10.22059/ijvm.2020.302556.1005087

Iranian Journal of Veterinary Medicine
Volume 15- Issue 01

Original Article
Online ISSN : 2252-0554

Molecular Detection of Avian *Metapneumovirus* in Semnan Broiler Farms

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Abstract

BACKGROUND: Avian *Metapneumovirus* (AMPV) causes mild to acute contagious infection of the upper respiratory tract in turkey and chicken with different mortality rate.

OBJECTIVES: This study was aimed to molecular detection and subtyping of AMPV infection in broiler flocks using RT-PCR method in Semnan province on samples from 2016 to2020.

METHODS: Sampling was carried out from the upper part of the trachea, choana, and sinuses of broiler chickens from the 85 broiler flocks. All flocks were more than 3 weeks of age. In total 10 swabs were taken from each flock, while each 5 were pooled as one sample (total two samples per flock). The samples were transferred to the laboratory for RNA extraction and RT-PCR amplification.

RESULTS: Out of 85 tested broiler flocks, 30 (35.3%) were positive for AMPV using the Nd/Nx primer set. In addition, 28 positive samples were found to be of subtype B using the Ga/G12 primer set and 2 remaining positive samples were non-subtype B, probably A, C or D subtypes.

CONCLUSIONS: Since AMPV vaccination was not performed in Semnan province, it can be concluded that some cases were infected with the natural viruses. Therefore, vaccination could be effective in controlling AMPV-induced respiratory distress.

KEYWORDS: Avian *Metapneumovirus*, Broiler flocks, Nd/Nx primer, RT-PCR, Subtype B

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Received: 2020-09-01

Accepted: 2020-12-15

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How to Cite This Article

Darebaghi, A ., Emadi Chashmi, H., Kafshdozan, Kh., Hosseini, H., A. (2021). Molecular Detection of Avian *Metapneumovirus* in Semnan Broiler Farms, 15(1), 27-34.

Introduction

Avian *Metapneumovirus* (AMPV) is a single-strand, negative-sense RNA virus belonging to *Paramixoviridae* family, *Pneumovirinae* subfamily, and *Metapneumovirus* genus. It causes contagious infection of the upper respiratory tract in turkey, chicken, and some poultry species with different mortality rate. In addition to economic losses to the broiler growers, AMPV infection increases feed conversion ratio, egg drop, incidence of eggshell breakage, and hatching rate (Umar *et al.*, 2016). According to the nucleotide and deduced amino acid sequence data, AMPV is divided into 4 subtypes: A, B, C and D. A, B, and D are closely similar. The respiratory distress and reproductive disorder are the main problems of this infectious agent. Secondary bacterial infections play an important role in exacerbating disorders and wild birds could be as natural reservoirs for this virus. The clinical diseases caused by AMPV previously referred to as avian infectious pneumoniae (APV), swollen head syndrome (SHS), turkey rhinotracheitis (TRT) and avian rhinotracheitis (ART) are the acute and highly contagious infection of the upper respiratory tract in turkey, chicken and some poultry species (Suarez *et al.*, 2020).

AMPV was first reported in South Africa in the late 1970s, initially detected in turkeys. In 1984, the virus was isolated in France and the UK. Then, it was isolated from chickens and caused the upper respiratory infection in poultry. It was also found that the virus in some poultry species causes SHS (Brown *et al.*, 2019; Mayahi *et al.*, 2017). In Iran, it has been first reported by Sheikhi and Masoudnia (2011), using serological ELISA test. Then, Hoseini and Ghalyanachi (2012) conducted the first molecular epidemiology study. There are considerable differences in the clinical signs and the mortality rates between experimentally and naturally infected chickens. Rhinotracheitis is a very important manifestation in AMPV, which targeted the respiratory tissue organs. On the other hand, concurrent infection with exacerbating agents such as *E. coli*, *Bordetella avium, Ornithobacterium rhinotracheale, Riemerella anatipestifer, Mycoplasma gallisepticum*, and lentogenic Newcastle disease increase the incidence of clinical signs significantly (Miller *et al.*, 2013; Umar *et al.*, 2016; Brown *et al.*, 2019). The transmission of the virus through the air and direct contact with wild birds is possible (Suarez *et al.*, 2020). In this regard, the present study was conducted with the aim of molecular detection and subtyping of *AMPV* infection in broiler flocks from 2016-2020 in Semnan province, Iran, using RT-PCR method.

Materials and Methods

Sample Collection

The lesion observation and necropsy time of upper respiratory tract are very important because AMPV is present in the sinuses and turbinates only for 6–7 days. For this study, sampling was carried out from 85 broiler flocks from the Semnan province of Iran with the respiratory clinical signs. The samples were taken from the upper part of the trachea, choana, and sinuses of dead broiler chickens. All flocks were older than 3 weeks of age, characterized by mild respiratory symptoms, SHS, and suspected to AMPV infection in autumn and winter seasons, between 2016 and 2020. For each flock, 10 swabs were taken while 5 of each were pooled and considered as one sample (total 170 samples, 2 samples from each flock). The samples were transferred to the Faculty of Veterinary Medicine, Semnan University for RNA extraction and RT-PCR amplification.

