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Original Article

Detection of Newcastle disease, H9N2 Avian Influenza, and Infectious Bronchitis Viruses in Respiratory Diseases in Backyard Chickens in Ahvaz, Iran, in 2014-2015

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ABSTRACT

Newcastle disease virus (NDV), avian influenza virus (AIV), and infectious bronchitis virus (IBV) are the most prevalent viral pathogens in the Iranian poultry industry. This study aimed to reveal the presence of these viruses in the backyard chickens in Ahvaz, located in the Southwest of Iran. A total of 100 chickens with respiratory signs and mortality were examined by taking the blood samples as well as tracheal and cloacal swabs. Most of the chickens had not received any vaccine. The blood samples were assessed for the antibodies against NDV and AIV by haemagglutination inhibition test, and against IBV by enzyme-linked immunosorbent assay. The swab samples were utilized for molecular detection using reverse transcription polymerase chain reaction (RT-PCR). Based on the results of the serologic test, 77%, 45%, and 38.4% of the birds were positive for NDV, AIV, and IBV, respectively. In the RT-PCR, 95% of the birds were positive for one of the three viruses. The detection rates of NDV, AIV, and IBV were 60%, 34%, and 55%, respectively. The coinfections of AIV/NDV, AIV/IBV, NDV/IBV, and AIV/NDV/ IBV were observed in 13%, 4%, 23%, and 7% of the sampled chickens, respectively. The results demonstrated that the Iranian backyard chickens were infected with NDV, AIV, and IBV. This could pose a threat to the commercial poultry; therefore, preventive measures need to be implemented in this regard.

Keywords: Backyard chickens, Infectious bronchitis, Influenza, Newcastle disease

Détection de la maladie de Newcastle, de la grippe aviaire (H9N2) et des virus de la bronchite infectieuse chez les poulets de basse-cour souffrant de maladies respiratoires entre 2014 et 2015 à Ahvaz (Iran)

Résumé: Le virus de la maladie de Newcastle (NDV), le virus de la grippe aviaire (AIV) et le virus de la bronchite infectieuse (IBV) sont les pathogènes viraux les plus répandus dans l'industrie avicoleiranienne. Notre objectif était d'évaluerla présence de virus dans les poulets de basse-cour à Ahvaz (sud-ouest de l'Iran). Des échantillons de sang et des écouvillons trachéaux et cloacaux ont été prélevés à partir de 100 poulets présentant des signes respiratoires et une mortalité La plupart des poulets examinés n'avaient reçu aucun vaccinLa présence d' anticorps dirigés contre le NDV et l'AIV dans les échantillonssanguins aété évaluéepar untest d'inhibition de l'hémagglutination, et par dosage immuno-enzymatique contre l'IBV; tandis que les échantillons sur écouvillon ont été utilisés pour les analysesmoléculaire en utilisant une réaction de polymérase en chaîne par transcription inverse (RT-PCR). Les résultats des analyses sérologiques montraient que 77% des oiseaux étaient positifs pour le virus de la maladie de Newcastle, 45% pour l'AIV et 38,4% pour l'IBV. Les résultats obtenus par RT-PCR ont révélés que 95% des oiseaux étaient positifs àl'un des trois virus testés dans cette étude. Le taux de détection du

NDV, de l'AIV et de l'IBV était respectivement de 60%, 34% et 55%. Des co-infection impliquantl'AIV /NDV,AIV/IBV et NDV/ IBV ontété respectivement observées dans 13,4et 23 pourcents des cas alors que 7% des poulets échantillonnés étaient porteurs des trois virus . Ces résultats montrent que les poulets de basse-cour iraniens sont infectés par le NDV, l'AIV et l'IBVetconstituent une menace non-négligeable pour la volaille commerciale qui doit être pris en compte lors des mesures préventives.
Mots-clés: poulets de basse-cour, bronchite infectieuse, grippe, Newcastle

