

V. Product performances

1. Evaluation studies done for the product

Performance Evaluation of Avian Influenza Virus Antigen Test kit

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Abstract

Vet-Smart AIV Ag Test kit is able to detect Avian Influenza Virus Ag, subtype H5N1 efficiently. These virus isolations were obtained from infected chicken and duck during avian flu epidemic in Thailand in 2004. The test kit required AIV Ag at least 10^7 ELD₅₀/ml to show positive result, whereas chick embryo technique, haemagglutination test and multiplex RT-PCR required virus at least 10^0 , 10^7 , and 10^4 ELD₅₀/ml, consequently. In addition, Vet-Smart AIV Ag took only 3 minutes to reveal the testing result, which became clearly distinguished within 30 minutes.

Key words: Influenza virus, avian species, antigen, test kit

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Introduction

Avian influenza is caused by influenza virus, in Orthomyxoviridae Family, contains RNA genome. This virus can be divided into three types: A, B and C per matrix protein (M) (Swayne and Halvorson, 2003). Only Influenza Virus Type A is communicable and highly pathogenic avian influenza virus (HPAI). The virus can be further classified into subtype per two envelope proteins: 15 varieties of haemagglutinin (H) and 9 varieties of neuraminidase (N) (Webster, 1997). Most of HPAI Type A that causes disease in chicken are H5 and H7. In Thailand, the first epidemic was reported in February 2004, from chicken farm, and it has been reported until now. The epidemic covered more than 30 provinces all over the country (www.oie.int). Many kinds of avian are susceptible to Influenza virus infection. Symptoms may be varied due to virus subtype and kind of infected animal (Perkin and Swayne, 2003). Avian Influenza not only caused a great damage to avian farm industry, but also caused panic in public health due to human being susceptibility to this avian flu subtype. Infected people has respiratory problem and can be fatal. Current laboratory diagnostic method is based on embryonated egg inoculation, followed by heamagglutination and subtyping with standard serum (OIE, 2000, Swayne *et al.* 1998). The whole process is time consuming, high cost, and requires classified laboratory and experienced technicians. Then the conventional method may not convenient for screening at field site. At present, a test kit has been developed named Vet-Smart AIV Ag, for primary diagnosis with ease and quick result. The kit is intended for AIV Type A Ag detection. From in-house preliminary study, the performance was acceptable. So, the objective of this study was to evaluate the performance of Vet-Smart AIV Ag in comparison to conventional laboratory method to support further development for better performance.

Objectives

To evaluate the performance of test kit in comparison of current standard laboratory methods.

Materials and Methods

1. Virus Preparation: Two samples of Influenza virus Type A were isolated from a dead chicken found in large epidemic in Bangkok (A/chicken/Bangkok/CUVDL02/04(H5N1)) and from a duck in Chonburi (A/duck/Chonburi/CUVDL01/04(H5N1)) in February 2004. Both isolations were classified to be AIV subtype H5N1. They were algorithmically ten-fold diluted in minimal essential medium (MEM) until virus concentration was 10^{-3} ELD₅₀/ml. For negative control, allantoic fluid from non-inoculated embryonated egg was also algorithmically diluted in MEM. These diluted preparations were divided into three aliquots for further testing as detailed below:
 - a. Embryonated egg inoculation: 100 ul of each diluted preparation was inoculated into allantoic cavity of 9-day-old embryonated egg of white leghorn layer chicken, two eggs per dilution. All inoculated eggs were incubated at 37 C. Fetus vitality was checked daily under lighting. Allantoic fluid from egg of dead fetus was collected on that day but that of live fetus was collected on the seventh day of incubation. The allantoic fluid was tested for AIV Ag by three techniques: polymerase chain reaction; PCR) with reverse transcriptase (RT-PCR), haemagglutination and Vet-Smart AIV Ag.
 - b. haemagglutination test: to measure AIV quantity by its haemagglutination ability. The sample was heat inactivated at 56 C for 90 minutes. Then it was algorithmically two-fold diluted from 1: 2 to 1: 256 with phosphate buffer saline (PBS). 1% Chicken RBC in PBS was added in each dilution. Mixed well, and left at RT for an hour. Record the most diluted titer that caused haemagglutination.
 - c. Multiplex reverse transcriptase PCR (multiplex RT-PCR): 200 ul of each serial diluted sample was heat inactivated at 56 C for 90 minutes. Then RNA was extracted with RNA extraction kit (QIAamp[®] Viral RNA Mini Kit (QIAGEN, Hilden)). After that, it was amplified by using RT-PCR technique in thermocycler with 3 sets of primer to amplify H5 protein gene, N1 protein gene, and M protein gene. The amplified product was resolved by agarose gel electrophoresis. The electrophoresis condition was 1% agarose gel, 100 V, for one hour. The agarose gel was stained with ethidium bromide. And amplified gene bands were detected under UV light.
 - d. Vet-Smart AIV Ag Test Kit
 - i. Dispensed 100 ul of each serial diluted sample to 1 gram of feces obtained from non-AIV infected chicken. Mixed well and performed testing per test procedure recommended by manufacturer. Collected sample by cloacal swab and dissolved the sample in provided diluent. Left for a while to let sediment fell down to the bottom. Took supernatant with provided dropper, and dispensed 8 drops into sample well. Left it on flat surface, at RT for 10, 30 and 60 minutes, recorded the result.