RNA Extraction

Swabs were first diluted with 0.50 mL phosphate buffer saline (PBS) and then RNA was extracted using super plus RNA extraction kit (maxcell, Iran) according to the manufacturer's instruction.

Primers

In this study, the Nd/Nx and Ga/G12 primers were used for detection of AMPV and subtype B, respectively (Table 1). These primers were previously used by Bayon-Auboyer *et al.* (2000).

Table 1. Primers Applied in Current Study

Primer	Gene	Sequence	Reference
Nd (forward)	N	5'- AGCAGGATGGAGAGCCTCTTTG -3'	Bäyon-Auboyer *et al.* 2000
Nx (reverse)	N	5'- CATGGCCCAACATTATGTT -3'	
Ga (forward)	G	5'- CCGGGACAAGTATCTCTATGG -3'	Bäyon- Auboyer *et al.* 2000
G_{12} (reverse)	G	5'- CAGTCGCCTGTAATCTTCTAGGG -3'	

CDNA Synthesize

The first-strand cDNA was synthesized by SinaClon First-Strand cDNA Synthesis Kit (Cat. No. RT520100).The mixture was subjected to 12 cycles of 21°C for 30 sec, 43°C for 4 min, and 54°C for 30 min followed by a final inactivation for 5 min at 95°C. The amplification of first-strand cDNA was performed in a thermal cycler (Lab gene Scientific Co., Zurich, Switzerland).

The PCR Amplification

The PCR Mix including 10 µL of Parstous PCR Mix® (Pars-Tous, Mashhad, Iran), 2 µL of forward and reverse primers, 5 µL of DEPC treated water (CinnaGen, Karaj, Iran) and 3 µL (200 ng) of each cDNA sample was prepared. The samples were subjected to 1 cycle of initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, annealing temperature for 30 sec (51°C for *geneN* and 54°C for *geneG*), and 72°C for 60 sec, followed by a final elongation step at 72°C for 5 min. The PCR products were electrophoresed on 2% agarose gel at 140 V for 40 min and then visualized by DNA safe stain (CinnaGen) (Table 2).

Table 2. Primer Set, Annealing Temperature and Expected Products Size

Primer Set	Gene	APMV Subtype	Annealing Temperature	Product Size
Nx/Nd	N	ALL	51	115
Ga/G12	G	B	54	312

Results

Using the Nd/Nx primer set, from 85 flocks and 170 samples, 30 flocks (35.3%) and 51 samples (30%) were positive for AMPV. They included 3 out of 20 (15%) broiler flocks from 2016, 13 out of 30 (43%) from 2017 to 2018 and 14 out of 35 (40%) from 2019 to 2020. Overall, all positive flocks for AMPV had a history of upper respiratory distress and SHS signs during the clinical examination. Twenty-eight flocks were found infected with subtype B. Interestingly, 2 flocks were positive for another subtype, probably belonging to A, C or D subtypes. The characteristics of positive flocks for the AMPV were shown in Table 3.

Table 3. Positive flocks and their details, WINT: Winter, AUT: Autumn, NON B: not belonging to B subtype

Flock Number	Age	Date Of Sampling	Subtype
1	32	WIN. 2016	B
2	28	WIN. 2016	B
3	39	AUT. 2016	B
4	27	WINT. 2017	B
5	38	WINT. 2017	B
6	45	WINT. 2017	B
7	43	AUT. 2017	B
8	28	AUT. 2017	B
9	36	WINT. 2018	B
10	45	WINT. 2018	B
11	47	WINT. 2018	B
12	29	WINT. 2018	B
13	21	WINT. 2018	B
14	26	AUT. 2018	B
15	28	AUT. 2018	B
16	34	AUT. 2018	B
17	49	WINT .2019	NON B
18	29	WINT. 2019	B
19	32	WINT. 2019	B
20	33	WINT. 2019	NON B
21	34	WINT. 2019	B
22	42	WINT. 2019	B
23	39	AUT. 2019	B
24	34	AUT. 2019	B
25	32	AUT. 2019	B
26	33	AUT. 2019	B
27	33	WINT. 2020	B
28	39	WINT. 2020	B
29	25	WINT. 2020	B
30	24	WINT. 2020	B

Discussion

In spite of insufficient information on AMPV and its prevalence in Semnan province, most broiler farms in the province suffered from acute respiratory distress with high mortality (Personal communication by local veterinarians). Although the AMPV was not solely responsible for this mortality, it could have exacerbated the problem. Overall, 35.3% of

broiler flocks were found positive for AMPV in the present study. Since there was no obvious and specific clinical sign for AMPV, it might be disguised as other familiar agents, such as Newcastle disease (ND), Infectious bronchitis virus (IBV) and Colibacillosis. Meanwhile, AMPV vaccination was not performed in Semnan province, therefore, it can be concluded that cases had been infected with the natural field viruses and that vaccination could be effective to control AMPV-induced respiratory distress.

