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Full Length Research Paper

Single step multiplex RT-PCR for detection and differential diagnosis of avian influenza, newcastle disease and infectious bursal disease viruses in chicken

Aris Haryanto*[1], Sri Handayani Irianingsih[2], Dini Wahyu Yudianingtyas[3], Nastiti Wijayanti[4] and Teguh Budipitojo[5]

[1]Department of Biochemistry, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta.
[2]Division of Virology, Animal Disease Investigation Center Wates, Daerah Istimewa Yogyakarta Province.
[3]Division of Virology and Biotechnology, Animal Disease Investigation Center, Maros, South Sulawesi Province.
[4]Department of Animal Physiology, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta.
[5]Department of Anatomy, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta.

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Avian Influenza (AI), Newcastle Disease (ND) and Infectious Bursal Disease (IBD) are highly contagious diseases with high occurrence in poultry. These 3 viral diseases are a major cause of disease problems in the poultry industry in Indonesia. The classical methods for detection and characterization of the etiological agents are by clinical sign, serological test, immunodiffusion test, pathology, histopathology and virus isolation. Since these conventional laboratory method have low sensitivity and specificity, the rapid diagnostic tool based on molecular technique are needed. Rapid detection and differential diagnosis for viral diseases have an important implication in clinical, economical and epidemiological aspects. RT-PCR amplification for diagnosis of viral disease in poultry industry is common used. This method can detect virus as etiological agent in poultry disease. Multiplex RT-PCR involves simultaneous amplification of more than one infectious agent using more than primer pair. In the present study, we developed a single step multiplex RT-PCR method, which can help in rapid detection and differentiation viruses as an etiological agent of AI, ND and IBD diseases. The method is highly sensitivity, specificity, fast and less expensive. The results showed that the single step multiplex RT-PCR method has been developed to rapid detection and differential diagnose for AI, ND and IBD viruses simultaneously in one step amplification reaction. This method is simple and easy for laboratory diagnosis application as well as specific and efficient to diagnose of viral diseases in poultry

Keywords: Single step multiplex RT-PCR, differential diagnosis, AI, ND, IBD viruses.

INTRODUCTION

Avian diseases are an important aspect in poultry industry because of high mortality due to viral infection. The etiology agent of viral disease in the poultry is complex, often involving more than one virus at the same time and case. AI, ND and IBD viruses are pathogenic and important in poultry disease because they can cause viral disease independently, in association with other virus and in association with bacterial agents (Ali and Reynolds, 2000).

Avian Influenza (AI) is a viral disease spread worldwide. This disease is caused by influenza A viruses of the family Orthomyxoviridae. Influenza viruses are classified into 16 subtypes on the basis of the surface glycoprotein Hemagglutinin (H1 to H16) and 9 subtypes

*Corresponding Author E-mail: arisharyanto@yahoo.com; Tel: 62-274-560865.

on the basis of the glycoprotein Neuraminidase (N1 to N9). Protein H and N of AI virus are highly variable; therefore, a great number of AI virus (AIV) subtypes occur (Fouchier *et al.,* 2005). Influenza virus type A causes widespread and fatal disease in birds as well as mammals, including humans. Amino acid sequence identity among subtypes of HA and NA ranges from 25-80% and 42-57%, respectively (Colman *et al.,* 1983). All influenza A virus subtypes have been found in aquatic and domesticated birds, and a few subtypes have been recovered from mammals (Pantin-Jackwood and Swayne, 2009). Influenza A viruses that have infected humans during the past 90 years have been limited to the H1, H2, and H3 subtypes. However, human infections with several AI subtypes such as H5N1, H7N7, and H9N2 have occurred, thus demonstrating direct crossing of the species barrier (Peiris *et al*., 2004; Koopmans *et al*., 2004; Butt *et al.,* 2005).

