Bangladesh J Microbiol, Volume 26, Number 1 & 2, June and December 2009, pp 21-25

Original Article

Development of Multiplex PCR for Simultaneous Detection of Avian Influenza and Newcastle Disease Viruses

Md. Rezaul Bari[1,2], Md. Taohidul Islam[1,3], Muhammad Tofazzal Hossain[1], Mohammad Mohiuddin[1], Mohammad Ali Zinnah[1] and Md. Alimul Islam[1*]

[1]Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh, [2]School of Agriculture and Rural Development, Bangladesh Open University, Gazipur-1705, Bangladesh, [3]Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

[Received 15 July 2009; Accepted 15 May 2010]

A study was undertaken to develop a rapid, sensitive and reliable molecular method for complete sub-typing of avian influenza virus (H5N1) either alone or simultaneous detection of avian influenza (H5N1) and Newcastle disease viruses prevalent among the poultry population of Bangladesh. Complimentary DNA of reference Newcastle disease (ND) and avian influenza (H5N1) viruses were used to develop a uniplex polymerase chain reaction then duplex and multiplex polymerase chain reaction. The band of the polymerase chain reaction products for F gene of Newcastle disease virus, hemagglutinin and neuraminidase genes of avian influenza virus appeared at 356 bp, 219 bp, and 616 bp on 2% NA agarose gel respectively. The newly developed and standardized multiplex polymerase chain reaction was applied on the field samples. For this, clinical and post-mortem samples were collected from naturally and experimentally Newcastle disease virus infected chickens and subsequently inoculated into 10-day-old chicken embryos for collection of allantoic fluid. All the samples showed the band at the position of 356 bp that indicated Newcastle disease virus positive. None of the samples were showed the band at the position 219 bp and 616 bp for hemagglutinin and neuraminidase genes of avian influenza virus respectively. Results of the present study clearly indicated that the multiplex polymerase chain reaction is a highly sensitive, specific and rapid test for complete sub-typing of avian influenza virus along with simultaneous detection of Newcastle disease virus.

Key words: Avian influenza virus, Newcastle disease virus, Polymerase chain reaction, Sub-typing

Introduction

Highly pathogenic avian influenza (HPAI) and Newcastle disease (ND) are considered as top ranking list. A viral diseases of the Office International des Epizooties which have been responsible for serious losses to the poultry industry[1-2]. Avian influenza also has caused severe human losses throughout the centuries[3]. Nevertheless, avian influenza has been transmitted to human directly, as seen in Hong Kong[4].

Three types of influenza viruses, types A, B, and C are known and they belong to a family of single-stranded negative-sense enveloped RNA viruses called *Orthomyxoviridae*. The viral genome is comprised of eight RNA segments (seven in type C). Influenza A viruses can be classified into subtypes based on antigenic difference in the two surface glycoproteins, name, hemagglutinin (HA) and neuraminidase (NA) which is required for viral attachment and cellular release. Other major viral proteins include the nucleoprotein (NP) which is the main structural protein, membrane proteins (M1 and M2), polymerase proteins (PA, PB1 and PB2), and non-structural proteins (NS1 and NS2). Currently, sixteen subtypes of HA (H1-H16) and nine NA (N1-N9) antigenic variants are known in influenza A virus mostly related with veterinary significance[5].

Newcastle Disease (ND) is caused by avian paramyxovirus serotype 1 (APMV-1) viruses, which, with viruses of the other eight APMV serotypes (APMV-2 to APMV-9), have been placed in the genus *Rubulavirus*, sub-family *Paramyxovirinae*, family *Paramyxoviridae*, in the current taxonomy[6]. The ND virus has a 15 kb RNA genome that codes for six viral proteins: an RNA-directed RNA polymerase (L), hemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P) and nucleoprotein (NP). The F glycoprotein is responsible for fusion between the

***Corresponding author:**
Dr. Md. Alimul Islam, Professor, Department of Microbiology and Hygiene, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh, Tel (Office): (091) 67401-6 (Extn 2386), Cell: 01714 325562, E-mail: alim_bau@yahoo.co.in

cellular and viral membranes, and subsequent virus genome penetration[7].

