**Iranian Journal of Virology 2009;3(2): 24-29**






## Original Article

# Identification of Different Serotypes of Infectious Bronchitis Viruses in Allantoic Fluid Samples with Single and Multiplex RT-PCR

**Rafiei MM[1], Vasfi-Marandi M[1*], Bozorgmehri-Fard MH[1], Ghadi S[1]**

1. Poultry Diseases Section, Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

## Abstract

**Background and Aims:** Infectious bronchitis virus (IBV) causes an acute, highly contagious respiratory and kidney disease of chickens which results in significant economic losses in commercial broilers, layers and breeders. Rapid identification of IBV serotypes involved in respiratory complex is a problem in the differential diagnosis.

**Materials and Methods:** In this study, a single and multiplex RT-PCR was used to detect group III, Massachusetts and 793/B serotypes of IBV that have been circulating in Iranian poultry industry since 1999.

**Results:** Only seven out of 49 allantoic fluid samples showed positive results in single RT-PCR using group specific primers. Two of these samples belonged to IBV positive group in VN test. Whereas; four samples belonged to H9N2 positive group and one sample belonged to IBV/AIV negative groups. The high sensitivity of single RT-PCR led to detect some of the missed IBV infections as compared with other virological tests. Three out of 7, IBV isolates identified with single RT-PCR, classified as 793/B serotype and four of them categorized as Massachusetts serotype in multiplex RT-PCR.

**Conclusion:** The results indicated that multiplex RT-PCR method, can be a valuable test for rapid identification of Massachusetts and 793/B serotypes in complex respiratory complex infection of chickens

**Keywords:** Infectious bronchitis virus (IBV); Avian influenza virus (H9N2); Massachusetts; 793/B serotype; multiplex RT- PCR

## Introduction

Infectious bronchitis virus (IBV) is a member of the Coronavirdiae family that are enveloped particles containing single-stranded RNA genome of positive polarity. The virus causes a highly contagious respiratory disease in chickens (1-3). The major clinical signs include tracheal rales, sneezing and coughing. The disease causes a high mortality rate in young chickens when it is complicated with secondary bacterial infections such as *E. coli* and *Mycoplasma* (1). Many different IBV serotypes and variants are recognized on the basis of antigenic variation, determined by virus neutralization (VN) or molecular analysis of IBV genome. Serotypes of IBV with economic importance such as Massachusetts, which is the serotype most commonly found worldwide; and those which were isolated in Connecticut, Arkansas and Delaware 072 in USA. From Australia and

***Corresponding author:** Mehdi Vasfi Marandi, Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
Tel: (+98) 21 61 11 71 50
Email: mvmarand@ut.ac.ir

European countries D274, D1466, 793/B (4/91 or CR88), and Italian 02 strains. Some IBV variants have been reported as epidemiologically important strains, but their significance in terms of IBV infection control, is less clear. More recently, in Europe, an IBV variant designated as D388 (QX) strain has been associated with false layers (4-21).

In addition to antigenic variation within IBVs, differences in virulence in these serotypes are observed. There have been reports from the USA and Europe of IBV isolated strains with increased virulence compared to the previous isolates of the same serotypes which suggests be variations in their pathogenesis. One such example of pathogenic variation is observed in 793/B serotype, which has been associated with both muscle myopathy and scouring in broilers, as well as with mortality in adult breeding hens (4-21).

Mixed IBV serotype infections such as Massachusetts and 793/B stains, may occur in field conditions. The multiplex reverse transcriptase polymerase chain reaction test (multiplex RT-PCR) is advantageous because it allows laboratory detection of multiple pathogenic agents through the use of one single test. This test, is cost effective, time saving, specific and sensitive. Furthermore, it has been used for screening and surveillance of infection poultry flocks (10, 11).

The goal of this study was to determine the IBVs by single RT-PCR based on detection of M and N genes within the allantoic fluids of inoculated embryonated eggs with samples from poultry farms during the years from 1999 to 2002 and distinguish their serotype by multiplex RT-PCR based on S1 gene.

