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Case Report

Detection and Molecular Characterization of Avian Metapneumovirus in Iran: The First Report

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Abstract

Avian pneumovirus (APV) causes turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) of chickens, which is usually accompanied by secondary bacterial infections that increase mortality. In Iran, some serological studies indicated that APV infection is endemic in commercial poultry industry. In this study we diagnosed APV genome molecularly in suspected broiler flock (4 weeks) in Iran and characterized APV genome based on G glycoprotein .In phylogenetic analysis PCRLAB/HG2010 is located in B subtype cluster and near APV strains from Nigeria, Japan and Brazil. It is the first molecular epidemiology study on APV in Iran. In conclusion, vaccination programs in the Iranian poultry industry should be adjusted to include the APV vaccine to aid in the control of this respiratory disease. It is also concluded that more work is required to isolate and characterized AVP in different geographical regions of Iran and different species.

Keywords: Avian Metapneumovirus; Iran; Phylogenetic analysis

Introduction

Avian pneumovirus (APV) causes turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) of chickens, which is usually accompanied by secondary bacterial infections that increase mortality (1, 2) . APV is a member of the *Paramyxoviridae* family, subfamily *Pneumovirinae*, and has been proposed as the type species for the newly defined genus *Metapneumovirus* (3, 4). APV is a negative-sense, nonsegmented single-stranded RNA virus that contains eight genes, namely, nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), second matrix (M2), small hydrophobic (SH), attachment protein (G), and RNA-dependent RNA polymerase (L) in the order 3 -N-P-MF-M2-SH-G-L-5 (3, 5). Two different subgroups, designated A and B, exist within a single serotype. Recently, subgroup C was reported in the United States and subgroup D was reported in France (4, 6, 7). One of the major surface glycoproteins of APV is the attachment (G) protein which, by analogy with RSV, has been proposed to be responsible for virus binding to its cell receptor. The APV G gene and its predicted protein have several features in common with their RSV counterparts (8). The very low sequence identity between the G glycoproteins of the two types was used to develop a nested reverse transcription–polymerase chain reaction (RT-PCR) based on subtype-specific primers in the second PCR which generated amplification products of 268 base pairs (bp) and 361bp for type A and B viruses, respectively(9-11). The virus was first reported in South Africa in 1978, but it has since been isolated in Europe, Israel, Asia, and recently in

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the United States. APV is now considered a major disease threat in both turkeys and chickens in many parts of the world (2, 3, 12). The initial virus infection is often complicated by secondary bacterial infection, resulting in high morbidity and variably mortality. In laying or breeding turkeys, the same virus may also cause substantial falls in egg production. Uncomplicated cases have low mortality (2 to 5%), but infections accompanied by concurrent secondary infections can result in up to 25% mortality (13-15). In Iran, some serological studies indicated that pneumovirus infection is endemic in commercial broiler ,layer, breeder(16) .In this report and study we detect and characterized APV type B based on G gene in Iran.

Methods

Case history

In late January 2010, an outbreak of SHS occurred on a single floor of a barn housing broiler chickens.

Alborz Province is one of the 31 provinces of Iran; centered in Karaj (In neiborhing of Tehran, Capital of Iran). During post mortem examination of the dead birds, the following gross lesions were observed, Slowen head syndrome, Hyperemia of trachea. The mortality rate was 10% No APV vaccines have been used in Iran.

RNA extraction

Oropharyngeal swabs from flock were swabbed with sterile swabs. Swabs were placed in PBS and were scraped on the side of the tube to facilitate removal of contents from the swab head. Extraction of RNA was performed on the pooled material for swabs from flock with a RNAeasy Mini Kit (QIAGEN, USA) according to the manufacturer's procedure.

