

Forewords

The document is the report of the joint-work between Balai Penelitian Veteriner, Bogor and PT. Elokarsa Utama, Jakarta in the study of sensitivity and specificity of *Rockeby Avian Influenza Virus Antigen Test Kit*.

The test was conducted for 5 days starting from 28 January to 2 February 2006 in the Virology Laboratory of Balai Penelitian Veteriner by Muharam Saepulloh, S.Si., M.Sc., assisted by 2 technicians (Pudji Kurniadhi and Nana Suryana). The budget required for the test is Rp. 8,820,500.- (eight million and eight hundred twenty thousand five hundred rupiah), sponsored by the collaborator organization, PT. Elo Karsa Utama.

It is our wish that the report will bring any benefit to PT. Elo Karsa Utama and farming industry especially poultry industry.

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SUMMARY

SENSITIVITY AND SPECIFICITY TEST OF ROCKEBY KIT TO DETECT AVIAN INFLUENZA OF TYPE A

Tested diagnostic materials	:	Rockeby Avian Influenza Virus Antigen Kit
Objective	:	To qualitatively detect avian influenza virus in avian feces
Test Site	:	Balai Penelitian Veterener, Bogor
Tester	:	Muharam Saepulloh, S.Si., M.Sc. assisted by two technicians, namely Pudji Kurniadi and Nana Suryana.

Test Results:

Rockeby Avian Influenza Virus Antigen Test Kit can be used to detect avian influenza (AI) virus of type A found in the sample of cloaca swab and feces of both healthy avian without any clinical symptoms and the sick or dead avian. Rockeby Kit is capable of detecting AI virus of type A up to $10^{6.33}$ EID₅₀/ml. However, it will fail in detecting the virus with concentration level below $10^{6.33}$ EID₅₀/ml. Rockeby Kit is capable of detecting faster than the pre-set 10 minute-limit and has clearer and sharper band integrity (positive) than that of other detector of AI virus of type A. The result of RT-PCT Matrix primary test shows that Rockeby Kit has a sufficiently high sensitivity level (90%) in detecting AI virus of type A. Rockeby Kit is also capable of detecting sub-type H5 and H7, however, the sensitivity level for sub-type H5 is lower, which is 30%. Rockeby Kit does not react against other non-avian influenza avian viruses. This data clearly confirms the used antibody monoclonal for the avian influenza virus is very specific and accurate. Therefore, the kit can detect only AI virus. The conclusion drawn states that Rockeby Kit can be used to detect Avian Influenza virus of type A found in the sample of avian feces and cloaca swab and (2) Rockeby Kit has a high accuracy and sensitivity in detecting avian influenza virus of type A.

SENSITIVITY AND SPECIFICITY TEST OF ROCKEBY BIT TO DETECT AVIAN INFLUENZA VIRUS OF TYPE A

INTRODUCTION

Avian Influenza or also known as Bid flu is a disease caused by a virus of *Orthomyxoviridae* Family, which infects domestic and wild avian. The symptom may range from the clinical symptom to high death rate (Lam and Krug, 1996). Based on the glycoprotein surface, avian influenza virus can be subdivided into hemagglutinin (HA) and neuraminidase (NA) (Easterday et al., 1977). Avian influenza virus of type A can infect bird, pig, horse and other species, including human. Avian influenza virus of type A comprises 15 hemagglutinin (HA) subtypes and 9 neuraminidase (NA) subtypes and it has been successfully isolated from various avian species (Rohm et al., 1996). The 15 HA subtypes of the influenza virus found in aquatic birds act as the carrier (reservoir) of all avian influenza virus of type A (Webster et al., 1997). Among the 15 HA subtypes of avian influenza virus of type A, subtype H5 and H7 are the most malignant and can cause death to the animal of avian species (Alexander, 1995). The major factor of the death is due to the inexistence of clinical symptom shown by the avian species, especially bird. As such, an early detection is highly required before the virus spreads vastly and infects the other animals.

