Biochemistry and Biotechnology Research
Vol. 2(2), pp. 27-31, May 2014
ISSN: 2354-2136
Full Length Research Paper

Detection and differentiation of pathogenic H5 and H7 influenza A virus subtypes in Indonesian poultry by multiplex reverse transcription-polymerase chain reaction

R. Wasito[1]*, H. Wuryastuty[1], G. Tjahyowati[2], S. H. Irianingsih[2], T. Tyasasmaya[1] and R. K. Maes[3]

[1]Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia.
[2]Disease Investigation Center, Wates, Yogyakarta, Indonesia.
[3]Diagnostic Center for Population and Animal Health, Michigan State University, 4125 Beaumont Rd., Lansing, M. 48910, USA.

Accepted 20 May, 2014

ABSTRACT

Influenza A viruses (AIV) are known to infect birds and have 16 hemagglutinin (H1-16) and 9 neuraminidase (N1-9) subtypes. The H5 and H7 hemagglutinin subtypes are circulating in poultry and continue to cause morbidity and mortality in Indonesian poultry operations. At present, the laboratory diagnosis of influenza A virus is still based on virus isolation and conventional RT-PCR, both of which are time consuming and a relatively expensive. Multiplex RT-PCR (mRT-PCR) is designed to amplify more than one target gene using multiple primers in one reaction. The mRT-PCR is designed to overcome the above-mentioned virus isolation and conventional RT-PCR shortcomings, while maintaining required sensitivity and specificity. In the present study, sera and lung tissues were obtained from poultry suggested of having AIV infection, based upon combination of pathological examination, serological testing and virus isolation. Viral nucleic acid were extracted from the specimens using commercial kit. Three separate sets of primers were used to amplify AIV matrix, H5 and H7 sequences. The respective sizes of the amplification products were 1023, 456 and 208 bp. Based on the results obtained, it was concluded that the mRT-PCR technique has high specificity to detect AIV and to differentiate between the pathogenic H5 and H7 hemagglutinin subtypes in chickens, quails and ducks. This technique could be applied to routine biosurveillance of influenza A virus in chickens in Indonesia.

Keywords: Biosurveillance, influenza A viruses, mRT-PCR, pathogenic hemagglutinin, poultry.

*Corresponding author. E-mail. wst_ugm@yahoo.com, hastari_ugm@yahoo.com. Tel: 062-0274-9061104.

INTRODUCTION

AVIAN influenza (AI) is caused by influenza type A virus, classified within the family of *Orthomyxoviridae.* AI occurs worldwide occurrence and has led to major economic losses for the poultry industry. At present, 16 hemagglutinin subtypes (HA1-16) and 9 neuraminidase subtypes (NA1-9) are recognized (Fouchier et al., 2005; OIE, 2009). All influenza type A virus are found in both aquatic and birds, but only a subset of the currently recognized several subtypes have been found in commercial poultry. The most important ones being H5, H7 and H9. All H5 and H7 subtypes may be pathogenic in humans and therefore specific diagnostic identification

to H5 and H7 subtypes is important for biosurveillance in birds (Koopman et al., 2004; Tran et al., 2004). Although, the H5 and H7 subtypes have also been identified in quail both serologically and by molecular diagnostic methods in Indonesia in 2007 (Wuryastuti, nd), the overall prevalence of influenza type A of the H7 subtype has not yet been investigated extensively.

AI virus is endemic causing high morbidity and mortality in commercial chicken flocks in Indonesia. To date, the routine laboratory diagnosis for viral diseases, including the AI virus is still based on virus isolation in embryonated chicken eggs, followed by hemaggulitination inhibition-based testing to differentiate between viruses subtypes. In terms of use in biosurveillance and disease investigation, these diagnostic techniques, which take more than one week to complete, are less than ideal. Meanwhile, the use of PCR as an alternative is limited by assay cost, sometimes the availability of adequate test sample volume and by diagnostic capacity if only one specific type or viral subtype being analyzed per PCR assay. To overcome these limitations, a multiplex reverse-transcriptase polymerase chain reaction (mRT-PCR) has been described (Rashid et al., 2009; Saberfar et al., 2009). The mRT-PCR is a molecular biology approach to simultaneously ampify more than one target sequence using more than one pair of primers in the reaction. Thus, the mRT-PCR has the potential to produce considerable savings of time, effort and even chemicals within laboratory without compromising test utility (Elnifro et al., 2000). In the field of infectious diseases, the mRT-PCR approach has been shown to be a valuable method for identification and differentiation of avian influenza viruses and other poultry respiratory pathogens (Rashid et al., 2009; Saberfar et al., 2009; Wang and Taubenger, 2010).

