JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.0160032

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

Vasudevan Gowthaman[1, 2]*, Shambu Dayal Singh[1], Kuldeep Dhama[1], Palani Srinivasan[2], Sellappan Saravanan[2], Thippichettypalayam R. Gopala Krishna Murthy[2] and Muthannan Andavar Ramakrishnan[3]

[1] Avian Diseases Section, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh – 243 122, India
[2#] Poultry Disease Diagnosis and Surveillance Laboratory, Veterinary College and Research Institute Campus, Namakkal, Tamil Nadu – 637 002, India
[3] Division of Virology, Indian Veterinary Research Institute, Mukteshwar, Uttarakhand-263 138, India

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

J. Poult. Sci., 54: 179–184, 2017

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

Received: March 8, 2016, Accepted: August 25, 2016
Released Online Advance Publication: October 25, 2016
Correspondence: Dr. Vasudevan Gowthaman, Poultry Disease Diagnosis and Surveillance Laboratory, Veterinary College and Research Institute Campus, Namakkal, Tamil Nadu – 637 002, India.
(E-mail: vetgowth@yahoo.co.uk)
* Present address

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 (ILTV), 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℃ for a maximum period of 5 days and chilled for overnight at 4℃. The embryos were candled every day and the dead embryos were chilled, and then the allantoic fluids were tested for hemagglutination (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 (Adzhar *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 (Scigenom, 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 *Dde*I, *Rsa*I, and *Eco*RI 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 m*l* PCR tube. The reaction mixture was incubated at 37℃ for overnight in digital water bath and stored at −20℃ 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).

Table 1. **The details of primers used for screening of different respiratory- immunosuppressive pathogens**

S. No	Name of disease	Target gene	Primer sequence- 5′- 3′ sequence	Product size (bp)	Reference
1.	Influenza A virus	M	CTT CTA ACC GAG GTC GAA ACG AGG GCA TTT TGG ACA AAKCGT	244	Ottiger, 2010
		Hemagglutinin	TATTCGTCTCAGGGAGCAAAAGCAGGGG TTYTGATGYCTGAADCCRTACCA	1232	Self-designed Self-designed
		Neuraminidase	TATTGGTCTAGGGAGCAAAAGCAGGAGT CCIIKCCARTTRTCYCTRCA	909	Hoffman *et al*., 2001 Alvarez *et al*., 2008
2.	ND	F	GCAGCTGCAGGGATTGTGGTG TCTTTGAGCAGGAGGATGTTG	356	Toyoda *et al*., 1987
3.	IB	S1	CCCCAATTTGAAAACTGAACA CCTACTAATTTACCACCAGA	1600	Adzhar *et al*., 1996
4.	ILT	US4	CTACGTGCT GGG CTCTAATCC AAA CTC TCG GGT GGC TAC TGC	588	Ottiger, 2010
5.	CAV	VP2	CTA AGA TCT GCA ACT GCG GA CCT TGG AAG CGG ATA GTC AT	419	Ottiger, 2010
6.	FAV	Hexon	CAARTTCAGRCAGACGGT TAGTGATGMCGSGACATCAT	897	Ottiger, 2010
7.	MG	16S	GAGCTAATCTGTAAAGTTGGTC GCTTCCTTGCGGTTAGCAAC	185	Lauerman 1998
8.	MS	16S	GAGAAGCAAAATAGTGATATCA CAGTCGTCTTCCGAAGTTAACAA	215	Lauerman *et al*., 1993
9.	APEC	16S	AGAGTTTGATCATGGCTCAG GGTTACCTTGTTACGACTT	1600	Mendoza-Espinoza *et al*., 2008

ND-Newcastle Disease, IB-Infectious Bronchitis, ILT- Infectious laryngotracheitis, CAV-Chicken anaemia virus, FAV-Fowl adenovirus, MG-*Mycoplasma gallisepticum*, MS-*Mycoplasma synoviae*, APEC-Avian pathogenic *Escherichia coli*.

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 (RRRKR*F) 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, inappetance, 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, spleenic 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, MG, 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

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

S. No	Farm Code	State	Flock size	Age (weeks)	Type of bird	Mortality (%)	Isolation of hemagglutinating virus		Co-infections detected
							LPAI	NDV	
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.	HR0002	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

and MG). The above results clearly indicate that the multiple etiological agents play a pivotal role in occurrence of respiratory diseases in poultry. A recent study of 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 ND presenst 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 reconfirms 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

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.

Acknowledgments

Authors are thankful to the Director IVRI for providing facilities and funds for carrying out the study.