Rockeby Kit is an immunoassay chromatography diagnostic tool to quantitatively detect avian influenza virus of type A found in the sample of cloaca sab and avian feces. A series of test conducted in the laboratory and field will then be performed to prove the capability of Rockeby Kit of detecting avian influenza virus of type A. The test focuses on the sensitivity and specificity featured by Rockeby Kit in laboratory as well as test of various samples taken from the field such as cloaca swab and avian feces. The sample would be taken from the healthy avian species and the ones showing the AI clinical symptoms.

MISSION AND OBJECTIVE

To prove the capability of ROCKEBY kit of detecting avian influenza virus of type A.

MATERIALS AND METHODS

A. Sample of feces and cloaca swab

To test the sensitivity of Rockeby Kit, a good sample taken from the healthy and sick avian species is required. Therefore, 10 samples of cloaca swab and feces taken from fresh avian species from Indramayu were then collected to detect any possible existence of avian influenza virus of type A.

B. Sensitivity Test

1. Rocheby Kit's capability of detecting various concentrations of avian influenza virus of type A.

A serial dissolution (multiple of 10) was performed to the AI virus of subtype H5N1 with pre-determined titer level. The multiplication level of the dissolution started initially at 10^{-1} and gradually increased to 10^{-4} . The dissolved virus would then be detected by Rocheby Kit for 3 times. Sensitivity would then be determined by the Rocheby Kit's capability of detecting the most dissolved virus and the existence of visible band.

2. Detection of AI virus by RT-PCT, ROCKEBY kit and other detecting kits.

The test used the original sample of cloaca swab and feces of various healthy and sic avian species. The sample would subsequently be tested using ROCKEBY kit, other kits called Kit A and test RT-PCT for the primary and H5 Matrix. The use of cloaca swab and feces sample was in line with the ROCKEBY kit's instruction of use, Kit A's instruction of use and procedure of sample taking for RNA isolation.

RNA Isolation

RNA was extracted using Trizol LS (Invitrogen Cat. No. 10296-010). In short, 250 μ l suspension of cloaca swab or feces was mixed with 750 μ l Trizol LS. After the mixture has perfectly blended, it was then kept in a room temperature for 5 minutes. The mixture was then extracted by adding 0.2 mL chloroform and centrifugalized 10,000 x g for 15 minutes at 4⁰C. RNA on the upper layer would be collected and precipitated by adding isopropanol with the same ratio of dosage. The RNA deposit would then be taken by centrifugalization 10,000 x g for 20 minutes at 4⁰C. The deposit was then bleached by ethanol 75% and finally dissolved by adding 10 μ l of Rnase-free water (Invitrogen).

Matrix and H5 Primary

The matrix and H5 primary used was in line with the Lee et al. procedure (2001) namely forward Matrix (M52C) 5'-CTT CTA ACC GAG GTC GAA ACG-3' and Reverse Matrix (M253R) 5'-AGG GCA TTT TGG ACA AAG/T CGT CTA-3'. H5 Primary implemented forward Matrix (H5-155f) 5'-ACA CAT GOY CAR GAC ATA CT-3' and H5 reverse (H5-699r) 5'CTY TGR TTY AGT GTT GAT GT-3' (Invitrogen Custom Primers).

Reverse Transcription-Polymerase Chain Reaction (RT-PCT)

RT-PCT used Superscript TM One step RT-PCT with Platinum [®] Taq (Invitrogen Cat. No. 10928-034) with total reaction of 50 μ l containing 25 μ l of 2X Reaction Mix, 10 μ l RNA, 1 μ l RT/Platinum [®] Taq Mix, Matrix Primary and H5 Primary respectively of 0.8 μ M. For RT-PCR Matrix Primary,