A variety of techniques are commonly used for the isolation and identification of AIV and NDV such as isolation in embryonated eggs, enzyme immunoassays, hemagglutination inhibition (HI) test, enzyme linked immunosorbent assay or direct immunofluorescence test[3, 8-9]. With the advancement of science, various molecular methods have been established for complete subtyping of AIV and simultaneous detection of highly pathogenic AIV and NDV[10-12]. Molecular methods of diagnosis can be used for type A influenza viruses for screening of samples using primers specific for nucleoprotein (NP) gene[13-14] or matrix (M) protein gene[15]. It can also be used for complete subtyping of AIV using HA and NA gene specific primers[14,16-17]. Methods such as Multiplex RT-PCR assays allow for the simultaneous amplification of several genes, thereby minimizing the use of reagents and decreasing personnel time[18].

Recently, Bangladesh is facing with a series of outbreak of avian influenza following emergence of HPAI virus (H5N1) at different location throughout the country such as at Rangpur, Narsingdi, Joupurhat, Jaldhaka Nilphamari, Sharishabari Jamalpur, Shankorpur Jessor, Savar Dhaka, Kashimpur Gazipur, Sonakanda Narayangonj, Hakimpur Dinajpur.

In the context of Bangladesh, ND of poultry is also a common disease and caused mostly by velogenic strains of NDV than mesogenic or lentogenic[19-20]. The mortality rate incase of very virulent (vv) NDV up to 100% in young chickens and in varies from 80-90% in adults[21]. Thereby, 40-60% of the total mortality of poultry population in Bangladesh caused by this disease[22] when appeared as in the epidemic form.

Hemagglutination (HA) and hemagglutination inhibition (HI) tests are used for the detection of NDV, which are time consuming, laborious and limited specificity and sensitivity. On the other hand, commercially available membrane immunoassay kits which is commonly known as rapid antigen test kit, has been used for primary screening of AIV, most of which can only detect type A group-specific antigen with the limitation of complete subtype determination and the sensitivity to the specific antigen varies from 60-70%. This assay is only applicable for initial screening of samples in the field level. Furthermore, membrane immunoassays are not so reliable test for routine surveillance upon which one can assure about the presence or absence of the antigen of AIV, as it is not able to detect a low level of infection. Presently, RT-PCR has been carried out for partial subtyping (only for HA) in different laboratories of Bangladesh.

Therefore, the present research work was carried out to develop a highly sensitive, specific and confirmatory molecular test for complete sub-typing of H5N1 and sumultaneous detection of avian influenza and Newcastle desease viruses.

Materials and methods

Reference viruses

Reference NDV of Komarov strain collected from the repository of the Department of Microbiology and Hygiene, BAU, Mymensingh and cDNA from highly pathogenic avian influenza virus H5N1 subtype kindly provided by Dr. Hiroshi Kida, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Japan were used in this study.

Clinical and post-mortem samples

Clinical (oro-nasal swab, faeces) and post-mortem samples (lungs, colon) were collected from a field outbreak of suspected ND and also from chickens of experimental infection with NDV.

Extraction of RNA

Extraction of total viral RNA of different samples was performed using QIAamp viral RNA Mini Kit (Qiagen, Germany) following manufacturer's protocol with all necessary safety precautions.

Primers

One set of primers[23] specified for amplification of 356 bp of F gene of NDV and two sets of AIV subtype specific primers[24] specified for amplification of 219 bp and 616 bp of HA (H5) and NA (N1) genes respectively were used in this study (Table 1).

Table 1. *List of primers used for AIV (H5N1) and NDV genome detection*

Primers	Sequence (5'-3')	Nucleotide Position	Amplicon Size (bp)
WHOH5-1/F(HA) (Forward primer)	GCCATTCCACAACATMCACCC	943-963	219
WHOH5-3/R(HA) (Reverse primer)	CTCCCCTGCRTTGCTATG	1142-1161	
WHON1-1/F(NA) (Forward primer)	TTGCTTGGTCGGCAAGTGC	490-508	616
WHON1-2/R(NA) (Reverse primer)	CCAGTCCACCCATTTGGATCC	1184-1105	
NDV (Forward primer)	GCAGCTGCAGGGATTGTGGT	158-177	356
NDV (Reverse primer)	TCTTTGAGCAGGAGGATGTTG	513-493	

Reverse transcription (RT)

Initially a volume of 4 µl of eluted RNA of NDV and 8.3 µl DEPC water were taken in a PCR tube, spinned and then placed in a forty eight wells thermocycler (MJ Mini thermocycler, BIORAD®, USA) following a thermal profile of 94° C for 5 minutes for linearization of coiled RNA followed by snap cooling. Then, the volume was made up to 25 µl by adding 12.7 µl of reaction mixture containing 4.0 µl 5x RT buffer, 2.0 µl 10 mM dNTP, 1.0 µl prime RNase inhibitor, 0.2 µl AMV-RT, 0.5 µl primer (RH 100 pmol) and 5.0 µl DEPC water. The RT was carried out at 42° C for 40 minutes followed by 85° C for 5 minutes. RT products were cooled on ice and stored at -20° C until use.

