International Journal of Animal and Veterinary Advances 3(5): 300-304, 2011
ISSN: 2041-2908

Submitted: July 13, 2011 Accepted: September 05, 2011 Published: October 15, 2011

Molecular Identification of Avian Respiratory Viral Pathogens in Commercial Broiler Chicken Flocks with Respiratory Disease in Shiraz-Iran During 2009-2010

[1]M.J. Mehrabanpour, [1]A. Rahimian, [3]A.H. Shoshtari, [2]P.D. Fazel,
[2]E. Kariminejhad and [1]Gh R. Moazeni Jula
[1]Department of virology, Razi Vaccine and Serum Research Institute, Shiraz, Iran
[2]Islamic Azad University Jahrom Branch, Jahrom, Iran
[3]Razi Vaccine and Serum Research Institute, Shiraz, Iran

Abstract: Newcastle disease and avian influenza A viruses (AIV) have been responsible for serious losses in the Iran poultry industry. During 2009-2010 the poultry industry in Fars province faced an almost heavy loss that was characterized by mild and in some flocks high mortality and respiratory distress. The aim of this study was designed to clarify the roles of the Newcastle Disease Virus (NDV) and AIV singly and jointly by RT-PCR assay in recent outbreak in Fars province in Iran. During the period of this study a total 44 commercial broiler flocks with high mortality in Fras province were visited. Samples were collected from chickens with respiratory distress. To identify AIV and NDV by RT-PCR we used specific primers based on conserved sequences of the NP gene, HA gene and F gene. The results show, that out of 18 samples were positive for NDV, 6 for AIV and 18 flocks were infected with both NDV and AIV and 2 flocks of these were negative for AIV and NDV. It is most likely that flocks used in this study were also naturally exposed to a virulent strain of NDV which is why the vaccines were not covered. The high rates of AIV in broiler flocks in this study, confirms the endemic nature of AIV (H9N2) in Fars province.

Key words: Avian influenza, avian respiratory disease, broiler flocks, Iran, Newcastle disease, RT-PCR

INTRODUCTION

Poultry industry in Iran is being confronted with numerous disease problems including infectious diseases (Siddique *et al*., 2008). Respiratory tract infections are of paramount problem in the poultry industry in Iran, because high mortality may occur in poorly managed cases (Roussan *et al*., 2008). The etiology of respiratory organisms is complex often involving more than one pathogen (Ahmed *et al*., 2009). These respiratory pathogens are of major importance because they can cause disease independently, in association with each other, or in association with bacterial or viral agent (Roussan *et al*., 2008). In the recent years, respiratory diseases are the main hazards to the industry causing considerable economic losses (Ahmad *et al*., 2008). Poultry industry has faced tough challenges its viral infections especially AI and ND (Ahmed *et al*., 2009). These are responsible for serious losses in the poultry industry (Hadipour *et al*., 2011). Newcastle disease is one of the most important viral diseases of poultry in Iran. It is an endemic and sometimes epizootic disease in chickens (Hadipour *et al*., 2011). During 1998-2000, H9N2 viruses (AIV) were reported in Middle Eastern countries and were responsible for widespread and serious disease in commercial chickens in Iran (Nilli and Asasi, 2003; Nilli and Asasi, 2001), Pakistan (Naeem *et al*., 1999; Naeem *et al*., 2003) The United Arab Emirates (Manvell *et al*., 2000), Saudi Arabia (Banks *et al*., 2000), Korea (Kwon *et al*., 2008) and Jordan (Monne *et al*., 2007; Hadipour *et al*., 2011). AIV is believed as one of the main causes of chicken respiratory diseases in Iran as indicated by many field reports (Hadipour *et al*., 2011). In addition to these other infections such as Infectious Bronchitis Virus (IBV), Infectious Laryngotracheitis Virus (ILTV) and pneumovirus are also of major concern to the respiratory tract of chickens (Ahmed *et al*., 2009).The disease rapidly spread to other flocks with cardinal signs of respiratory distress, poor growth , decreased production and at last mortality (Hadipour *et al*., 2011). Dissemination of various strains of these viruses by migratory birds has been noted several times, while showing no detectable clinical signs of the disease (Hadipour *et al*., 2011). To control infection and transmission most respiratory viruses in the operation , an intensive vaccination program was implemented in poultry industry for many years with live and inactivated vaccines (Zhang *et al*., 2008; Ahmed *et al*., 2009). Despite the vaccination effort and the strict biosecurity measures employed; infectious viruses were still isolated