Fouchier et al. (2000) procedure was implemented with amplification process by using *Thermal Cycle* (Hybaid) machine as follows: cDNA synthesis and pre-denaturation of 1 cycle at 42⁰C for 30 minutes and at 95⁰C for 4 minutes; PCR amplification of 40 cycles at 95⁰C for 1 minute (*Denaturation*), at 45⁰C for 1 minute (*Annealing*), 72⁰C for 3 minutes (*Extension*). RT-PCT of HT Primary implemented Lee et al. (2001) procedure with amplification as follows: cDNA synthesis and pre-denaturation of 1 cycle at 42⁰C for 45 minutes and 95⁰C for 3 minutes; PCR amplification of 35 cycles at 95⁰C for 30 seconds (*Denaturation*), at 55⁰C for 40 seconds (*Annealing*) and 72⁰C for 40 seconds (*Extension*), 72⁰C for 10 minutes (*Final Extension*). Each reaction would then be analyzed using electrophoresis of 50 Volt for 2.5 hours with agarose concentration of 2% (Invitrogen) with ethidium bromide coloring and subsequently viewed using UV illuminator viewer.

C. Specificity Test

1. ROCKEBY Kit's reactivity in detecting subtype H5 and H7 of avian influenza virus

The used antigens for avian influenza virus of subtype H were H5N1, H5N2, H5N3, H5N7, H7N3 and H7N7. The six antigens (Inactive) of AI virus were dissolved by using the liquid found in the kit. The solution would then be dropped as stated in the procedure provided by the kit. The result would be positive if Rockeby kit could detect both the viruses which was indicated by the appearance of a purple band in the "test" column.

2. Rockeby Kit's reactivity in detecting other avian viruses

The test was meant to identify ROCKEBY kit's capability of detecting other avian viruses such as *Newcastle Disease* (ND), *Infectious Bronchitis* (IB), *Infectious laryngotracheitis* (ILT), *Egg Drop Syndrome* (EDS) and *Infectious Bursal disease* (IBD). Rockeby Kit was acknowledged to possess specificity towards avian influenza of type A, if the avian virus could not be detected (negative). The test required avian virus which was taken from a vaccine with guaranteed purity. The avian virus vaccine was obtained commercially.

RESULT AND EXPLANATION

The result of specificity test of Rockeby Kit against various dissolved avian influenza virus of type A subtype H5N1 can be found in Table 1 and Image 1. The result clearly shows that Rockeby kit can detect only AI virus at 10^{6.33} EID₅₀/ml concentrations. The AI virus will not be detected if the concentration value is lower. According to Webster and Bean Jr (1998), AI virus replication takes place in the digestive system of Aquatic birds, which clinically will not indicate any symptom of disease. However, it can secrete AI virus with concentration of up to 10^{8.7} EID₅₀/gram feces). Therefore, Rockeby kit's capability of detecting an AI virus of 10^{6.33} EID₅₀/ml concentrations found in cloaca swab and feces is sufficient. However, to achieve a perfect result, the test should not be limited to cloaca swab only, it should also be

performed to feces, by taking sample two times, namely cloaca swab and feces (mixed) to be tested by Rockeby kit.

Table 1. ROCKEBY Kit's capability in detecting any dissolved avian influenza virus of type A.

No.	Dissolved Virus (H5N1)	Concentration of H5N1 Virus (EID ₅₀ /ml)	Test Result		
			Repetition 1	Repetition 2	Repetition 3
1	10 ⁰	10 ^{8.33}	+++	+++	+++
2	10 ⁻¹	10 ^{7.33}	++	++	++
3	10 ⁻²	10 ^{6.33}	+	+	+
4	10 ⁻³	10 ^{5.33}	-	-	-

Below is the comparison of Rockeby Kit against other kits used for quick test of avian influenza virus of type A, in this case is referred to as kit A. A parallel test of 10 cloaca and avian feces samples was performed using Rockeby Kit and Kit A. The result is shown in table 2 and image 2. The reading clearly shows Rockeby Kit is more sensitive and quicker to react against the sample than Kit A. It is indicated by the intensity purple band and speed in detecting AI virus of type A. The clear intensity of purple band is very helpful to the surveillance officers. Dissatisfaction and misinterpretation were very common as the result of blurry positive result. Detection speed is also none the less as essential as the band intensity. Rockeby kit could detect AI virus of type A found in the sample within the time limit (10 minutes). Rockeby Kit's sensitivity remained high when the time limit for both kits was due.

