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Full Length Article

Molecular Identification of Agents Causing Respiratory Infections in Chickens from Southern Region of Pakistan from October 2007 to February 2008

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ABSTRACT

Livestock and poultry contribute more than 49% of the agricultural GDP of Pakistan and play an important part in the alleviation of poverty and improvement in quality of life. Poultry industry is the second largest and organized industry of the country, progressing with an average rate of 8-10% yearly. However, viral and bacterial diseases are the major constraints in its growth. Respiratory infections are of paramount importance as high mortality may occur in poor management. Therefore, the etiology of respiratory disease is complex, often involving more than one pathogen at the same time. A wide variety of pathogens have been associated with respiratory infection in poultry, including Avian Pneumovirus (APV), Avian Influenza Virus (AIV), Infectious Bronchitis (IBV), Newcastle Disease Virus (NDV), Infectious Laryngotracheal virus (ILTV) and *Mycoplasma gallisepticum* (MG). As the Avian Influenza and Newcastle disease are endemic in the country, therefore, this study was planned to evaluate their status during the winter outbreaks. Tracheal samples were collected from poultry farms in southern region of Pakistan, experiencing heavy mortality of poultry flock. The samples were subjected to RNA extraction followed by RT-PCR. Out of 50 samples, 20 samples were positive for NDV, 28 for AIV and 2 were negative for both. Further, serotyping of 28 AIV isolates showed that, 6 were positive for H9, 20 for H5 and 2 for H7. Thus, it can be concluded that molecular techniques help in rapid identification of the agents causing infections. Further, the southern region of Pakistan had major infection resulting from AIV (H5) during October 2007 to February 2008.

Key Words: Newcastle disease virus (NDV); Avian influenza virus (AIV); Reverse transcription-polymerase chain reaction (RT-PCR); Avian influenza virus serotype H5; Avian influenza virus serotype H7; Avian influenza virus serotype H9

INTRODUCTION

Poultry sector is one of the most vibrant segments of agriculture sector of Pakistan that generates an employment (direct/indirect) and income for about 1.5 million people. The current investment in this industry is about Rs. 200 billion with an annual growth of 8-10% (Government of Pakistan, 2007). The sector has faced tough challenges through its viral infections especially Avian Influenza (AI) and Newcastle disease (ND). 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. Pneumovirus and ILTV have been found in tissues of the respiratory tract, whereas IBV, NDV and AIV also invade other tissues such as the kidneys, reproductive system and the gastrointestinal tract and the central nervous system by NDV and AIV (Swayne *et al.,* 1998). To control most respiratory viruses, live and inactivated vaccines have been developed and used by the poultry industry for many years. In most cases, live vaccines are prepared with attenuated strains of the respective virus and therefore, these viruses replicate in the tissues of the respiratory tract, inducing a reaction that is known as post vaccination reaction. Moreover, promising results have also been obtained using hyper immune yolk to protect against viral infection (Muhammad *et al.,* 2001).

The etiology of respiratory organisms is complex often involving more than one pathogen (Yashpal *et al.,* 2004). Therefore, these are of most importance as they cause diseases independently, in association with other or with bacterial organisms (Yashpal *et al.,* 2004). Poultry industry in Pakistan during the year 2004-05 had suffered an enormous loss of about Rs. 5.4 billion due to these respiratory viral infections. The NDV and AIV are endemic in the country with a quiet different situation in comparison

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to other viral infections (personal communication). The Avian Influenza which first appeared in 1995 as H7N3 was identified from an isolated area (Naeem & Hussain, 1995) in Punjab. The next epidemic of HPAI H7N3 occurred in 2003-2004 in commercial layers and broiler-breeders. As a results mass vaccination was carried out and acceptable protective titers against H7N3 was recorded during a study in 2005 (Numan *et al.,* 2005) In general, the highly pathogenic form of the disease is easily diagnosed mainly because of the high mortality and typical lesions observed. Since both these organisms are endemic in the country, therefore present study was designed to evaluate their status during the winter outbreaks in 2007-2008.

MATERIALS AND METHODS

Sample collection. Fifty tissue samples preferably tracheal were collected from poultry farms experiencing relatively high mortality in the vicinity of Karachi from October 2007–February 2008. Properly labeled samples were immediately transferred to liquid nitrogen container and transported to the laboratory for further processing.

