FULL PAPER *Virology*

Development of a Pen-Site Test Kit for the Rapid Diagnosis of H7 Highly Pathogenic Avian Influenza

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ABSTRACT. As well as H5 highly pathogenic avian influenza viruses (HPAIV), H7 HPAIV strains have caused serious damages in poultry industries worldwide. Cases of bird-to-human transmission of H7 HPAIV have also been reported [11]. On the outbreak of avian influenza, rapid diagnosis is critical not only for the control of HPAI but also for human health. In the present study, a rapid diagnosis kit based on immunochromatography for the detection of H7 hemagglutinin (HA) antigen of influenza A virus was developed using 2 monoclonal antibodies that recognize different epitopes on the H7 HAs. The kit detected each of the tested 15 H7 influenza virus strains and did not react with influenza A viruses of the other subtypes than H7 or other avian viral and bacterial pathogens. The kit detected H7 HA antigen in the swabs and tissue homogenates of the chickens experimentally infected with HPAIV strain A/chicken/Netherlands/2586/03 (H7N7). The results indicate that the present kit is specific and sensitive enough for the diagnosis of HPAI caused by H7 viruses, thus, recommended for the field application as a pen-site test kit.

KEY WORDS: avian influenza virus, diagnosis kit, H7 HA subtype, immunochromatography.

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Avian influenza viruses belong to genus Influenza A virus of family *Orthomyxoviridae*. Influenza A viruses are divided into 16 HA and 9 neuraminidase (NA) subtypes on the basis of their antigenic specificity [10]. In addition, they are grouped on the basis of their pathogenicity for chicken into HPAIV, in which mortality may be as high as 100%, and low pathogenic avian influenza viruses (LPAIV) causing much milder respiratory disease [1]. Since they are antigenically and genetically stasis in their natural hosts, feral water birds that do not show clinical signs, they can be designated as apathogenic avian influenza viruses (APAIV) [15, 16]. HPAIV have been restricted to H5 and H7 HA subtypes, although not all viruses of these subtypes are highly pathogenic for chickens. Feral water birds are endemically infected with the APAIV strains of H5 and H7 HA subtypes. The available evidence indicates that these APAIV strains are first transmitted to domestic water fowls, quails or turkeys, and then in live bird markets to chickens. During multiple infections in chicken population, they may acquire pathogenicity for this species to become HPAIV. Consequently outbreaks of HPAI in poultry are difficult to prevent [1, 22]. Besides causing havoc in poultry, infrequent chicken-to-human and human-to-human transmissions of H7 HPAIV have been reported [8, 11].

Rapid diagnosis of HPAI on the outbreak is critical for its control. WHO, OIE and FAO have also emphasized the need for early detection, reporting and introduction of measures to control this menace. Although virus isolation is the standard test for diagnosis of avian influenza [18], it requires time and bio-safety level 3 (BSL-3) facilities [21]. To supplement this method other rapid diagnostic tests have been developed which detect either the viral nucleic acids or viral antigens. Nucleic acid based molecular diagnostic techniques share the principle of amplifying the nucleic acid to detection levels. Most common techniques are reverse transcriptase polymerase chain reaction (RT-PCR) [17], RT-PCR with enzyme linked immunosorbant assay (ELISA) [9], real-time reverse transcriptase polymerase chain reaction (RRT-PCR) [20], nucleic acid sequence-based amplification (NASBA) [6, 7] and loop mediated isothermal amplification (LAMP) [13, 14]. Some of the common techniques used for the detection of viral antigens are indirect fluorescence antibody test (IFA) and ELISA [12]. Although these techniques provide results in shorter time than the classic method of virus isolation, they require extensive laboratory set up, special equipment and skilled personnel. To overcome these defects, an antigen capture method based on immunochromatography was used in the present study. The main advantage of using this test is that no extensive laboratory set up, special equipment or skilled personnel are required and the result can be obtained within 15–30 min [2–5, 23].