## Methods

**Viruses**

The IBV strains used in this study were the 793/B Iranian reference strain isolated in 1998, attenuated vaccine CR88 (Merial), 4/91 (Intervet) strains, and inactivated antigens of M41 (PA0121), D247 (PA0715) and D1466 (PA0716) provided by Central Veterinary Laboratory Agency (OIE international reference laboratory; Weybridge, UK). These IBV strains were used as positive control in RT-PCR tests.

**Allantoic fluid samples**

Allantoic fluids of inoculated eggs with 49 tissue samples including tracheal and cloacal swabs, lung, secal tonsil and kidneys during the H9N2 avian influenza virus (AIV) outbreaks between 1998-2002, were used for virus isolation (17, 18). A collection of 23 out of 49 allantoic fluid samples contained H9N2 serotype based on HI test, and two of them were IBV isolates based on VN test using group specific antiserum. Besides, 26 out of 49 allantoic fluids used in this study were apparently negative for AIV, NDV and IBV in HI and VN tests.

**Viral RNA extraction and RT test**

Viral RNA was extracted from 200 µl of allantoic fluid using RNX-plus™ viral RNA extraction kit (Cinnagen, Iran) according to the manufacture procedures. Extracted RNA was dissolved in 20 µl DEPC water. The reverse transcriptation (RT) reaction was performed using ReverAid™ first strand cDNA synthesis kit (Fermentas, UK) with oligo $(dt)_{18}$ primers.

**Single RT-PCR**

A single RT-PCR test based on detection of M and N genes of IBV was performed using oligonucleotide primers of MIBVPCR-(5'-TAAGCTTTCAGTGGCTTGCTAAGTGTGAACC-3') and IBVPCR-(5'TGGATCCACCGCTACCTTCAAACTTGGGCGG-3'). Using these primers made it possible to distinguish all Coronaviruses belonging to group III. These primers amplify a fragment of 1020 bp length using the modified procedure described by Jackwood (1998) (13). The PCR reaction mixture containing 2.5 µl of cDNA and 22.5 µl of master mix composed of 2.5 µl 10X buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 mM of each primers, 1.25 units of Taq DNA polymerase and 17 µl DD water in total volume of 25 µl was used. The PCR test was preformed with one initial cycle of 94ºC for 5 min, 52 ºC for 2 min and 72ºC for 2 min, followed by 35 cycles: of 94ºC for 1 min, 52 ºC for 2 min and 72ºC for 2 min with final extension at 72ºC for 15 min of using a

master cycler gradient termocycler (Eppendorf).

**Multiplex RT-PCR**

A multiplex RT-PCR with oligonucleotide primers of XCE3-(5'-CAGATTGCTTACAACCACC-3'), MCE1+ (5'-AATACTTTTACGTTACAC-3') and BCE1+ (5'-AAGTGCCTTTAGGCCTGG-3') on the basis of S1 gene was used to differentiate Massachusetts and 793/B serotypes according to the procedure described by Adhar with some modification [17]. These primers amplify two fragments of 274 and 154 bp length for Massachusetts and 793/B serotypes respectively. Fifty microliters of PCR reaction containing 5 µl of cDNA and 45 µl of master mix consisting 5 µl 10X buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 1 µl of 10 mM of each primers, 2.5 units of Taq DNA polymerase and 34 µl DD water, was used. The PCR amplification was preformed for an initial cycle of: 94ºC for 10 min, 48ºC for 2 min and 72ºC for 2 min, followed by 35 cycles of: 94ºC for 1 min, 48ºC for 2 min and 72ºC for 2 min with extension at 72ºC for 15 min.

**Agarose gel electrophoreses**

The PCR products were analyzed by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide and visualized in a by UV transilluminator (M-15 UVP co. USA).