Polymerase Chain Reaction (PCR)

Standardization of uniplex PCR

Uniplex PCR was carried out for F gene of NDV, H5 and N1 of AIV separately in 50 µl reaction volume. In all cases, reaction mixture contained 10X LA buffer 5.0 µl, 25 mM MgCl2 2.0 µl, 10 mM dNTP 2.0 µl, LA-Taq 0.2 µl, cDNA 1.5 µl while concentration of primers varied from 0.3 µl to 0.8 µl. In case of NDV, 0.8 µl each of primers (NDVF-100 pmol and

NDVR-100 pmol) were used while 0.3 µl each of primers specific for H5 (WHOH5-1/F - 100pmol and WHOH5-3/R - 100pmol) and N1 (WHON1-1/F - 100pmol and WHON1-2/R - 100pmol) of AIV were used. In each case, DEPC water was added to make the volume up to 50 µl. In all cases, the reaction mixture was subjected to following thermal cyclic conditions: one cycle at 94^0 C for 2 min, 30 cycles each at 94^0 C for 30 sec, 45^0 C for 45 sec, and 60^0 C for 1 min followed by one cycle at 60^0 C for 5 min.

Duplex PCR for simultaneous detection of HA (H5) and NA (N1) of AIV

Duplex PCR for simultaneous detection of HA (H5) and NA (N1) of AIV was also carried out with same reaction mixtures and following the same thermal profile as of uniplex PCR.

Multiplex PCR (mPCR)

Optimization of mPCR was done by varying reaction components and cyclic conditions. The volume of WHON1-1/F (100 pmol) and WHON1-2/R (100 pmol) was 0.3 µl each while the volume of primers for NDV, H5 and rest of the reaction mixture and cyclic conditions were as same as uniplex PCR.

Agarose gel electrophoresis

PCR products were analyzed on 2% agarose gel and visualized by ethidium bromide staining (0.5 µg/mL) under UV-transilluminator.

Results

This paper describes the development of a multiplex PCR for simultaneous detection of avian influenza and Newcastle disease viruses using the cDNA of H5N1 and field samples of NDV.

Standardization of uniplex PCR

A clear and distinct band of PCR product of the reference NDV appeared at the position at 356 bp on 2% agarose gel after electrophoresis (Fig. 1).

HA (H5) and NA (N1) genes of AIV were determined using uniplex PCR. The PCR products of HA and NA genes amplified by specific primers were appeared at 219 bp and 616 bp position, respectively after 2% agarose gel electrophoresis (Fig. 2).

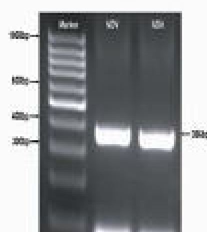

Figure 1. *Uniplex PCR product of 356 bp of NDV after 2% agarose gel electrophoresis*

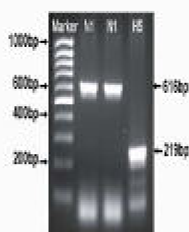

Figure 2. *Uniplex PCR products of 219 bp and 616 bp of HA (H5) and NA (N1) genes respectively of AIV after 2% agarose gel electrophoresis*

Standardization of duplex PCR for the detection of HA (H5) and NA (N1) genes of AIV

Specific primers were used for HA and NA genes in a single tube with other reagents for standardization of duplex PCR. The band of the PCR products appeared at 219 bp for HA gene and 616 bp for NA gene in a single column as appeared in uniplex PCR (Fig. 3).

