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## Research Paper

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

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## ABSTRACT

**Introduction:** Rhinotracheitis is an acute respiratory infection in turkeys and is considered a relatively significant economic disease caused by Avian metapneumovirus (AMPV) in poultry species. AMPV is responsible for turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) in 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.

**Materials & Methods:** 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 central Iran (Hamadan, Qazvin, Zanjan and Isfahan) to identify and isolate the virus. These samples were tested using specific primers and reverse transcriptase-polymerase chain reaction (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.

**Results:** Out of 87 flocks, twenty-eight samples tested 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 detected in 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.

**Conclusion:** The results of this study confirm that PCR with specific primers is an effective and reliable method for identifying and differentiating avian metapneumovirus.

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## 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. AMPV has been identified in various regions worldwide 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 turkeys and chickens. The first isolation of aMPV was reported 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, 8]. Due to the high genetic variability of 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 transmission mode 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. In some cases, dry swabs were also collected for polymerase chain reaction (PCR) analysis

### 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 both 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 resulting cDNA samples were stored at -30 °C for diagnostic tests and for determination of aMPV subtypes.

### 2.3. Primer design

Based on previously conducted studies 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 presented in Table 4.

### 2.4. 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 primer pair are listed in Table 5, and the optimized conditions for each primer pair are detailed in Table 6.

**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 |

**Table 2.** cDNA synthesis reaction volumes

| Material/Primers | Volume (µL) |
|---|---|
| 2X reaction buffer | 10 |
| Sam script enzyme | 1 |
| RNA sample | Varies based on RNA concentration |
| Nuclease-free water | Varies based on the sample amount |
| Total reaction volume | 20 |

### 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. 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 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

**Table 3.** cDNa synthesis reaction conditions

| Condition | Tem (˚C) | Time (m) |
|---|---|---|
| Incubation | 25 | 10 |
| Incubation | 47 | 60 |
| Reaction termination | 85 | 5 |

**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.

samples containing subtypes A and B of the virus, respectively (Figure 1C).

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

## 4. Discussion

AMPV, also known as avian pneumovirus, is a major pathogen causes respiratory disease and egg production

**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 | °C | Time/ Second | °C | Time/ Second | °C | Time/ Second | °C | Time/ Second | °C | Time/ Second | °C |
| 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 |