**Analytical sensitivity and specificity of RT-PCR**

The sensitivity of the RT-PCR assay was determined according to the standard method (17). Briefly, serial dilutions of the Massachusetts or 793/B IBV viruses in sterile PBS solution ranging from $10^{-1}$ to $10^{-9}$ were prepared and each dilution, inoculated into five 9-day-old embryonated chicken eggs. The $EID_{50}$ titration was determined and all dilutions were examined by the single and multiplex RT-PCR assays for detection of Massachusetts and 793/B serotypes. To evaluate the specificity of the RT-PCR, allantoic fluids containing NDV (La Sota strain), H9N2 subtype of AIV (ZMT-101 strain) and ILTV (Razi strain) were used as control both in single and multiplex RT-PCR.

## Results

A coronavirus group III specific single RT-PCR based on conserved regions of M and N genes with 1020 bp was carried out on 49 allantoic fluids as well as the reference antigens of M41 (PA0121), D247 (PA0715) and D1466 (PA0716) with MIBVPCR and NIBVPCR primers. Only seven out of 49 allantoic fluid samples showed positive results in single RT-PCR. Two of them belonged to IBV positive group in VN test; whereas; four samples belonged to H9N2 positive group and one sample belonged to IBV/AIV negative groups (Fig. 1 and Table 1). The detection limit of the RT-PCR was determined to be approximately equivalent to $10^{3.7}$ $EID_{50}$. In the single RT-PCR test the NDV, AIV and ILTV strains were not amplified.

A multiplex RT-PCR was optimized to differentiate Massachusetts and 793/B serotypes by using reference inactivated strains of M41 (PA0121), D247 (PA0715) and D1466 (PA0716). Vaccine CR88 (Merial), Using this test, vaccine strain CR88 (Merial) 4/91 (Intervet) strains and the 793/B reference strain isolated in Iran could be differentiated. Amplified samples showed bands of 274 bp belonging to Massachusetts and abound of 154 bp belonging to 793/B strains. No PCR bands were seen in extracted RNA or DNA from ND, AI and ILT viruses (Fig. 2).

The detection limit of the multiplex RT-PCR was determined to be approximately equivalent to $10^{2.5}$ $EID_{50}$. No spurious PCR amplification between these two serotypes was observed

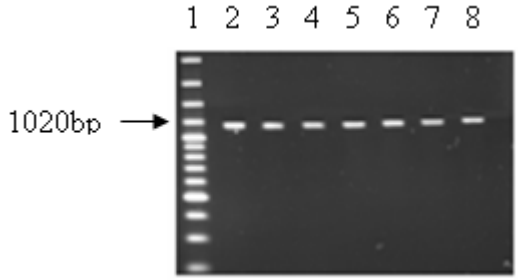


**Fig. 1:** Agarose gel electrophoresis of IBV specific products amplified by single RT-PCR. Lane 1 shows molecular size marker (100 bp plus DNA ladder fermentas); and Lane 2 to 8 show a 1020 bp band related to M and N genes of coronavirus group III.

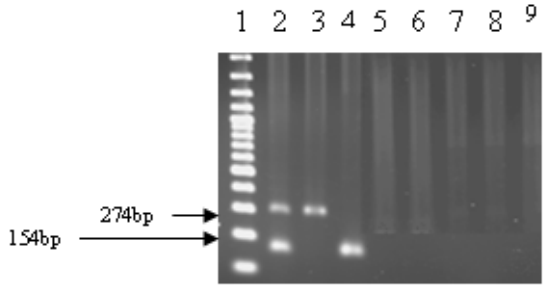


**Fig. 2:** Agarose gel electrophoresis of IBV specific products amplified by multiplex RT-PCR using reference IBV strains. Lane 1 shows molecular size marker; Lane 2 M41, 793/B; Lane 3 M41; Lane 4 793/B; Lane 5 D247; Lane 6 D1466; Lane 7 AIV-H9N2; Lane 8 NDV; Lane 9 ILT.