Newcastle Disease (ND) is an infectious viral disease of poultry which has a serious economic impact on poultry industry. The etiological agent of ND disease is a member of avian paramyxoviruses (APMV), which are classified into the *Avulavirus* genus and *Paramyxoviridae* family (Abolnik *et al*., 2004). It has been reported that Paramyxovirus isolated from avian species were grouped into 9 serotypes APMV-1 to APMV-9. ND virus is referred to as APMV-1 (Alexander, 2003). ND virus is an enveloped virus which contains a nonsegmented, single stranded RNA genome with negative polarity. The whole genome is 15 kb encodes 6 major proteins, namely: a large RNA polymerase (L), Hemagglutinin-Neuraminidase (H/N), Fusion protein (F), matrix protein (M), phosphoprotein (P) and Nucleoprotein (NP) (Aldous and Alexander, 2001; dee Leeuw and Peeters, 1999).

The etiologic agent of infectious bursal disease (IBD) is IBD virus. This virus caused an acute and highly contagious disease affecting young chickens with characterized by immunosuppression and a high rate of mortality. IBD virus is belong to the Birnaviridae family with a genome consisting of 2 segments of double-stranded RNA. This virus is a nonenveloped icosahedral virus with a diameter of 55–60 nm. The smaller segment of RNA encodes viral protein (VP) 1, which is the RNA polymerase of the virus. The large segment encodes a polyprotein that is processed into 3 structural proteins, VP2, VP3, and VP4. The large segment also encodes the structural protein VP5 in another reading frame, of these different genes, VP2 gene encodes for the major antigenic protein and its hypervariable region is what confers variability among different IBD virus strains (Azad *et al.,* 1985; Hudson *et al*., 1986; Spies *et al.,* 1987; Mundt *et al*., 1995). The structural protein VP2 has been identified as the major host-protective immunogen of IBD virus (Becht *et al*., 1988).

Rapid detection of virus as etiological agent in poultry disease has important clinical economical and epidemiological implication. So far, the viral genome amplification by RT-PCR method for the diagnosis of viral disease in poultry is common in use. RT-PCR method normally can detect only a single virus in given reaction condition at a time. It cannot detect more than one virus is involved, whereas the multiplex RT-PCR technique involve simultaneous amplification of more than one virus or more than one gene using more than one primer set. This technique has advantage to combine the sensitivity and rapidly of PCR and it can eliminate the need to test clinical samples separately for each virus. Multiplex RT-PCR has been used successfully for typing and subtyping Avian Influenza (AI) virus (Stockton *et al.,* 1998; Munch *et al.,* 2001), and for detection of dual infections, such as Newcastle Disease (ND) and Avian Pneumovirus (APV) (Ali and Reynolds, 2000). In the current study, detection and differential diagnosis by single step multiplex RT-PCR method to amplify 3 viral genes was developed to determine etiologic agent of Avian Influenza Virus (AIV), Newcastle Disease Virus (NDV) and Infectious Bursal Disease Virus (IBDV) for rapid, efficient and simultaneous amplification.

MATERIALS AND METHODS

Sample collection

Samples of AI, ND and IBD viruses were isolated from specific pathogen free (SPF) embryonated chicken eggs which were submitted to the Laboratory of Biotechnology and Virology at Animal Disease Investigation Center (ADIC) Wates, Daerah Istimewa Yogyakarta Province in Indonesia. Based on the serological tests, the research samples are classified into 3 categories: AI positive test, ND positive test and IBD positive test.

Extraction of RNA

Viral RNA of AI, ND and IBD in 200 µl of chorioallantois fluid from infected chicken eggs were extracted using QIAamp® viral RNA mini kit 250 (QIAGEN). The viral RNA was eluted in final volume of 50 µl. The procedure of viral RNA extraction was performed following manufacture's procedure.

Primer design

For amplification by single step multiplex RT-PCR, the specific oligonucleotides primers for amplification of M gene for AI virus was designed by AAHL (2004), F gene for ND virus was designed based on Kho *et al.* (2000) and VP2 gene for IBD virus was designed by Lee *et al.* (1994). Sequence of oligonucleotides primers are presented in the Table 1.

Table 1. Sequence of specific primers for amplification of M gene for AI virus, F gene for ND virus and VP2 gene of IBD virus.