Most of the commercially available rapid diagnosis kits detect the nucleoprotein (NP) antigen, whose antigenicity is common among type A influenza viruses [2–4]. Therefore, availability of a rapid diagnosis kit for the HA antigen subtype determination particularly of HPAIV will be a useful adjunct to the rapid diagnosis of HPAI and consequent implementation of rapid control measures. Rapid diagnosis

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kit for the HPAI caused by H5 avian influenza viruses has been described [23]. Here we report an immunochromatography test kit for the rapid diagnosis of H7 avian influenza virus infection.

MATERIALS AND METHODS

Viruses and bacteria: In this study 33 strains of influenza A viruses were used (Table 1). These viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs for 48 hr at 35°C. The infectious allantoic fluid was harvested and was used as virus strain stock. Other viruses and bacteria used included avian paramyxovirus (APMV) strains APMV-1/ruddy shelduck/Mongolia/19/06, APMV-4/duck/Hokkaido/w337/06, APMV-7/pintail/Hokkaido/U6/06 from our repository and infectious bronchitis virus strain B42, infectious laryngotracheitis virus strain NS175, fowl pox virus strain Sishui, avian adenovirus-7, *Staphylococcus aureus* strain Hyogo, *Escherichia coli* and *Mycoplasma gallisepticum* strain C5PT purchased from the Japanese Association of Veterinary Biologics (Tokyo, Japan).

Development of the kit for rapid detection of H7 HA antigen: Hybridoma cell lines producing monoclonal antibodies (MAbs) against the H7 HA antigen of A/duck/Hokkaido/Vac-2/04 (H7N7) were established and examined for reactivity with 21 different strains of influenza viruses of H7 HA by immunofluorescent antibody test (personal communication). The immunochromatography kit was manufactured under the contract with BL Corporation Ltd. (Shizuoka, Japan). Details in the preparation of the kit are proprietary. In brief, the kit consists of a plastic support on which a nitrocellulose membrane is mounted. Anti-mouse immunoglobulin antibodies and anti-H7 HA MAbs were immobilized onto nitrocellulose membrane as capture antibodies in the control and test judgment regions, respectively. A reagent pad containing colloidal-gold-labeled anti H7 HA MAbs was mounted on the nitrocellulose membrane between test judgment region and sample port (sample-dropping region) (Fig. 1).

Test procedure: The efficacy of the present kit was compared with that of an NP antigen detection kit, Capilia® Flu A+B (BL Co., Ltd., Japan). The samples were prepared in the extraction buffer (BL Co., Ltd., Japan). The immunochromatography test was performed by adding 100 μl of test solution in the sample port of each kit. Results were read visually after 15 min of incubation at room temperature. A single colored line appearing in the control judgment region indicates the absence of H7 HA antigen. The concurrent presence of colored lines in both control and test judgment areas indicate the presence of H7 HA antigen in the samples.

Specificity and sensitivity of the kit: Specificity of the kit was determined by using reference influenza virus strains of 16 HA subtypes (H1-H16), 14 additional strains of H7 influenza viruses, 3 strains of highly pathogenic avian H5N1 influenza viruses and other avian bacterial and viral pathogens mentioned earlier.

Sensitivity of the kit was assessed and compared with that of Capilia® Flu A+B kit using A/chicken/Netherlands/2586/03 (H7N7), A/duck/Hokkaido/Vac-2/04 (H7N7), A/seal/Massacheussettes/1/81 (H7N7) and A/chicken/Pakistan/447/95 (H7N3) influenza viruses. Serial four-fold dilutions of each virus were tested. In parallel, these viruses were inoculated into embryonated chicken eggs to determine the 50% egg infectious dose (EID_{50}). The sensitivity was the lowest virus titer detectable by the kit and was expressed as $\log_{10}$ EID_{50}/test.