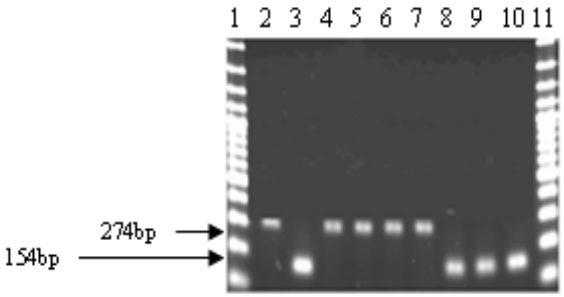


**Fig. 3:** Agarose gel electrophoresis of IBV specific products amplified by Multiplex RT-PCR using field IBV isolates. Lane 1 and 11 show molecular size markers and Lane 2 M41; Lane 3 793/B; Lane 4-7 and Lane 8-10 demonstrate respectively Mass and 793/B similar bands.

using various amounts of both RNA mixtures. Three out of 7 isolates amplified with M and N genes of IBV in single RT-PCR, classified as 793/B serotype and four of them categorized as Massachusetts serotype in multiplex RT-PCR (Fig. 3 and Table 1).

## Discussion

Mixed serotype of viral respiratory infections is usual in poultry farms. In many field conditions, one or more mixed pathogenic viruses are involved in respiratory complex that makes it difficult to determine the main viral causative agent which produces severe disease. Because of the similarity in clinical singes of respiratory diseases, sensitive and specific diagnostic methods, for rapid identification of the involved pathogens are needed. Therefore, various diagnostic methods such as various serological tests have been developed but there tests are expensive and

**Table 1:** This table shows the result of virus isolates with single and multiplex RT-PCR by using 49 allantoic fluid samples.

| allantoic fluid samples<br>test | total | IBV(-)<br>AIV(-) | AIV(+) | IBV(+) |
|---|---|---|---|---|
| Virus isolation | 49 | 24 | 23† | 2†† |
| Single RT-PCR | 49 | 23 | - | 7* |
| Multiplex RT-PCR | 49 | 23 | - | 3(793/B)**<br>4(Mass)** |

† Samples contained H9N2 subtype based on HI test

†† Samples contained IBV isolates based on VN test

* Samples showed positive results in single RT-PCR by using M & N specific primers

** Serotype of IBV isolates are detected with multiplex RT-PCR by using Mass and 793/B serotypes specific primers.

time consuming.

Therefore, it is important to identify the involved serotypes in a short time. The RT-PCR-RFLP methods described by Kwon (14) and Lin (16-18) are capable of identifying different serotypes of IBV. However, usually more procedural steps are required to identify the PCR product that makes such tests somewhat cumbersome and time consuming. Most recently, the serotype specific single RT-PCR based on S1 gene developed by Keeler (16) greatly improved the speed of serotyping and diagnosis of IBV, but it still needs to conduct several individual RT-PCR tests to identify only one serotype. So, with multiplex RT-PCR, different serotypes of IBV in one sample could be identified. The multiplex RT-PCR optimized in this study, was able to detect IBV Massachusetts and 793/B serotypes simultaneously in one reaction. These two serotypes are circulating in Iranian poultry farms since 1998. Therefore, rapid identification of involved serotypes is important for early detection and effective prevention of the disease. It has been proven that multiplex RT-PCR to be sensitive, specific, and can be useful in diagnosis, screening and surveillance of poultry flocks. Besides; this test has the added benefits of being time saving, cost effective, as only one sample from a unique source, has to be processed and less reagents are used in multiplex RT-PCR when compared with several single RT- PCR or RFLP-RT-PCR test including limitation procedures virus isolation (22).

Mixed infections of different serotypes of IBV may occur due to the extensive use of multiple live vaccines. In order to test the limitation of the technique in the diagnosis of mixed Massachusetts and 793/B infections, different titers of Massachusetts and 793/B viruses were used. This multiplex RT-PCR was able to detect about all of the manually mixed serotype of Massachusetts and 793/B. Besides one field sample showed both 274 and 154 bp. The band of 274 bp disappeared in multiplex RT-PCR, Massachusetts serotype of IBV was neutralized with specific antiserum before egg inoculation. This suggests that the appearance of the two bonds was due to co-infection of flock.

The development of multiplex RT-PCR for other serotypes of IBVs, would be dramatically shortens the IBV diagnosis process, especially when mixed infections of other IBV serotypes including Connecticut, Arkansas etc, are involved in outbreaks.

