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Reverse transcriptase-polymerase chain reaction detection in chicken flocks in Iran

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ABSTRACT

Avian metapneumovirus causes acute and highly contagious upper respiratory tract infections in chickens and turkeys. Avian metapneumovirus plays an important role in respiratory diseases; it may be involved in multifactorial disease. The aim of this study was the detection and molecular characterization of avian metapneumovirus from commercial chicken flocks in Iran. Clinical samples from 50 commercial chicken flocks with respiratory signs such as swollen infraorbital sinuses, nasal discharges, coughing, tracheal rales and foamy conjunctivitis were collected and brought to the Razi vaccine and serum research Institute for RT-PCR. Samples included the choenal cleft, trachea and turbinate's swabs. The G genes of Positive samples were sequenced. Of the 50 chicken flocks, 8 flocks were positive by RT-PCR (16%). Partial sequence analysis of the G gene confirmed that the positive samples belonged to subtype B. Phylogenetic tree demonstrated that Iranian strains formed one group apart from subtype B vaccine strain used in Iran. Comparison of nucleotide sequences of the Ggene of the Iranian aMPV samples with subtype B isolates from other countries revealed 95.2% to 97.4% identity. Nucleotide sequences showed 96.9 to97.9% identities among the Iranian samples and 95.5 % identity with the subtype B vaccine strains used in Iran.

1. Introduction

AMPV causes upper respiratory tract infection in turkey and chicken and can result in increased mortality when combined with secondary bacterial infections. In chickens, aMPV infection is often associated with the swollen head syndrome (O'Brien, 1985; Cook et al., 1988; Perelman et al., 1988; Jones et al., 1991) However, the disease could not be reproduced using aMPV alone and chicken flocks can be infected without showing clinical signs.

AMPV is classified in the genus *Metapneumovirus*, within the subfamily *Pneumovirinae* of the *Paramyxoviridae* family (Pringle et al., 1998) .The aMPV genome consists of approximately 13 kb of a non-segmented, linear, negative-sense strand RNA, which encodes eight genes: nucleocapsid (N), phosphor protein (P), matrix (M), fusion (F), second matrix (M2), small hydrophobic (SH), surface glycoprotein (G), and RNA dependent RNA polymerase (L). The disease was first reported from South Africa in 1980 (Buys and Du Preez, 1980) and later from many European countries (Naylor and Jones, 1993; Jones, 1996). In the US, APV was first isolated from affected turkeys in Colorado (Kleven, 1997) and Minnesota (Goyal et al., 2000). The disease is characterized by depression, sneezing, coughing tracheal rales, nasal and ocular discharges, swollen infraorbital sinuses and foamy conjunctivitis, with morbidity of up to 100%. Mortality may reach 30% in cases complicated by secondary bacterial infections. (Gulati et al., 2001; Jirjis et al., 2002; Gough, 2003). The highly variable attachment G gene, encoding a surface glycoprotein and serving as one of the major antigens of the metapneumoviruses, is a good candidate for type differentiation and for epidemiological studies (Bäyon- Auboyer et al., 1999; Alvarez, et al., 2003). aMPV subgroup A was first described in England, and subgroup B in France; A and B, mostly found in Europe, Africa and Asia; The recent emergence of a third subtype, C, in the US (Seal, 2000) and of a fourth, subtype D, in France (Bäyon-Auboyer et al., 2000) In Iran, although clinical diseases similar to SHS have reported in chicken by field veterinarian and antibodies to aMPV have been detected in some province ,there have been no reports detecting and and molecular characterization of avian metapneumovirus from commercial chicken flocks in Iran (Rahimi, 2011). In this study, we report detection and characterization of aMPV from chicken flocks in Iran.

2. Materials and Methods

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Clinical samples from 50 commercial chicken flocks (43 broiler flocks, 5 layer and 2 broiler breeder flocks) with respiratory signs such as , sneezing, coughing, tracheal rales, nasal and ocular discharges, swollen infraorbital sinuses and foamy conjunctivitis were collected and brought frozen to the laboratory for molecular examination. Samples included the trachea, turbinate's and choenal cleft swabs. Swabs placed in PBS. Samples were collected from the beginning of October 2010 to September 2012.Flocks were non vaccinated for aMPV.

2.1. AMPV reference viruses

aMPV subtype B (Nemovac; Merial, France and Hipraviar-SHS, Spain) vaccine strains that were available and used in Iran were used as refrence for RT-PCR.