Experimental infection of chickens with H7 HPAIV: Four-week-old chickens (Boris-brown) were purchased from Hokuren Central Breeding Farm (Hokkaido, Japan). The chickens were infected intranasally with 100 μl of infectious allantoic fluid containing 100 CLD_{50} of A/chicken/Netherlands/2586/03 (H7N7). The chickens, in this study, were housed in self-contained isolator units (Tokiwa

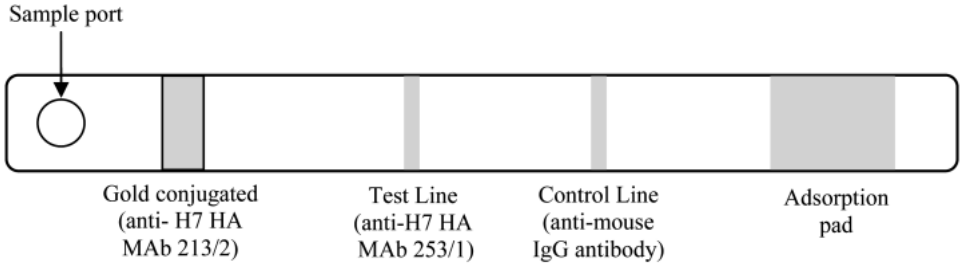

Fig. 1. Schematic diagram of H7 HA antigen detection kit showing different components. Anti-mouse immunoglobulin antibodies and anti-H7 HA MAb 253/1 are striped on the nitrocellulose membrane in the control and test judgment regions as capture antibodies. A reagent pad containing anti-H7 HA MAb 213/2, conjugated to colloid gold particles is placed between sample port and test judgment regions. An adsorption pad is placed next to the control region which absorbs the extra sample after it has migrated through the membrane. When test sample containing antigen is dropped in the sample port, it migrates on the nitrocellulose membrane. The H7 HA antigen in the sample binds to the colloidal-gold-labeled MAbs and this complex is arrested by the immobilized MAbs in the test judgment region producing a colored line. Some of the MAbs are arrested by the anti-mouse Immunoglobulin G (IgG) thus producing a colored line in the control judgment region.

Kagaku Kikai Co., Ltd., Japan) in a BSL-3 facility at Graduate School of Veterinary Medicine, Hokkaido University, Japan. This study was conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

Tracheal and cloacal swabs were collected daily for 6 days post-inoculation (p.i.) and were either placed in the extraction buffer or transport medium (MEM containing 10,000 U/m*l* Penicillin G, 10 mg/m*l* Streptomycin, 0.3 mg/m*l* Gentamicin, 250 U/m*l* Nystatin and 0.5% bovine serum albumin) for titration of infectivity. The swab samples collected in the extraction buffer were then treated with an enzyme pad (BL Co., Ltd., Japan) for 15 min at room temperature. Then immunochromatography test was performed using the present kit and Capilia® Flu A+B kit as explained earlier. In addition to swab samples, trachea, kidney and colon were also collected daily for 6 days p.i. from dead or sacrificed chickens. Ten percent tissue homogenates were prepared in transport medium as test samples for titration of infectivity. The test samples were diluted 5-fold with extraction buffer, treated with enzyme pad as for swabs, and applied to the sample port of the kit. The results were recorded after 15 min of incubation at room temperature.

The infectivity titers of the virus in the swabs and supernatants of the tissue homogenates were calculated by fifty percent end point method [19] and expressed as EID_{50}/m*l* of swab or EID_{50}/g of tissue.

RESULTS

Selection of monoclonal antibodies for the kit: To develop an immunochromatography kit for rapid diagnosis of avian influenza caused by influenza viruses of H7 subtypes, MAbs were produced against the H7 HA of A/duck/Hokkaido/Vac-2/04 (H7N7) (personal communication). Two MAbs, 213/2 and 253/1 were selected on the basis of sensitivity in immunochromatography assay. These MAbs were tested as both conjugate and capture antibodies. In the present kit, MAb 213/2 is conjugated to gold-colloid particles and MAb 253/1 is immobilized to the nitrocellulose membrane, as the capture antibody, by overnight drying at room temperature.

