Evaluation of Rapid Detection Kit against Avian Influenza A Virus and H5 Subtype for Field Sample

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Abstract

Avian influenza virus is a poultry viral disease, which causes high economic losses. Various efforts have been made to control the disease. One effort required fast and accurate screening diagnostic test. This study aimed to determine the potential of a rapid test kit, namely AIV/H5 Anigen Rapid Test for the detection of AI virus types A and subtype H5 in the fields. Some tests were carried out, e.g. the potential test, cross-reaction test, sensitivity and specificity test. The potential test was done to evaluate detection limits of the kit, by having the test of a serial dilution of AI virus positive control. Cross-reaction test was done to detect antigens other than AI virus H5N1, e.g. IB virus of Massachusetts strain, IBV strain 4-91, Newcastle Disease virus, and *Escherichia coli*. Sensitivity and specificity test were applied to the field samples which clinically and laboratory were confirmed as H5N1 positive. To confirm the result of rapid test was then being done by reverse transcriptase polymerase chain reaction. Based on these results it can be concluded that Anigen Kit AIV/H5 Ag Rapid Test can detect antigen-containing samples having AI virus HA titer up to 2^6 of type A virus, and up to 2^5 for subtype H5 virus. Anigen Kit AIV/H5 Ag Rapid Test showed no cross-reactions with Infectious Bronchitis virus, Newcastle Disease virus, and *Escherichia coli*. Anigen A Rapid Test Kit AIV Ag showed a sensitivity of 50% and specificity of 100%, while Anigen Ag Rapid Test Kit AIV/H5 showed a sensitivity of 25% and specificity of 100%.

Keywords: Avian influenza, rapid detection kit, specificity and sensitivity

Introduction

Highly pathogenic avian influenza (AI) H5N1 subtype has been firstly reported to cause the mortality of commercial poultry farms in West Java and Central Java in 2003 (Asmara *et al*., 2005; Damayanti *et al*., 2004; Dharmayanti *et al*., 2004; Wiyono *et al*., 2004; Wibowo *et al*., 2007). The outbreak was continued to spread to other provinces and it has been declared to be endemic of AI infection in all provinces except Maluku and Gorontalo provinces (NCAIPIP, 2008). In the year of 2011, AI outbreaks had been reported in Gorontalo, where previously known as free AI cases. The AI infection is still existing sporadically throughout Indonesia, as reported by Wibawa *et al*. (2012), Hidayanto *et al*. (2015), and Mahardika *et al*. (2016). A further report of H5N1 virus in Indonesia still belong to HPAI virus (Wibowo *et al*., 2006; Dharmayanti and Indriani, 2007; Wibowo *et al*., 2013; Srihanto *et al*., 2015; Susanti *et al*., 2008).

Avian influenza virus is a member of the family *Orthomyxoviridae*, genus Influenza A virus. The viral genome is segmented and consists of single-stranded-RNA, negative sense, and has eight segments gene which responsible for internal and surface proteins. The surface protein consists of hemagglutinin (H/HA), neuraminidase (N/NA), and matrix

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(M2). Internal proteins are nucleoprotein (NP), polymerase complex (PB1, PB2, PA), matrix (M1), NS-1, and NS-2. Influenza A virus is classified into type A, B, and C based on matrix or nucleoprotein antigenic character. Influenza viruses A are further divided into subtypes based on the antigenicity of protein H/HA and N/NA (Cox and Kawaoka, 1998). The cumulative studies reported that 16 HA and 9 NA subtypes had been recognized (Fourchier *et al.*, 2005). A putative 17th HA was recently discovered from fruit bat (Tong *et al.*, 2012). Avian influenza virus H5N1 subtype has a broad range of hospes, including: variety of avian species (Anonymous, 2003), dogs and cats (Songserm *et al.*, 2006), exotic animals such as leopard and tiger (Kheawacharoen *et al.*, 2004), and humans (Gambaryan *et al.*, 2005).