Based on the information provided by the Disease Investigation Center, Wates, Yogyakarta, Central Java, Indonesia, the laboratoric diagnosis for the AI virus samples for the routine surveillance program is still based on virus isolation in embryonated chicken eggs, followed by HI-based testing to differentiate between viruses subtypes. Serotyping is routinely required to identify influenza type A virus of subtype H5N1. In addition, uniplex RT-PCR is currently required to confirm the presence of subtype H7 if serotyping test is positive this subtype.

MATERIALS AND METHODS

Sources of samples

In the present study, sera and lungs tissues, in the total of fifty (50) collected from chicken (25), quail (15) and duck (10) suggested of having AIV infection pathologically and were AIV positive samples based on RT-PCR were used as samples in this studies. AIV (H5N1) and AIV (H7N1) originally isolate from previous field cases among poultry in Yogyakarta were uesed for positive control.

Extraction RNA procedure

Viral genomic RNA was extracted from chorio-allantoic fluid by using high pure viral nucleic acid kit (Roche). Two hundreds microliter of each sample was pipetted into a sterile 1.5 ml Eppendorf tube. Two hundreds µl of binding buffer, supplemented with poly A and 50 µl of proteinase K solution was added into the Eppendorf tube. The mixture was vortexed and incubated in a waterbath at 72°C for 10 min. After incubation, 100 µl of binding buffer was added and the mixture was put into a high pure filter tube, collection tube assembly and then centrifugated at 8,000 rpm for 1 min. The flowthrough and the collection tube were discarded and replaced with a new collection tube. Inhibitor removal buffer (500 µl) was added to the filter tube and the assembly was then centrifugated at 8,000 rpm for 1 min. The flowthrough and the collection tube were discarded and was replaced with a new collection tube. Wash buffer (450 µl) containing 70% ethanol, was added to the filter tube and the assembly was centrifugated at 8,000 rpm for 1 min. The flowthrough and the collection tube were again discarded. The filter tube was combined with a new collection tube and 450 µl of wash buffer was again added to the filter tube, followed by centrifugation at 8,000 rpm for 1 min. The flowthrough was discarded, but the filter tube-collection tube assembly was left in the centrifuge and re-spin for 30 s at maximum speed (12,00 rpm) to remove wash buffer. The filter tube was then placed in a sterile Eppendorf tube and 50 µl of elution buffer was added into the filter tube. The tube assembly was centrifugated at 8,000 rpm for 1 min. The centrifugation was repeated once more at 12,000 rpm for 30 s to maximize the collection of RNA. Subsequently, the filter tube was discarded and the Eppendorf containing the purified RNA was kept at -20°C until *m*RT-PCR test was performed.

One hundred mg of the lung tissue was added to a sterile, 2 ml Eppendorf tube. One ml of Trizol solution was added to the tube and the tissue was homogenized in a Heidalp-p7 homogenizer for 3 min. The homogenate was then incubated at room temperature for 5 to 10 min and 200 µl of chloroform was added to the homogenate slowly. The tube was then vigorously shaken for 15 s to obtain complete mixing of the contents. This mixture was incubated at room temperature for 10 minutes and was then centrifugated at 12,000 rpm at 4°C for 15 min. Clear supernatant (400 µl) was collected and was added to a new, sterile Eppendorf tube containing 500 µl of isopropanol and 0.5 µl of glycogen solutions. This mixture was centrifugated at 12,000 rpm, at 4°C for 15 min. After centrifugation, the supernatant was discarded and the pellet was washed by adding 1000 µl of 70% ethanol. The washed pellet was centrifugated at 7,500 rpm at 4°C for 5 min. This washing procedure was done twice. After the washings, the supernatant was discarded and the pellet was air dried at room temperature. The dried pellet was diluted into the 50 µl of RNase-free H_20. The Eppendorf tube containing the filtrate was kept at -20°C until the mRT-PCR was performed.