2.2. RNA extraction and RT-PCR

In this study we are following 3 goals. These goals included detection and subtyping of aMPV and then nucleotide sequences of the G gene. The APV primers used in this study were previously evaluated by Bäyon – Auboyer et al. (1999) and are listed in Table 1.The viral RNA was extracted from choenal cleft, nasal turbinate and tracheal swab using the high pure viral nucleic asid kit (Roche™).Purified genomic RNA was used to generate DNA by (RT-PCR) according to the manufacture's procedure. One step RT-PCR was performed using transcript or one-step RT-PCR kit according to the manufacture's instruction (Roche™). Each RT-PCR reaction consisted of D.W 27.5 µI, Buffer(5X)10 µI , D.T.T(0.01mM/ml) 2.5 µI, Forward Primer(10pmol/ml)2 µI, Reverse primer (10pmol/ml)2 µI, dNTP(2.5Mm)1 µI, Enzyme(20-25U/µl)1 µI, RNA Template (1 µl/pg)4 µI. The RT-PCR was performed using specific primers (G_a- G_y) as described by Bäyon-Auboyer et al. (1999) and are listed in Table 1.G_a and G_y primers were general primers designed to hybridize to both subtybe A and B for the initial RT-PCR. Reverse transcription was carried out at 45˚C for 45 min and 94˚C for 3 min, followed by 34 cycles of 94˚C for 60 sec, 54˚C for 60 sec and 68˚C for 60 sec, all followed by a further extension step of 10 min. RT-PCR products were visualized after electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml).

Table 1: Polymerase chain reaction primers used for aMPV molecular detection, typing and sequencing

Primer	RT-PCR Purpose	Size of product
G_a/G_y	Detection	448 bp
G_a/G_z	Sequencing	1196 bp
G_a/G_{12}	Typing (B)	312 bp

2.3. Sequence analysis

The PCR products were applied to Low Melting Point (LMP) agarose and the distinct bands were purified from gel for sequencing (MWG Co., Germany). The PCR products were purified using high pure product purification kit (Roche Germany). Nucleotide sequences of the G genes were done by G_a- G_z primers and comfort read methods. Sequences of the G genes were edited with the Editseq (DNASTAR Laser gene Software package Version 7.1.0) .Then, Nucleotide sequences were aligned by Clustal W Method. Phylogenetic tree was performed with the Mega program version 5.05 and established their relationship to the vaccine strains and other isolates of other countries.

3. Results

3.1. Screening of the flocks by RT-PCR

As shown in Table 2 of the50 chicken floks, 8 Broiler flocks were positive by RT-PCR with Ga – Gy primers. All layer and broiler breeder flocks that examined in this study were negative by RT-PCR. Four PCR products were successfully sequenced and they all clustered with aMPV subtype B strains (Fig. 1). All clinical specimens tested were collected from non-vaccinated flocks.

3.2. Sequencing and phylogenetic analysis

The 4 G genes of positive samples were sequenced. Partial sequence analysis of the G gene confirmed that the positive samples belonged to subtype B. Phylogenetic tree demonstrated that these Iranian strains formed one group apart from subtype B vaccine strains used in Iran. Sequence analysis of the G gene confirmed the results of the RT-PCR subtyping. The nucleotide sequences of the G gene of the other subtypes received the following Gen Bank accession numbers:

Subtype A. CVL/14 from England (accession number L34032), 1556 from France (L34030), LAH (AY640317).

Subtype B. 872S from Spain (L34034), 6574 from Hungary (L34033), 2119 from Italy(L34031), VCO3/6061 from France(AB548428),

Chicken/10/2008(JN651917),chicken/22/2010(JN651921),chicken/12/2010(JN651920)fromRussia, chicken/05/2009(JN651948) from Ukraine

Subtype C. Colorado 1 from USA (AY579780), Minnesota 2a from USA (AY198393).

Subtype D. Fr/85/1 (AJ288946) and Fr/85/2 (AJ251085) from France.

Comparison of nucleotide sequences of the G gene of the Iranian aMPV samples with subtype B isolates from other countries revealed 95.2 - 97.4% identity. Nucleotide sequences showed 96.9-97.9%

identity among the Iranian samples and 95.5% identity with the subtype B vaccine strains used in Iran.