The outbreak of the disease caused high economic losses due to morbidity and mortality of bird, eradication cost, and export-import restriction for poultry product. Various attempts have been made to control the disease. The diagnosis could be achieved by in vitro, in vivo, and molecular assay. Molecular diagnosis is the direct detection at the molecular level or nucleic acid genome contained in the sample. Some of the advantages of modern diagnostic techniques are: fast, accurate, sensitive and specific, but requires a more expensive and specific equipment, technically complicated, and standardized reagents (Charlton *et al.*, 2009; Suarez, 2003). One of the control strategies required rapid and precise diagnosis. In the field, the condition of diagnosis could not be performed accurately, due to some constraints. Common field diagnosis is screening to have a quick response. However; the result should be followed by definite laboratory testing.

Rapid test kit has been developed, based on immunochromatography assay. The kit uses cellulose membrane as a carrier and colloidal gold-labeled antibody as a tracer. This method has been widely used as a diagnostic tool for infectious diseases in humans and animals, as well as for detection of bioactive molecules, hormones, and hapten. Kang *et al.* (2007) reported rapid immunochromatography method for detecting rabies virus. According to Peng *et al.* (2008), immunochromatography strip model has been used for the diagnosis of AI virus H9 subtype in poultry. Rapid test kit has some advantages especially it is easy to perform, does not need a special skill, can be used immediately in the field situation and possible to differentiate AI virus subtypes. According to Tsuda *et al.* (2007), the technique also could be used to detect the H5 subtype AI virus in poultry.

One of the AI diagnostic kits is Anigen AIV A/H5 Ag rapid test kit which produced for field AI detection, both A type and H5 subtypes applied directly from feces samples. Technically, the rapid test kit does not need special skills, test result could be observed in short time and could be applied directly to the field condition. This study aimed to evaluate the potential, sensitivity, and specificity of those diagnostic kits.

Materials and Methods

The materials were Anigen A/H5 Rapid AIV Ag test kit, RNA isolation kits, and reverse transcriptase-polymerase chain reaction (RT-PCR) kits. Cloacal swabs samples were obtained from chicken with symptoms to AI infection. Positive control of AI virus and *Escherichia coli* have been characterized by Department of Microbiology, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Newcastle disease virus and Infectious Bronchitis virus were obtained from virus vaccine. Phosphate buffered saline (PBS) was used as negative control. Other materials were viral transport media, cloacal swab, and conical tube.

Limit detection kits

For analysis of the kit limit detection, positive control AI virus was used as the samples. Positive control of AI virus suspension was diluted in a serial manner, to reach following dilution: 2^1, 2^2, 2^3, 2^4, 2^5, 2^6, 2^7, 2^8. Each dilution was tested with rapid test kits AIV/H5 Ag and confirmed by using RT-PCR.

Cross-reaction test

To determine the possible reaction with other antigens in the poultry, rapid test kits were examined to the potential cross reaction with Newcastle Disease virus, Infectious Bronchitis virus, and *E. coli*. Each isolate was tested using the rapid test kit AIV/H5 Ag according to the manufacturer's instructions and was performed with five repetitions.

Field rapid test kits application

The sample used to perform the test of Anigen^R rapid test kit AIV A was obtained from Lampung and Yogyakarta, Central Java. Samples taken from Lampung were cloacal swabs and organ samples where the cases had been positively confirmed by RT-PCR. Samples from Yogyakarta obtained from cases of AI at a poultry farm by the end of 2010 when this research was being conducted. The case was clinically characteristic to AI infection and already confirmed by RT-PCR. Cloacal swabs were collected at day 6 when the case was clinically observed.

A cloacal swabs samples of chickens with AI symptoms were tested with rapid test kits Anigen A/H5 AIV Ag according to manufacturer's instructions. Principally, the cloacal swab was added in the assay buffer, mixed thoroughly and allowing the debris to settle down in a few second. Using the disposable dropper provided by the kits, the supernatant was dropped onto the sample window of the slide provided and allowed to flow by capillary action of the horizontal surface along the chromatography strip. To confirm the result, we also used positive control virus of Indonesian isolate.