Specificity and sensitivity of the kit: The specificity of the kit was evaluated by using reference influenza virus strains of H1-H16 HA subtypes and other avian viruses and bacteria. The kit was also evaluated using 14 strains of H7 subtype influenza viruses. The kit detected each of the H7 influenza viruses and did not show any non-specific reaction with the influenza viruses of other HA subtypes (Table 1), other avian viruses and bacteria (data not shown).

The sensitivity of the kit was, then, evaluated using four H7 influenza virus strains. Four-fold serial dilutions of infectious allantoic fluid were prepared and applied to the sample port of the present kit and Capilia® Flu A+B kit. The detection limit of the present kit ranged $10^{3.7}$–$10^{5.3}$ EID_{50}/test while detection limit for Capilia® Flu A+B kit ranged $10^{3.1}$–$10^{4.4}$ EID_{50}/test (Table 2).

Table 1. Specificity of the kit for the detection of the H7 HA antigen

Viruses	Subtypes	Results
A/duck/Tottori/723/80	H1N1	–
A/duck/Hokkaido/17/01	H2N3	–
A/duck/Mongolia/4/03	H3N8	–
A/duck/Czechoslovakia/56	H4N6	–
A/duck/Pennsylvania/10218/84	H5N2	–
A/chicken/Yamaguchi/7/04	H5N1	–
A/whooper swan/Mongolia/2/06	H5N1	–
A/duck/Yokohama/aq-10/03	H5N1	–
A/turkey/Massachusetts/3740/65	H6N2	–
A/seal/Massachusetts/1/80	H7N7	+
A/chicken/Netherlands/2586/03	H7N7	+
A/duck/Hokkaido/Vac-2/04	H7N7	+
A/turkey/England/63	H7N3	+
A/turkey/Italy/4580/99	H7N1	+
A/chicken/Pakistan/447/95	H7N3	+
A/duck/Mongolia/47/01	H7N1	+
A/duck/Hokkaido/143/03	H7N7	+
A/gull/Shimane/91/88	H7N8	+
A/duck/Taiwan/4201/99	H7N7	+
A/swan/Shimane/42/99	H7N8	+
A/duck/Hong Kong/47/76	H7N2	+
A/turkey/Tennessee/1/79	H7N3	+
A/duck/Hokkaido/W128/04	H7N7	+
A/duck/Hokkaido/W182/04	H7N7	+
A/turkey/Ontario/6118/68	H8N4	–
A/turkey/Wisconsin/1/66	H9N2	–
A/chicken/Germany/N/49	H10N7	–
A/duck/England/1/56	H11N6	–
A/duck/Alberta/60/76	H12N5	–
A/gull/Maryland/704/77	H13N6	–
A/mallard/Astrakhan/263/82	H14N5	–
A/duck/Australia/341/83	H15N8	–
A/black-headed gull/Sweden/5/99	H16N3	–

Detection of H7 HA antigen from swabs and tissues of experimentally infected chickens: The applicability of the present kit for the diagnosis of H7 influenza virus infection was evaluated for the chickens infected with A/chicken/Netherlands/2586/03 (H7N7). The infected chickens started showing respiratory signs on day 4 p.i. Later, some chickens developed paralysis and two died. The viruses were first recovered from the respiratory and intestinal tracts on day 1 p.i and later from the kidneys on day 2 p.i (Table 3). During first 3 days p.i., overt clinical signs were not observed and the virus titers were low in the swab and organ samples. Therefore, during this phase of infection, H7 HA antigen was not detected by the kit. In comparison, Capilia® Flu A+B detected NP antigen in some samples (Table 3). From day 4–6 p.i, chickens showed clinical signs and the present kit detected H7 HA antigen from both swab samples and tissue homogenates (Table 3). It is apparent from the results that H7 rapid diagnosis kit detected antigen from the clinically sick chickens thus demonstrating its usefulness for pen-site diagnosis.