RNA extraction. RNA was extracted using TRI Reagent (Molecular bioproducts, USA) as per manufacturer's protocol. Briefly, 30 mg of tissue was homogenized with 1 mL of TRI reagent followed by centrifugation at 10,000 rpm for 10 min. The supernatant was transferred to a fresh DEPC treated tube and added with 200 µl of chilled chloroform. The mixture was left at room temperature for 15 min. The same was later centrifuged at 10,000 rpm for 15 min at 4°C. The upper aqueous phase was than transferred to a fresh tube and 500 µl of chilled isopropanol was added followed by centrifugation at 10,000 rpm for 10 min at 4°C. Supernatant was discarded and pellet was first washed with 100% followed by 70% ice-cold ethanol. Finally the pellet was re-suspended in 100 µl DEPC treated water and stored at -70°C for further analysis. Moreover, the extracted RNA was analyzed for its quality and quantity using Nano-drop method (Nano1000, Thermo Scientific, USA). Briefly, the equipment was first activated with 2 µl of RNA/DNA free water. Later the same amount of sample was placed on the machine pedestal and the setting for "RNA" selected. The system measures the quality and quantity of RNA in the given sample.

Primers. The primers specific to Newcastle disease virus, avian influenza virus and its sero-types were used in the present study to amplify the highly conserved regions of the F gene, NP gene and HA gene (Table I), respectively as described previously (Chaharaein *et al*., 2009; Kho *et al.,* 2000; Spackman *et al.,* 2002). These primers have been designed based on the nucleotide sequences of NDV and AIV available with the Gene Bank.

RT-PCR and gel electrophoresis. One-step RT-PCR was performed using Qaigen One step RT-PCR kit (Qaigen, GmbH, Germany) as per manufacturer's instructions. Briefly, 1µl each of dNTP's mixture (320µM each dNTP), enzyme mix, forward and reverse primer (20 pmol each), 5µl of 5 X reaction buffer, 3µl of template RNA and 13µl of Rnase free water was added to make a reaction volume of 25µl. The tube was than vortexed and spin for 3-5 sec before being placed over the thermalcycler for reaction. The amplification took place under the following conditions: RT at 48°C for 45 min, one cycle at 94°C for 2 min, 40 cycles of heat denaturation at 94°C for 30 s, primer annealing at 58°C for NP, 57°C for H5, 59°C for H7, 60°C for H9 and 58°C for NDV for 1 min, primer extension at 68°C for 1 min and one cycle of final extension step at 68°C for 7 min in automated thermal cycler (Eppendorf, Germany). The products were analyzed on 1.5% agarose (Invitrogen Life Technologies, USA) gel prepared in 1 X TBE buffer containing 0.5 ug mL^{-1} of ethidium bromide (Invitrogen Life Technologies, USA) at 120 volts for 45 min. All the amplification were supported by positive and negative controls for the viruses certified from South East Poultry Research Lab (SEPRL), Athens, GA-USA and National Agriculture Research Council (NARC), Islamabad, Pakistan. The bands were visualized using Quantity One software on Chemi Doc (Biorad, UK) and the product size calculated using 100 bp step ladder (Qaigen, GmbH, Germany).

RESULTS AND DISCUSSION

The endemic nature of Newcastle disease virus and Avian Influenza in Pakistan has caused severe economic losses to the poultry industry of Pakistan in past years and is continuing. These viral infections are also a threat to the human population engaged with the rearing of live birds and its business (Suarez, 2000; Lewis, 2006). Since the outbreak of Newcastle disease in late 70's in Pakistan (Rehmani, 1979) its vaccination is a routine practice. Lentogenic as well as Mesogenic strains are routinely applied for its prevention (Rehmani, 1989). The Avian Influenza outbreaks in the country in 90's have worsened the situation (Naeem & Hussain, 1995). The results of this study are also endorsing the arguments of the endemic nature of these organisms. The results show that out of 50 samples, 40% were positive for NDV, 56% for AIV and 4% were negative to both. Further the serotyping of 56% AIV isolates showed that 22% were H9, 72% were H5 and 6% were H7. This shows that in spite of the vaccination of Newcastle disease since late 70's its outbreaks are still occurring (Ph.D. thesis), which points out the mutation, which might be occurring in the virus providing room for its escape from vaccination. Therefore, the velogenic form of disease is still in circulation and prepares grounds for the other respiratory pathogens to attack along with it since its infection occurs throughout the year. The high percentage of Avian Influenza cases 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

Table I. Primers used in present study

Name	Sequence	Expected size of PCR product (bp)
Newcastle Disease virus	5'GGGAGGCATACAACAGGACA 3'	238
Newcastle Disease virus	5' TGGTTGCAGCAATGCTCTC 3'	
Avian Influenza virus (NP)	5' TGTACGGACTTGCTGTGGCC 3'	106
Avian Influenza virus (NP)	5' GAGACTGAAGACCTGGCTGTT 3'	
Avian Influenza virus (H5)	5' ACAAAGCTCTATCAAAACCCAAC 3'	499
Avian Influenza virus (H5)	5' TACCCATACCAACCATCTACCAT 3'	
Avian Influenza virus (H7)	5' CAGGCGGAATTGATAAGGAG 3'	409
Avian Influenza virus (H7)	5' TGCCCCATTGAAACTGAAAG 3'	
Avian Influenza virus (H9)	5' ATCGGCTGTTAATGGAATGTGTT 3'	221
Avian Influenza virus (H9)	5' TGGGCGTCTTGAATAGGGTAA 3'	