Laboratory testing

Sensitivity and specificity of rapid test kits Anigen A/H5 AIV Ag were determined by comparing the results of the rapid test of field sample with RT-PCR assay. Cloacal swab samples was placed in the conical tube filled with PBS, antibiotic, and antifungal. The mixtures were centrifuged, and the supernatant was collected and used for RNA isolation.

RNA extraction

Viral RNA was extracted from the supernatant of cloacal swab using RNA extraction kit Invitrogen Pure Link™ Micro to Midi 50xRxn Total RNA Purification System according to the manufacturer's protocol. To purify total RNA from 0.2 ml supernatant of the cloacal swab, in a 1.5 ml RNAse-free microcentrifuge tube was added 0.2 ml of RNA lysis solution containing 1% (v/v) 2-mercaptoethanol. The mixture was vortexed thoroughly to disrupt and lyse blood cells and followed by a centrifugation at 12,000 x g for 2 minutes at room temperature. The supernatant was transferred to clean 1.5 ml RNA-se free microcentrifuge tube and 200 µl of 100% ethanol was added. Any precipitate was dispersed by vortexing or pipetting up and down several times. The sample was transferred to the RNA spin cartridge, centrifuged at 12,000 x g for 15 seconds at room temperature. The flow-through was discarded and 700 µl of Wash Buffer I was added to the spin cartridge and centrifuged at 12,000xg for 15 seconds at room temperature. The spin cartridge was placed into a clean RNA Wash Tube and 500 µl of Wash Buffer II with ethanol was added to the spin cartridge then centrifuged at 12,000xg for 15 seconds at room temperature. The flow-through was discarded. This step was repeated once. The spin cartridge was centrifuged at 12,000 x g for 1 minute at room temperature to dry the membrane, and the cartridge was moved into an RNA Recovery Tube. Fifty µl of RNAse-free water was added to elute the RNA and incubated for 1 minute at room temperature then centrifuged for 2 minutes at 12,000xg at room temperature. This step was repeated once, after that the cartridge was discarded and elutes was stored at -4°C.

Thermocycling

One step Reverse transcriptase-PCR (RT-PCR) was carried out in Gene Amp® PCR System 2400 machine and performed using Invitrogen Superscript™ III One-Step RT-PCR System. Primers designed for this research was for H5 and N1. The design for H5 was H5-F: 5′-ggagactcagcaatcccatgaaaag-

3' and H5-R: 5'-ccataccaaccgtctaccattcc-3', which has 246 bp expected product size (Ito *et al.*, 2001). Meanwhile, N1 identification used a primer designed by Payungporn *et al.*, (2004), which has nucleotide sequence: NA-F: 5'-gtttgagtctgttgcttggtc-3' and NA-F: 5'-tgatagtgtctgttattatgcc-3' which has 131 bp expected band. The PCR condition for H5 and N1 were initially done with a reverse transcription step at 50°C for 30 minutes. After a hot start step at 94°C for 5 minutes, amplification was performed as follows: denaturation (94°C for 30 seconds), annealing (50°C for 1 minute for HA, 47°C minute for NA) and extension (68°C for 45 seconds), ended by final extension at 68°C for 5 minutes. Total cycles performed in PCR were 40 times for HA, and 35 times for NA.

Electrophoresis of PCR Product

PCR products were run for electrophoresis in 1.5% agarose gel in 1x TBE. Electrophoresis tank used MSMIDIDUO, Cleaver Scientific Ltd. The gel and was run at 100V for 40 minutes. The gel was exposed under UV transilluminator at 302 nm wave length to check the result and then followed by determining the size of PCR product fragment by comparing the PCR products with the ladder.