Multiplex RT-PCR procedure

The mRT-PCR assay was carried out with three sets of primers and thus referred to as a trivalent mRT-PCR. The H5 and H7-specific primer sets were obtained from the literature (Webster and Kawaoka, 1987; Ng et al., 2006; Anonymous, 2007), and synthesized by Integrated DNA Technologies, Inc. (Table 1). The primers were diluted in buffer to a new concentration of 20 pMol/µl, aliquoted and was kept at -20°C until used for mRT-PCR analysis.

Amplification condition

Multiplex RT-PCR (mRT-PCR) used in the present study is one

Table 1. Primer base sequence for mRT-PCR.

No.	Gene target	Primer sequence	PCR product (bp)
1	Gene matrix	MF: 5'-GAA GGT AGA TAT TGA AAG ATG-3' MR: 5'-GAA ACA AGG TAG TTT TTT ACT C-3'	1023
2	Gene H5	H5F: 5'- ACT ATG AAG AAT TGA AAC ACC T-3' H5R: 5'-GCA ATG AAA TTT CCA TTA CTC TC-3'	456
3	Gene H7	H7F: 5'-GGG GCT TTC ATA GCT CCA GAT CGT GC-3' H7R: 5'-TCT CCT TGT GCA TTT TGA TGC-3'	208

step procedure consisted of reverse transcription and amplification in one tube. Superscript III One-step RT-PCR kit with platinum Taq DNA polymerase (Invitrogen) which is commercially available was used. The 25 µl 2x reaction mix containing buffer for reverse transcription and PCR, deoxyribonucleotide triphosphate and buffer solution, 2,5 µl Mg^{++}, 1 µl each primer [20 pMol], 2 µl RT/platinum Taq mix, 5 µl AIV RNA and nuclease free water was pipetted into each PCR tube until the final total volume is 50 µl. The mRT-PCR amplification was done by using thermocycle machine with cycle conditions as follows: cDNA synthesis at 45°C for 45 min, early denaturation at 96°C for 5 min, then 40 cycles and each cycle consisted of denaturation at 96°C for 1 min, annealing at 53°C for 1 min and extention at 72°C for 1 min, and followed by final extention at 72°C for 10 min. The products of amplified cDNA bands were visualized by using gel agarose 1.5% with ethidium bromide and DNA markers 100 bp (Vivantis) at 100 V for 25 min. The amplification results were stained with ethidium bromide and the photographs were taken by using Gel Logic 100 Imaging System.

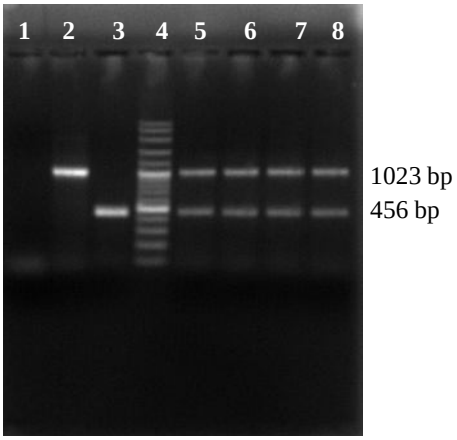

Figure 1. mRT-PCR product of AIV subtype H5. Lane 1 represent AIV negative control. Lane 2 represent AIV positive control (1023 bp). Lane 3 represent AIV H5 subtype positive control (456 bp). Lane 4 represent 100 bp DNA marker. Lane 5, 6, 7 and 8 represent samples positive for AIV subtype H5 from chicken.