Corresponding Author: M.J. Mehrabanpour, Department of virology, Razi Vaccine and Serum Research Institute, Shiraz, Iran

Table1: primers used for RT-PCR reaction

Name	Primer	Primer sequences	PCR product
NDV	ndv1F	5'-TTGATGGCAGGCCTCTTG-<C>3	69 bp
NDV	ndv2R	5'-GGAGGATGTTGGCAGCAT-<T>	
AIV	NP-1200F	5'-CAG (A/G) TACTGGGC (A/T/C)3	30 bp
AIV	NP-152R	5'-GCATTGTCTCCGAAGAAATAAG	
AIV	H5-155F	5'-ACACATGCYCARGACATACT	545 bp
AIV	H5-699R	5'-CTYTGRTTYAGTGTTGATGT	
AIV	H9-F	5'-CT(C/T) CACACAGA (A/G) CACAAATGG	488 bp
AIV	H9-9R	5'-GTCACACTTGTTGTTGT (A/G) TC	
AIV	H7-12F	5'-GGGATACAAAATGAAYACTC	634 bp
AIV	H7-645R	5'-CCATABARYYTRGTCTGYTC	

Codes for mixed bases position: R: A/G; Y: C/T; B: G/C

from chickens (Zhang *et al*., 2008). The aim of this study was designed to clarify the roles of NDV and AIV (H9N2) singly or jointly by RT-PCR assays, in recent outbreaks of respiratory disease in broiler chicken in Fars province during November 2010 until May 2011. So it was conducted to investigate the problem for suggesting proper preventive and therapeutic measures.

MATERIALS AND METHODS

Source and collection of specimens: During the period from November 2010 to May 2011, a total of 44 commercial broiler chicken flocks with high mortality around shiraz city, were visited and detailed history of the flocks including age, breed ,feed and egg productions, medication, vaccination schedule and management conditions of the farms were noted. Samples were collected from dead and morbid birds showing signs of respiratory distress for laboratory investigations. All of these flocks received of influenza A, Newcastle and infectious bronchitis viruses' vaccines. The following types of samples were collected from the diseased flocks:

- Organs including trachea and lung
- Cloacal swabs by using sterile swabs

The tissue and swab samples were transported in cold chain system to the laboratory and stored at -70ºC until used.

Processing of samples and virus isolation: For checking bacterial and fungal contamination, swabs and tissue samples were placed into tubes containing 1 mL PBS solution (pH 7.2) and antibiotics (10.000 I U/mL penicillin, 1 mg/mL streptomycin sulphate, 1 mg/mL gentamicin sulphate (Noroozian *et al*., 2007). Frozen tissues were thawed; grinded and squashed in a sterile pestle and a 10% suspension with transport medium were made (Numan *et al*., 2008). Homogenate was centrifuged at 3000 rpm for 15 min and supernatant was collected. Frozen cloacal swabs were thawed, vortexes and pooled from each flock and centrifuged at 3000 rpm for 15 min (Numan *et al*., 2008; Noroozian *et al*., 2007). Supernatants were collected and filtered through 0.22 µm syringe filter (Gelman, USA). A part of filtered material was stored at -70ºC for PCR testing until used (Numan *et al*., 2008). Filtered material was stored at -70ºC for PCR testing until used.

RNA extraction: Viral RNA was extracted from all filtered material samples using viral Gene-spin TM viral DNA/RNA extraction kit (Vetek, Korea) according to the manufacturer's instructions.