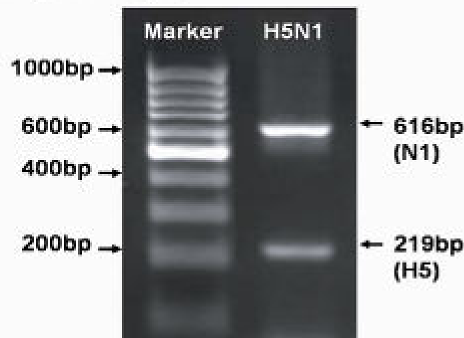

Figure 3. *Duplex PCR products of 219 bp and 616 bp of HA (H5) and NA (N1) genes of AIV respectively in a same column after 2% agarose gel electrophoresis*

Standardization of multiplex PCR

A multiplex PCR assay was standardized with three sets of primers specific for F gene of NDV, HA (H5) and NA (N1) genes of AIV in a single tube reaction with other reagents as mentioned in materials and methods section. The PCR products were found to be appeared at 356 bp, 219 bp, and 616 bp as band for reference NDV, HA (H5) gene and NA (N1) genes respectively in a single column (Fig. 4).

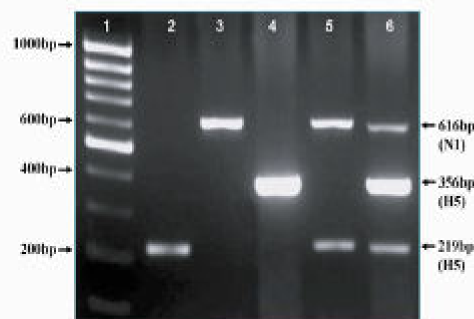

Figure 4. *Multiplex PCR products of 219 bp, 616 bp and 356 bp of HA (H5) and NA (N1) genes of AIV and F gene of NDV respectively after 2% agarose gel electrophoresis*

Lane 1: DNA marker (100 bp); Lane 2: PCR product of 219 bp HA gene of AIV; Lane 3: PCR product of 616 bp NA gene of AIV; Lane 4: PCR product of 356 bp F gene of NDV; Lane 5: PCR products of 219 bp and 616 bp HA and NA genes of AIV respectively; Lane 6: mPCR products HA and NA genes of AIV and F gene of NDV.

Detection of viruses in clinical and post-mortem samples using the newly developed multiplex PCR

The newly developed multiplex PCR was applied on the clinical and post-mortem samples collected from a field outbreak of suspected ND and also from chickens of experimental infection with NDV. Clear and distinct band of PCR product was found at 356 bp position after 2% agarose gel electrophoresis in all cases which was indicative of NDV infection. No samples of that outbreak were positive for AIV (H5N1) (Fig. 5).

Figure 5. *Multiplex PCR products visualized on 2% agarose gel after electrophoresis obtained from clinical and post-mortem samples using three sets of primers*

Lane 1: DNA marker (100 bp); Lane 2-3: Multiplex PCR products of reference HA and NA genes of AIV and F gene of NDV; Lane 4: Negative control (PBS); Lane 5: mPCR product obtained from sample of experimentally NDV infected chickens; Lane 6-7: mPCR products obtained from samples of field outbreak of suspected ND.

Discussion

The present research work was undertaken to develop and standardize molecular methods e.g. multiplex PCR for simultaneous detection of avian influenza and Newcastle disease viruses using cDNA of AIV (H5N1) and reference NDV.

The primer sets for the genome detection of NDV and AIV (H5N1) were highly specific, no cross reactivity was noticed between the nucleic acid amplification of the two viruses. Specific primers were used for the amplification of cDNA of reference NDV and the PCR product gave as band at 356 bp position in the uniplex PCR which corresponds to the findings of earlier researchers[25-26].

Reference cDNA of AIV (H5N1) was used for the detection of HA (H5) and NA (N1) genes in uniplex, duplex and multiplex PCR in this study. In every case the band of PCR product for HA (H5) gene appeared at the position 219 bp and for NA (N1) gene at 616 bp. Many researchers[13,14,16,17,27] also detected PCR product of different sub-types of AIV using cDNA as a template in the PCR assay.

As AIV (H5N1) and NDV are the viruses of two different families, usually separate sets of primers were required for the amplifications of their individual gene. It was found that the bands of the amplified products HA (H5), NA (N1) of AIV (H5N1) and NDV were always appeared at 219 bp, 616 bp, and 356 bp respectively in each occasion. The use of multiplex PCR for the detection of AIV and NDV in a single tube reaction was also previously described[28-34].

The bands of NDV were found more dense and distinct than the bands of AIV though minimum amount of cDNA of NDV were used compared to that of cDNA of AIV (H5N1). This might be due to the variation of virus concentration of NDV and the RNA used for the synthesis of cDNA of NDV compared to the cDNA of AIV during PCR assay.

While the goal of development of molecular based test was to achieve a simpler, more reliable, rapid test, this was combined with maximum operator safety. Separation and visualization of the PCR products were carried out by agarose gel electrophoresis stained with ethidium bromide that was a mutagenic compound as reported earlier[35-36].