Table II. Summary of the samples analyzed against Newcastle disease virus, Avian Influenza virus and its serotypes

SAMPLE STATUS	NDV	AIV (NP)	AIV (H5)	AIV (H7)	AIV (H9)
Positive	20	28	20	2	6
Negative	30	22	30	48	44

Fig. 1. RT-PCR amplification of H gene (AIV), An expected size PCR product for 499 bp for H5, 409 bp for H7 and 221 bp for H9 was detected from different samples. Lane 1 = Gene Ruler™ 100 bp DNA Ladder (Fermentas, USA), Lane 2-4 = Field samples

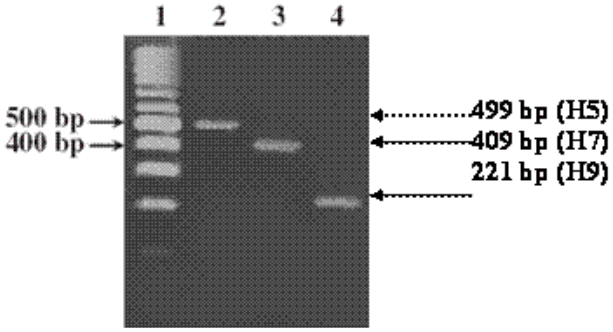
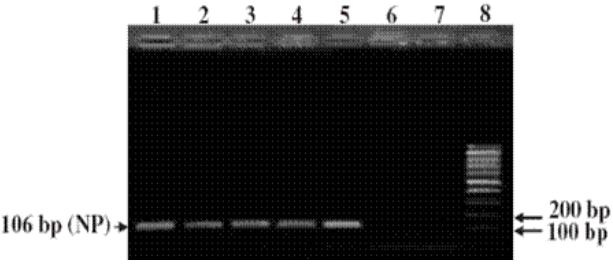
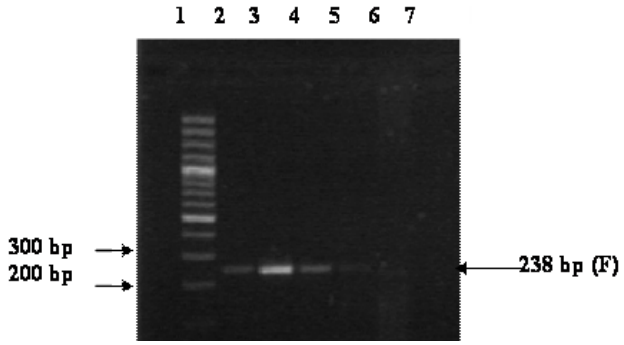


Fig. 2. RT-PCR amplification of NP gene (AIV) from different samples, An expected size PCR product, 106 bp of NP gene was detected from different samples. Lane 1–4 = field samples, Lane 5 = positive control, Lane 6-7 = NDV and IBDV (negative control), respectively, and Lane 8 = Gene Ruler™ 100 bp DNA Ladder (Fermentas, USA)



the etiology of respiratory organisms is very complex and it often involves more than one pathogen (Yashpal *et al.*, 2004). Further the southern region of Pakistan during October 2007 to February 2008 was badly hit by both the Avian Influenza virus and Newcastle disease. In addition, to the above arguments it is also concluded that the primer sets

Fig. 3. RT-PCR amplification of F gene (NDV), An expected size PCR product for 238 bp NDV was detected from different samples. Lane 1 = Gene Ruler™ 100 bp DNA Ladder (Fermentas, USA), Lane 2 = Positive control, Lane 3 -7 = Field samples



tested in this study can be used for the samples from Pakistan and also endorses the homogeneity in genome and circulating (endemic) nature of these viruses, which are being transported through the migratory birds from country to country (Webster *et al.*, 1992). Further, the extensive import of live vaccine from around the world has also worsened the situation as this serves as an incubator for the virus to intermingle and reform into a new subtype (Suarez, 2000). Moreover, the Biosecurity measures that need to be practiced, while carrying out a live vaccination or even a vaccination are rarely inpractice in country and have contributed in creating such complicated situations. Further, it is also concluded that molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) and real-time reverse transcription polymerase chain reaction RRT-PCR help in rapid and accurate identification of the etiological agents responsible for an infection.

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