RT-PCR

The APV detection primers used in this study were previously evaluated by Bayon-Auboyer et al. (1999) and are listed in Table 1. The screening of flocks was performed with the primer pair Nd/Nx. he APV molecular typing primers used in this study were evaluated previously (17)and are listed in Table 1. The Ga-G2-, Ga-G12 primers are specific for APV subtypes A and B respectively (18). RT-PCR program were performed as described above except for the annealing temperatures. Primers specific for each subtype, their annealing temperature, and the expected band size are listed in Table 2. Reverse transcription was done by using Random Hexamer with Revert Aid first strand cDNA synthesis Kit (Fermentas Co, Canada). The PCR was carried out in a 50-µl reaction volume consisting of 5 µl 10x PCR buffer, 1 µl 10 mM dNTPs, 1.25 µl of each primer (10 pmol/µl), 0.25 µl *Taq* DNA polymerase (5U/µl), 1.5 µl 50 mM $MgCl_2$, 33.75 µl of dH_2O, and 6 µl cDNA dilution, and was programmed as following condition: 94˚C for 3 min followed by 35 cycles of 95˚C for 30 sec, different annealing temperature (Table 2) for 30 sec, 72˚C for 60 sec, and a final extension at 72˚C for 15 min. A 5 uL aliquot of the PCR reaction mixture was loaded onto a 1% agarosegelandelectrophoresedfor40minin TAE buffercontaining 0.5 mg/mL ethidium bromide.

Bioinformatics analysis

The PCR products were purified using a purification kit (Qiangen, Valencia, CA, USA) prior to sequencing to Bioneer Company (Korea). Cycle sequencing was performed using the purified PCR products with the ABI Prism Ready Reaction Dideoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).Nucleotide sequence editing, analysis, prediction of amino acid sequences, and alignments were conducted with CLC Main workbench software. The genomic sequence of PCRLAB/HG2010 was compared with other APV genomic sequence data available in GenBank .A phylogenetic tree was created with the program MEGA 5 (Molecular Evolutionary Genetics Analysis, Version 3.1) using the Kimura two-parameter model and the neighbor-joining algorithm with 1000 bootstraps. All branches that were supported by >50% bootstrap replicates were considered as the same group in the trees.

Gene Submission

The sequence of G protein gene of PCRLAB/HG2010 has been deposit under accession number JX131351 in Genebank.

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Table 1. Polymerase chain reaction primers used for avian pneumovirus molecular detection and typing (18).

Primer Name	Gene Name	Sequence (5'--→3')
Nd	N	AGC AGG ATG GAG AGC CTC TTT G
Nx	N	CAT GGC CCA ACA TTA TGT T
Ga	G	CCG GGA CAA GTA TCT CTA TGG
G2-	G	CCA CAC TTG AAA GAT CTA CCC
G12-	G	CAG TCG CCT GTA ATC TTC TAG GG

Table 2. Polymerase chain reaction primer pairs used for avian pneumovirus (APV) molecular detection and typing, annealing temperature, and expected band size (18).

Primer	APV type	Annealing temperature	Band size, bp
Nd/Nx	All	51	115
Ga/G2-	A	54	504
Ga/G2-	B	54	312

Results

We obtained the type (115bp) and subtype B (312bp) in just one swab sample. The G protein gene of PCRLAB/HG2010 has high identities to VCO3/60616[Japan, 2010] (96%), Nigeria/NIR89/2006 [Nigeria, 2006] (98%) and Turkey rhinotracheitis virus (strain 2119) [96%]. Based on phylogenetic analsysis (NJ method) PCRLAB/HG2010 has been located in Type B cluster.