In conclusion, results of the present study clearly indicated that the newly developed multiplex PCR is highly sensitive, specific and rapid for complete sub-typing of AIV and simultaneous detection of NDV and AIV in a single tube reaction. However, it would be worthwhile to evaluate this mPCR on AIV suspected samples from different parts of Bangladesh.

References

1. Swayne DE. 1997. Pathology of H5N2 Mexican avian influenza virus infections of chickens. *Vet Pathol.* **34**: 557-567.
2. Yang CY, Chang PC, Hwang JM and Shieh HK. 1997. Nucleotide sequence and phylogenetic analysis of Newcastle disease virus isolates from recent outbreaks in Taiwan. *Avian Dis.* **41**: 365-373.
3. Johnston SLG & Siegel CS. 1991. A comparison of direct immunofluorescence, shell vial culture, and conventional cell culture for the rapid detection of influenza A and B. *Diagn Microbiol Infect Dis.* **14**: 131-134.
4. Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C and Huang J. 1998. Characterization of an avian Influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* **279**: 393-396.
5. Swayne DE and Suarez DL. 2000. Highly pathogenic avian influenza. *Rev Sci Tech.* **19**: 463-482.
6. Rima BK, Alexander DJ, Billeter MA, Collins PL, Kingsbury DW and Lipkind MA. 1995. The Paramyxoviridae. In *Virus Taxonomy, Classification and Nomenclature of Viruses.* Sixth Report of the International Committee on Taxonomy of Viruses (Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA and Summers MD eds), pp. 268-274. Vienna and New York, Springer Verlag.
7. Glickman RL, Syddall RJ, Iorio RM, Sheehan JP and Bratt MA. 1988. Quantitative basic residue requirements in the cleavage-activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. *J Virol.* **62**: 354-356.
8. Coonrod JD, Karathanasis P, Betts RF, and Donofrio JC. 1988. Enzyme-linked immuno sorbent assay of core antigens for clinical diagnosis of influenza. *J Med Virol.* **25**: 399-409.
9. Gleaves CA and Brown JA. 1993. Detection of influenza A in clinical specimens and cell culture fluid by a commercial EIA. *Clin Diagn Virol.* **1**: 123-127.

10. Bressoud A, Whitcomb J, Pourzand C, Haller O and Cerutti P. 1990. Rapid detection of influenza virus H1 by the polymerase chain reaction. *Biochem Biophys Res Commun.* **167**: 425-430.

11. Donofrio JC, Coonrod JD, Davidson JN and Betts RF. 1992. Detection of influenza A and B in respiratory secretions with the polymerase chain reaction. *PCR Methods Applic.* **1**: 263-268.

12. Wright KE, Wilson GAR, Novosad D, Dimock C, Tan D and Weber JM. 1995. Typing and s ubtyping of influenza viruses in clinical samples by PCR. *J Clin Microbiol.* **33**: 1180-1184.

13. Altmuller A, Kunerl M, Muller K, Hinshaw VS, Fitch WM and Scholtissek C. 1991. Genetic relatedness of the nucleoprotein (NP) of recent swine, turkey and human influenza a virus (H1N1) isolates. *Virus Res.* **22**: 79-87.

14. Lee MS, Chang PC, Shien JH, Cheng MC and Shieh HK. 2001. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. *J Virol Methods.* **97**: 13-22.

15. Fouchier RAM, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SAG, Munster V, Kuiken T and Rimmelzwaan GF. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc Natl Acad Sci.* **101**: 1356-1361.

16. Munch M, Nielsen LP, Handberg KJ and Jørgensen PH. 2001. Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription-PCR and PCR ELISA. *Arch Virol.* **146**: 87-97.

17. Senne DA, Panigrahy B, Kawaoka Y, Pearson JE, Suss J, Lipkind M, Kida H and Webster RG. 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. *Avian Dis.* **40**: 425-437.

18. Renshaw MA, Ellingsen D, Costner B, Benson J, Heit JA and Hooper WC. 2001. Fluorescent multiplex polymerase chain reaction analysis of four genes associated with inpaired fibrinolysis, and myocardial infarction. *Blood Coagul Fibrinolysis.* **12**: 245-251.

19. Chowdhury MSI, Chowdhury TIMFR, Sarker AJ and Amin MM. 1981. Determination of an optimum age for primary Newcastle disease vaccination of chicks having maternal antibody. *Bangl Vet J.* **15**: 19-27.

