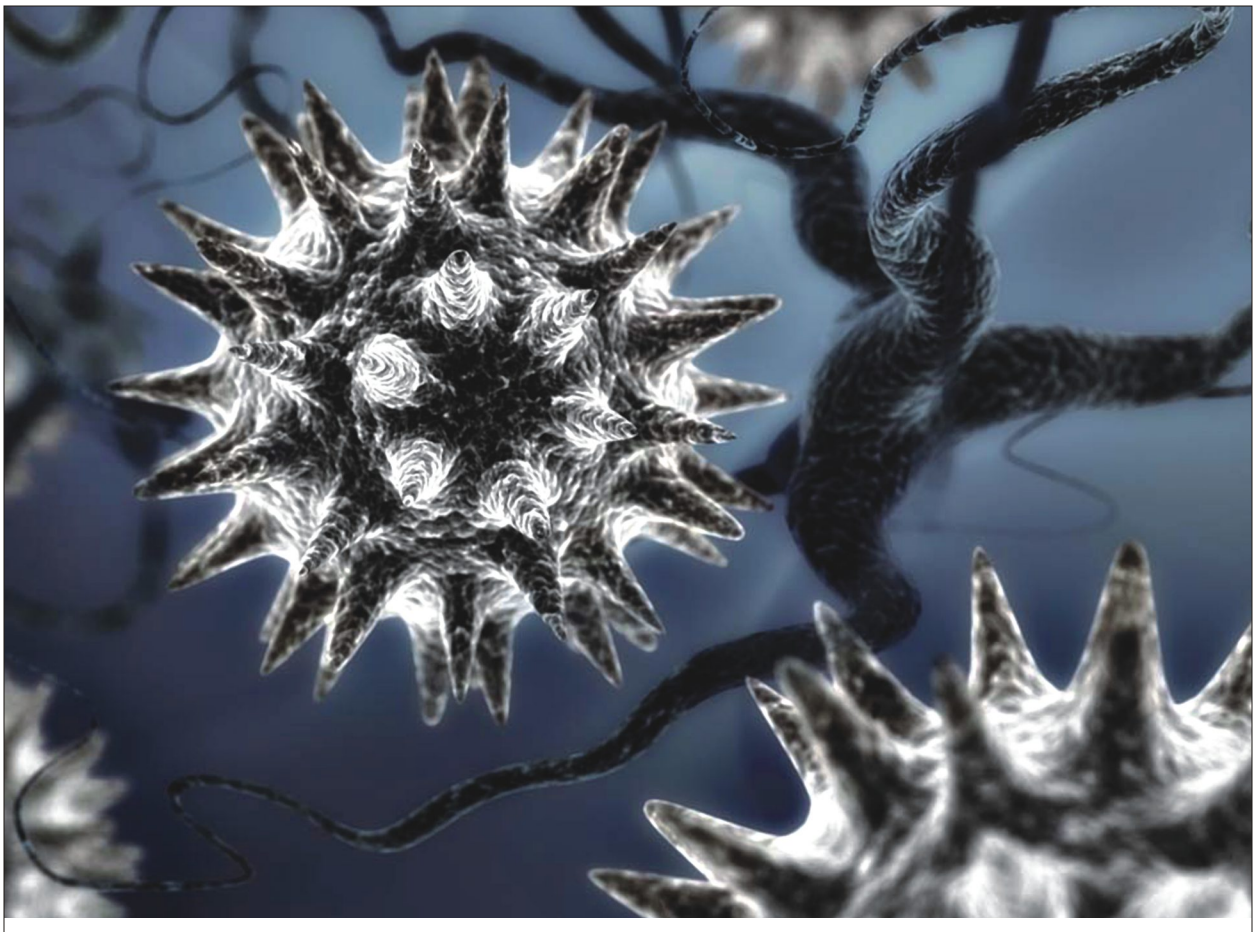

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Research Article
Serological and Molecular Analysis of Avian Influenza Virus Subtype H5 Isolated from Aceh Province in Indonesia