Table 2. Sensitivity Comparison of Rockeby Kit and Kit A.

No.	Avian Species	Status	Sample Type	Rockeby Kit		Kit A	
				Reaction Time	Result	Reaction Time	Result
1.	Pigeon	Normal	Cloaca swab and feces	3 min, 1 sec.	++	4 min, 41 sec.	+
2.	Chicken	Sick	Cloaca swab	1 min, 30 sec.	++	4 min, 7 sec.	++
3.	Turkey	Dead	Cloaca swab	56 sec.	++	2 min.	++
4.	Pearl Chicken	Dead	Cloaca swab	10 sec.	++	3 min.	+
5.	Pigeon	Normal	Cloaca swab and feces	42 sec.	++	2 min, 26 sec.	+
6.	Chicken	Sick	Cloaca swab	7 min.	++	15 min.	+
7.	Pigeon	Normal	Cloaca swab	1 min, 2 sec.	+++	1 min, 11 sec.	+++
8.	Pigeon	Normal	Cloaca swab	29 sec.	+++	54 sec.	+
9.	Pigeon	Normal	Cloaca swab	20 sec.	+++	20 sec.	+++
10.	Chicken	Sick	Cloaca swab and feces	37 sec.	++	41 sec.	+

Rockeby kit's capability of detecting avian influenza virus of type A subtype H5 and H7 is shown in table 3 image 3. The monoclonal antibody against avian influenza A used by Rockeby Kit could react against various avian influenza virus of type A subtype H5N1, H5N2, H5N3, H5N9, H7N3 and H7N7 with a very clear intensity of purple band. Hence, Rockeby Kit is proven to be a suitable product for surveillance which requires quick and accurate detection as well as follow-up action as a preventive measure against the spread of avian influenza disease. Despite Rockeby Kit's capability of detecting subtype H5 and H7, subsequent test was still necessary to confirm the result such as virus isolation and detection by a more specific molecular biology. The consolidation of results of the three tests generated a very satisfactory result and therefore, a decision is required to establish the action to be taken.

Table 3. Result test of ROCKEBY kit's capability of detecting AI virus subtype H5 and H7.

No.	AI Virus Antigen	Result
1	H5N1	Positive
2	H5N2	Positive
3	H5N3	Positive
4	H5N9	Positive
5	H7N3	Positive
6	H7N7	Positive

However, Rockeby kit did not react against other non-avian influenza virus of type A as shown in Table 4 and Image 4. The result states the monoclonal antibody against avian influenza of type A used by Rockeby kit possesses such an excellent level of purity that none of non-AI virus of type A (ND, IB, IBD, ILT and EDS) is detectable. The purity level of monoclonal antibody used in a diagnostic tool is absolutely required and one of the conditions that must be met by the tool itself. The test must be performed considering the magnificent number of avian virus which can infect the avian species.

Table 4. Result of ROCKEBY Kit specificity test against other non-avian influenza virus of type A.

No.	Virus	Vaccine Name	Result
1	ND	Avinew (Merial, France)	Negative
2	IB	Bioral H-120 (Merial, France)	Negative
3	ILT	ILT-Bal, Singapore	Negative
4	EDS	Newvaxidrop (Merial, France)	Negative
5	IBD	BUR 706 (Merial, France)	Negative

Table 5 displays the result of detection of AI virus of type A found in the cloaca swab and feces sample taken from various avian species in Indramayu using RT-PCR, Rockeby kit and kit A. Out of 10 tested samples, Rockeby Kit and kit A showed similar positive result in detecting all the samples, namely 100% (10/10). However, the positive findings detected by RT-PCT Matrix primary is only 90% (9/10) and RT-PCR H5 primary is 30% (3/10) (Image 5). The result clearly proves that Rockeby kit is suitable for a quick test tool to detect the avian influenza virus of type A found in the avian cloaca swab and feces sample due to its high sensitivity level (90%) for RT-PCR matrix primary. However, it is less sensitive (30%) when used to detect AI virus of subtype H5.

