DOES CONTROL OF ANIMAL INFECTIOUS RISKS OFFER A NEW INTERNATIONAL PERSPECTIVE?

DEVELOPMENT OF MULTIPLEX-RT-PCR FOR RAPID DETECTION AND SUBTYPING OF AVIAN INFLUENZA VIRUSES TYPE A

BROOMAND CH.1,2*, AINI I.3, ABDULRAHMAN O.3, KHATIJAH Y.4, SHRIFAH S.H.5

1. Razi Vaccine and Serum Research Institute, Karaj, Iran.
2. Agriculture & Natural Resources Research Centre of Kermanshah, Iran
   PO Box 67145-1661
3. Faculty of Veterinary Medicine, 4. Department of Biochemistry and Microbiology, Faculty Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor Darul Ehsan, Malaysia,
   5. Veterinary Research Institute, Ipoh, Prak, Malaysia

ABSTRACT

A multiplex reverse transcription polymerase chain reaction (RT-PCR) was developed and optimized to simultaneously detect 3 subtypes (H5, H7 and H9) of avian influenza virus type A. The developed method was specific to amplify only the HA gene of H5, H7 and H9 of Influenza type A and no amplification with other subtypes of AIV and other avian infectious viruses such as Newcastle disease virus, infectious bronchitis virus, infectious bursal disease virus was observed. The multiplex RT-PCR assay developed in this study was as sensitive as conventional RT-PCR and virus isolation. Therefore this method is reliable, rapid, sensitive and specific and could be used as a tool for detecting and subtyping of AIV, in the control program of AIV.

INTRODUCTION

Avian influenza (AI) is a viral disease affecting the respiratory, digestive and/or nervous system of many species of birds, both domestic and wild (Alexander, 2000). Influenza viruses are of three types A, B, and C. The typing is based on antigenic differences between their nucleoprotein (NP) and matrix (M) proteins of the virus (Lamb and Krug, 1996). Influenza A viruses are further subdivided by antigenic characterization of the haemagglutinin (HA) and NA surface glycoproteins that project from the surface of the virion (Jeffery and Layne, 2001). Currently, 16 HA and 9 NA subtypes are known.

*Contact author: Email: bchaharaein@gmail.com

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and appear in varied combinations (Fouchier, 2005). Within one subtype, a spectrum from low-pathogenic (LPAI) to highly pathogenic (HPAI) strains may exist. In chicken, until now only H5 and H7 subtypes have been characterized as HPAI (Senne et al., 1996). Highly pathogenic avian influenza viruses have the potential to cause severe disease in domestic poultry, which can lead to substantial economic losses. On the other hand, while most of the LPAI outbreaks are either subclinical or cause mild non-pathognomonic respiratory clinical signs in infected chickens, there is a great risk for them to become HPAI (Horimoto and Y. Kawaoka., 1995; Garcia et al., 1996; Banks et al., 2001; Shult and Carol, 2003). Development of a simple, rapid and reliable method of virus detection and identification is of great importance to detect type and subtypes of AIVs. Therefore the objective of this study was to establish a sensitive and specific Multiplex-PCR method for rapid detection of three subtypes (H5, H7 and H9) of AIVs.

MATERIALS AND METHODS

In this study, 33 strains of AIVs including 14 HA subtypes in different combination with 7 NA subtypes were used. Extraction of viral RNA was done using Trizol Reagent® LS Kit (Molecular Research Centre, Inc., USA) as described by Lee et al. (2001) with slight modification. Nucleotide sequences of viral NP and HA (H9, H5 and H7) genes were retrieved from the Gene Bank of the National Centre of Biotechnology Information (NCBI), USA, and aligned using Megalign software package (Dnastar Inc., Madison). Four different sets of primers specific for amplification of NP and HA (H9, H5 and H7 subtypes) of influenza type A were designed based on more than 300 conserved sequences of the genes. The functionality and specificity of all primer were tested in single reactions before combining them in a multiplex PCR assay. The Multiplex-PCR was carried out according to the manufacturer instructions (QIAGEN). In brief, the 50µl reaction contained 14µl nuclease free water, 25µl QIAGEN Multiplex PCR Master Mix, 2µl of each cDNA (0.5µg), 5µl of 10x primer mix (0.2µM of each primer of Table 1). Samples were amplified in thermalcycler (PTC-200 DNA Engine, MJ Research Inc, Massachusetts) by the following conditions: Initial activation step at 95°C for 15 min. After an initial activation step, 40 cycles of heat denaturation at 94°C for 30 sec, primer annealing at 60°C for 90 sec, and primer extension at 72°C for 90 sec were followed by a final primer extension step at 72°C for 10 min. In order to determine the
sensitivity of the multiplex PCR, serial ten-fold dilutions of the mixture of 100 ng of each subtype was used as template RNA. The sensitivity of RT-PCR using single primer pairs was compared with the multiplex assay. In order to determine the specificity of the assays, in all the RT-PCR and multiplex PCR reaction, 2µl of RNA from NDV, IBV and IBDV were included. A volume of 2µl RNase free water was also included as negative control.

RESULTS AND DISCUSSION

The NP one tube RT-PCR was able to amplify a fragment of 107 bp from NP gene of 32 strains of 14 different HA subtypes (H1-H5 and H7-H15) of influenza A viruses. The designed primer set specific for HA subtypes of H5, H7 and H9 successfully amplified the fragments of 499bp, 409bp and 244 bp from HA gene of H5, H7 and H9 subtypes. Specificity of multiplex RT-PCR were tested by including RNA of other subtypes of AIV and NDV, IBV and IBDV. No PCR amplifications were observed.

In this study, a multiplex reverse transcription polymerase chain reaction (RT-PCR) was developed and optimized to simultaneously detect 3 subtypes (H5, H7 and H9) of avian influenza virus type A. The amplification of three HA genes in a single step covering 3 subtypes of influenza viruses, which are known to cause significant clinical influenza disease in humans and chicken was successfully achieved. Multiplex RT-PCR which was developed, successfully amplified the HA gene of H5, H7 and H9 subtypes. The sensitivity of multiplex RT-PCR was compared with the conventional assay, and there was no significant difference between the two assays. There was also no loss of sensitivity when multiple primer sets were used together, compared to the same primer sets used individually, and no apparent decrease in the yield of the PCR product. These results were in accordance with the published studies (Harris et al., 1998; Stockton et al., 1998; Pang et al., 2002; Johnson et al., 2003). In conclusion, the established multiplex RT-PCR assay in this study was reliable, rapid, sensitive and specific and could be used as a tool for detecting and subtyping of AIV, in the control program of AIV. The whole process from extracting RNA to analysis of PCR product by agarose gel electrophoresis can be completed in 6 hours and provides a rapid means of identification of the type and subtypes of 3 important (H5, H7 and H9) influenza viruses, which is beneficial for the identification, and surveillance of influenza viruses. A considerable advantage of subtyping of AIV by this method is decreasing
the time as compared to standard method like virus inoculation in SPF embryonated eggs.

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REFERENCES


