Molecular Survey of Respiratory and Immunosuppressive Pathogens Associated with Low Pathogenic Avian Influenza H9N2 Subtype and Virulent Newcastle Disease Viruses in Commercial Chicken Flocks

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The study was carried out in 48 poultry flocks to elucidate the roles of various complicating pathogens involved along with Newcastle disease (ND)/ low pathogenic avian influenza (LPAI) outbreaks. Necropsy was conducted and samples were collected for the isolation of Newcastle disease virus (NDV), Influenza A virus, infectious bronchitis virus (IBV), pathogenic bacteria; molecular detection of infectious laryngotracheitis virus (ILTV), fowl adeno virus (FAV), chicken anaemia virus (CAV), Mycoplasma synoviae (MS) and Mycoplasma gallisepticum (MG). The isolation results confirmed that 18/48 flocks (37%) were positive for the presence of hemagglutinating agents. Out of 18 hemagglutination (HA) positive flocks, 11 flocks (61%) were positive for both avian influenza virus (AIV) and NDV; 4 flocks (22%) were positive for NDV; and 3 flocks (17%) were positive for AIV. Sequence analysis of hemagglutinin and neuraminidase genes of AIV revealed that all were belonging to LPAI-H9N2 subtype. Sequence analysis of F gene of NDV revealed that they belong to virulent type. The PCR results confirmed the presence of three to seven etiological agents (CAV, FAV, ILTV, MG, MS and avian pathogenic E. coli along with LPAI/NDV from all the 18 HA-positive flocks. The detection rate of triple, quadruple, quintuple, sextuple and sevenfold infections was 17% (3 flocks), 28% (5 flocks), 11% (2 flocks) 28% (5 flocks) and 17% (3 flocks), respectively. In conclusion, the disease complex involved more than one pathogen, primarily resulting from the interplay between LPAI-H9N2 and NDV; subsequently this could be exacerbated by co-infection with other agents which may cause exacerbated outbreaks that may otherwise go undetected in field.

Key words: low pathogenic avian influenza, molecular survey, respiratory complications, velogenic Newcastle disease

Introduction

Among respiratory diseases, Newcastle disease (ND) and avian influenza (AI) are the highly infectious and dynamically evolving diseases of poultry birds causing high morbidity and mortality. The clinical signs and gross lesions of these two diseases often overlap with each other. In India, Newcastle disease virus (NDV) was first reported in 1927 (Edwards 1928) and since then several outbreaks have been reported frequently despite of strict vaccination programs (Jakhesara et al., 2016; Khorajiya et al., 2015). The low pathogenic avian influenza (LPAI) H9N2 subtype was first reported in India during 2003 and then subsequently reported from several northern states (Nagarajan et al., 2009; Kale et al., 2013; Jakhesara et al., 2014). In the recent past, field outbreaks resembling ND or AI complicated with various agents such as Escherichia coli, mycoplasma, etc. have been flaring up in Indian poultry industry (Vegad et al., 2008; Vegad 2014). NDV and avian influenza virus (AIV) often
prepare grounds for the various respiratory/immunosuppressive pathogens to complicate the outcome of the disease process (Ahmed et al., 2009). In addition to AIV and NDV, many infectious pathogens viz., infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILT), mycoplasmosis, chicken anaemia virus (CAV), fowl adenovirus (FAV) and avian pathogenic Escherichia coli (APEC) involve respiratory and/or immune system and produce similar clinical signs. This further adds to the complexity of the problem regarding correct identification and differentiation of various respiratory-immunosuppressive agents complicating LPAI-ND disease complex. Several diagnostic methods such as isolation and serology are applied for detecting and differentiating various respiratory-immunosuppressive infections in poultry (Luciano et al., 2011; Pawar et al., 2012; Bodhidatta et al., 2013; Saadat et al., 2014). However, the conventional diagnostic methods are time consuming and labor intensive. Furthermore, nonspecific reactions or cross-reactions (Nascimento et al., 2006) frequently hinder serologic assays. Rapid and sensitive techniques to identify and distinguish respiratory disease pathogens are vital for implementing appropriate preventive and control measures to mitigate economic losses. Molecular methods, such as polymerase chain reaction (PCR) techniques and others, have been frequently used in the field for rapid and sensitive detection of avian pathogens (Rodriguez-Sanchez et al., 2008; Hoffmann et al., 2009). The present study aimed to carry out molecular survey for elucidating the roles of various complicating pathogens involved along with ND/LPAI outbreaks in poultry.