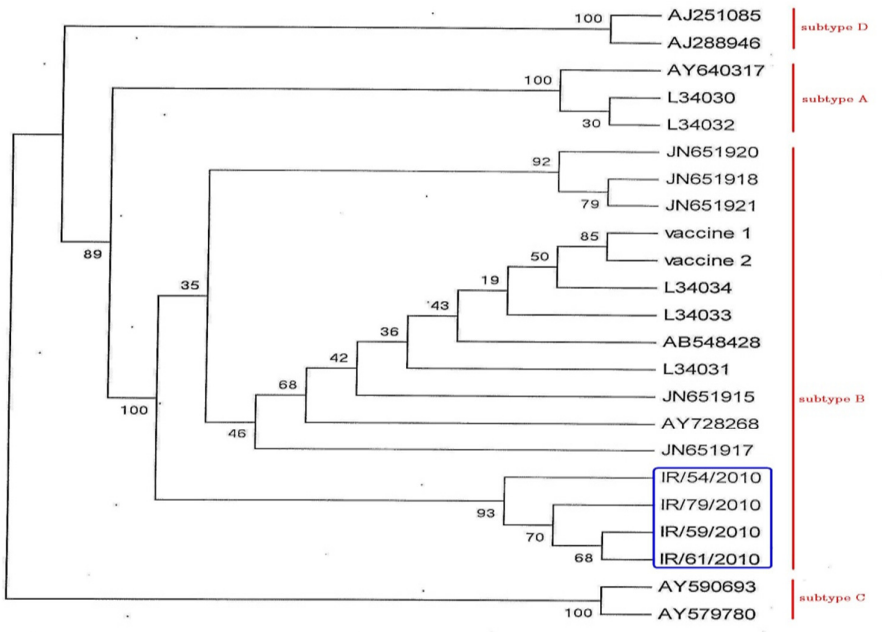

Fig. 1: Phylogenetic relationships among aMPVs. Following alignment of sequences from the G gene. Bootstrap confidence levels are presented

Table 2: Results of aMPV detection from field samples

Chicken	Flocks	Samples	Positive RT-PCR	Detection rate(%)
Broiler	43	422	8(B type)	18.6%
Layer	5	48	0	0 %
Broiler breeder	2	18	0	0 %
Total	50	488	8	16%

4. Discussion

In Iran because of various infections with different respiratory viruses the entrancing of a new virus such as aMPV in form of primary or secondary cause of respiratory syndrome will complicate hygine problems in commercial chicken flocks. In this study we tried to demonstrate the presence of aMPV by detection of virus by RT-PCR method. Because of isolation of virus is difficult and time consuming work therefore molecular method for detection and subtyping of the virus is very important. In this study we detected ¸ subtyped of aMPV and sequenced of G gene of the virus by RT-PCR and the last we determined virus position among other viruses from different regions of the world and two subtype B live vaccine that were used in Iran. Unfortunately, positive control for other subtypes was unavailable for use in this study. The results indicated presence of aMPV in commercial chicken flocks in Iran. Although two subtype B vaccines are available in Iran, but during the time the samples were collected in all studied flocks vaccines against aMPV were never used. In our study the next step was the subtyping the virus by subtype specific primers. The result indicated all Iranian strains belonged to B subtype. Gharaibeh and Algharaibeh (2007) examined chicken flocks with respiratory disease by RT-PCR for detection of aMPV .Avian metapneumovirus nucleic acid was detected in 20 flocks and all of the isolates were subtype B.

Clinical signs of flocks that were positive by RT-PCR include swelling of per orbital tissue and infraorbital sinuses, coughing, tracheal rales, nose discharge and swelling of the head. For the differential diagnosis, the samples tested for detection of ND, IB and AI viruses. NDV, IBV and AI virus were not detected in any of 8 positive samples (data not shown). Although our positive samples were not tested for detection of bacterial again, but it seems main factor that cause clinical signs are avian metapneumovirus. However should not take no notice roles of bacterial disease in complication of clinical signs. When Chacon et al. (2007) observed the signs and lesions in the field and the detection of aMPV by nested-PCR, which was confirmed by virus

isolation, suggested that aMPV might have been responsible for the disease outbreaks. However, as the clinical signs observed could have been produced by other pathogens, differential diagnosis with other respiratory agents was performed .NDV, IBV, MG and ILTV were not detected in any of the flocks. The sequences of the four strains from chicken flocks were aligned with previously published sequences of all subtypes of aMPV and were confirmed as B subtype. Furthermore two subtype B vaccines used in Iran were sequenced. Phylogenic tree revealed vaccine strains formed a small branch apart from four Iranian strains. These findings demonstrated Iranian strains were wild strains. In one recent study for the first time presence of aMPV in turkey flocks in Iran was reported (Mahmoodzadeh et al., 2012).Phylogenic analyses and sequence of this virus indicated turkey virus are much closed to the chicken viruses of our study. Therefor it concludes the origin of chicken aMPV is turkey viruses. It will pave the way for further epidemiological studies on this virus.

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