INTRODUCTION

Newcastle disease virus (NDV), avian influenza virus (AIV), and infectious bronchitis virus (IBV) are of major importance in the respiratory diseases of the poultry. These viruses can be found independently, in association with each other, or along with other bacterial or viral agents (Malik et al., 2004). In Iran, Newcastle disease (ND) is an endemic and sometimes epizootic disease in the commercial chickens (Mehrabanpour et al., 2011). Vasfi Marandi and Bozorgmehri Fard (2002) documented the etiology of the outbreak of this disease by the isolation of H9N2 subtype AIV in Iran. The experimental studies in specific-pathogen-free broiler chickens have indicated that the Iranian H9N2 isolates are not highly pathogenic (Vasfi Marandi and Bozorgmehri Fard, 2001). However, the frequent field observations have shown that the respiratory infection in the commercial chickens is associated with severe hyperemia, leakage of air passage, and tubular cast formation in the tracheal bifurcation, which may extend to the lower bronchi (Nili and Asasi, 2002). Furthermore, in spite of regular vaccinations with Massachusetts strains, virulent IBVs are one of the main problems in the poultry industry of Iran (Saify abad Shapouri et al., 2004a; Boroomand et al., 2011). Furthermore, it seems that H9N2 viruses cause high mortality in the affected commercial birds when they are associated with vaccinal or virulent IBVs (Seifi et al., 2010). Backyard chickens provide an important source of income and high-quality animal protein in the rural socioeconomics with little or no capital investment (Baba et al., 1998). In the Southwest of Iran, millions of chicks are annually produced by the breeder flock of local chickens, some of which are very near to the commercial chicken flocks, and distributed to the rural communities. The backyard chickens can play a role in the epidemiology of many bacterial and viral agents. However, to the best of knowledge, there is a paucity of literature on the diseases of these birds in Iran. Regarding this, the present study was conducted to find the evidence of infection with NDV, AIV (H9N2 subtype), and IBV in the backyard chickens breeding in the Southwest of Iran.

MATERIALS AND METHODS

Sample collection. For the purpose of the study, a total of 100 backyard chickens, including hens, cocks, and chicks, aged 3-18 months were collected from different parts of Ahvaz in Khuzestan province, Iran, from February 2014 to November 2015. The chickens were obtained from 25 local farms with a range of 3-5 birds per farm. When sampled, the birds had a history of respiratory signs, such as dyspnea, sneezing, coughing, and wet rales, and in some cases with mortality for at least three to five days. Blood samples were obtained via brachial vein, and the collected sera were assessed for specific antibodies against the viruses. Simultaneously, tracheal swab (TS) and cloacal swab (CS) were taken from the birds and processed by molecular technique.

Serological examination. The antibodies against NDV and AIV were measured by haemagglutination inhibition (HI) test in 96-well microplates with 4 HAU of antigen (i.e., killed Iranian isolate of H9N2 AIV, and

live NDV LaSota vaccine strain) according to the procedure of the World Organization for Animal Health (2015). The HI titers were regarded as positive if there was a complete inhibition at a serum dilution of 1/16 or higher. The antibody against IBV was detected using the commercial enzyme-linked immunosorbent assay kit (BioChek, Netherlands) according to the manufacturer's instruction.

RNA extraction. The RNA extraction from the swab samples was performed using the RNX plus solution (CinnaGen Molecular Biology and Diagnostic Sale Office, Tehran, Iran) based on the manufacturer's protocol. The isolated RNAs were directly used for the reverse transcription polymerase chain reaction (RT-PCR) or stored at -80 °C.

Reverse transcription polymerase chain reaction and gel electrophoresis. The primers employed in the RT-PCR (Table 1) were described in the previous studies. These primers were used to amplify a 330 bp fragment of the fusion protein (F) gene of NDV (Mehrabanpour et al., 2011), a 488 bp fragment of the H9 gene of AIV (Lee et al., 2001) and a 466 bp fragment of the S1 gene of IBV (Adzhar et al., 1997).

The complementary DNA (cDNA) was synthesized using BioNeer RT PreMix Kit (BioNeer Co., South Korea) according to the manufacturer's instruction. The preparation of cDNA was accomplished by using 5 µL of total RNA and 1 µL of each 20 pmol primer. The same primers were used in the RT- PCR. The master mix for ND and IBV was composed of 2 µL PCR buffer 10X, 0.6 µL MgCl2 (50 mM), 0.2 µL deoxynucleotide triphosphate (dNTPs; 10 mM), 1 µL of each 20 pmol primer, 5 µL cDNA, and 10 µL distilled water. At the end, 0.2 µL Taq DNA polymerase (5 IU/µL) was added. The master mix used for AIV contained 2 µL PCR buffer 10X, 0.3 µL MgCl2 (50 mM), 0.2 µL dNTPs (10 mM), 1 µL of each 20 pmol primer, 5 µL cDNA, 10.3 µL distilled water, and the final addition of 0.2 µL Taq DNA polymerase (5 IU/µL). The PCR conditions for the amplification of F, H9, and S1 genes are listed in Table 2. These procedures were performed in a thermal cycler (Quanta Biotech, Germany). Finally, the PCR products were separated in 1% agarose gel containing safe stain (CinnaGen,Tehran, Iran) using an ultraviolet trans-illuminator (UVITEC, EU).

RESULTS

Serology assay. The serological findings showed that 77%, 45%, and 38.4% of the birds were positive for NDV, AIV, and IBV, respectively.