RESULTS AND DISCUSSION

The results of the present study indicated that the mRT-PCR used successfully amplified influenza type A virus (AIV) and differentially diagnosed the most pathogenic AIV hemagglutinin subtypes H5 and H7 in poultry simultaneously. As demonstrated in Figures 1 to 3 AIV matrix, H5 and H7 gene-specific PCR amplification products had respective sizes of 1023, 456 and 208 bp. Review of mRT-PCR results indicated that AIV infection in both layer and broiler chickens were mostly due to the infection with AIV strains of H5 subtype (Figure 4). It was reported that most of the poultry cases of AIV in 2005 in Indonesia appeared to be AIV H5N1 subtype (Wuryastuti et al., 2005). This AIV H5N1 type is highly pathogenic with a mortality rate of 75% or above. As of 2013, there had been no direct evidence for the presence of highly pathogenic AIV H5N1. Instead, the AIV H5N1 in Indonesian poultry had lower pathogenicity. In the present study, 5 of 15 lungs tissues specimens obtained from quail were H7 hemagglutinin subtype positive, but one of them yielded a mixture of both H5 and H7-specific mRT-PCR amplification products. Pertaining to the lungs tissue specimens obtained from the ducks, 3 of 10 were AIV H7 subtype positive and one specimen was positive for both the AIV H5 and H7 subtypes. Spread of the AIV H7 subtype occurred more rapidly in quails than in other poultry species (Perez et al., 2003). The mRT-PCR did not amplify the gene matrix of all specimens, although repeated amplification had been done to the same negative mRT-PCR sample, and likewise, no amplification bands of the sizes (1023, 456 and 208 bp) could be amplified for RNA for AIV by those specific primers. The AIV matrix gene is a determinant of the AIV type which is the highest numbers in the AIV protein structure. The amplification efficiency of a primers set can be influenced by target size (Maes, 2012-2013). This was potentially substantiated since the use of primers that amplified a smaller fragment of AIV matrix gene-specific target resulted in amplification of the matrix gene in all samples.

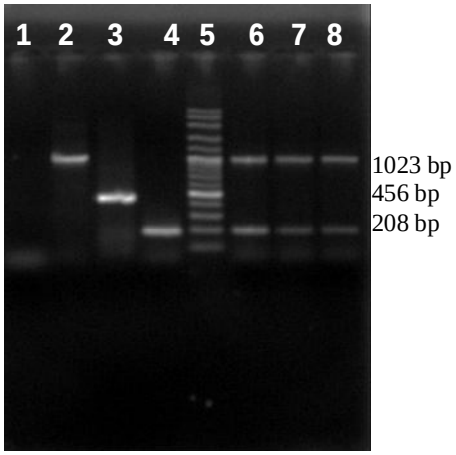

Figure 2. mRT-PCR product of AIV subtype H7. Lane 1 represent AIV negative control. Lane 2 represent AIV positive control (1023 bp). Lane 3 represent AIV H5 subtype positive control (456 bp). Lane 4 represent AIV H7 subtype positive control (208 bp). Lane 5 represent 100 bp DNA marker. Lane 6, 7 and 8 represent samples positive for AIV subtype H7 from duck.

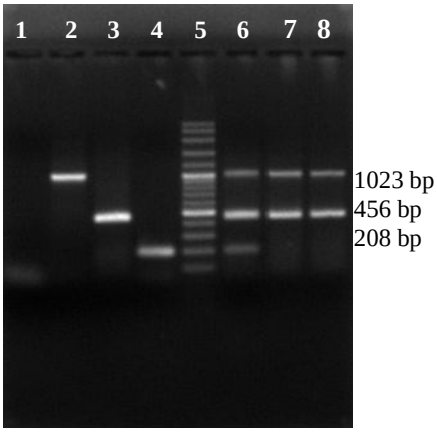

Figure 3. mRT-PCR product of AIV subtype H5 and H7. Lane 1 represent AIV negative control. Lane 2 represent AIV positive control (1023 bp). Lane 3 represent AIV H5 subtype positive control (456 bp). Lane 4 represent AIV H7 subtype positive control (208 bp). Lane 5 represent 100 bp DNA marker. Lane 6, 7 and 8 represent samples positive for AIV subtype H5 and H7 from quail.

The efficiency of mRT-PCR depends on rate of annealing of the primers to target genes according to oligonucleotide sequence of its interest and the rate of the annealed primers rate to be elongated based on the DNA sequence of its interest during the early, middle and late of amplification cycles. Poor design primers, non-optimal supporting buffer and annealing temperature are the factors that may inhibit optimal annealing rate. Rapid elongation between elongated primers and its annealed target sequence depends on enzyme activity and availability of important components, such as dNTP and biological properties of DNA targets. That is why modifications to improve mRT-PCR performance is mainly aimed to factors that influence annealing and/or rapidity of extension (Elnifro et al., 2000). The optimization of of mRT-PCR amplification has several constraints, such as poor sensitivity and specificity and/or preferential amplification of certain specific targets (Mutter and Boynton, 1995).