- ii. Dispensed 100 ul of each serial diluted sample into provided diluent. Left for a while to let sediment fell down to the bottom. Took supernatant with provided dropper, and dispensed 8 drops into sample well. Left it on flat surface, at RT for 10, 30 and 60 minutes, recorded the result.
2. Performance of AIV Ag detection was compared between Vet-Smart AIV Ag and standard method of embryonated egg inoculation followed by multiplex RT-PCR of allantoic fluid from dead fetus egg.

Results

From the study it was found that Vet-Smart AIV Ag Test Kit was able to detect antigen of HPAI (H5N1) in allantoic fluid at the virus level of 10^7 ELD₅₀/ml. The result was clearly seen at RT (25° C) within 30 minutes (Fig. 1) as shown in Table 1. At the virus level from 10^8 ELD₅₀/ml, the result could be seen within 3 minutes. When the sample was mixed with 1 gram of feces, the kit could detect positive at virus level of 10^9 ELD₅₀/ml within 30 minutes (Fig. 2) without any false positive result as shown in Table 2. Whereas HA and multiplex RT-PCR could detect antigen of HPAI (H5N1) in C25SL02P3 isolation at level of 10^7 and 10^6 ELD₅₀/ml, consequently and in C17Dx01P3 isolation at the level of 10^7 and 10^4 ELD₅₀/ml as shown in Table 3. With embryonated egg inoculation method, the virus isolations were quantified. It was found that C25SL02P3 and C17Dx01 P3 contained virus at level of 10^9 ELD₅₀/ml and 10^{10} ELD₅₀/ml, consequently. Allantoic fluid obtained from all dead fetus eggs showed positive result to both HA and Vet-Smart. The same result was observed when some samples were tested by RT-PCR. For allantoic fluid which was obtained from live fetus egg after seven days of incubation, all gave negative results to HA, Vet-Smart and RT-PCR as shown in Table 4.

Table 1 : Number of positive results per tested samples with Vet-Smart at different result reading durations.

Virus level (ELD ₅₀ /ml)	Result reading duration (min)						
	10		30		60		
	C25SL02P3	C17Dx01P3	C25SL02P3	C17Dx01P3	C25SL02P3	C17Dx01P3	Negative control
10 ⁹	2/2	2/2	2/2	2/2	2/2	2/2	0/2
10 ⁸	1/2	2/2	2/2	2/2	2/2	2/2	0/2
10 ⁷	0/2	0/2	2/2	2/2	2/2	2/2	0/2
10 ⁶	0/2	0/2	0/2	0/2	0/2	0/2	0/2
10 ⁵ -10 ⁻³	0/18	0/18	0/18	0/18	0/18	0/18	0/18

Table 2 : Number of positive results per tested samples with Vet-Smart at different result reading durations when mix the sample with 1 gram of feces.

Virus level (ELD ₅₀ /gram of feces)	Result reading duration (min)						
	10		30		60		
	C25SL02P3	C17Dx01P3	C25SL02P3	C17Dx01P3	C25SL02P3	C17Dx01P3	Negative control
10 ⁸	1/2	0/2	2/2	2/2	2/2	2/2	0/2
10 ⁷	0/2	0/2	0/2	0/2	0/2	0/2	0/2
10 ⁶	0/2	0/2	0/2	0/2	0/2	0/2	0/2
10 ⁵	0/2	0/2	0/2	0/2	0/2	0/2	0/2
10 ⁴ -10 ⁻⁴	0/18	0/18	0/18	0/18	0/18	0/18	0/18

Table 3 : Results of HA and RT-PCR in each level of virus of two isolations.