Materials and Methods

Case History, Pathological Studies and Sample Collection

This study was carried out between January 2010 and January 2012 in 48 commercial poultry flocks with respiratory complications from different poultry producing states of India viz., Haryana, Tamil Nadu, Uttar Pradesh and Uttarakhand. The flocks consisted of broilers, growers and layers aged between 3 and 76-weeks, and reared under standard management practices. Necropsy examination was conducted on freshly dead birds and 10 birds/flock were sampled. Pooled tissue samples such as trachea, lungs, kidneys, spleen and oviduct from all the birds (5 bird/pool; 2 pools/flock) were collected for the isolation of NDV, Influenza A, IBV, and for direct PCR detection of ILTV, FAV, CAV, MS and MG. Heart blood swabs from all the birds were collected aseptically for isolation of pathogenic bacteria.

Virus Isolation

The tissues were processed (10% homogenate in phosphate buffered saline) and inoculated into 9–11 days-old embryonated chicken eggs through allantoic route, and the eggs were incubated at 37°C for a maximum period of 5 days and chilled for overnight at 4°C. The embryos were candled every day and the dead embryos were chilled, and then the allantoic fluids were tested for haemagglutination (HA) activity. Three blind passages were carried out before deciding the negativity of the samples.

Screening of NDV, AIV and IBV

Viral RNA from HA positive amnio allantoic fluid was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and cDNA was synthesized by random primers using SuperScript® First-Strand Synthesis System. (Invitrogen, Darmstadt, Germany). PCR reaction was carried out with HotStarTaq Master Mix (Qiagen, Hilden, Germany). The presence of AIV, NDV and IBV was confirmed by PCR targeting M gene (Ottiger 2010), F gene (Toyoda et al., 1989) and S1 gene (Adzhari et al., 1996), respectively. Typing of influenza A viruses and NDV was done by further PCR amplification, sequencing of the hemagglutinin and neuraminidase (Hoffmann et al., 2001; Alvarez et al., 2008), F genes of AIV and NDV, respectively. Two micro litres of the PCR product were analyzed by electrophoresis in 1.5% agarose gel. PCR products were purified employing Exo-SAP-IT (Affymetrix, USA) and sequenced through commercial source (Seigenom, Cochin).

Screening of ILTV, FAV, CAV and Mycoplasma

Tissues from the LPAI/NDV positive flocks were subjected to further screening of ILTV, FAV, CAV and mycoplasma. DNA from tissues was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). The presence of ILT, FAV and CAV was confirmed by PCR targeting of the envelope glycoprotein-G, hexon and VP2 genes, respectively (Ottiger 2010). The presence of Mycoplasma synoviae (MS) and Mycoplasma gallisepticum (MG) was confirmed by targeting the 16S gene (Lauerman et al., 1993; Lauerman 1998). The details of primers used for screening of different respiratory-immunosuppressive pathogens are provided in Table. 1.

Bacterial Isolation and Molecular Detection

All the swabs collected were processed as per standard methods for bacterial isolation as described by American Association of Avian Pathologists (Glisson et al., 2008). Bacterial DNA from the culture was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, USA). The presence of APEC, Avibacterium paragallinarum (IC) and Ornithobacterium rhinotracheale (ORT) was detected by PCR targeting 16S gene (Mendoza-Espinoza et al., 2008), followed by restriction fragment length polymorphism (RFLP) with DdeI, Rsal, and EcoRI enzymes (Thermo Scientific, Waltham, USA) as per the manufacturer’s instructions. For digestion, ~ 0.5 to 1 μg of purified PCR products were taken in a 10 μl reaction mix in a 0.2 ml PCR tube. The reaction mixture was incubated at 37°C for overnight in digital water bath and stored at −20°C until it was subjected to agarose gel electrophoresis. Digested products along with DNA ladder were electrophoresed in 3% agarose gel in modified TAE buffer at 60 volts for 3 hrs using submarine electrophoresis apparatus (Atto, Japan). Following electrophoresis, the gel was visualized under UV illumination and photographed. The size of restriction fragment was ascertained by comparison with standard DNA molecular weight marker (Fermentas, EU).
Results