Table 5. Result of detection of AI virus of type A found in the avian cloaca swab and feces sample by using Korean ROCKEBY kit and RT-PCT

No.	Origin of sample	Species	Number of sample	Rockeby Kit		Kit A.		RT-PCR Matrix		RT-PCT H5	
				Number of +ve	% +ve	Number of +ve	% +ve	Number of +ve	% +ve	Number of +ve	% +ve
1	Indramayu	Pigeon	5	5	100	5	100	4	80	0	0
2	Indramayu	Turkey	1	1	100	1	100	1	100	1	100
3	Indramayu	Pearl Chicken	1	1	100	1	100	1	100	1	100
4	Indramayu	Chicken	3	3	100	3	100	2	66.66	1	33.33
		Total	10	10	100	10	100	9	90	3	30

CONCLUSION

The laboratory test of Rocheby kit has shown:

1. Rocheby Kit is capable of detecting avian influenza virus of type A up to $10^{6.33}$ EID₅₀/ml. However, it cannot detect any virus with lower concentration.
2. Rocheby Kit is capable of detecting in a shorter time and provides positive result indicated by the clear and sharp band.
3. Rocheby Kit is capable of detecting subtype H5 and H7 despite its lower accuracy level.
4. Rocheby Kit possesses high level of sensitivity and accuracy in detecting avian influenza virus of type A and is capable of detecting reliably the virus found in both healthy and sick avian species.

SUGGESTIONS

To achieve more perfect results, the followings are required:

1. Field test is absolutely required by testing more than 100 samples on the healthy and sick avian species.
2. Field test must be complemented by a confirmatory result through RT-PCT test on the matrix and H5 primaries. As such, Rocheby Kit's sensitivity and specificity in detecting AI virus of type A will be accurate.

Rockeby Kit's Sensitivity

Ulangan ke-1: Repetition 1

Ulangan ke-2: Repetition 2

Ulangan ke-3: Repetition 3

Kesimpulan: Conclusion

Rockeby Kit is capable of detecting avian influenza virus of type A up to $10^{6.33}$ EID₅₀/ml.

Gambar 1. Kemampuan kit Rockeby...

Image 1. Rockeby kit's capability of detecting various concentration of AI virus of type A subtype H5N1.

Rockeby's Sensitivity vs Other Kits' Sensitivity

1. Sample #21 is taken from a pigeon's feces and cloaca swab. Above) Rockeby, the reading shows 3 min. 1 sec. ++. Below) Kit A, the reading shows 4 min. 41 sec. +.
2. Sample #27 is taken from a sick chicken's cloaca swab. Above) Rockeby, the reading shows 1 min. 30 sec. ++. Below) Kit A, the reading shows 4 min. 7 sec. +.
3. Sample #44 is taken from a dead turkey's cloaca swab. Above) Rockeby, the reading shows 56 sec. ++. Below) Kit A, the reading shows 2 min. ++.
4. Sample #45 is taken from a pearl chicken's cloaca swab. Above) Rockeby, the reading shows 10 sec. ++. Below) Kit A, the reading shows 3 min. +.
5. Sample #46 is taken from a pigeon's feces and cloaca swab. Above) Rockeby, the reading shows 42 sec. ++. Below) Kit A, the reading shows 2 min. 26 sec. +.

Gambar 2a. Uji sensitivitas kit Rockeby dibandingkan dengan kit A
Image 2a. Sensitivity test of Rockeby Kit versus Kit A.

6. Sample #47 is taken from a sick chicken's cloaca swab. Above) Rocheby, the reading shows 7 min. ++. Below) Kit A, the reading shows 15 min. +.
7. Sample #51 is taken from a pigeon's feces and cloaca swab. Above) Rocheby, the reading shows 1 min. 2 sec. +++. Below) Kit A, the reading shows 1 min. 11 sec. +++.
8. Sample #56 is taken from a pigeon's feces. Above) Rocheby, the reading shows 29 sec. +++. Below) Kit A, the reading shows 54 sec. +.
9. Sample #70 is taken from a pigeon's feces and cloaca swab. Above) Rocheby, the reading shows 20 sec. +++. Below) Kit A, the reading shows 20 sec. +++.
10. Sample #72 is taken from a sick chicken's cloaca swab and feces. Above) Rocheby, the reading shows 37 sec. ++. Below) Kit A, the reading shows 41 sec. +.