Pimer design, RT _ PCR and gel electrophoresis: To identify NDV, AIV and its subtypes by RT-PCR ,we used from specific primers based on conserved sequences of the NP gene of viruses of avian, human, swine and equine origins, HA gene and F gene of Newcastle virus (Table 1) respectively as described previously(Lee *et al*., 2001). RT-PCR was carried out in 8 µL reaction mixture containing (dNTP, enzyme mix, reaction buffer and stabilizing buffer), 1 µL from each forward and reverse primer, 2 µL of template RNA, 0.25 RNase inhibitor and 8 µL of RNase free water was added to make a reaction volume of 20 µL (Lee *et al*., 2001). The tube was than vortexes and spin for 3-5 sec and before being placed over the thermal cycler for reaction. The PCR condition for the amplification was: RT at 45ºC for 45 min, one cycle at 95ºC for 3 min, 40 cycles of heat denaturation at 95ºC for 30 sec, primer annealing at 55ºC for 1 min, primer extension at 72ºC for 1min and one cycle of final extension step at 72ºC for 10 min in automated thermal cycler (Ahmed *et al*., 2009; Lee *et al*., 2001). The PCR condition for the amplification of NP, H9 and H7 was the same as above, except that the annealing temperature for H5was 58ºC (Lee *et al*., 2001). The RT-PCR products were checked by 1.5% agarose gel electrophoresis and analyzed under UV light (Kang *et al*., 2006).

RESULTS AND DISCUSSION

Respiratory diseases in poultry have been reported to be caused by mix or single infections with several agents

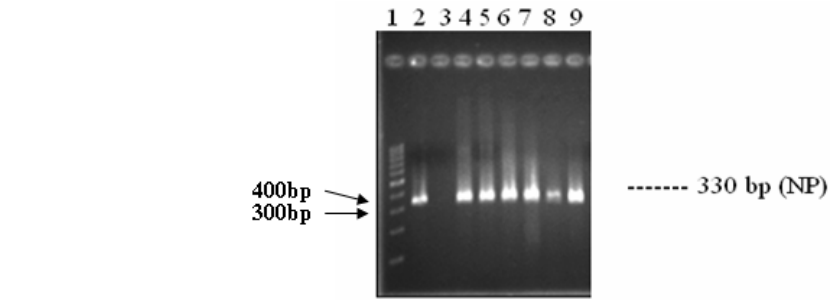

Fig. 1: RT-PCR products of NP gene (AIV), an expected size PCR product, 330 bp of NP gene were detected. Lane 1= Marker 100 bp DNA ladder, Lane 2 = positive control, Lane 3 = negative control, Lane 4-9 = field samples

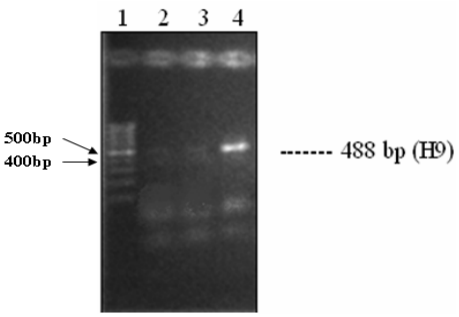

Fig. 2: RT-PCR products of HA gene for AIV, An expected size PCR product for 488bp for H9, 545 for H5 and 634 for H7.Lane 1 = Marker 100 bp DNA ladder, Lane 2 = for H5, Lane 3 = for H7, Lane 4 = for H9