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Abstract

Background: Avian influenza is an infectious viral disease of birds which can spread to domestic poultry (chicken, duck and quail) and cause large-scale outbreaks of serious disease. Avian Influenza Virus (AIV) has also been reported to cross the species barrier and cause disease or subclinical infections in other mammals and humans. Based on the antigenic differences in nucleoprotein (NP) and Matrix (M) proteins, AIV is classified into A, B and C types. All influenza viruses infected and isolated from avian are classified as A type. Further subtyping of AIV is based on antigenic differences between the two surface glycoproteins hemagglutinin (H) and neuraminidase (N). **Objective:** The aim of this study was to identify AIV type A, subtype of H5 by conducting the serological and molecular analysis toward different poultry species which were recently infected by AIV. **Materials and Methods:** All of 37 samples collected from tracheal, cloacal swabs and visceral organs from various species of poultry (chickens, ducks and quails) were used in this study. The samples were inoculated into Specific Antibody Negative (SAN) embryonated chicken eggs of 9-11 days of age for serological and molecular analysis. For serological analysis, all 37 samples were tested by Hemagglutination Activity (HA) and Hemagglutination Inhibition (HI) tests. For molecular analysis of Matrix (M) and Hemaglutinin 5 (H5) genes, the RNA viruses were extracted and amplified by RT-PCR method. The amplification products were then separated on 1% agarose gel electrophoresis and sybrsafe staining. **Results:** Serological analysis by HA and HI tests shown that 7 samples from total of 37 samples were positively infected by AIV type A. Molecular analysis by RT-PCR amplification to amplify matrix and hemaglutinin genes followed by separation of RT-PCR products on 1% agarose gel electrophoresis were obtained DNA fragments in size of 276 and 1.725 bp, respectively in 7 tested samples. **Conclusion:** It could be concluded that the infectious agent which cause of the death of various species of poultry in Aceh province, Indonesia were AIV type A, subtype H5.

Key words: Avian influenza virus, H5 subtype, serological and molecular analysis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Influenza viruses are pleomorphic and enveloped RNA viruses belonging to the family of Orthomyxoviridae. Protruding from the lipid envelope are two distinct glycoproteins, the hemagglutinin and neuraminidase. The genome of influenza viruses is segmented, consisting of 8 single-stranded, negative sense RNA molecules, which encode 10 proteins. The RNA segments are contained within the viral envelope in association with the nucleoprotein (NP) and three subunits of viral polymerase (PA, PB-1 and PB-2), which together form the ribonucleoprotein (RNP) complex. They are responsible for RNA replication and transcription. Additional proteins contained within the virion include M2 and the viral Nuclear Export Protein (NEP), which function in assembly and budding and export of RNP from the nucleus, respectively. Based on antigenic differences in nucleoprotein (NP) and Matrix (M) proteins, influenza viruses are classified as types A, B and C. All avian influenza viruses are classified as type A. Further subtyping of influenza A viruses is based on antigenic differences between the two surface glycoproteins hemagglutinin and neuraminidase[1].

Influenza A virus can infect various species of birds, mammals and human and it can lead a major pathogenic role in spreading influenza pandemic worldwide. Influenza A virus which recently has been found in the form of 18 H (H1-H18) and 11 N (N1-N11)[2,3]. The natural hosts of type A influenza virus is wild birds and water fowls. In its natural host, this virus is stabilized and not infectious. Based on the level of infection, AI virus can be grouped into two levels of infection: Highly Pathogenic Avian Influenza (HPAI) and Low Pathogenic Avian Influenza (LPAI). The HPAI is a highly pathogenic infection[4] that can lead to death up to 100%.

The factor which influencescases of AIV infection in Indonesia is the un-optimal treatment to prevent the infection of AI viruses. It can be seen from the distribution pattern of poultry in the markets which are not fully controlled, low biosecurity on poultry farms, especially in sectors 3 and 4 of poultry industry, the spread of AI viruses from wild waterfowl and the weakness of vaccination strategies[5].

Identification and characterization of AI virus can be conducted in some methods, either conventionally or molecular methods. The use of electron microscopy, tissue culture, isolation of the virus in SPF-embryonated chicken eggs and serological examinations are commonly performed[6]. According to OIE[7] the virus isolation from SPF-embryonated chicken eggs is the gold standard for diagnosis of AI virus. However, this method has not been able to answer the whole biological properties of AI virus. Therefore, AI virus isolation in embryonated chicken eggs must still be followed by serological test, such as Hemagglutination Activity (HA) and Hemagglutination Inhibition (HI) tests, which will also be continued by molecular testing method using RT-PCR amplification.