Determine of sensitivity and specificity

Sensitivity and specificity test were analyzed by using a 2X2 table (Martin *et al.*, 1988) and comparing the result of the rapid test with RT-PCR assay.

Results and Discussion

The potency of Anigen rapid tests as detection kits

The potency of Anigen rapid test kit AIV A/H5 Ag to detect AI antigen were evaluated by serial dilution of AI virus positive control which has been characterized in the Microbiology Laboratory. A positive result was observed a band line appear both in the test sample (T) and control zone (C) (Figure 1). However, the sample is considered negative if only one line band appears in the control zone (C) of the membrane (Figure 1).

The results showed that Anigen[R] rapid test kit AIVA Ag is capable of detecting AI virus type A antigen up to 2^6 dilutions. This is equivalent to HA titers 2^4 with a reaction time less than 20 minutes. Anigen[R] rapid H5 AIV Ag test kit exhibited potential detection of H5 subtypes antigen up to dilution 2^5, equivalent to HA titer of 2^5 with a reaction time less than 20 minutes. The results showed different results if compared to other rapid tests, such as Vet. Smart AIV kit (manufactured by Pacific Biotech Co. Ltd.). This rapid test reported in Thailand by using Thailand AI virus isolates demonstrated that the potential detection of the kits against AI virus H5N1 subtype up to 10^7 ELD-$_{50}$/ml (Tantilertcharoen *et al.*, 2016).

Figure 1. Rapid test positive (code 5) and negative (code 8).

Cross-reaction test

To determine cross-reactions to other pathogens in chicken, the kits were tested to ND virus, IB virus, and *Escherichia coli*. The test was performed in five repetitions for each virus and bacterium. The results indicated that the kit showed no cross-reaction to those pathogens. This result is similar to Tsuda *et al.* (2007), who developed an immunochromatographic kit for rapid diagnosis of H5 avian influenza virus infection. In that study, cross-reaction of the kit was evaluated against avian Paramyxovirus, Infectious Brochitis virus, and other viruses but also against *Staphylococcus* sp.

Sensitivity and specificity of the test

Samples of rapid test kits for typing and subtyping were obtained from cases of AI infection, which clinically characteristic to AI infection and already confirmed by RT-

PCR assay. The number of positive and negative by rapid test antigen detection and RT-PCR on the same group of the sample are shown in Table 1 and 2. Sensitivity test was calculated by Martin *et al.* (1988) presented in a 2x2 table.

Table 1. Anigen Rapid Test kit AIV AAg vs RT PCR (for typing).

	RT-PCR positive	RT-PCR negative	Total
Rapid positive	5	0	5
Rapid negative	5	7	12
Total	10	7	17

Typing calculation of sensitivity was 5/10 = 50%, while the specificity was 7/7 = 100%.

Table 2. Anigen Rapid Test kit AIV H5Ag vs RT PCR (for subtyping).

	RT-PCR positive	RT-PCR negative	Total
Rapid positive	7	0	7
Rapid negative	21	3	24
Total	28	3	31

Subtyping calculation of sensitivity was 7/28 = 25%, while the specificity was 3/2 = 100%.

Sensitivity test for AI typing kit by the Anigen A Rapid Test kit AIV Ag showed better results compared to AI subtyping kit by Anigen Rapid Test kit AIV H5 Ag. The value of sensitivity was 50% and 25% respectively. Rapid test specificity both typing and subtyping indicated higher results (100%) compared to sensitivity test. Sensitivity and specificity test which was conducted by AI reference laboratory in Geelong, Australia, reported that various rapid test kits indicated similar results to our study (Sellect, pers. comm.).