Virus Isolation and Confirmation

A total of 48 poultry flocks were screened for the presence of hemagglutinating viruses by inoculating the samples in to embryonated chicken eggs followed by HA and RT-PCR of amnio allantoic fluid. The results confirmed that 18 out of 48 (37%) flocks were positive for the presence of haemagglutinating agents. Out of 18 HA-positive flocks, 11 flocks (61.11%) were co-infected with both AIV and NDV, 4 flocks (22%) were positive for NDV alone, and 3 flocks (17%) were positive for AIV alone as detected by isolation as well as PCR. None of the flocks was found positive for IBV. Samples from all the 18 HA-positive flocks were further processed for genetic and phylogenetic analysis. Sequence analysis of hemagglutinin and neuraminidase genes of AIV revealed that all were belonging to low pathogenic H9N2 subtype (RSSR*G in hemagglutinin gene) since it was lacking multiple basic amino acids at hemagglutinin cleavage site. Sequence analysis of F gene of NDV revealed that they belong to virulent type (RRRRK*R) since it contained multi basic amino acids at fusion protein cleavage type.

Clinical Disease

The clinical disease of LPAI/NDV positive flocks was characterized by dullness, inappetence, stunted growth, diarrhoea, gasping, rales, progressive neurological signs, drop in egg production and deterioration of egg quality. Predominant postmortem lesions included emaciation, tracheitis, pulmonary congestion/ oedema, airsacculitis, petechiae in serosal layer of heart and abdominal fat, pericarditis, perihepatitis, haemorrhagic proventriculitis, button ulcers in the small intestine, splenic atrophy, egg peritonitis, oophoritis, and nephritis-nephrosis complex.

Differential Diagnosis

In order to determine synergism between LPAI/NDV and other respiratory-immunosuppressive pathogens, the clinical materials of all the 18 HA positive flocks were subjected to isolation followed by PCR confirmation of IC, ORT, APEC and direct tissue PCR analysis for ILTV, CAV, FAV, MG, MS. The results showed the presence of three to seven etiological agents (CAV, FAV, ILT, APEC, MS and APEC) along with LPAI/NDV from all the 18 HA-positive flocks. The detection rate of triple, quadruple, quintuple, sextuple and sevenfold infections was 17% (3 flocks), 28% (5 flocks), 11% (2 flocks), 28% (5 flocks) and 17% (3 flocks), respectively. Among the 18 LPAI/NDV-positive flocks, APEC and CAV in 16 flocks (89%), MS in 11 flocks (61%), MG in 9 flocks (50%), FAV in 7 flocks (39%) and ILT in 2 flocks (11%) were detected. Epidemiological details and co-infections along with LPAI/NDV are presented in Table 2.