The detection of AI virus by HA/HI methods is a diagnostic method which is routinely performed in the laboratories to detect and determine the type and subtype of influenza viruses. Furthermore, the direct use of molecular techniques to detect the virus in infected allantoic fluids contribute to more rapid, precise and accurate identification and genetic characterization study of type A influenza virus[7].

The objective of this study was to analyze and identify AI virus by serological and molecular methods toward suspected poultry from some poultry species which infected by AI virus in the Aceh province. The data of this study can be used as a basic database for further consideration of the government and stakeholders to determine the policy for preventing and controlling of AI virus infection in poultry industry in Indonesia, especially in Aceh province and surrounding areas.

MATERIALS AND METHODS

Samples collection: The trachea swab, cloaca swab and organ samples were collected from different species of poultry (chickens, quails and ducks) originated from endemic areas of AI virus infection in Aceh province. Samples were collected from 7 districts in the Aceh province, including Aceh Timur, Aceh Tamiang, Aceh Utara, Bireuen, Aceh Tengah, Bener Meriah and Aceh Besar. After collection, the samples were transported to the laboratory of Microbiology in Syiah Kuala University, Aceh. All of samples were packed in ice and transported to the laboratory of Virology, Animals Diseases Investigation Center (ADIC) in Wates, Yogyakarta and were stored at -20°C until used.

Virus isolation: The swabs and organ samples were made as viral suspension by adding a solution of Phosphate Buffered Saline (PBS), pH 7.0-7.4 and antibiotics. Then 0.2 mL of the viral suspension was incubated at room temperature for 1-2 h and inoculated through allantoic cavity route in 3 embryonated chicken eggs of 9-11 days old. After inoculation, the SPF-embryonated chicken eggs were incubated at 37°C for 4 days. The embryos will be infected and died within 24 h. The died embryos containing eggs were chilled and collected. Similarly, the live embryos containing eggs after 5 days also selected and collected the allantoic

fluid. They preserved as a source of AI viruses. The collected allantoic fluids were kept in sterile eppendorf tubes. They were stored at -20°C until used.

Preparation of 2 and 0.5% chicken Red Blood Corpuscles (RBC) suspension: Samples of poultry blood were collected from wing vein with sterile syringe and needlecontaining anti-coagulant (4% trisodium citrate) at the rate of 1 mL for 10 mL of blood. Following collection, blood sample was mixed with Phosphate Buffered Solution (PBS) and centrifuged at 1,000 rpm for 10 min. The supernatant was discarded, pellet was resuspended with PBS and centrifuged at 1,000 rpm for 10 min. This process was followed for three times and finally the supernatant was discarded. About 1 mL of chicken RBC was mixed with 49 mL of PBS to prepare 2% RBC suspension. For plate Hemagglutination Activity (HA) and plate Hemagglutination Inhibition (HI) test 10 mL of 2% RBC suspension was mixed with 30 mL of PBS to make 0.5% chicken RBC and stored at 4-8°C for 3 days.

Hemagglutination Activity (HA) test: This test was performed to detect the hemagglutinating viruses in the collected samples. The procedure of plate hemagglutination activity test was as follows: (1) About 25 µL of PBS was dispensed in each well of a plastic U-bottomed microtitre plate. (2) About 25 µL of AF was placed in the first well and mixed well. (3) From first well 25 µL of mixture was transferred into second well to make two fold dilutions. This process was continued up to the last well (11th) and from there 25 µL of mixture was discarded. Well 12 was used as control. (4) About 25 µL of 0.5% (v/v) chicken RBC was dispensed into each well. (5) The plate was tapped gently and was allowed to keep at room temperature for about 15 min. (6) The HA was determined by tilting the plate and observing the hemagglutination of the RBC. (7) A uniform layer of hemagglutination covering the bottom of well of the plate was considered as positive HA and a sharp buttoning of RBC at the bottom of well of the plate was considered as negative[7,8].

