Genetic Characterization and Subtype Isolation of Circulating Metapneumovirus in Commercial Turkeys in the Central Region

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ABSTRACT:

Rhinotracheitis is an acute respiratory infection in turkeys, considered a relatively significant economic disease caused by Avian metapneumovirus (AMPV) in poultry species. AMPV causes turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) of chickens, which is usually accompanied by secondary bacterial infections that increase mortality. This virus belongs to the genus *Metapneumovirus* within the subfamily Pneumoviridae of the family Paramyxoviridae. AMPV has been identified in Africa, Asia, Europe, North America, and South America. In turkeys, depending on the country, AMPV may be the most important viral pathogen causing substantial economic losses. AMPV also causes disease in domesticated poultry. In the present study, a total of 208 samples were collected from 87 flocks of broilers, layers, broiler turkeys, breeder chickens, and indigenous chickens from the geographical region of central Iran (Hamadan, Qazvin, Zanjan and Isfahan) to identify and isolate the virus. These samples were tested using specific primers and RT-PCR techniques. Additionally, 4 positive samples were partially sequenced. Positive samples were inoculated into embryonated eggs for virus isolation, and evaluations were conducted post-inoculation. Twenty-eight samples from 87 flocks were positive. Positive samples were mainly swabs from the upper respiratory tract. The positive samples predominantly identified subtype B of the virus, with one sample also testing positive for subtype A. Positive samples were identified from turkeys, broilers, and layer chickens. Both subtypes A and B of the virus were positive and isolated after 3 to 5 sequential inoculations in embryonated eggs. The results of this study confirm that the use of PCR with specific primers is an effective and reliable method for identifying and differentiating avian metapneumovirus.

Keywords: Rhinotracheitis, Turkey, *Metapneumovirus*, Isolation, Genetic Characteristics

1. Introduction

Rhinotracheitis disease in turkeys, caused by avian metapneumovirus (aMPV), is one of the major challenges in the poultry industry. This virus can cause severe respiratory problems in turkeys, leading to significant economic losses in the industry. Avian metapneumovirus has been identified in various regions around the world and can rapidly spread among poultry flocks. Previously known as avian pneumovirus and avian rhinotracheitis, aMPV is an acute and highly contagious upper respiratory tract infection affecting in turkeys and chickens. The first isolation of aMPV was described and conducted in the late 1970s from turkeys with respiratory symptoms in South Africa (1). Shortly after the first report, this disease was also reported in chickens (2). Currently, aMPV is reported worldwide. The diagnosis of infection is complicated by non-specific lesions and clinical signs. In the field, several other respiratory diseases can be mistaken for Turkey Rhinotracheitis (TRT).

aMPV consists of a negative-sense RNA genome containing 8 genes (5'-N-P-M-F-M2-SH-G-L-3'), which encode 9 proteins (3). Significant heterogeneity in the SH, M, N, P, and F genes can be used to differentiate between virus subtypes (4-6). However, the most variable gene is the glycoprotein (G) gene, which has been used to determine virus subtypes. Based on nucleotide sequence divergence in the G gene and antigenic differences, aMPV has been classified into four subtypes: aMPV-A, aMPV-B, aMPV-C, and aMPV-D (7 and 8). Due to the high genetic variability in the G gene, this gene has been analyzed to determine molecular relationships and to differentiate pathogenic strains in the field from vaccine strains.

Genetic characterization and isolation of circulating subtypes of metapneumovirus in turkeys can provide a better understanding of the epidemiology and pathogenesis of this virus. This information can be used to develop effective vaccines and management strategies for controlling and preventing the disease. In this study, the genetic characteristics and isolation of different subtypes of metapneumovirus in turkeys from the central province were investigated. Using advanced molecular techniques, efforts were made to identify genetic differences and the mode of transmission of this virus among different flocks. This research not only aids in the better understanding of the genetic characteristics of the virus but also leads to the development of more accurate and rapid diagnostic methods. Additionally, the obtained information can improve the management of health and treatment of turkey flocks and reduce the economic losses associated with this disease.

2. Materials and Methods

2.1. Sample Collection

For virus isolation, it is crucial that samples be collected at the early stages of infection. Ideally, live birds in the acute phase of the disease should be sampled using sterile swabs from the upper respiratory tract in various regions as shown in Table 1. Samples from live birds in this study were collected from nasal secretions, swabs from the hard palate cleft (choanal cleft), and scraping tissue from sinuses and turbinates. Additionally, samples were taken from the trachea and lungs of affected turkey poults (dead bird). Swabs were transported to the laboratory in viral transport medium (VTM bulk with batch number 150130, Vista Biotech Company) on ice, and in some cases, dry swabs were also collected for PCR analysis.