The results of this study showed that the subtyping sensitivity was low (25%) compared to the similar research on rapid tests which have been conducted in Indonesia by Loth *et al.* (2008). It was reported that the detection kit Anigen AIV rapid test kit had a higher sensitivity of 69%. Rapid detection test of Flu DetectTM capture antigen test kit had a sensitivity of 71%. According to Boland *et al.* (2006) and the report was cited by Loth *et al.* (2008) stated that Anigen sensitivity reached 76%, for field application where samples obtained from AI infected chickens in Indonesia. Another study conducted by Chua *et al.* (2007) showed that the sensitivity of the 5 types of rapid detection test kits for AI (brand-name not specified) generally showed similar results which ranges from 36.3% to 51.4%. According to that study, the result was more variable depending on the type of sample. Fecal sample indicated sensitivity value of 0% to 12.5%, while the cloacal swab samples were 31.5% to 47.5%. Pulmonary organ sample exhibited varied sensitivity results, ranges 34.4% to 81.3%, while the swab brain reaches 40 to 93.3% of sensitivity.

Specificity test calculation according the Martin's table showed that the test indicated very good result, reaching 100%. The results are relevance to the report of Loth *et al.* (2008) that specificity of rapid AIV Anigen[R] reaches 98%. According to the study of others rapid test kit that was Flu Detect[TM] strip antigen capture test has similar sensitivity of 98%. Specificity tests of the rapid test conducted in Indonesia applied to sample obtained from AI infected chicken reported by Boland *et al.* (2006) cited by Loth *et al.* (2008) was reached 97%.

Analysis for some possible causes of the low sensitivity of the rapid test has been reported by Chua *et al.* (2002), stated that the result of the rapid test kit depending on the type of sample. According to that study, feces samples showed the lowest specificity, while cloacal samples exhibited various specificity, ranged from 31.5% to 49.4%. The best specificity obtained from brain swab ranging from 40 to 93.3%, especially in the case of died birds with symptoms of encephalitis. Specimens from chicken origin showed the sensitivity varies between 37.6% to 60.6%, while samples from the dead waterfowl were varies ranges from 30.7% to 49.2%.

Basically, the sensitivity of antigen detection kits depending on the concentration of virus in the sample. The concentration of

antigen is mainly correlated with the titer of virus in the sample. Artificial infection of H5N1 virus in ducks showed replication occurs in a few days at the beginning of infection. However, viral titers generally are found in the trachea and cloaca of chicken higher than the duck (Chua *et al.* 2008). According to Loth *et al.* (2008) type of sample for the application of rapid test kit should be taken into consideration, particularly it should be obtained from the sick birds or birds that had just died due to AI virus infection. In principle, there should be enough virus titer in a sample for rapid positive results. Additionally, cloaca swab samples should be taken without any fecal, mucus, and blood contamination, due to the potential interference of the rapid test kit process (Loth *et al.*, 2008; Tsuda *et al.*, 2008). Interesting report from Yamammoto *et al.* (2008) suggested that feathers can be considered useful samples for early AI virus detection in duck. The reason is that larger amounts of AI viruses can be isolated for a longer time from feathers than from swabs.

Further analysis of low sensitivity in this study, it may due to the AI virus master seed which be used to produce antibodies in the producing country is different with AI virus circulating in Indonesia. Smith *et al.* (2006) and Chen *et al.* (2006) analyzed that there are three genetically distinct sub-populations of AI viruses in Indonesia and distributed in several different geographic areas, although all type the AI virus can be found on the island of Java. The difference means that AI viruses Indonesia origin have antigenic variation, which can be measured from antibodies titer difference from each sub-population (Smith *et al.*, 2006).

Conclusions

Based on these results, it could be concluded that Anigen Rapid Test Kid AIV/H5 Ag are able to detect AI antigen-containing samples having HA titer up to 2^6 to determine type A AI virus and HA titer up to 2^5 for H5 subtype detection. Anigen Rapid Test Kit AIV/H5 Ag showed no cross-reactions with Infectious Bronchitis virus, Newcastle disease

and *Escherichia coli*. Sensitivity of Anigen A Rapid Test Kit AIV Ag was 50% and specificity test was 100%, while Anigen Ag Rapid Test Kit AIV/H5 showed a sensitivity test of 25% and a specificity of 100%.

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