Discussion

Avian metapneumoviruses (known as avian pneumoviruses or APVs) cause respiratory diseases and/or egg drops in species such as turkey, chicken, Muscovy or Pekin duck (19). First reported in the late 1970s in South Africa, and subsequently in France and the UK , APVs have now been described worldwide(4). Clinical signs are not pathognomonic for a diagnosis of APV.A diagnosis may be made by

		1	2	3	4	5	6	7	8	9	10
PCRLAB/HG2010	1		0.08	0.06	0.05	0.07	0.41	0.14	0.09	0.06	0.75
Vaccine Starin Hi	2	92.68		0.03	0.06	0.09	0.44	0.14	0.10	0.07	0.78
Vaccine Starin Me	3	94.27	97.13		0.06	0.08	0.43	0.14	0.09	0.06	0.74
VCO3/60616(AB548428)	4	94.90	94.27	94.59		0.03	0.38	0.11	0.06	0.02	0.72
chicken/Nigeria/NI39/2006	5	93.63	91.72	92.68	97.13		0.37	0.10	0.05	0.02	0.76
aMPV/B/Brazil-07/USP-06(EU140747)	6	68.47	66.88	67.52	70.06	70.87		0.30	0.31	0.37	1.24
Isr/1708/02(AY728268)	7	87.58	86.94	87.58	90.13	90.94	75.09		0.04	0.10	0.88
aMPV/B/Russia/chicken/02/2007(N651915)	8	91.08	90.76	91.40	93.95	94.82	74.66	95.95		0.05	0.81
Turkey 2119	9	94.27	92.99	93.95	98.41	98.07	70.74	91.00	94.86		0.73
chicken/A/BR/121/95	10	52.50	51.56	52.81	53.75	52.20	39.43	48.26	50.47	53.46	

Fig. 1. The percent of Identities and distance between Iranian Avian Metapnemovirus (PCRLAB/HG 2010) and other Avian Metapnemovirus acquired form Gene Bank and Vaccine strains.

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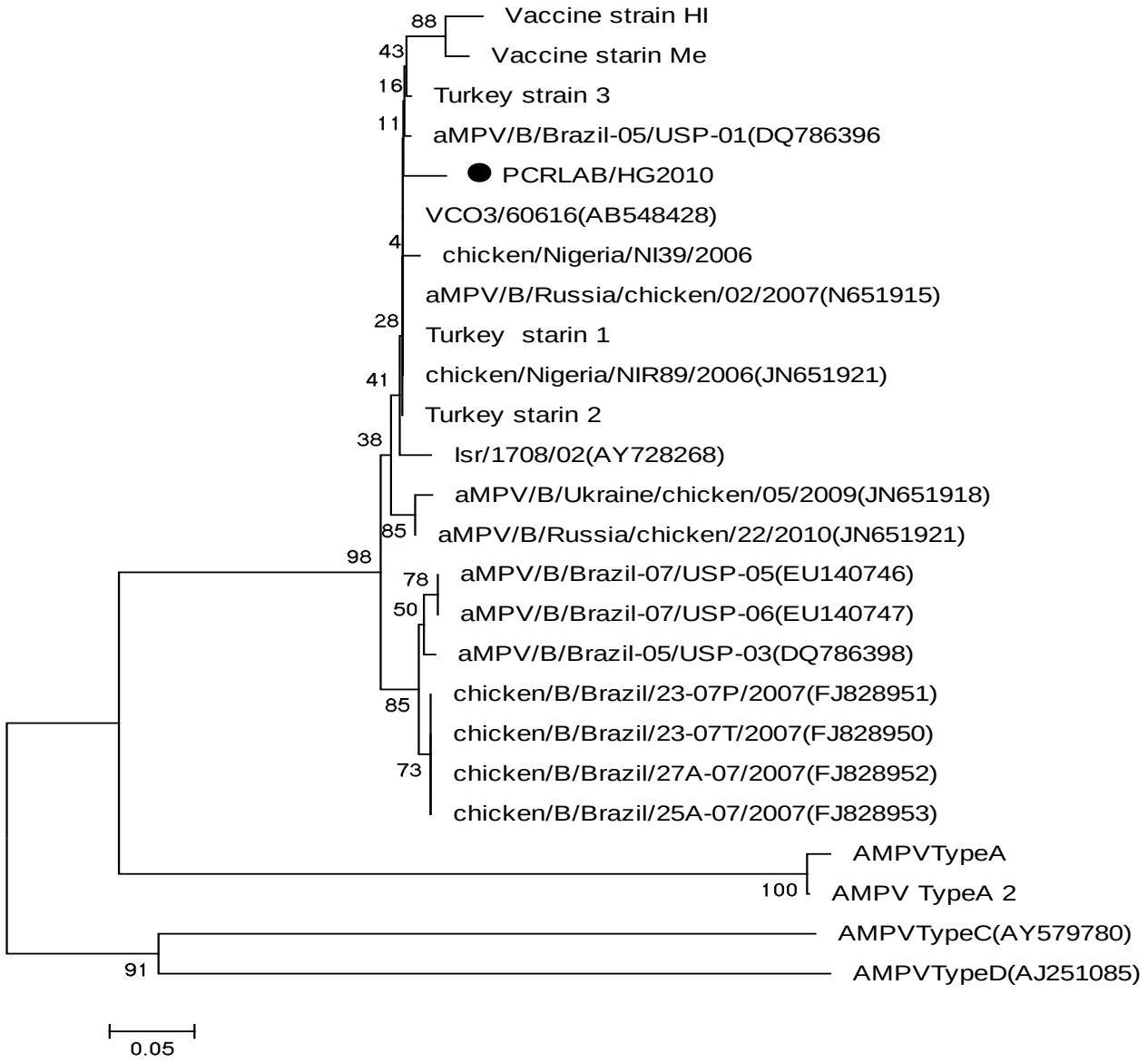