Gene Target	Oligonucleotide Sequence	Product
Matrix (M)	MF: 5'-GCACTTGAATTGTGGATTCTTAGTC-3'	200 bp
AI virus	MR: 5'-AGTAGAAACAAGGTAGTTTTTTACTCC-3'	
Fusion Protein (F)	FF: 5'-TACACCTCATCCCAGACAGGGTC-3'	532 bp
ND virus	FR: 5'-AGGCAGGGGAAGTGATTTGTGGC-3	
Viral Protein (VP2)	VP2F: 5'-GTCTACACCATAACTGCCGCAGATGAT-3'	447 bp
IBD virus	VP2R:5'-GGCTACTAGTGTGACGGGGCGGAGGGCACC-3'	

Amplification of viral genes by single step multiplex RT-PCR

Amplification of RNA viruses by RT-PCR were performed simultaneously in single step multiplex RT-PCR, which targeted to amplify M gene of AI virus, F gene of ND virus and VP2 gene of IBD virus using SuperScript™ III One-Step RT-PCR kit with Platinum®Tag (INVITROGEN). The primers used in this study are listed in Table. The multiplex RT-PCR was carried out in a reaction volume of 25 µl containing 12,5 µl of RT buffer (12,5 mM $MgCl_2$), 1 µl of deoxynucleoside triphosphates (dNTP) mixture (10 mM of each dNTP), 1 µl of enzyme mix, 1 µl of each primer: 1 µl MF and 1 µl MR for AI virus, 1 µl FF and FR for ND virus, 1 µl VP2F and 1 µl VP2R for IBD virus (10 pmol for each primer), 1 µl of RNA (500 ng), and 3,5 µl of nuclease-free water. Reverse transcription was carried out at 48ºC for 30 minutes followed by initial denaturation at 95ºC for 3 minutes. The PCR conditions used were 35 cycles of 94º C for 20 seconds (denaturation), 50ºC for 30 seconds (annealing), and 72ºC for 20 seconds (extension). After 35 cycles of PCR (INFINIGEN), the final extension was carried out at 72ºC for 5 minutes and soak at 4ºC for 30 minutes.

Agarose Gel Electrophoresis

A total of 12,5 µl RT-PCR product, loading buffer and H_2O were loaded onto 1,5% agarose gel. Then it was electrophoresed in Tris Boric Acid-EDTA (TBE) buffer at 80 volts for 45 minutes. After that, the DNA fragments of RT-PCR products were stained with Sybersafe (INVITROGEN) and visualized by UV transilluminator in the dark room.

RESULTS AND DISCUSSION

Molecular diagnosis method, such as multiplex RT-PCR has enabled rapid results. This method is not dependent on time consuming methods of propagation and virus isolation. Multiplex RT-PCR method allows for the simultaneous amplification of several genes and several viruses, thereby this method optimizing the use of reagents and decreasing personnel time (Renshaw *et al.*, 2001). Most multiplex assay for avian viruses are directed to the detection of these agents in poultry (Ali and Reynolds, 2000), while there remains a need for rapid screening of possibly differently introduced viruses that share the same reservoirs in the wild. A single step multiplexes RT-PCR for AI, ND and IBD viruses described in present work. Early detection and differential diagnosis of AI, ND and IBD is necessary for the control of these viral diseases. The PCR has applied to the rapid detection and differential diagnosis of AI, ND and IBD. It resulted in a very specific and sensitive assay (Wu *et al.,* 1992). In this study, the RT-PCR method offers several advantages. First, there is no need to grow or isolate virus from clinical specimens before RT-PCR amplification, which significantly reduces the time and labor. Second, the RT-PCR results in exponential amplification of a specific virus complementary DNA (cDNA) sequence flanked by a pair of primers selected from a known cDNA sequence of each viral genome.

Detection and differential diagnosis of AI, ND and IBD viruses using RT-PCR amplification is not new for diagnosis of viral disease in poultry. A lot of this diagnosis tools have been developed to AI, ND and IBD viruses individually in different tube, different reaction and different RT-PCR condition. In this study, we have developed a technique for the simultaneous detection and differential diagnosis of 3 viruses as etiological agent of AI, ND and IBD disease by single step multiplex RT-PCR using the selected primers sets produced virus-specific products of expected DNA fragment size in RT-PCR. The amplification was performed simultaneously by single step multiplex RT-PCR for AI, ND and IBD viruses. Single step multiplex RT-PCR amplication results for M gene of AI virus, F gene of ND virus and VP2 gene of IBD virus respectively, are depicted in Figure 1.