## Acknowledgments

This research was supported by a grant from the Research Deputy of Faculty of Veterinary Medicine, University of Tehran.

## References

1. Cavanagh D, Elus MM, Cook JK. Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo. Avian Pathol. 1997;26(1):63-74.

2. Boltz DA, Nakai M, Bahra JM. Avian infectious bronchitis virus: a possible cause of reduced fertility in the rooster. Avian Dis. 2004;48(4):909-15.

3. Williams AK, Wang L, Sneed LW, Collisson EW. Comparative analyses of the nucleocapsid genes of several strains of infectious bronchitis virus and other coronaviruses. Virus Res. 1992;25(3):213-22.

4. Cavanagh D. Coronaviruses in poultry and other birds. Avian Pathol. 2005;34(6):439-48.

5. Cavanagh D. Coronavirus IBV: further evidence that the surface projections are associated with two glycopolypeptides. J Gen Virol. 1983;64 (Pt 8):1787-91.

6. Cavanagh D, Davis PJ, Darbyshire JH, Peters RW. Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutin-ation inhibiting antibody, or induce chicken tracheal protection. J Gen Virol. 1986;67 ( Pt 7):1435-42.

7. Koch G, Hartog L, Kant A, van Roozelaar DJ. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. J Gen Virol. 1990;71 (Pt 9):1929-35.

8. Parr RL, Collissor EW. Epitopes on the spike protein of a nephropathogenic strain of infectious bronchitis virus. Arch Virol. 1993;133(3-4):369-83.
9. Cavanagh D. Coronavirus avian infectious bronchitis virus. Vet Res. 2007;38(2):281-97.
10. Pang Y, Wang H, Girshick T, Xie Z, Khan MI. Development and application of a multiplex polymerase chain reaction for avian respiratory agents. Avian Dis. 2002;46(3):691-9.
11. Soumet C, Ermel G, Rose N, Rose V, Drouin P, Salvat G, et al. Evaluation of a multiplex PCR assay for simultaneous identification of Salmonella sp., Salmonella enteritidis and Salmonella typhimurium from environmental swabs of poultry houses. Lett Appl Microbiol. 1999;28(2):113-7.
12. Adzhar A, Shaw K, Britton P, Cavanagh D. Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction. Avian Pathol. 1996;25(4):817-36.
13. Jackwood MW, Kwon HM, Hilt DA. Infectious bronchitis virus detection in allantoic fluid using the polymerase chain reaction and a DNA probe. Avian Dis. 1992;36(2):403-9.
14. Kwon HM, Jackwood MW, Gelb J, Jr. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 1993;37(1):194-202.
15. Lin Z, Kato A, Kudou Y, Ueda S. A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism. Arch Virol. 1991;116(1-4):19-31.
16. Keeler CL, Jr., Reed KL, Nix WA, Gelb J, Jr. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Dis. 1998 Apr-;42(2):275-84.
17. Akbari-Azad G, Vasfi-Marandi M, Keyvani H. Isolation and molecular identification of infectious bronchitis viruses in poultry farms of Iran. J Fac Vet Med. 2004;59(3):259-64.
18. Vasfi-Marandi M, Bozorgmehri-Fard MH. Isolation and identification of infectious bronchitis viruses in chickens between 1997-2000 in Iran. J Fac Vet Med. 2001;59(3):119-24.
19. Ren X, Yin J, Ma D, Li G. Characterization and membrane gene-based phylogenetic analysis of avian infectious bronchitis virus Chinese strain HH06. Virus Genes. 2009;38(1):39-45.
20. Bochkov YA, Tosi G, Massi P, Drygin VV. Phylogenetic analysis of partial S1 and N gene sequences of infectious bronchitis virus isolates from Italy revealed genetic diversity and recombination. Virus Genes. 2007;35(1):65-71.
21. Fabricant J. The early history of infectious bronchitis. Avian Dis. 1998;42(4):648-50.
22. Wang X, Khan MI. A multiplex PCR for Massachusetts and Arkansas serotypes of infectious bronchitis virus. Mol Cell Probes. 1999;13(1):1-7.