Table 2. Detection limit of the kit to detect the antigen of H7 subtype viruses

Kit name	Detection limit ($log_{10}EID_{50}$/test)			
	A/chicken/Netherlands /2586/03 (H7N7)	A/chicken/Pakistan /447/95 (H7N3)	A/seal/Massachusetts /1/80 (H7N7)	A/duck/Hokkaido /Vac-2/04 (H7N7)
H7 HA antigen detection kit	5.3	3.7	3.9	4.1
Capilia® Flu A+B kit	4.1	3.1	4.2	4.4

Table 3. Antigen detection and virus titers in the swabs and tissue homogenates of chickens infected with A/chickens/Netherlands/2586/03 (H7N7)

Days p.i.	Clinical signs[a)]	Swab samples		Tissue homogenates		
		Trachea	Cloaca	Trachea	Kidney	Colon
0	–	–[b)]/–[c)]/–[d)]	–/–/–	–/–/–	–/–/–	–/–/–
	–	–/–/–	–/–/–	–/–/–	–/–/–	–/–/–
1	–	–/–/1.8	–/–/–	–/–/–	–/–/–	–/–/–
	–	–/–/2.5	+/+/2.3	–/–/2.5	–/–/–	–/–/–
	–	–/+/4.5	–/–/–	–/–/1.5	–/–/–	–/–/1.8
2	–	–/–/1.5	–/–/3.5	–/–/3.3	–/–/3.5	–/–/3.5
	–	–/–/2.3	–/–/–	–/–/2.8	–/–/2.8	–/–/2.8
	–	–/–/3.5	–/–/1.5	–/–/3.8	–/+/3.8	–/+/3.3
3	+	–/+/3.5	–/–/2.8	–/+/3.5	–/+/3.8	–/+/3.3
	+	–/–/1.5	–/–/1.5	–/–/–	–/–/3.3	–/–/3.5
	+	–/+/4.5	–/–/1.8	–/+/4.5	–/+/3.8	–/–/3.5
4	++	+/+/5.8	–/+/2.5	+/+/5.5	+/+/5.5	–/+/4.8
	++	–/+/3.5	–/–/–	–/+/3.8	–/+/3.8	–/–/2.5
	++	–/+/3.5	–/–/2.3	–/+/4.3	–/+/3.8	–/+/4.5
5	+++[†]	+/+/4.8	–/+/3.3	+/+/4.5	+/+/6.8	+/+/5.5
	++	–/+/2.8	+/–/–	–/+/3.8	+/+/6.5	+/+/6.5
	++	–/+/4.5	+/+/2.5	–/+/4.5	+/+/7.8	–/+/5.5
6	+++[†]	–/+/2.3	+/–/2.5	–/+/4.3	+/+/4.5	+/+/4.5
	+++	–/–/–	+/–/2.3	–/–/3.3	+/+/7.5	+/+/4.5
	++	–/+/3.5	–/–/2.8	–/+/3.3	+/+/6.5	+/+/4.8

a) Clinical signs recorded on the day of sampling, –: No clinical signs, +: Huddling together but alert, ++: Gasping, ruffled feathers, reduced feed intake and depressed, +++: In addition to respiratory signs, chicken developed paralysis. b) Result of H7 HA antigen detection kit, c) Result of Capilia® Flu A+B diagnosis kit, d) Virus titers of swab samples (log_{10} EID_{50}/m*l*) and tissue homogenates (log_{10} EID_{50}/g). –: <1.5 log_{10} EID_{50}/m*l* or g. † Dead chicken.