Amplification viral genome using RT-PCR for detection of AI, ND and IBD viruses have already done individually in different amplification reaction. The expected RT-PCR product showed as DNA fragments which have size of nucleotides as described before by AAHL (2004) for AI virus, Kho et al. (2000) for ND virus and Lee et al. (1994) for IBD viruses. To do more effective, efficient and easier, this study has been developed a modification technique for the simultaneous detection of AI, ND and IBD viruses

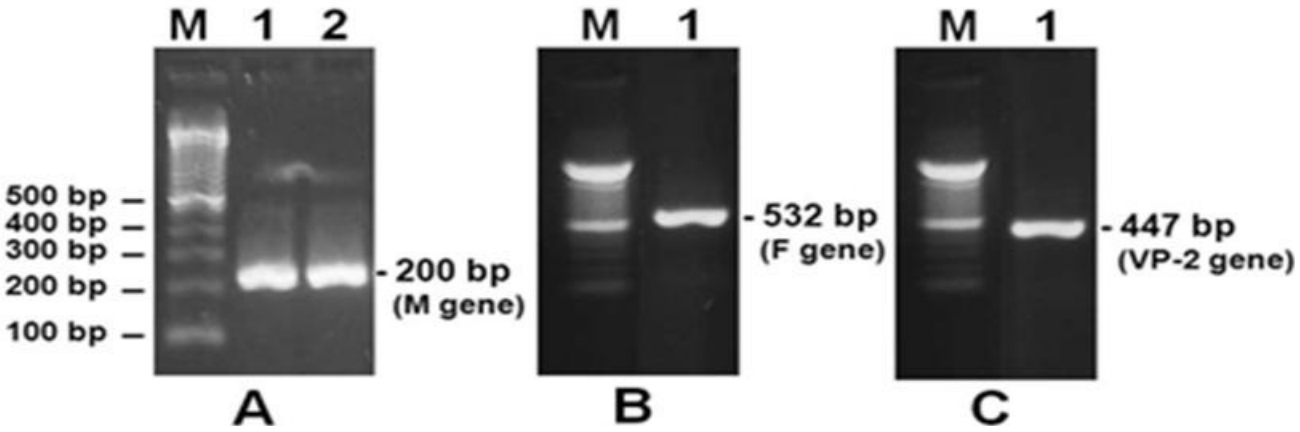

Figure 1. Agarose gel electrophoresis of RT-PCR products for M gene of AI virus, F gene of ND virus and VP2 gene of IBD virus. (A) RT-PCR amplification of M gene AI virus with expected products in size of 200 bp. (B) RT-PCR amplification of F gene ND virus with expected products in size of 532 bp. (C) RT-PCR amplification of VP2 gene IBD virus with expected products in size of 447 bp. (M) Marker DNA 100 bp ladder; 1 and 2 are samples

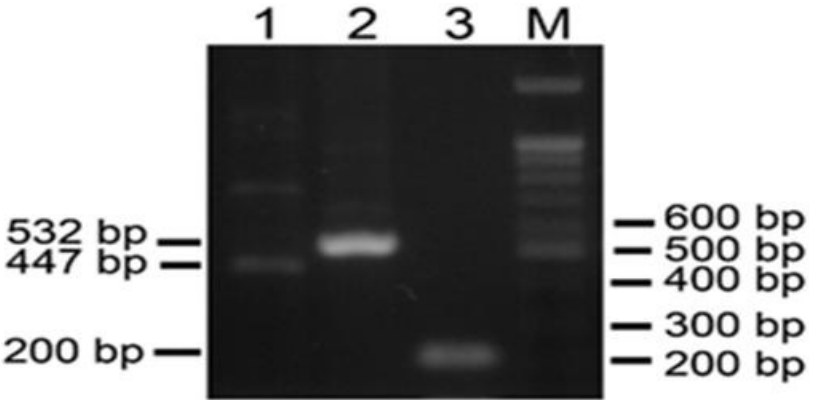

Figure 2. Agarose gel electrophoresis of simplex RT-PCR products for M gene of AI virus, F gene of ND virus and VP2 gene of IBD virus. Lane 1 is VP2 gene of IBD virus with expected products in size of 447 bp. Lane 2 is F gene of ND virus with expected products in size of 532 bp. Lane 3 is M gene of AI virus with expected products in size of 200 bp. M is Marker DNA 100 bp ladder.