Virus level (ELD ₅₀ /ml)	C25SL02P3		C17Dx01P3	
	HA	RT-PCR	HA	RT-PCR
10 ⁹	128	+	128	*
10 ⁸	64	+	16	*
10 ⁷	8	+	1	+
10 ⁶	<1	+	<1	+
10 ⁵	<1	-	<1	+
10 ⁴	<1	-	<1	+
10 ³ -10 ⁻³	<1	-	<1	-

* not done

Table 4 : Positive results per tested samples with Vet-Smart, HA and RT-PCR in allantoic fluid.

Virus level (ELD ₅₀ /ml)	Vet-Smart with direct sample		Dead fetus egg		allantoic fluid sample					
					HA		Vet-Smart		RT-PCR	
	C25SL02P3	C17Dx01P3	C25SL02P3	C17Dx01P3	C25SL02P3	C17Dx01P3	C25SL02P3	C17Dx01P3	C25SL02P3	C17Dx01P3
10 ⁹	2/2	2/2	2/2	2/2	≥256	≥256	2/2	2/2	2/2**	2/2**
10 ⁸	2/2	2/2	2/2	2/2	≥256	≥256	2/2	2/2	2/2**	-
10 ⁷	2/2	2/2	2/2	2/2	≥256	≥256	2/2	2/2	-	-
10 ⁶	0/2	0/2	2/2	2/2	≥256	≥256	2/2	2/2	-	-
10 ⁵	0/2	0/2	2/2	2/2	≥256	≥256	2/2	2/2	-	-
10 ⁴	0/2	0/2	2/2	2/2	≥256	≥256	2/2	2/2	-	-
10 ³	0/2	0/2	2/2	2/2	≥256	≥256	2/2	2/2	-	-
10 ²	0/2	0/2	2/2	2/2	≥256	≥256	2/2	2/2	-	-
10 ¹	0/2	0/2	2/2	2/2	≥256	≥256	2/2	2/2	-	-
10 ⁰	0/2	0/2	1/2	1/2	≥256*	>256*	1/2*	1/2*	1/2*	1/2*
10 ⁻¹	0/2	0/2	0/2	0/2	<1	<1	0/2	0/2	0/2**	-
10 ⁻²	0/2	0/2	0/2	0/2	<1	<1	0/2	0/2	0/2**	-
10 ⁻³	0/2	0/2	0/2	0/2	<1	<1	0/2	0/2	0/2**	-
negative control	0/26		0/26		<1	<1	0/26		0/26**	

* Positive results were found only in dead fetus egg

** two samples were combined into one – not done

Discussion and Conclusions

Vet-Smart AIV Ag Test Kit was able to detect HPAI (H5N1) at the level of 10⁷ELD₅₀/ml. This performance is comparable to that of HA, whereas RT-PCR was able to detect the lowest virus level at 10⁴ELD₅₀/ml. However, Vet-Smart AIV Ag Test Kit took less time to perform the testing and gave the results faster, only 30 minutes. Moreover, Vet-Smart AIV Ag Test Kit was able to detect antigen of HPAI (H5N1) in allantoic fluid obtained from inoculated embryonated egg at the same virus level as HA and RT-PCR. From all of these findings, it seems that Vet-Smart AIV Ag Test Kit can be used as an efficient tool for laboratory diagnosis of AIV infection with ease and short resulting time. Even though, we have the limitation of making chicken infection in laboratory to demonstrate kit performance of cloacal swab from real specimen, the kit may be used as primary screening for ill or died avians in the field conveniently. The recommended sample is cloacal swab, since it was reported that ill or died chickens shed the virus HPAI (H5N1) via cloaca. One collection of cloacal swab obtained virus at least 10⁸ELD₅₀/ml (Ref. Vet. Rachoj Tantilertcharoen, Veterinarian Diagnostic Laboratory) which was enough for the test kit sensitivity. However, the further study should be performed with direct cloacal swab from ill and died chickens to confirm sensitivity and specificity.