References

Adzhar A, Shaw K, Britton P and Cavanagh D. Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction. Avian Pathology, 25: 817−836. 1996.

Ahmed A, Khan T, Kanwal B, Raza Y, Akeam M, Rehmani S, Lone N and Kazmi S. Molecular identification of agents causing respiratory infections in chickens from southern region of pakistan from october 2007 to february 2008. International Journal of Agriculture and Biology, 11: 325−328. 2009.

Alvarez AC, Brunck ME, Boyd V, Lai R, Virtue E, Chen W, Bletchly C, Heine HG and Barnard R. A broad spectrum, one-step reverse-transcription PCR amplification of the neuraminidase gene from multiple subtypes of influenza A virus. Virology Journal, 5: 77. 2008.

Bodhidatta L, Srijan A, Serichantalergs O, Bangtrakulnonth A, Wongstitwilairung B, McDaniel P and Mason CJ. Bacterial pathogens isolated from raw meat and poultry compared with pathogens isolated from children in the same area of rural Thailand. The Southeast Asian Journal of Tropical Medicine and Public Health, 44: 259−272. 2013.

Bradbury JM. Avian mycoplasma infections: prototype of mixed infections with mycoplasmas, bacteria and viruses. Annales De Microbiologie, 135A: 83−89. 1984.

Edwards J. Annual Report of Imperial Institute of Veterinary Research, Mukteswar, India. IVRI, 14−15. 1928.

Glisson JR. Bacterial respiratory disease of poultry. Poultry Science, 77: 1139−1142. 1998.

Glisson J, Jackwood M, Pearson J, Reed, W, Swayne D and Woolcock P. Isolation, Identification, and Characterization of Avian Pathogens. American Association of Avian Pathologists 5th Ed. 2008

Gowthaman V, Singh SD, Dhama K, Barathidasan R, Kumar MA, Desingu PA, Mahajan NK and Ramakrishnan MA. Fowl adenovirus (FAdV) in India: evidence for emerging role as primary respiratory pathogen in chickens. Pakistan Journal of Biological Sciences, 15: 900−903. 2012.

Hoffmann B, Beer M, Reid SM, Mertens P, Oura CAL, van Rijn PA, Slomka MJ, Banks J, Brown IH, Alexander DJ and King DP. A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. Veterinary Microbiology, 139: 1−23. 2009.

Hoffmann E, Stech J, Guan Y, Webster RG and Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Archives of Virology, 146: 2275−2289. 2001.

Jakhesara SJ, Bhatt VD, Patel NV, Prajapati KS and Joshi CG. Isolation and characterization of H9N2 influenza virus isolates from poultry respiratory disease outbreak. SpringerPlus, 3: 196. 2014.

Jakhesara SJ, Prasad VVSP, Pal JK, Jhala MK, Prajapati KS and Joshi CG. Pathotypic and sequence characterization of Newcastle disease viruses from vaccinated chickens reveals circulation of genotype II, IV and XIII and in India. Transboundary and Emerging Diseases, 63: 523−539. 2016.

Kale SD, Mishra AC and Pawar SD. Suitability of specimen types for isolation of avian influenza viruses from poultry. Indian Journal of Virology, 24: 391−393. 2013.

Khorajiya JH, Sunanda Pandey, Ghodasara PD, Joshi BP, Prajapati KS, Ghodasara DJ and Mathakiya RA. Patho-epidemiological study on Genotype-XIII Newcastle disease virus infection in commercial vaccinated layer farms. Veterinary World, 8: 372−381. 2015.

Lauerman L. Mycoplasma PCR Assays. : Nucleic acid amplification assays for diagnosis of animal diseases. L. H. Laureman, ed. American Association of Veterinary Laboratory Diagnostics, Auburn, AL. USA, 41−52. 1998.

Lauerman LH, Hoerr FJ, Sharpton AR, Shah SM and van Santen VL. Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. Avian Diseases, 37: 829−834. 1993.

Luciano RL, Cardoso ALSP, Stoppa GFZ, Kanashiro AMI, de Castro AGM and Tessari ENC. Comparative study of serological tests for *Mycoplasma synoviae* diagnosis in commercial poultry breeders. Veterinary Medicine International, 304349. doi:10.4061/2011/304349. 2011.

Mendoza-Espinoza A, Koga Y and Zavaleta AI. Amplified 16S ribosomal DNA restriction analysis for identification of Avibacterium paragallinarum. Avian Diseases, 52: 54−58. 2008.