Table 1: The geographical distribution and types of production units in the central region of the country where samples were collected.

Poultry type	Region	Number of Flock	Number of Sample	Swap	Tissue(Trachia)
Broiler	Markazi	40	85	40	45
Broiler	Hamadan	10	30	15	15
Broiler	Qazvin	3	10	5	5
Layers	Markazi	3	10	10	-
Breeders	Markazi	1	3	3	-
Broiler Turkeys	Markazi	15	52	45	7
Broiler Turkeys	Isfahan	5	15	15	-
Broiler Turkeys	Zanjan	1	3	2	-
Total		78	208	135	72

2.2. RNA Extraction and cDNA Synthesis

The extraction process was carried out immediately after the samples were received. RNA extraction was performed using the Simbio extraction kit from Korea, following the kit's instructions. The extracted RNA samples were evaluated qualitatively and quantitatively using a NanoDrop, and were immediately converted to cDNA using the Simbio cDNA synthesis kit from Korea according to the instructions (Tables 2 and 3). The cDNA samples were stored at -30°C for diagnostic tests and the determination of aMPV subtypes.

Table 2: cDNA Synthesis Reaction Volumes.

Material/primers	Volume (µL)
2X Reaction Buffer	10
SamScript Enzyme	1
RNA sample	Varies based on RNA concentration
Nuclease-free Water	Varies based on the sample amount
Total Reaction Volume	20

Table 3: cDNA Synthesis Reaction Conditions.

Condition	Tem(°C)	Time (minutes)
Incubation	25	10
Incubation	47	60
Reaction Termination	85	5

2.3. Primer Design

Based on the studies conducted and sequences registered on the NCBI website, four pairs of specific primers from conserved regions of the virus were designed for virus identification on the N, F, and G genes. Additionally, two pairs of primers on the G gene were designed to determine subtypes A and B of the virus and were ordered from Metabion Company for synthesis. The primer specifications and their sequences are provided in Table 4.

Table 4: Sequences of the Primers Used

Primer Name	Primer Type	Sequence
Nd	F	5'–AGCAGGATGGAGAGCCTCTTTG–3
Nc	F	5'–TTCTTTGAATTGTTTGAGAAGA–3
Nx	R	5'–CAT-GGC-CCA-ACA-TTA-TGT-T–3
Ga	F	5'-CCGGGACAAGTATCTCTATGG-3
G2	R	5'-CCACACTTGAAAGATCTACCC-3
G12	R	5'-CAGTCGCCTGTAATCTTCTAGGG-3
Gy	R	5'-TCTCGCTGACAAATTGGTCCTGA-3
F5	F	5'-CCTCGAAATAGGGAATGTTGAGAAC-3
F3	R	5'-CCTATGGAGCAACTTACAC-3

F=forward, R=reverse

2.4. Polymerase Chain Reaction (PCR)

After preparing the cDNA from the samples, the positive samples were first identified using PCR with the designed specific primers for detection (Nd/Nx, Nc/Nx, F5+/F3-, and Ga/Gy). Then, the positive samples were tested for subtypes A and B using PCR with the specific primers Ga/G2 and Ga/G12. The reaction volumes for each pair of primers used are listed in Table 5, and the optimized conditions for each pair of primers are detailed in Table 6.

Table 5: PCR Reaction Volumes

Material/Primer((µL)	Nd/Nx	Nc/Nx	F5+/F3	Ga/Gy	Ga/G2	Ga/G12
Master Mix	10	10	10	10	10	10
Forward Primer	1	1	1	1	1	1
Reverse Primer	1	1	1	1	1	1
cDNA Sample	1	1	1	1	1	1
Nuclease-free Water	7	7	7	7	7	7
Total Reaction Volume	20	20	20	20	20	20

Table 6: PCR Reaction Conditions

Primers	Ga/Gy		F5+/F3		Nc/Nx		Nd/Nx		Ga/G12		Ga/G2	
Condition	Time / second	℃	Time / second	℃	Time / second	℃	Time / second	℃	Time / second	℃	Time / second	℃
Denaturation	60	95	60	95	60	95	60	95	60	95	60	95
|Annealing	45	55	45	57	45	51	45	51	45	55	45	55
Number of Cycles	35	35	35	35	35	35	35	35	35	35	35	35
|Extension	10	72	10	72	10	72	10	72	10	72	10	72

2.5. Sequencing Products

PCR products obtained from the Ga/G2 primer pair with a size of 504 base pairs and the Ga/G12 primer pair with a length of 312 base pairs were extracted from the gel and purified using a gel purification kit. The samples were then sent to Pishgam Company for sequencing. The sequencing results of the positive samples were aligned using BioEdit software and compared with 34 sequences retrieved from the NCBI website. A phylogenetic tree of the available sequences was constructed.