Discussion

Out of a total of 48 poultry flocks surveyed, 18 (37.5%) of the flocks were found positive for virulent NDV and/ or LPAI-H9N2 along with concurrent infections of three to seven pathogens (NDV/LPAI, CAV, FAV, ILT, APEC, MS...
and NDV). The above results clearly indicate that the multiple etiological agents play a pivotal role in occurrence of respiratory diseases in poultry. A recent study by Smietanka et al. (2014) in Poland revealed the presence of DNA/RNA of Ornithobacterium rhinotracheale, Bordetella avium and, less frequently, of astro, rota, reo, parvo and adenoviruses along with H9N2 LPAI in infected turkeys. A survey conducted for respiratory pathogens in 115 broiler chicken flocks in Jordan by PCR/RT-PCR revealed presence of the following etiological agents viz., AIV (H9N2), NDV, avian metapneumo virus (aMPV), IBV, and MG with different combinations (Roussan et al., 2008). Earlier studies by (Springer et al., 1974) on the synergistic role of IBV, MS and E. coli in the airsacculitis syndrome revealed that the severity of the lesions was higher with the presence of three agents than of one or two. With these agents alone it is often not likely to reproduce the clinical signs seen in the field, experimentally. Therefore, it is hypothesized that the development of respiratory disease is often a consequence of dual infections by respiratory viruses with certain bacteria. The results are in agreement with the fact that etiology of respiratory disease complex (RDC) in poultry is very complex and often involves more than one pathogen at a time (Smietanka et al., 2014; Roussan et al., 2015). In addition to this, our study denotes that immunosuppressive agents like CAV and FAV are also involved in the etiology of RDC which prepares grounds for predisposition to the other respiratory pathogens to attack along with it. Due to the extensive use of multiple live vaccines, lack of biosecurity, high geographic populations, and housing densities, mixed infections of different respiratory agents may occur (Glisson 1998). Virulent NDV was detected in 11 LPAI positive cases (61%). This confirms the co-existence of virulent NDV and LPAI-H9N2 subtype under field conditions. Although, several reports are available on concurrent infections of poultry with various viral and bacterial agents, especially in RDC cases, studies on co-occurrence of LPAI and NDV in domestic poultry are very limited (Roussan et al., 2008; Al-Mohana et al., 2013). The concurrent infections of poultry with LPAI and NDV present an overlapping clinical picture often misleading the identification and diagnosis of both of these viruses (Shortridge 1982). ILT was detected in two flocks (11%). The ILTV and AIV coinfection observed in the present study is uncommon. CAV was detected in all the 18 NDV/LPAI positive cases. This suggests that CAV might be involved in causing subclinical infections. Because of the widespread practice of vaccination for breeders and the presence of maternal antibodies, the clinical form of CAV is rare (Sommer and Cardona 2003). The subclinical CAV with other respiratory pathogens, measurably decreased the performance of birds, leads to vaccine failures and contributed to concurrent infections. Similar observations were also reported by several researchers in CAV subclinical cases with other agents (Rimondi et al., 2014). FAV could be detected in 7 flocks (38.88%) and this observation confirms the earlier report of avian adeno virus association with respiratory disease (Gowthaman et al., 2012). MS and MG could be detected in 11 flocks (61.11%) and 9 flocks (50%), respectively. Synergism has been demonstrated between MG and other respiratory pathogens (Bradbury 1984). Recent findings of (Roussan et al., 2015) revealed 12.9 and 5.7% of broiler flocks in Jordan to be co-infected with LPAI-H9 subtype and MS; and LPAI- H9 subtype and MG, respectively. Out of forty eight flocks tested, thirty were