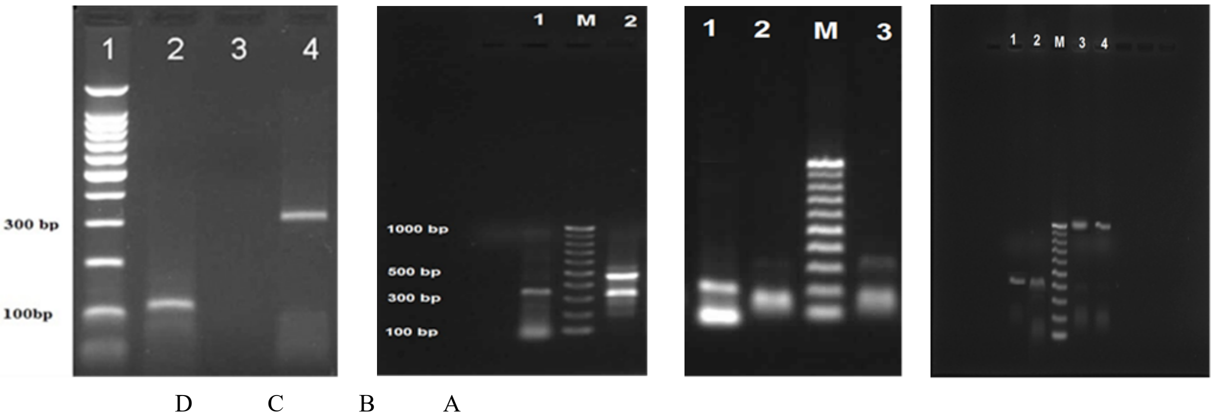


**Figure 1.** Electrophoresis on 1.5% agarose gel, 100 bp ladder

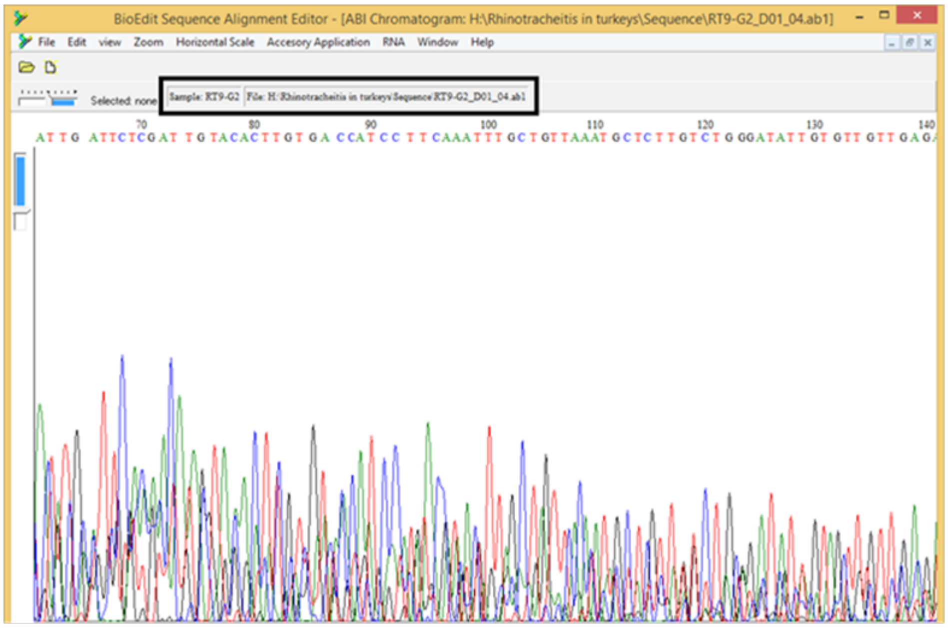
Note: A) Lanes 1 and 2: PCR products with primer set Ga/Gy, Lanes 3 and 4: Positive samples with primer set F5/F3; B) Lane 1: Product of positive samples with primer set Nc/Nx, Lanes 2 and 3, positive samples with primer set Nd/Nx; C) Lane 1: A positive sample with primer set Ga/G12 (subtype A) and a positive sample with primer set Ga/G2 (subtype B; D) Lane 2: Positive sample with primer set Nd/Nx, Lane 4: Positive sample with primer set Ga/G2.

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, PCR using four primer pairs (Nd/Nx, Nc/Nx, F5+/F3-, and Ga/Gy) was introduced as a suitable and specific method for identifying AMPV 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, respectively.

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 AMPV 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



Sample: RT9-G2 File: H:\Rhinotracheitis in turkeys\Sequence\RT9-G2_D01_04.ab1

ATTG ATTCTCGAT TGTACACTTGTGA CCATCC TTCAAATTTGCTGTTAAATGCTCTTGTCTG GGATATTGTGTTGTTGAGA

**Figure 2.** Sequence image of one of the positive samples of subtype A

3 total sequences

Mode: Select / Slide

Selection: 0
Position:

Sequence Mask: None
Numbering Mask: None

Start ruler at: 1

```
Avian metapneumovirus isolate aMPV/B/Turkey/IR/RT3/G/2024    gggacaagtatctctatgggggcagagctctacaccgtagagggggtgagctcatctgaaatagtcctcaatcaagtcc
Avian metapneumovirus isolate aMPV/B/Chicken//IR/RT9/G/2024  ...............................................................................
Avian metapneumovirus isolate aMPV/B/Chicken/IR/RT7/G/2024   ......................................g........................................
```

**Figure 3.** Sequence alignment of three subtype B samples from positive AMPV cases

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, who 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 AMPV. 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.

## 5. Conclusion

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 AMPV. 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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### Compliance with ethical guidelines

This study was approved by the Scientific Committee of Razi Vaccine and Serum Research Institute, Karaj, Iran (Code: 2-98-18-061-001166).

### Data availability

The data supporting the findings of this study are available upon request from the corresponding author.

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### Authors' contributions

Study design and methodology: Seyed Davood Hosseini and Morteza Mahdavi; Supervision, data analysis and interpretation: Seyed Davood Hosseini; Writing: Esmaeil Asli; Project administration, technical, and material support: Saeedeh Ebrahimi and Mohsen Lotfi; Final approval: All authors.

### Conflict of interest

The authors declared no conflict of interest.

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