Plate Hemagglutination Inhibition (HI) test for detection of H5N1: (1) About 25 µL of PBS was dispensed into each well of a plastic U-bottomed microtitre plate, except well 1 and 7 of each raw. (2) About 25 µL of known positive antiserum (H5N1) was placed into first and second wells of each raw of the plate. (3) From well 2 and 8 of each raw two-fold dilution was made across the plate. (4) About 25 µL of HA positive virus was added into each well and left for a minimum of 30 min at room temperature. (5) About 25 µL of 0.5% (v/v) chicken RBC was added to each well. After gentle mixing the plate was allowed to keep for about 40 min at room temperature. (6) In each raw two wells (6 and 12) were kept as control. (7) The hemagglutination inhibition activity was observed after 40 min and compared with control one[7,8].

Extraction of RNA viruses: RNA viral extraction from chorioallantoic liquid was performed using Purelink™ micro-to-Midi Total RNA Purification System from Invitrogen, following the recommended standard procedures of the company. The RNA extraction was used as a template for RT-PCR and stored at -20°C before used.

Primer design: Amplification of M gene was applied with single-step method of RT-PCR and using a primer pair specific to influenza type A which is the reverse and forward. The specific primers for amplifying H5 gene was recommended by Payungporn *et al.*[9] which were designed based on genetic information of the Indonesian virus strains deposited in the National Center of Biotechnology Information (NCBI) database. Primer design applied primer3 version 4.0.0 online (http://primer3.ut.ee/).

Amplification by RT-PCR method: After obtaining the RNA template from viral RNA isolation process, all isolates were amplified against M and H5 gene using RT-PCR. Prior to amplification, a 22.5 µL master mix was prepared for each sample, 12.5 µL 1x buffer mix reaction was inserted into the tube, then added with 1 µL primer forward and 1 µL primer reverse of specified to the M gene with a concentration of 10 pmol μL^{-1} and added with 8 µL dH_2O and 1 µL Taq Superscript™ III Polymerase enzyme. Master mix was added to 2.5 µL RNA template so that the total volume of the reaction to the sample was 25 µL.

The PCR reaction mixture consisting of master mix and templates were ready to be amplified. The cDNA synthesis process was conducted by reverse transcription process at 48°C for 45 min in one cycle and initial denaturation at 94°C for 5 min in one cycle. The process was continued with PCR amplification of 40 cycles with denaturation temperature of 94°C, respectively for 30 sec, annealing at 47°C for 60 sec, extension at 68°C for 30 sec and a final extension at 68°C for 10 min. The results of RT-PCR performed directly in electrophoresis.

Analysis of RT-PCR products by electrophoresis: The PCR amplification products were analyzed by electrophoresis on agarose gel 1% with addition sybrsafe. After all the wells

are filled with samples, markers and positive controls, running electrophoresis was performed at 135 V for 45 min. The results of electrophoresis were read on transilluminator viewer.

Data analysis: Data were obtained from the inoculation of the virus in embryonated chicken eggs. The results of HA/HI test were tabulated based on the infected area/endemic. The data were analyzed descriptively.

RESULTS AND DISCUSSION

The swab and organs samples from various species of suspected poultry infected by AI virus in 7 districts/cities in the province of Aceh were obtained total of 37 samples. They shown in Table 1.

The positive result of virus inoculation into SPF embryonated chicken eggs and HA/HI tests were followed by a screening to determine the type of AI virus by amplification of the Matrix gene. Amplification was conducted with single-step method of RT-PCR and using a primer pair specific to influenza type A. The results of amplification of matrix gene were electrophoresed on agarose gel 2%. The results obtained from the process were in the form of a band deoxyribonucleic acid (DNA) at 276 bp for all isolates as shown in Fig. 1.

For this amplification, appearing of M gene DNA fragments in size of 276 bp showed that the all 7 samples infected by AI virus type A. By the same methods but using different primers design, the same amplification product of RT-PCR also reported by Haryanto *et al.*[10] for M gene of AIV isolated from Yogyakarta and Central Java regions, viral protein-2 (VP-2) gene for Avian Encephalomyelitis (AE) virus[11] and Fusion (F) protein encoding gene for Newcastle Disease (ND) virus[12].