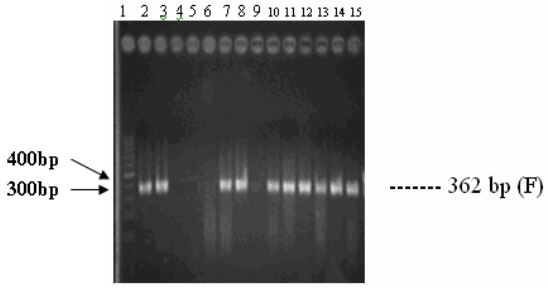

Fig. 3: RT-PCR products of F gene for NDV, An expected size PCR product, 362 bp were detected. Lane 1 = Marker 100 bp DNA ladder, Lane 2 = positive control, Lane 3-7-8-10 to 15 = field positive samples, Lane 4-5-6 & 9 = field negative samples

(Roussan *et al*., 2008; Watanabe *et al*., 1977; Sakuma *et al*., 1981; Malik *et al*., 2004). Avian influenza and Newcastle are the major case of acute respiratory and reproductive tract infection of chickens and every year bring about high morbidity and mortality worldwide (Siddique *et al*., 2008). Clinically it is impossible to detect these viruses from each other. Therefore, it is necessary to develop a diagnostic test for the rapid identification of these viruses directly from clinical specimens (Siddique *et al*., 2008). Further, we and other researchers conclude that molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) help in rapid and accurate identification of the etiological agents responsible for an infection (Ahmed *et al*., 2009). RT-PCR has the ability to even detect a single virus particle, whether active or inactive (Siddique *et al*., 2008). It also provides better detection of the virus from the clinical samples which might otherwise appear negative due to inappropriate sampling or less of infectivity during shipment (Siddique *et al*., 2008). It is common practice in Iran, as in other countries, to vaccinate broiler flocks against IBV, NDV and AIV. Despite the use of IBV, NDV and AIV vaccines, it is common to find NDV, IBV and AIV infections in vaccinated broiler flocks (Roussan *et al*., 2008). However; it is most likely that the flocks used in this study were also naturally exposed to virulent strains of NDV which is why the vaccines were not covered. The high rates of NDV and AIV infections in broiler flocks suggested that NDV and AIV are the most important causes of respiratory disease in this study. Table 2, represent the cause of respiratory infection in broiler chicken flock included in this study according to the results obtained from RT-PCR. A total of 44 commercial broiler flocks with a history of respiratory disease were received . The results show that out of 18 samples, for NDV (Fig. 3) and 6 for AIV were positive (Fig. 1, 2). Further the serotyping of 6 AIV isolates showed that all of them were H9 and we didn't find any H5 or H7 (Fig. 1, 2).

Table 2: Causes of respiratory infection in broiler chicken flocks

Type of respiratoryinfected	infection flocks
AIV	6
NDV	18
NDV+ AIV	18
Non NDV,AIV	2

AIV: avian influenza virus; NDV: Newcastle disease virus

The high percentage of AIV and NDV reported in this study addresses the strong need for more aggressive monitoring and vaccination of the susceptible and already vaccinated poultry flocks. Therefore, the present study is conclusive with this fact that the etiology of respiratory

organisms is very complex and it usually involves more than one pathogen. In addition, to the above arguments it is also concluded that the primer set tested in this study can be used for the samples from Iran and also endorses he homogeneity in genome and circulating nature of these viruses, which are being transported thorough the migratory birds from country (Ahmed *et al*., 2009). Our data showed that these respiratory pathogens were the most important causes of respiratory disease in broiler chicken in Iran. Further studies are necessary to assess circulating strains, economic losses caused by infections and co infections of this pathogen, and the costs and be benefits counter measures .Furthermore, farmers need to be educated about the signs and the importance of these pathogens (Roussan *et al*., 2008).

CONCLUSION

In this study, NDV viruses and AIV viruses of nHPAI isolated from poultry chicken in broiler farm in Shiraz. The high rates of NDV and AIV infection in broiler flocks during 2009-2010 suggested that NDV and AIV are most important causes of respiratory disease in this study.

ACKNOWLEDGMENT

This study has been supported by of Razi vaccine and serum research institute Karaj-Iran (project No 128418890289010). We are grateful to the management of Shiraz veterinary organization and special thanks to the Dr. Rezaeianzadeh for sampling assistance

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