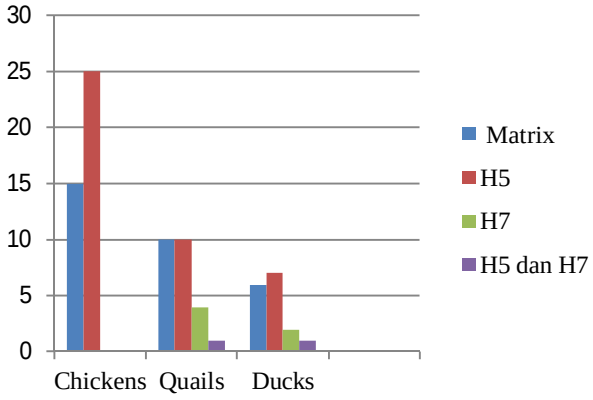

Figure 4. The recapitulation of mRT-PCR. AIV infections in chickens were mostly due to the H5 subtype.

False negatives are often revealed in mRT-PCR amplification due to the presence of more than one set of primers in mRT-PCR that will be able to increase primarily formation of primer dimers (Edwards and Gibbs, 1994; Brownie et al., 1997). These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Thus, the optimization of mRT-PCR should aim to minimize or reduce such nonspecific interactions. In addition, special attention to primer design parameters, such as primers homology with their target nucleic acid sequences, their length, the GC content, and their concentration have to be considered (Wu et al., 1991; Dieffenbach et al., 1993; Shuber et al., 1995; Mitsuhashi, 1996; Nicodeme and Steyaert, 1997; Robertson and Walsh-Weller, 1998). Ideally, all the primer pairs in a mRT-PCR should enable similar amplification effciencies for their respective target. This may be achieved through the utilization of primers with nearly identical optimum annealing temperatures (primer length of 18 to 30 bp or more and a GC content of 35 to 60% may prove satisfactory (Wu et al., 1991; Dieffenbach et al., 1993; Shuber et al., 1995). Preferential

amplification of one target sequence over another is a known phenomenon in mRT-PCRs that is designed to amplify more than one target simultaneously (Walsh et al., 1992; Mutter and Boynton, 1995; Polz and Cavanaugh, 1998). In the present study, although all of the positive RT-PCR results were compatible both with clinical signs and pathological lesions in AIV infected-poultry, positive mRT-PCR results were not obtained under all conditions. A variety of parameters, such as specimen collection, specimen storage and storage and extraction method used can all have an effect on amplification efficiency of an mRT-PCR. Primer-specific parameters do however have the most profound effect and should therefore be optimized very carefully (Edwards and Gibbs, 1994; Brownie et al., 1997; Wu et al., 1991; Dieffenbach et al., 1993; Shuber et al., 1995; Mitsuhashi, 1996; Nicodeme and Steyaert, 1997; Robertson and Walsh-Weller, 1998). Since the H5 and H7 hemagglutinin subtypes are circulating. Our data indicated that mRT-PCR successfully amplified influenza type A virus (AIV) and diagnosed differentially the most AIV pathogenic hemagglutinin subtypes H5 and H7 in poultry simultaneously. The results of the present study are very important since the most factor in the outbreaks of AIV in birds, especially chickens, quails and ducks in Indonesia was due to H5 infection and/or a mixed infection of H5 and H7 hemagglutinin subtypes that are presently circulating. The multiplex approach in this study can play a vital and efficient role in the biosurveillance of AIV in Indonesia in order to reduce the risk of those subtypes to become pathogenic in human beings.

ACKNOWLEDGMENT

We gratefully acknowledge the DIKTI, Kemendiknas RI and Gadjah Mada University for financial support via Penelitian Unggulan Perguruan Tinggi, Gadjah Mada University (LPPM-UGM/1348/LIT/2013). We are grateful for the advice provided by Prof. Roger K. Maes, Michigan State University, East Lansing, MI, USA during the preparation of this manuscript.

REFERENCES

Anonymous, 2007. One step RT PCR for detection of H5 & H7 avian influenza & cleavage site sequencing. Avian Influenza Community Reference Laboratory. pp. 1-10.

Brownie J, Shawecross S, Theaker J, Whitcombe D, Ferrie R, Newton RC, Little S, 1997. The elimination of primer-dimer accumulation in PCR. Nucleic Acids Res, 25:3235-3241.