3. Results

The polymerase chain reaction (PCR) along with three pairs of primers Nd/Nx, Nc/Nx, F5+/F3-, and Ga/Gy is a suitable and specific method for virus identification and distinguishing positive and negative samples. The PCR with each of the mentioned primer pairs resulted in bands of sizes 115 bp, 150 bp, 1030 bp, and 448 bp, respectively, in positive samples, as shown in Figure 1. Using Two Specific Primer Pairs Ga/G2 and Ga/G12 PCR on all positive samples with general primer sets from the previous stage resulted in bands of sizes 504 bp and 312 bp for samples containing subtypes A and B of the virus, respectively (Figure 1, part C).

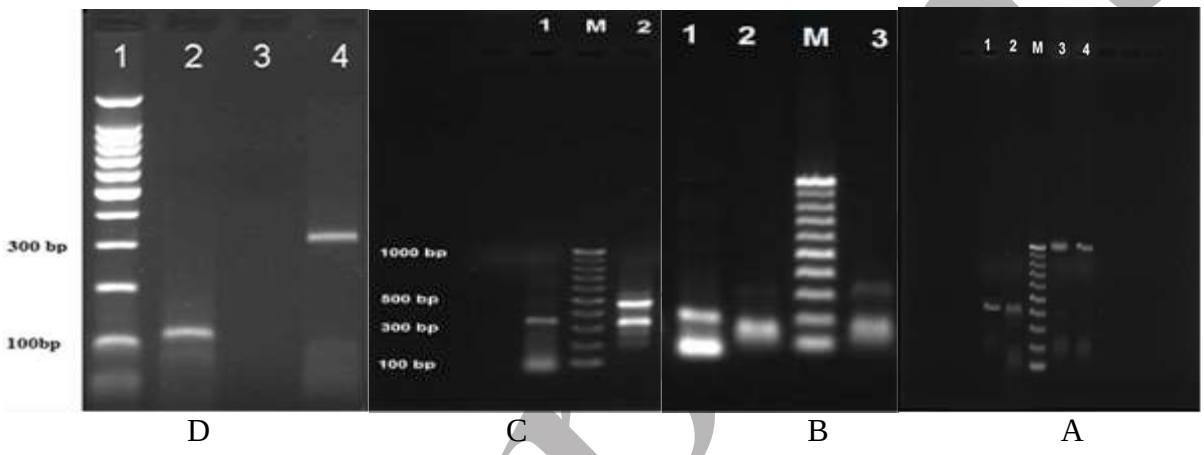

Figure 1: Electrophoresis on 1.5% Agarose Gel, 100bp ladder. **A:** Lanes 1 and 2, PCR products with primer set Ga/Gy; lanes 3 and 4, positive samples with primer set F5/F3. **B:** Lane 1, product of positive samples with primer set Nc/Nx; lanes 2 and 3, positive samples with primer set Nd/Nx. **C:** Lane 1, a positive sample with primer set Ga/G12 (Subtype A) and a positive sample with primer set Ga/G2 (Subtype B). **D:** Lane 2, positive sample with primer set Nd/Nx and lane 4, positive sample with primer set Ga/G2.

The sequencing results of the PCR products of the positive samples and the alignment of the sequences with those available in NCBI confirmed the PCR results. The identification of both subtypes A and B of the virus was validated (Figures 2 and 3).