20. Islam MA, Ito T, Takakuwa H, Takada A, Itakura C and Kida H. 1994. Acquisition of pathogenicity of a Newcastle disease virus isolated from a Japanese quail by intracerebral passage in chickens. *Jpn J Vet Res.* **42**: 147-156.

21. Eisa M and Omer EA. 1984. A natural outbreak of Newcastle disease in pigeons in Sudan. *Vet Rec.* **114**: 297.

22. Talha AFSM, Hossain MM, Chowdhury EH, Bari ASM, Islam MR and Das PM. 2001. Poultry diseases occurring in Mymensingh district of Bangladesh. *Bangl Vet.* **18**: 20-23.

23. Nanthakumar T, Kataria RS, Tiwari AK, Butchiah G and Kataria JM. 2000. Pathotyping of Newcastle disease viruses by RT-PCR and restriction enzyme analysis. *Vet Res Commun.* **24**: 275-286.

24. WHO. 2005. Recommended laboratory tests to identify avian influenza A virus in specimens from humans. World Health Organization, Geneva. available at: *http://www.who.int/csr/disease/avian_influenza/guidelines/avian_labtests2.pdf*. pp. 1-7.

25. Gohm DS, Thur B and Hofmann MA. 2000. Detection of Newcastle disease virus in organs and faeces of experimentally infected chickens using RT-PCR. *Avian Pathol.* **29**: 143-152.

26. Singh K, Jindal N, Gupta SL, Gupta AK and Mittal D. 2005. Detection of Newcastle disease virus genome from the field outbreaks in poultry by reverse transcription-polymerase chain reaction. *Int J Poult Sci.* **4**: 472-475.

27. Starick E, Römer-Oberdörfer A and Werner O. 2000. Type- and subtype-specific RT-PCR assays for avian influenza A viruses (AIV). *J Vet Med. B.* **47**: 295-301.

28. Farkas T, Antal M, Sámi L, Germán P, Kecskeméti S, Kardos G, Belák S and Kiss I. 2007. Rapid and simultaneous detection of avian influenza and Newcastle disease viruses by duplex polymerase chain reaction assay. *Zoonoses and Public Health* **54**: 38-43.

29. Malik YS, Patnayak DP and Goyal SM. 2004. Detection of three avian respiratory viruses by single-tube multiplex reverse transcription-polymerase chain reaction assay. *J Vet Diagn Invest.* **16**: 244-248.

30. Soares PB, Demétrio C, Sanfilippo L, Kawanoto AH, Brentano L and Durigon EL. 2005. Standardization of a duplex RT-PCR for the detection of Influenza A and Newcastle disease viruses in migratory birds. *J Virol Methods.* **123**: 125-130.

31. Thontiravong A, Payungporn S, Keawcharoen J, Chutinimitkul S, Wattanodorn S, Damrongwatanapokin S, Chaisingh A, Theamboonlers A, Poovorawan Y and Oraveerakul K. 2007. The single-step multiplex reverse transcription- polymerase chain reaction assay for detecting H5 and H7 avian influenza A viruses. *Tohoku J Exp Med.* **211**: 75-79.

32. Wang Z, Vreede FT, Mitchell JO and Viljoen GJ. 2001. Rapid detection and differentiation of Newcastle disease virus isolates by a triple one-step RT-PCR. *Onderstepoort J Vet Res.* **68**: 131-134.

33. Xie Z, Pang YS, Liu J, Deng X, Tang X, Sun J and Khan MI. 2006. A multiplex RT-PCR for detection of type A influenza virus and differentiation of avian H5, H7, and H9 hemagglutinin subtypes. *Mol Cell Probes.* **20**: 245-249.

34. Zhu RN, Qian Y, Wang F and Liu CG. 2003. Application of molecular biological techniques in the surveillance of influenza viruses in infants and young children. *Zhonghua Liu Xing Bing Xue Za Zhi.* **24**: 9-14.

35. Aldous EW and Alexander DJ. 2008. Newcastle disease in pheasants (Phasianus colchicus): a review. *Vet J.* **175**: 181-185.

36. Alexander DJ. 1997. Newcastle Disease and Other Paramyxovirus Infections. In *Diseases of Poultry* (Calnek BW, Barnes HJ, Beard CW, McDougal LR, and Saif YM eds), 10th edn, pp. 541-569. Iowa State University Press, Ames, IA.