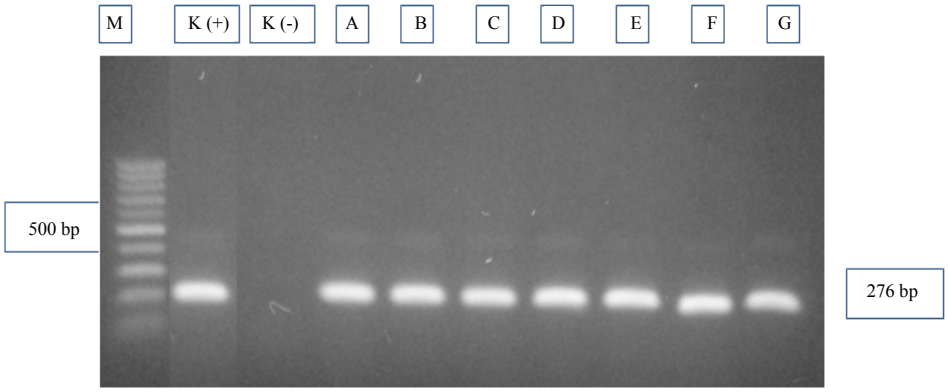

Fig. 1: Electrophoresis of RT-PCR products of matrix gene (276 bp) from the 7 positive samples. M: Markers 100 bp DNA ladder, K (+): Positive control, K (-): Negative control, samples A-G: Virus isolates originated from quail, sample D is virus isolate originated from layer

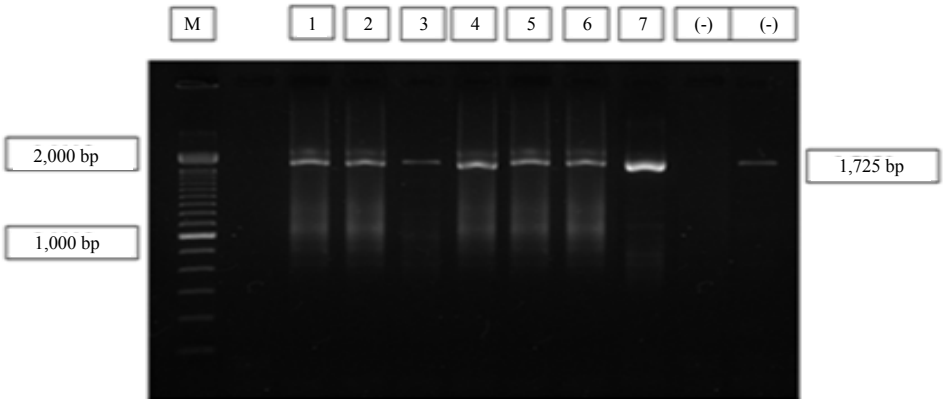

Fig. 2: Electrophoresis of RT-PCR products of H5 gene (1,725 bp) from the 7 samples. M: Marker 100 bp DNA ladder, K (+): Positive control, K (-): Negative control, sample 1-7: Virus originated from quail, sample 3 is virus originated from layer

Table 1: Data of collected field samples isolated from different species of poultry which were suspected by AI virus in the province of Aceh, Indonesia

	Species and No. of samples			
Origin of samples	Chicken	Duck	Quail	Total No. of sample
Aceh Timur	3	1	-	4
Aceh Tamiang	2	1	-	3
Aceh Utara	2	1	6	9
Bireuen	2	2	9	13
Aceh Besar	3	2	-	5
Aceh Tengah	1	1	-	2
Bener Meriah	1	-	-	1
Total	14	8	15	37

Table 2: Data of originated 7 virus isolates and results of serological analysis by HA and HI tests

		Anti-AI	Anti-ND
Origin of isolates	HA test	HI serum	HI serum
Layer from Aceh Besar	+	+	-
Quail from Aceh Utara	+	+	-
Quail from Aceh Utara	+	+	-
Quail from Aceh Utara	+	+	-
Quail from Bireuen	+	+	-
Quail from Bireuen	+	+	-
Quail from Bireuen	+	+	-

HA: Haemaglutination, HI: Hemagglutination inhibition, AI: Avian influenza, ND: Newcastle disease, RT-PCR: Reverse transcription polymerase chain reaction, M: Matrix, (+): Positive result, (-): Negative result

Table 3: Result of RT-PCR amplification using specific primers for M and H5 genes of AIV

Origin of isolates	RT-PCR product of M gene	RT-PCR product of H5 gene
Layer from Aceh Besar	+	+
Quail from Aceh Utara	+	+
Quail from Aceh Utara	+	+
Quail from Aceh Utara	+	+
Quail from Bireuen	+	+
Quail from Bireuen	+	+
Quail from Bireuen	+	+