DISCUSSION

Most of the commercially available diagnosis kits are to diagnose influenza A or B virus infection by detecting NP antigen in the samples, hence do not identify the HA subtype of the influenza A viruses [2–5]. In the present study, a rapid diagnosis kit based on immunochromatography was developed and evaluated for the diagnosis of H7 influenza virus infection. Epitope mapping of the MAbs, used in the present kit, was done by analysing escape mutants selected in the presence of MAbs. Escape mutants produced in the presence of MAb 213/2 had amino acid substitutions at S145N and S152Y while those produced in the presence of MAb 253/1 had amino acid substitution at A169D (Amino acid numbering started from start codon). The binding sites for these MAbs are located in the globular head of the HA molecule. These MAbs bound each H7 HA antigen of the tested influenza virus strains currently circulating in Eurasia and North America (personal communication). The kit detected specifically the H7 HA antigen and did not react with any of the tested influenza viruses of the other HA subtypes including recently isolated H5N1 subtype HPAIV (Table 1) neither with other viruses nor bacteria. Since HPAIV are restricted to H5 and H7 HA of influenza A viruses, the kit developed for the detection of H5 HA antigen [23] and the present kit for the detection of H7 HA antigen must be useful for the control of HPAI.

The sensitivity of the present kit was determined and compared with that of Capilia® Flu A+B kit using both HPAIV and LPAIV (Table 2). The detection limit of the present kit ranged from $10^{3.7}$ to $10^{5.3}$ EID_{50}/test while detection limit for Capilia® Flu A+B kit ranged from $10^{3.1}$ to $10^{4.4}$ EID_{50}/test. Tsuda *et al.* [23] reported the detection limit of the H5 rapid diagnosis kit ranging from $10^{3.5}$ to $10^{5.5}$/test. Similarly Chua *et al.* [5] reported the detection limits of $10^{3.8}$/0.1 m*l* and $10^{4.8}$/0.1 m*l* for NP antigen detecting immu-

nochromatography kits. The detection limit of the present kit is, thus, comparable to the commercially available kits.

The field application for the present kit was evaluated by determining the capability of the present kit to detect the H7 HA antigen in the samples collected from the chickens inoculated intranasally with A/chicken/Netherlands/2586/03 (H7N7). As shown in Table 3, virus titers were higher in the tissue homogenates ($10^{1.5}$–$10^{7.8}$ EID_{50}/g) than in the swab samples ($10^{1.5}$–$10^{5.8}$ EID_{50}/m*l*) and virus titers were higher in tracheal swabs ($10^{1.5}$–$10^{5.8}$ EID_{50}/m*l*) than the cloacal swabs ($10^{1.5}$–$10^{3.5}$ EID_{50}/m*l*). Bai *et al.* [3] and Tsuda *et al.* [23] also reported that HPAIV cause systemic infection and organs like kidney and colon have higher virus titers than swab samples. On day 5 p.i., H7 HA antigen was detected by the present kit, but virus was not isolated from this sample. Furthermore, NP antigen was also not detected by the Capilia® Flu A+B kit. These results suggest that it was a false positive reaction. This false positive result could be due to the presence of sticky materials like mucus or other fecal materials in the cloacal swab samples. Briefly, the present kit detected H7 HA antigen in both of swab samples and tissue homogenates of at least $10^{4.5}$ EID_{50}/m*l* or g infectivity titer.

The sensitivity of the present kit is lower than that of Capilia® Flu A+B kit, however, samples positive by the Capilia® Flu A+B can be immediately sub-typed using present kit at pen-site. It is assumed that even the detection of a small number of positive influenza samples should be enough for the diagnosis of H7 influenza virus infection in birds as a flock. Other rapid subtype determination techniques like RT-PCR [17] may be available. Such facilities are, however, not always available particularly in remote areas and other developing countries. Therefore, under such circumstances, the present kit would be a proper adjunct for pen-site diagnosis.

The present results indicate that the kit developed should be a useful tool for the diagnosis and control of HPAI caused by H7 subtype influenza viruses.

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