Molecular assay. The molecular results are illustrated in figures 1, 2 and 3. The results of the RT-PCR revealed that 95% of the birds were infected with one or more of the respiratory viruses (Table 3). Therefore, the use of RT-PCR resulted in the identification of a higher number of birds as positive, compared to the serological examination. The detection rates of NDV, AIV, and IBV were respectively 60% (i.e., 11%, 20%, and 29% in the TS, CS, and both swabs, respectively), 34% (i.e., 12%, 10%, and 12% in the TS, CS, and both swabs, respectively), and 55% (15%, 30%, and 10% in the TS, CS, and both swabs, respectively). The coinfections of NDV/AIV, NDV/IBV, AIV/IBV, and NDV/AIV/ IBV were observed in 13%, 23%, 4%, and 7% of the sampled chickens, respectively.

DISCUSSION

In the current study, 77% of the backyard chickens were seropositive for NDV, and 60% of them were recognized to harbor the virus, which is consistent with the results reported in the previous studies. In Zimbabwe, Kelly et al. (1994) found that 27% of the native birds were seropositive for NDV. Furthermore, in a serological survey performed by Gutierrez-Ruiz et al. (2000) in Mexico, 2.2% of backyard chickens had antibodies for NDV. (Abdu et al., 2002) examined the incidence rate of various diseases in the Nigerian local poultry and found that 21.8% of the diseases were due to NDV outbreak. Sharma et al. (2006) reported a high

seroprevalence rate of NDV in the backyard chickens of Grenada (99%). In this study, 45% of the backyard chickens were seropositive for AIV, and 34% of them were identified to carry the virus. The role of backyard chickens and wild birds as reservoirs for the spread of AIV to the commercial birds has been well documented before. Accordingly, Douglas et al. (2007) reported the isolation and characterization of AIV from the wild water fowl in Barbados. Furthermore, Senne (2010) reported about the AIV isolated from the live bird market, village poultry, and fighting cocks in Haiti for the first time. In a survey conducted by Lefrancois et al. (2010) on the wild and domestic birds living in Guadeloupe, Martinique, St. Lucia, and the Dominican Republic, all samples were observed to be negative for AIV. In Grenada, 18.8% of the backyard chickens had antibodies against AIV, whereas turkeys, ducks, pigeons, and guinea fowl were found to be seronegative for AIV. In the same study, AIV ribonucleic acid was not detected in the seropositive chickens (Sabarinath et al., 2011). In a recent study carried out on the commercial birds in Grenada, (Sharma et al., 2014) could not detect any antibodies against AIV. We found that the number of the NDV and AIV seropositive birds

Table 1. Target genes and related primers used in polymerase chain reaction

Oligonucleotide	Gene	Sequence	Product size (bp)	Reference
NDV Forward	F	TT GAT GGC AGG CCT CTT G-<C>	330	Mehrabanpour et
NDV Reverse	F	GG AGG ATG TTG GCA GCA T-<T>		al., 2011
H9 Forward	H9	CTY CAC ACA GAR CAC AAT GG	488	Lee et al., 2001
H9 Reverse	H9	GTC ACA CTT GTT GTT GTR TC		
IBV (XCE-1)	S1	CTC TAT AAA CAC CCT TAC A	466	Adzhar et al.,
IBV (XCE-2)	S1	CAC TGG TAA TTT TTC AGA TGG		1997

NDV: Newcastle disease virus, IBV: infectious bronchitis virus

Table 2. Reverse transcription polymerase chain reaction programs of Newcastle disease virus for 40 cycles and avian influenza and infectious bronchitis viruses for 35 cycles

Phase	Temperature (°C)		Time	
	NDV	AIV and IBV	NDV	AIV and IBV
Early denaturation	95	94	3 min	5 min
Denaturation	95	94	30 sec	30 sec
Annealing	55	50	1 min	30 sec
Elongation	72	72	1 min	35 sec
Final elongation	72	72	10 min	4 min

NDV: Newcastle disease virus, AIV: avian influenza virus, IBV: infectious bronchitis virus

Table 3. Detection rate of respiratory viruses by reverse transcription polymerase chain reaction in backyard chickens (n=100) of Ahvaz, Iran

Type of virus	NDV	AIV	IBV	NDV + AIV	NDV + IBV	AIV + IBV	NDV + AIV + IBV	Total
No. of positive birds	17	10	21	13	23	4	7	95