Dieffenbach CW, Lowe TMJ, Dveksler GS, 1993. General concepts for PCR primer design. PCR Meth Appl, 3:S30-S37.

Edwards MC, Gibbs RA, 1994. Multiplex PCR: Advantages, development, and applications. Genome Res, 3:S65-S75.

Elnifro EM, Ashshi AM, Cooper RJ, Klapper PE, 2000. Multiplex PCR: Optimization and application in diagnostic virology. Clin Microbiol Rev, 13:559-570.

Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD, 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol, 79:2814-2822.

Koopman M, Wilbrink B, Conyn M, 2004. Transmission of H7N7 avian influenza A virus to humanbeing during a large outbreak in commercial poultry farm in the Netherlands. Lancet, 63:587-593.

Maes RK, 2012-2013. Virology Section, Department of Microbiology and Public Health, College of Veterinary Medicine, Michigan State University, E. Lansing, MI, USA, private communication.

Mitsuhashi M, 1996. Technical report: Part 2. Basic requirements for designing optimal PCR primers. J Clin Lab Anal. 10:285-293.

Mutter GL, Boynton KA, 1995. PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. Nucleic Acid Res, 23:1411-1418.

Ng L, Barr I, Nguye T, Noor SM, Tan R, Agathe LV, Gupta S, Khalil H, To TL, Hassan SS, Ren E, 2006. Specific detection of H5N1 avian influenza A virus in field specimens by a one-step RT PCR assay. BMC Infect Dis, 10:6-40.

Nicodeme P, Steyaert J, 1997. Selecting optimal oligonucleotide primers for multiplex PCR. Intel Syst Mol Biol, 51:210-213.

OIE, 2009. Avian influenza. OIE Chapter 2.3.4. Terrestrial Manual 2009. Version adopted by the World Assembly of Delegates of the OIE in May 2009, pp. 1-20.

Perez DN, Lim W, Seller JP, Yi G, Peiris M, Shortridge KF, Webster RG, 2003. Role of quail in the interspecies transmission of H9 influenza A viruses. Molecular changes on HA that correspond to adaptation from ducks to chickens. J Virol, 7:3148-3156.

Polz MF, Cavanaugh CM, 1998. Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microbiol, 64: 3724-3730.

Rashid S, Naeem K, Ahmed Z, Saddique N, Abbas MA, Malik SA, 2009. Multiplex polymerase chain reaction for the detection and differentiation of avian influenza viruses and other poultry respiratory pathogens. Poultry Sci, 88:2526-2531.

Robertson JM, Walsh-Weller J, 1998. An introduction to PCR primer design and optimization of amplification reactions. Meth Mol Biol, 98:121-154.

Saberfar E, Najafi A, Goodarzi Z, Lashini H, 2009. Multiplex reverse transcription-PCR assay for detection of type A influenza virus plus differentiation of avian H7 and H9 hemagglutinin subtypes in Iran. Iranian J. Publ. Health, 38:29-34.

Shuber AP, Grondin VJ, Klinger KW, 1995. A simplified procedure for developing multiplex PCRs. Genome Res, 5:488-493.

Tran TH, Nguyen TL, Luong TS, Pham PM, Nguyen VC, 2004. Avian influenza (H5N1) in 10 patients in Vietnam. N Engl J Med, 350:1171-1172.

Walsh PS, Erlich HA, Higuchi R, 1992. Preferential PCR amplification of alleles: H. A. Mechanisms and solutions. PCR Meth Appl, 1:241-250.

Wang R, Taubenger JK, 2010. Methods for molecular surveillance of influenza. Expert Rev Anti Infect Ther, 8:517-527.

Webster RG, Kawaoka Y, 1987. Avian influenza. Crit Rev Poult Biol, 1:212-246.

Wu DY, Ugozzoli I, Pal BK, Qian J, Wallace RB, 1991. The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. DNA Cell Biol, 10:233-238.

Wuryastuti H, nd. H5 and H7 subtypes in quails. Unpublished work.

Wuryastuti H, Widyarini S, Yanuartono, Adji D, Gesit T, Wasito DR, 2005. Kajian *avian influenza* (AI) pada unggas". Laporan Kajian AI. Kerjasama antara Lembaga Penelitian Universitas Gadjah Mada, Yogyakarta dan Direktorat Jenderal Bina Produksi Peternakan, Departemen Pertanian, Jakarta.