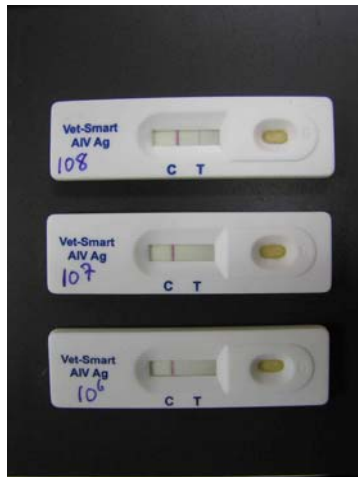


Figure 1

Test result of Vet-Smart with HPAI (H5N1)

in direct sample with feces

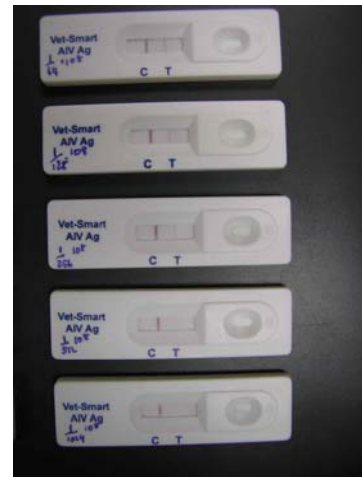


Figure 2

Test result of Vet-Smart with HPAI

(H5N1)

in diluted samples

Reference :

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2. Diagnostic value of test kit compared with other techniques

Preliminary Report on

Performance of Vet-Smart Avian Influenza Antigen Test in Cloacal Swab

Introduction : The first Avian Flu epidemic in Thailand in 2004 urged every related organizations, government agencies, medical and veterinary school staffs to work together to calm down the panicking people and put the situation under control. The conventional method of egg inoculation takes around 4 days to get the results, which could be done only in authorized lab centers. Due to slow time results and overload of samples sent in from various farms all over the country lead us to produce the easy-to-use kit for screening the positive cases of infected chickens. Only positive samples could be sent to lab centers for confirmation. This rapid test could help for controlling the epidemic of the disease as well as minimized the infected areas.

Materials and Methods :

- Materials :**
- A. Three known positive cloacal swabs of Avian Influenza Virus Infection by HAI after egg inoculation stored in Phosphate Saline Buffer
 - B. Twenty five healthy and eighteen sick chickens previously Avian Influenza Virus Infection confirmed by HAI after egg inoculation
- Method :** Testing protocol mentioned in package insert of Vet-Smart Avian Influenza Antigen Test was followed strictly as detailed here

For material A :

1. Immersed cloacal swab into a vial of supplied diluent
2. Swirled the swab until sample dissolved out
3. Left the vial stand until large particles settled down to the bottom of the vial
4. Removed test device from foil pouch and placed it on testing table
5. Took the supernatant from extracted sample with supplied disposable dropper
6. Dispensed eight drops of sample into sample well (S)
7. Read the result at 10 minutes
8. Negative result : presence of only one band at C
9. Positive result : presence of bands at C and T

For material B :

1. Swabbed cloacal area of studied chicken with sterilized cotton swab supplied in the kit

2. The same as mentioned above in No.2 – No.9

- Results :**
1. Testing with three known positive cloacal swabs stored in PBS, two samples showed very weak positive results. The other was found negative.
 2. Testing with direct fresh cloacal swabs collected from twenty five healthy and eighteen known positive chicken, the results are shown in the table below

Testing Method	Reference Method		Total
	Positive	Negative	
Vet-Smart			
Positive	18	0	18
Negative	0	25	25
Total	18	25	43

Discussion : Vet- Smart Avian Influenza Virus Antigen Test has been coated with monoclonal antibody against nucleoprotein of Avian Influenza Type A for the purpose of Avian Flu A antigen detection rapid testkit. The study shows that the recommended sample for testing is direct fresh cloacal swab, which yield 100% (18/18) sensitivity and 100% (25/25) specificity. For the cloacal swab previously stored in PBS yields only 67% (2/3) sensitivity. The direct fresh cloacal swab is preferred than cloacal swab stored in other solution to avoid sample dilution before testing which may cause weak positive result or even false negative. Therefore, Vet-Smart Avian Influenza Virus Antigen Test is suitable to be adopted in chicken with avian flu symptoms for screening of avian influenza virus infection.

Pacific Biotech Co.,Ltd.

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