NDV: Newcastle disease virus, AIV: avian influenza virus, IBV: infectious bronchitis virus

was higher than that of the infected ones, which is likely due to the previous exposure of some birds to these viruses. In the current study, 38.4% of the backyard chickens had antibodies against IBV, and 55% of them were found to be positive in the RT-PCR. Some of the IBV-infected birds were negative in serology, which may be due to being at the early stage of infection. Vasfi Marandi and Bozorgmehri Fard (2001) isolated IBV from 37 out of 546 tissue samples obtained from commercial flocks during 1997-2000 in Iran. Moreover, Saify abad Shapouri et al. (2004b) investigated the prevalence of IBV type 4/91 in commercial broiler flocks in 16 provinces of Iran by means of RT-PCR technique and detected the virus in 33 samples (42.8%). In a study performed by Mahzounieh et al. (2006), 85.3% of the domestic village chickens in the central part of Iran were seropositive for IBV, which is more than the rate obtained in the present study. This may be due to the different prevalence rates of IBV infection among the commercial chickens in various parts of Iran. In Mexico, the backyard (free-range) village chickens had the IBV seroprevalence rate of 56.5% (Gutierrez-Ruiz et al., 2000). An IBV infection rate of 43% was reported by Thekisoe et al. (2003) in the free-ranging chickens of South Africa. The present study revealed that 47% of the backyard chickens were simultaneously infected with two or three viruses (Table 3). Accordingly, similar coinfections have been reported in other countries. In this regard, Owoade et al. (2006) reported the simultaneous presence of IBV, avian metapneumovirus, infectious laryngotracheitis, and avian leukosis virus on the same premises in the Nigerian poultry. Furthermore, the co-existence of AIV, NDV, IBV, egg drop syndrome virus, and reovirus in chickens on small holdings in Bangladesh was demonstrated by Biswas et al. (2009). In a study conducted by Shadmanesh and Mokhtari (2013), antibodies against NDV, AIV, Salmonella, Mycoplasma gallisepticum, and M. synoviae were detected among the native hens in Iran. In addition, the coinfection with NDV, AIV, and chicken infectious anemia virus among the backyard chickens in Grenada were reported by Sharma et al. (2015). The NDV, AIV, and IBV cause serious loss in production and/or profitability through causing mortality. Regarding this, further studies are needed to clearly assess the effect of infection with these viruses on the health and performance of the backyard chickens.

As the backyard chickens examined in this study had no history of immunization with live vaccines, the respiratory signs, together with the positive serology and virus detection, suggested their exposure to the field strains of NDV, AIV, and IBV. This creates a risk for the commercial chickens and other avian species, and necessitates the implementation of preventive measures for these birds.

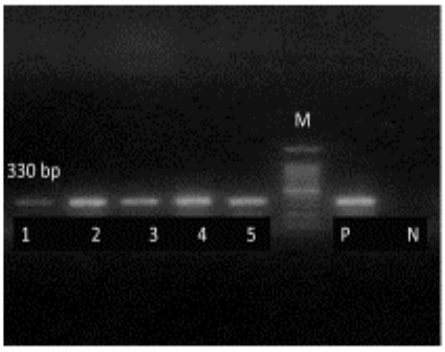

Figure 1. Electrophoresis analysis (1% agarose gel) of Newcastle disease virus, Lane M: marker (100 bp), lane P: positive control (330 bp), lane N: negative control, lane 1-5: positive samples

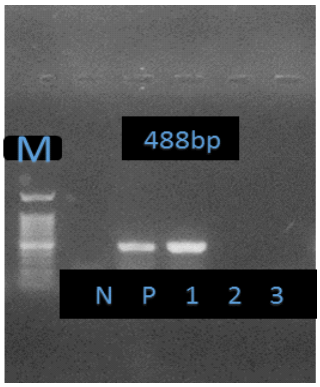

Figure 2. Electrophoresis analysis (1% agarose gel (of avian influenza virus, Lane M: marker (100 bp), lane P: positive control (488 bp), lane N: negative **control,** lane 1: positive sample, lanes 2 and 3: negative samples Ethics

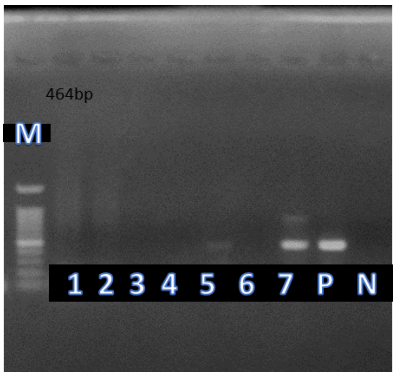

Figure 3. Electrophoresis analysis (1% agarose gel (of infectious bronchitis virus, Lane M: Marker (100 bp), lane P: positive control (464 bp), lane N: negative control, lanes 5 and 7: positive samples, lanes 1-4 and 6: negative samples

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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