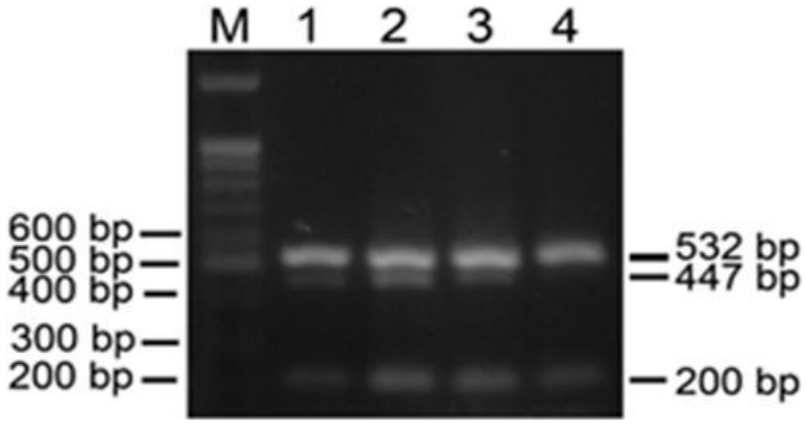

Figure 3. Agarose gel electrophoresis of single step multiplex RT-PCR products for M gene of AI virus, F gene of ND virus and VP2 gene of IBD virus. An expected product of M gene ND virus is fragment DNA in size of 200 bp, F gene of ND virus is in size of 532 bp and VP2 gene of IBD virus is in size of 447 bp. M is Marker DNA 100 bp ladder; 1, 2, 3, 4 are samples.

in a single step multiplex RT-PCR reaction. The each primer set produced virus specific products of expected size of 200 bp for M gene of AI virus, 532 bp for VP2 gene of ND virus and 447 bp for F gene of IBD virus respectively. For RT-PCR amplification, the target sequences selected were Matrix (M) gene for AI virus, Fusion Protein (F) gene for ND virus and Viral Protein 2 (VP2) for IBD virus. First, the amplification is conducted as simplex RT-PCR in individual reaction for different reaction and different amplification condition. The electrophoresis of simplex RT-PCR product more detail presented in Figure 2.

Multiplex RT-PCR was designed to simultaneously amplify three genes from three different viruses which commonly infected in poultry in a single step reaction to detect M gene of AI virus, F gene of ND virus and VP2 gene of IBD virus using specific nucleotide primers for each gene (see Table). Conventional methods for detection and differential diagnosis of AI, ND and IBD require the inoculation and propagation into embryonated fowl eggs or tissue culture followed serological tests (Wei *et al*., 2006). Result of amplification using single step multiplex RT-PCR method of M gene for AI virus, F gene of ND virus and VP2 gene of IBD virus respectively, are presented in Figure 3.

Based on this results, it indicates that detection and differential diagnosis of 3 viruses, as etiological agent of AI, ND and IBD diseases by using single step multiplex RT-PCR holds potential and reliable as a rapid diagnostic method for the simultaneous detection of 3 viruses with material genetic of RNA in clinical and field samples from poultry (Malik *et al*., 2004). This single step multiplex RT-PCR method could be used to investigate the presence of each AI, ND and IBD for rapidly detect and differential diagnosis of each virus from clinical specimens (Chang *et al*., 2008).

A multiplex RT-PCR that can rapidly differentiate between these infectious agents will be very important for the control of disease transmission not only in poultries but also in humans, along with the identification of three most common pathogen viruses which often found as mixed infections in poultry disease, and hence economic losses will be reduced in poultry (Rashid *et al*., 2009). In conclusion, the single step multiplex RT-PCR method has been developed to rapid detect and differential diagnose for AI, ND and IBD virus simultaneously one step amplification reaction. This method is a simple and easy for laboratory diagnosis application as well as specific and efficient to diagnose of viral diseases in poultry.

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