Al-Mohana A, Kadhimv H, Al-Charrakh A, Al-Habubi Z, Nasir F, Al-Hilali A and Hadi Z. Molecular diagnosis of avian respiratory diseases in commercial broiler chicken flocks in province of Najaf, Iraq. Scientific Research and Essays, 8: 1191−1195. 2013.

Nagarajan S, Rajukumar K, Tosh C, Ramaswamy V, Purohit K, Saxena G, Behera P, Pattnaik B, Pradhan HK and Dubey SC. Isolation and pathotyping of H9N2 avian influenza viruses in Indian poultry. Veterinary Microbiology, 133: 154−163. 2009.

Nascimento E, Polo P, Pereira V, Barreto M, Nascimento M, Zuanaze MAF, Corrêa AR and Silva R. Serologic response of SPF chickens to live vaccines and other strains of *Mycoplasma gallisepticum*. Brazilian Journal of Poultry Science, 8: 45−50. 2006.

Ottiger HP. Development, standardization and assessment of PCR systems for purity testing of avian viral vaccines. Biologicals, 38: 381−388. 2010.

Pawar SD, Kale SD, Rawankar AS, Koratkar SS, Raut CG, Pande SA, Mullick J and Mishra AC. Avian influenza surveillance reveals presence of low pathogenic avian influenza viruses in poultry during 2009−2011 in the West Bengal State, India. Virology Journal, 9: 151. 2012.

Rimondi A, Pinto S, Olivera V, Dibárbora M, Pérez-Filgueira M, Craig MI and Pereda A. Comparative histopathological and immunological study of two field strains of chicken anemia virus. Veterinary Research, 45: 102. 2014.

Rodriguez-Sanchez B, Sanchez-Vizcaino JM, Uttenthal A, Rasmussen TB, Hakhverdyan M, King DP, Ferris NP, Ebert K, Reid SM, Kiss I, Brocchi E, Cordioli P, Hjerner B, McMenamy M, McKillen J, Ahmed JS and Belak S. Improved diagnosis for nine viral diseases considered as notifiable by the world organization for animal health. Transboundary and Emerging Diseases, 55: 215−225. 2008.

Roussan DA, Haddad R and Khawaldeh G. Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. Poultry Science, 87: 444−448. 2008.

Roussan DA, Khawaldeh G and Shaheen IA. A survey of Mycoplasma gallisepticum and Mycoplasma synovaie with avian influenza H9 subtype in meat-type chicken in Jordan between 2011−2015. Poultry Science, 94: 1499−1503. 2015.

Saadat Y, Ghafouri SA, Tehrani F and Langeroudi AG. An active serological survey of antibodies to newcastle disease and avian influenza (H9N2) viruses in the unvaccinated backyard poultry in Bushehr province, Iran, 2012−2013. Asian Pacific Journal of Tropical Biomedicine, 4: 213−216. 2014.

Shortridge KF. Avian influenza A viruses of southern China and Hong Kong: ecological aspects and implications for man. Bulletin of the World Health Organization, 60: 129−135. 1982.

Smietanka K, Minta Z, Swiętoń E, Olszewska M, Jóźwiak M, Domańska-Blicharz K, Wyrostek K, Tomczyk G and Pikuła A. Avian influenza H9N2 subtype in Poland--characterization of the isolates and evidence of concomitant infections. Avian Pathology, 43: 427−436. 2014.

Sommer F and Cardona C. Chicken anemia virus in broilers: dynamics of the infection in two commercial broiler flocks. Avian Diseases, 47: 1466−1473. 2003.

Springer WT, Luskus C and Pourciau SS. Infectious bronchitis and mixed infections of *Mycoplasma synoviae* and *Escherichia coli* in gnotobiotic chickens. I. Synergistic role in the airsacculitis syndrome. Infection and Immunity, 10: 578−589. 1974.

Toyoda T, Sakaguchi T, Hirota H, Gotoh B, Kuma K, Miyata T and Nagai Y. Newcastle disease virus evolution. II. Lack of gene recombination in generating virulent and avirulent strains. Virology, 169: 273−282. 1989.

Vegad J. Drift variants of low pathogenic avian influenza virus: observations from India. World's Poultry Science Journal, 70: 767−774. 2014.

Vegad J, Bhindwale S, Mishra M, Sharma R, Vegard J, Bhindwale S, Mishra B and Sharma R. LaSota vaccine for Newcastle disease: observations from India on its adverse effects due to complicating pathogens. World's Poultry Science Journal, 64: 401−404. 2008.