Gambar 2b. Uji sensitivitas kit Rocheby dibandingkan dengan kit A.

Image 2b. Sensitivity test of Rocheby Kit versus Kit A.

Kesimpulan: Dalam mendeteksi....

Conclusion: In detecting the same set of samples, Rocheby kit is more sensitive and faster. The intensity of the purple band is also clearer.

Rockeby Kit's Specificity

No.	AI Virus Antigen	Result
1	H5N1	Positive
2	H5N2	Positive
3	H5N3	Positive
4	H5N9	Positive
5	H7N3	Positive
6	H7N7	Positive

Kesimpulan: Kit Rockeby...

Conclusion: Rockeby kit possesses an excellent capability of detecting avian influenza virus of type A.

Gambar 3: Reaktifitas kit Rockeby dalam mendeteksi subtype H5 dan H7.

Image 3: Rockeby Kit's reactivity in detecting subtype H5 and H7.

No.	Virus	Vaccine Name	Result
1	ND	Avinew (Merial, France)	Negative
2	IB	Bioral H-120 (Merial, France)	Negative
3	ILT	ILT-Bal, Singapore	Negative
4	EDS	Newvaxidrop (Merial, France)	Negative
5	IBD	BUR 706 (Merial, France)	Negative

Kesimpulan: Spesifitas kit Rockeby....

Conclusion: Rockeby kit's specificity is excellent because it can react only against avian influenza virus of type A. It will not react against other avian viruses.

Gambar 4: Reaktifitas kit Rockeby dalam mendeteksi virus unggas lain.

Image 4: Rockeby Kit's reactivity in detecting other avian viruses.

Detection of avian influenza virus of type A using Matrix primary on avian feces and cloaca swab sample from Indramayu. 1) and 14) Molecular weight 100bp, 2) negative control dH2O, 3) Isolate positive control H5N1 4) sample #21 pigeon's feces, 5) sample #27 sick chicken's cloaca swab, 6) sample #44 dead turkey's cloaca swab, 7) sample #45 dead pearl chicken's cloaca swab, 8) sample #46 pigeon's feces, 9) sample #47 chicken's feces, 10) sample #51 pigeon's feces and cloaca swab, 11) sample #56 pigeon's feces and cloaca swab, 12) sample #70 pigeon's feces and cloaca swab and 13) sample #72 sick chicken's cloaca swab.

Detection of avian influenza virus of type A using H5 primary on avian feces and cloaca swab sample from Indramayu. 1) and 14) Molecular weight 100bp, 2) negative control dH2O, 3) Isolate positive control H5N1 4) sample #21 pigeon's feces, 5) sample #27 sick chicken's cloaca swab, 6) sample #44 dead turkey's cloaca swab, 7) sample #45 dead pearl chicken's cloaca swab, 8) sample #46 pigeon's feces, 9) sample #47 chicken's feces, 10) sample #51 pigeon's feces and cloaca swab, 11) sample #56 pigeon's feces and cloaca swab, 12) sample #70 pigeon's feces and cloaca swab and 13) sample #72 sick chicken's cloaca swab.

Kesimpulan: Dari sepuluh....

Conclusion: Out of ten samples of avian feces and cloaca swab which were positively detected by Rocheby Kit, only 90% (9/10) was found positive of RT-PCT matrix primary and 30% (3/10) was found positive of RT-PCT H5 primary.

Gambar 5. Deteksi virus Avian Influenza A dengan menggunakan RT-PCT primer matriks dan H5

Image 5. Detection of Avian Influenza virus of type A using RT-PCT matrix and H5 primaries.