from which viruses were recovered.

Table 3

Discussion

Rapid diagnosis kits that detect the NP antigen do not identify subtypes of influenza A virus (1, 2, 4). In the present study, a rapid diagnosis kit to detect the H5 HA antigen was developed using anti-H5 HA MAb 64/1. For epitope mapping,

an escape mutant virus was selected in the presence of MAb 64/1 and the HA gene of the mutant was sequenced (Soda et al., submitted to Vaccine). The location of the amino acid substitution in the HA molecule of the mutant was Ser 157 to Pro (HA1). Ser 157 is located in the vicinity of the receptor-binding site of the globular head of the HA molecule (11, 14, 19). Anti-H5 HA MAb 64/1 recognized each of the H5 influenza viruses including strains of Eurasian and North American lineages, indicating that this epitope is highly conserved in H5 influenza viruses. The kit, on the other hand, did not react with influenza A viruses of the other HA subtypes (Table 1), influenza B virus strains, Newcastle disease virus, or other agents of avian respiratory disease such as avian paramyxoviruses, bronchitis coronavirus, laryngotracheitis herpesvirus, avian poxvirus, avian adenovirus, mycoplasma gallisepticum, and staphylococcus aureus (data not shown). The present rapid diagnosis kit, therefore, specifically detects H5 influenza virus antigens. It is known that the HA of avian influenza viruses are antigenically and genetically conserved in wild ducks (15). Antigenic variation of the HA of avian influenza virus may occur during circulation in chicken populations (5, 19). Although the epitope in the vicinity of the receptor-binding site appeared highly conserved, it is recommended that the reactivity of the MAb be carefully tested with the latest H5 influenza viruses.

Diagnostic systems for H5N1 influenza virus infection such as H5-RT-LAMP one-step RT-PCR, NASBA, microsphere immunoassay, and latex agglutination test have been developed. These detect H5 HA specific sequence of virus RNA with H5 HA gene specific primer pairs (7, 12, 21), or detect antibodies to H5 HA of

influenza virus (9, 22). However, it takes several hours to obtain results and specific reagents and instruments are needed. The present rapid diagnosis kit provides results within 15min and does not require specific reagents except those in the kit. Furthermore, performance of the test and interpretation of the results do not require any specific skill. The present kit detected the H5 HA antigens with high specificity and sensitivity.

The chickens infected with A/chicken/Yamaguchi/7/04 (H5N1) at $10^{8.0}$ EID₅₀ died on day 2, and viruses were recovered from each of the tissues tested (13). In this study, HA antigen was detected by the present rapid diagnosis kit. The kit detected the H5 HA antigens from the tracheal swabs and tissue homogenates of the trachea, kidney, and colon of chickens from which viruses were recovered at high titers from 2 to 4 days p.i. (Table 3). On the other hand, H5 HA antigen in the cloacal swabs was detected from two samples out of seven tested. Viral titers were lower in the cloacal swabs than tracheal swabs and tissues. The mucus and feces in the cloacal swabs may inhibit the binding of the antibodies to the HA. The present kit may need further improvement for the detection of the HA antigen in the cloacal swabs. In chickens, the HPAIV induces a systemic infection and low pathogenic influenza virus replicates mainly in tissues such as respiratory organs (1, 2, 4), so that the tracheal swabs should be used for the diagnosis of influenza virus infection in chickens. These results indicate that tracheal swabs or tissue homogenates are more suitable for the detection of antigen.

The present rapid diagnosis kit should enable to detect H5 HA antigen from infected chickens. The kit is, thus, useful for the diagnosis of H5 influenza virus

infection and for the control of HPAI.

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Figure Legends

FIG. 1. A rapid diagnosis kit to detect of H5 influenza virus was designed, and showed positive results. The inscriptions “C” and “T” indicate control line and test line, respectively.

TABLE 1. Specificity of rapid diagnosis kit with influenza A viruses

Viruses	Subtypes	Results
Duck/Tottori/723/80	H1N1	—
Duck/Hokkaido/17/01	H2N3	—
Duck/Mongolia/4/03	H3N8	—
Duck/Czechoslovakia/56	H4N6	—
Duck/Pennsylvania/10218/84	H5N2	+
Chicken/Ibaraki/1/05	H5N2	+
Tern/South Africa/61 a	H5N3	+
Swan/Shimane/449/83 a	H5N3	+
Hong Kong/156/97 a	H5N1	+
Hong Kong/483/97 a	H5N1	+
Viet Nam/1194/04 a	H5N1	+
Chicken/Yamaguchi/7/04 a	H5N1	+
Duck/Yokohama/aq-10/03 a	H5N1	+
Chicken/Suphanburi/1/04 a	H5N1	+
Whooper swan/Mongolia/3/05 a	H5N1	+
Whooper swan/Mongolia/2/06 a	H5N1	+
Common goldeneye/Mongolia/12/06 a	H5N1	+
Turkey/Massachusetts/3740/65	H6N2	—
Seal/Massachusetts/1/80	H7N7	—
Turkey/Ontario/6118/68	H8N4	—
Turkey/Wisconsin/1/66	H9N2	—
Chicken/Germany/N/49	H10N7	—
Duck/England/1/56	H11N6	—
Duck/Alberta/60/76	H12N5	—
Gull/Maryland/704/77	H13N6	—
Mallard/Astrakhan/263/82	H14N5	—
Duck/Australia/341/83	H15N8	—
Black-headed gull/Sweden/2/99	H16N3	—

^a Highly pathogenic avian influenza virus

TABLE 2. Sensitivity of rapid diagnosis kit

	Swan/Mongolia/06 (H5N1)	Ck/Yamaguchi/04 (H5N1)	Dk/Pennsylvania/84 (H5N2)	Ck/Ibaraki/05 (H5N2)
Detection limits (log ₁₀ EID ₅₀ /test)	3.5	5.0	4.4	5.5

TABLE 3. Antigen detection from the swabs and tissue homogenates of chickens infected with A/chicken/Yamaguchi/7/04 (H5N1)

Days p.i.	Swabs		Tissue homogenates		
	Trachea	Cloaca	Trachea	Kidney	Colon
1	- / 2.5 ^a	- / - ^c	- / 0.8	- / 1.8	- / 1.3
	- / 1.3	- / -	- / ≤ -0.9	- / -	- / -
2	+ / 5.5 ^b	- / 4.3	+ / 4.8	+ / 6.8	+ / 5.6
	+ / 5.5 ^b	- / 4.8	+ / 5.6	+ / 5.8	+ / 5.6
3	+ / 3.5 ^b	+ / 4.5	+ / 4.6	+ / 5.8	+ / 5.6
	+ / 4.3 ^b	- / 2.5	+ / 4.8	+ / 6.8	+ / 4.3
	+ / 3.5 ^b	- / 3.0	+ / 3.8	+ / 7.1	+ / 5.8
	- / 2.8 ^b	- / 3.5	+ / 4.3	+ / 5.8	+ / 5.1
4	+ / 3.0 ^b	+ / 3.5	+ / 4.6	+ / 6.8	+ / 5.6

^a Results of H5HA rapid diagnosis kit / Virus titers ($\log_{10}$ EID₅₀/test) measured in eggs.

^b dead.

^c - ; $< -0.5 \log_{10}$ EID₅₀/test

FIG. 1 Tsuda et. al.,