Figure 2: Sequence Image of One of the Positive Samples of Subtype A

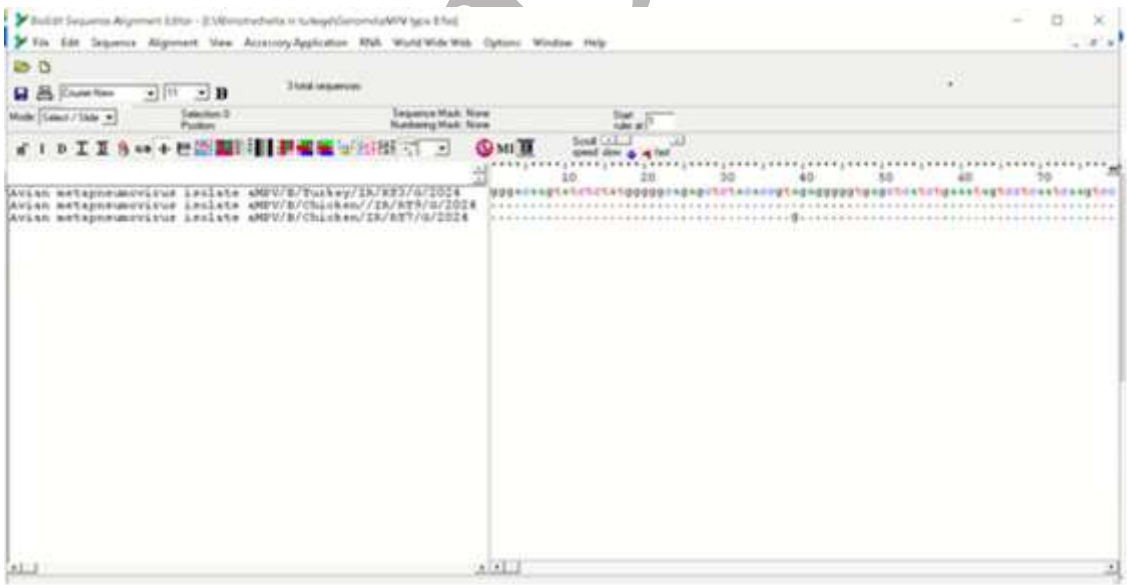

Figure 3: Sequence alignment of three Subtype B Samples from Positive aMPV Cases

4. Discussion

Avian metapneumovirus (AMPV), also known as avian pneumovirus, causes respiratory disease and egg production decline syndrome in various poultry species including chickens, turkeys, and ducks. The virus was first reported in 1970 in South Africa and subsequently in France, the United Kingdom, and other parts of the world. Clinical symptoms are not reliable for diagnosing the virus; however, serological and molecular methods, as well as isolation, can be used for diagnosis.

In the present study, polymerase chain reaction (PCR) using four primer pairs (Nd/Nx, Nc/Nx, F5+/F3-, and Ga/Gy) was introduced as a suitable and specific method for identifying avian metapneumovirus and distinguishing positive and negative samples. The results demonstrated that these primers could produce distinct bands of sizes 115 bp, 150 bp, 1030 bp, and 448 bp. Similar studies by other researchers also support these methods. For instance, a study by Mayahi et al (9) showed that the use of specific primers in PCR could effectively identify avian metapneumovirus and produce similar distinct bands. The results of this study also indicated the high capability of these primers in accurately identifying the virus.

In this research, the use of two specific primer pairs, Ga/G2 and Ga/G12, was able to produce distinct bands of sizes 504 bp and 312 bp in samples containing subtypes A and B of the virus. These findings are consistent with other results, indicating the high accuracy of these primers in differentiating various virus subtypes (7, 9-11).

The sequencing results of the PCR products of the positive samples and the alignment of the sequences with those available in NCBI also confirmed the PCR results. These findings are in agreement with the research conducted by Hosseini et al. in 2012, which showed that sequencing PCR products could help confirm the accuracy of the results and accurately identify virus subtypes (10). Overall, the results of this study indicate the effectiveness and high reliability of PCR using specific primers for identifying and differentiating avian metapneumovirus. This method can be effectively used in the diagnosis and control of the disease in poultry flocks and has yielded results consistent with previous research.

In summary, the results of this study confirm that the use of PCR with specific primers is an effective and reliable method for identifying and differentiating avian metapneumovirus. This method can be effectively used in the diagnosis and control of the disease in poultry flocks and has produced results similar to previous studies.

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Authors' Contribution

SDH and MM designed and planned the study protocol. SDH supervised the project, analyzed the data and interpreted the results. EA contributed in preparing the draft of the manuscript. SE and ML performed administrative, technical, and material support. All authors read and approved the final manuscript.

Ethics

The current study was approved by and carried out under the license 2-98-18-061-001166 of the Scientific Committee of Razi vaccine and serum Research Institute, Karaj, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Data Availability

The authors confirm that all data produced or analysed in this study are included in the paper. However, the data can be requested from the corresponding author, provided it is reasonable and approved by the funding sources.

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