### Table 2. Epidemiological details and co-infections along with low pathogenic avian influenza and Newcastle disease

| S. No | Farm Code | State      | Flock size ( chicks) | Age (weeks) | Type of bird | Mortality (%) | Isolation of hemagglutinating virus | Co-infections detected |
|-------|-----------|------------|----------------------|-------------|--------------|---------------|-------------------------------------|------------------------|
| 1     | NKL-1     | Tamil Nadu | 100,000              | 76          | Layer       | 4.9           | +                                   | +                      | CAV, MS, MG & APEC    |
| 2     | NKL-2     | Tamil Nadu | 15,000               | 23          | Layer       | 2.0           | -                                   | +                      | CAV, MS & APEC        |
| 3     | NKL-3     | Tamil Nadu | 40,000               | 34          | Layer       | 4.0           | +                                   | +                      | ILT, CAV, MS, MG & APEC |
| 4     | NKL-4     | Tamil Nadu | 6,000                | 31          | Layer       | 2.6           | +                                   | +                      | CAV & APEC            |
| 5     | NKL-5     | Tamil Nadu | 100,000              | 30          | Layer       | 10.0          | +                                   | -                      | CAV & APEC            |
| 6     | NKL-8     | Tamil Nadu | 50,000               | 8           | Layer       | 22.02         | +                                   | +                      | FAV, CAV, MS, MG & APEC |
| 7     | NKL-9     | Tamil Nadu | 25,000               | 7           | Layer       | 15.41         | +                                   | +                      | FAV, CAV, MS & APEC    |
| 8     | NKL-10    | Tamil Nadu | 15,000               | 8           | Layer       | 20.00         | +                                   | +                      | CAV, MS, MG & APEC    |
| 9     | NKL-11    | Tamil Nadu | 3,800                | 6           | Layer       | 40.82         | +                                   | +                      | CAV & APEC            |
| 10    | NKL-12    | Tamil Nadu | 18,000               | 8           | Layer       | 44.40         | +                                   | +                      | ILT, CAV & APEC       |
| 11    | NKL-14    | Tamil Nadu | 5,400                | 4           | Layer       | 31.44         | +                                   | +                      | FAV, CAV & APEC       |
| 12    | NKL-15    | Tamil Nadu | 4,500                | 14          | Layer       | 27.60         | +                                   | +                      | CAV, MS, MG & APEC    |
| 13    | BLY-43    | Uttar Pradesh| 2,000               | 35          | Breeder     | 80.00         | -                                   | +                      | ILT, FAV, CAV, MS, MG & APEC |
| 14    | BLY-006   | Uttar Pradesh| 2,400               | 3           | Broiler     | 42.04         | -                                   | +                      | CAV, MG& APEC         |
| 15    | HR-0001   | Haryana    | NA                   | NA          | Broiler     | NA            | +                                   | -                      | FAV, CAV, MG & APEC    |
| 16    | HR-0002   | Haryana    | NA                   | NA          | Broiler     | NA            | +                                   | -                      | FAV, CAV & APEC       |
| 17    | BLY-007   | Uttar Pradesh| 4,000               | 4           | Broiler     | 23.02         | -                                   | +                      | CAV & APEC            |
| 18    | BLY-008   | Uttar Pradesh| 2,000               | 3           | Broiler     | 31.00         | +                                   | +                      | CAV & APEC            |

NA-Not available
negative for NDV/LPAI, the mortality in these flock might be due to other pathogens like pneumovirus, ILTV, mycoplasma, E. coli and other managemental factors. However, determination of the exact cause of the respiratory disease, other than the LPAI/NDV, was not the aim of this study.

The PCR tests demonstrated the presence of DNA of CAV, FAV, ILT, MS, MG and APEC from the NDV/LPAI infected flocks. Although no virus and mycoplasma culture were performed, however based on overall findings we speculate that these secondary pathogens could play a role in the pathogenicity of the NDV/LPAI infections. However, these finding needs to be evaluated carefully since no experimental attempts/demonstration of organisms in tissues were made. In order to fully prove the hypothesis about the existence of synergism after simultaneous infection with NDV, LPAI and other pathogens, an experimental study is highly desirable. Nevertheless, we consider that this possibility is reasonably based on the field observations (variable mortality rates in NDV/LPAI H9N2-infected flocks).

In conclusion, the disease complex primarily resulted from the interplay between LPAI-H9N2 and NDV; subsequently this could be exacerbated by co-infection with other agents such as, ILTV, APEC, and mycoplasmas. Immunosuppression caused by CAV, FAV also plays a relevant role in the outcome of respiratory disease complex and is a constant unseen element in etiopathogenesis of RDC in poultry. Hence in addition to NDV and LPAI H9N2, several other avian pathogens (dual or multiple pathogen coinfections) may cause exacerbated outbreaks that may otherwise go undetected. A proper monitoring and prevention of these other agents is very imperative to formulate suitable control strategies against LPAI/NDV in field conditions. Also, it is suggested that while conducting passive and active surveillance for disease causative agents in poultry flocks, the possibilities that more than one pathogen may be involved in disease/outbreak outcome need to be taken care of fully.

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