Virus isolation was applied toward embryonated chicken eggs, 9-11 days of age, which were incubated for 4 days at 37°C. Chorioallantoic fluids were taken aseptically from the product of isolation to be used in rapid agglutination test on a glass plate using Red Blood Cells (RBC) 2% chicken erythrocytes. Among 37 samples which were tested using method of isolation and rapid agglutination, 7 positive samples were found to agglutinate red blood cells to rapid agglutination test. It was detected the presence of AI virus in the chorioallantoic fluid. According to Stevens *et al.*[13] the HA protein contained in influenza virus will be attached to the erythrocyte receptors on various species of poultry and mammalian. Therefore, the test was used as the foundation for detecting the presence of the AI virus in chorioallantoic fluid. The next step was to perform confirmation test to determine the titer value using 2 times dilution on the plate with the addition of 0.5% chicken red blood cells by the same volume in all samples[7]. The obtained data can be seen on Table 2.

Based on HA/HI test using specific antigen AI and ND, it can be ascertained that all the 7 samples were positive for AI viruses. The presence of the agglutination from HA tests and agglutination inhibition in HI tests showed specific reaction between AI viruses against anti-H5N1 antibodies. The quantities of the virus titer were characterized by faster and greater toward agglutination reaction. Meanwhile, the presence of precipitation line in Immune Diffusion (ID) test showed strong character of the AI virus. AI virus antigenic relationship was shown by the continuous lines of precipitation[7].

According to OIE[7] HI test has a high sensitivity because it can detect antigens HA, especially subtypes H5 of AI virus. HI test is more specific in detecting antigens HA belonging to subtype H5 but it is difficult to determine whether the AI virus was originated from subtype H5N1, H5N2 or H5N9. If the result is only based on the HI test, it will be difficult to determine the subtype. Therefore, the diagnostic confirmation by RT-PCR or genetic sequencing becomes absolutely necessary to characterize the subtype[14] of H5N1. The data of RT-PCR products for M and H5 genes of AI virus shown in Table 3.

Based on the results of electrophoresis (Fig. 1 and Table 3), RT-PCR amplification were well performed without any contamination. The result was consistent with Helmi *et al.*[15] reported took employing samples screening of avian influenza outbreak in the Aceh province in 2006 and 2008 which were RT-PCR amplified using specific primer for Matrix gene. It generated DNA fragments in size of 276 bp only in the group of AI virus subtype A.

Amplification of H5 gene AI virus from sample by RT-PCR method showed that all samples were H5 subtype which indicated by generating of RT-PCR products in size of 1.726 bp in samples No. 1-7 as depicted in Fig. 2.

Amplification of M and H5 gene for AI virus were performed by anone step multiplex RT-PCR method. The appearance of M gene fragments showed that the AI virus positive influenza virus type A, whereas the presence of H5 gene indicated the virus is influenza virus type A subtype H5. Although using different primers design, the RT-PCR amplification product in accordance with that done by Haryanto *et al.*[10]. The similar amplification product was also demonstrated by Haryanto *et al.*[16] with the emergence of three DNA fragment, respectively 276 bp for M gene, 189 bp for the H5 gene and 131 bp for N1 gene in the AI virus isolated from chickens in Maros, South Sulawesi province, Indonesia. The matrix protein of AI virus consists of two types including the matrix protein M1 and M2 with "Open reading frame" (ORF) starting from 26-781 nucleotides encoding for M1

proteins, while the nucleotides 26-51 and from 740-1007 encoding for M2. The M1 and M2 proteins have a role in the preparation of AI virions. The M1 protein is not only as a structural component of the virus but also has a role in early infection in M1 protein separation and RNP to get into the cytoplasm of the tropism cells. On the other hand, the M2 protein along with the HA and NA proteins build the viral envelope structure and also as an ion channels[17].

CONCLUSION

Identification of AI viruses can be conducted conventionally through HA and HI tests and molecularly through gene amplification by RT-PCR method. Based on HA and HI as well as a molecular analysis by RT-PCR amplification in all samples from Aceh province, it can be concluded that the infectious agents which cause of death of various poultry species were the AIV type A, subtype H5.

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