Fig. 2. Nucleic acid Phylogenetic relationships of G glycoprotein gene of Avian metapneomovirus genome isolated from Iran. The Phylogenetic tree was generated using Neighboring Joining model with MEGA (version 5.1 beta). Numbers below branches indicate bootstrap value from 1000 replicates, bootstrap values. Horizontal distances are proportional to the minimum number of Nucleic acid differences required to join nodes. The vertical lines are for spacing branches and labels. Analysis was based on complete open reading frames of all gene segments. The scale bar represents the distance unit between sequence pairs. The virus genome characterized in this report is indicated as Black Circle. The sequences obtained from Gene Bank.

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serology, PCR or virus isolation. Virus isolation of APV is time consuming and difficult. Current serology tests include enzyme linked immunosorbent assay (ELISA), or virus neutralization (VN) or immunofluorescence (FA). The polymerase chain reaction (PCR) procedure detects virus nucleic acid. PCR is used to sample respiratory tissues (such as, tracheal swabs and turbinates).Samples should be fresh, refrigerated and not frozen. For any diagnostic test, sample both affected and unaffected birds within a sick flock. Tracheal swabs and turbinates are considered to be the most

appropriate sources of APV.Non type of vaccines doesn't authorized for use in Commercial farms in Iran. The putative attachment glycoprotein of APVs, G, is the most variable protein between APV subgroups. This study reports the first sequences of the putative APV G genes In Iran. Only a few sequences of APV, from only a few countries, are available. The molecular epidemiology may be biased by the minimal sequences of APV that have been reported, and because those sequences came from only a limited number of countries. As reported in Europe, and because of our geographic location, we expected to diagnose APV subtypes A, B, or both in Iran. Characterization of the Iranian strain showed that detected virus were APV subtype B. Similarly, APV subtype B was characterized earlier in Israeli and Jordanian poultry(20) .This similarity with Brazil stains which probably reflects the policy of that country to import chicken from most major poultry producing countries in Europe and Asia . The present study was the first study and an initiative to record the molecular based presence of APV virus in local area On the basis of these results it is concluded that avian pneumovirus type B is present in Iran . In previous studies, serologically has been approved that AVP infection is endemic in commercial broiler ,layer, breeder(16) . It is also concluded that more work is required to isolate and characterized AVP in different geographical regions of Iran and different species such as Turkey flocks. Isolation of APV will allow the possibility of making autogenous vaccines. In conclusion, vaccination programs in the Iranian poultry industry should be adjusted to include the APV vaccine to aid in the control of this respiratory disease.

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