Sheikhi and Masoudian, (2011) investigated the AMPV using serological monitoring of broiler breeder flocks in 11 provinces. In those investigated provinces, the neighboring provinces of Semnan including Tehran, Qom, and Mazandaran were serologically positive for AMPV. Homayounfar *et al.* (2013) detected the AMPV using RT-PCR in poultry flocks in the East and West Azerbaijan provinces. They showed that 5 laying flocks, 2 broiler breeder flocks and also 8 out of 43 tested-broiler flocks were positive for the AMPV which is indicative of 16% of the total samples. That was very close to the finding (15%) of our study in 2016. In the present investigation during 2016-2020, infection rate increased to approximately 40%. Seifi and Boromand (2015) detected 8 positive flocks with 23% prevalence based on the detection of N-gene. They also tested the Ab levels of these flocks for AMPV demonstrating 28.5% of serologic prevalence. Hesami *et al.* (2013) reported a prevalence of 28% for AMPV in Ahvaz. Despite the routine live vaccination in some broiler flocks of Mazandaran and Golestan provinces, the existence of wild strains was confirmed by RT-PCR (Ghalyanachi *et al.*, 2013). Motamed Chaboki *et al.* (2018) reported a prevalence of 36% for AMPV in live bird markets in Gilan province, and suggested the live market could be as a reservoir for AMPV. Zahrabadi *et al.* (2017) also detected 65% of broiler flocks infected with AMPV in Qazvin province and all detected cases were subtype B. Mayahi *et al.* (2017) investigated the AMPV in turkey flocks using samples from slaughterhouse. They reported 4.1% infection in turkey flocks, and all detected cases were from subtype B. The incidence rate in Mayahi *et al.* (2017) study seems to be largely related to the sampling procedure, because the time of sampling is very important, and the virus is only detectable for 6-7 days in the upper respiratory tract. That is why sampling in acute phase of AMPV is recommended (Suarez *et al.*, 2020). In contrast to the high prevalence of AMPV in chicken farms in Iran, in the neighboring countries such as Pakistan, Turkey and Egypt, AMPV prevalences have been reported 2.2%, 7.2%, and 12.5%, respectively (Bayrakt *et al.*, 2018; Umar *et al.*, 2019; Abdelmoez *et al.*2019). This difference between Iran and the neighboring countries is controversial. Lax biosecurity, intensive flocks, and wild birds are very important in AMPV epidemiology. Since AMPV is an airborne disease and broiler flocks in Semnan are mostly located in the flat areas, the virus could easily be transmitted to the farms. Interesting data in the present study was that 2 samples were infected by a subtype other than subtype B. In the recent studies by Ghalyanchi *et al.* (2013), Hoseini *et al.* (2017), Mayahi *et al.* (2017), and Zahrabadi *et al.* (2017), all isolated AMPV belonged to the subtype B. Further investigations are needed to identify these 2 non-subtype B isolates detected in the study with using specific primer set for subtypes A, C and D or sequencing and phylogenetic analysis. In our study, for the first time, a different subtype from subtype B was identified and isolated from Iran. Overall, it seems that AMPV prevalence in Iran is significantly higher than the neighboring countries, and has had an increasing trend from its first report in 2010. Following other studies in Iran, high rate of AMPV prevalence in Semnan province (35.3%) and more importantly, its subtype(s) other than subtype B need political management and improved preventive strategies.

Further investigations are recommended to clear AMPV subtypes circulating in Iran and especially Semnan province.

Conclusion

Due to the nature of AMPV and existence of many exacerbating infectious agents, it can be concluded that vaccination could be an effective way in controlling AMPV-induced respiratory distress.

Acknowledgments

The authors would appreciate the Microbiology Laboratory of Semnan University for providing laboratory services.

Conflict of Interest

The authors declared no conflict of interest.

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Iranian Journal of Veterinary Medicine
Abstracts in Persian Language
10.22059/ijvm.2020.302556.1005087
Online ISSN 2252-0554

مجله طب دامی ایران، ۱۳۹۹، دوره ۱۵، شماره ۱، ۲۶-۳۴

تشخیص مولکولی متاپنوموویروس پرندگان در گله‌های طیور گوشتی سمنان با RT-PCR

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(دریافت مقاله: ۱۱، شهریور ماه ۱۳۹۹، پذیرش نهایی: ۲۵ آذر ماه ۱۳۹۹)

زمینه مطالعه: متاپنوموویروس پرندگان عامل عفونت‌های خفیف تا حاد با تلفات متغییر در قسمت فوقانی دستگاه تنفس بوقلمون و ماکیان است.

هدف: تشخیص مولکولی و تشخیص تحت تیپ متاپنوموویروس پرندگان در گله‌های گوشتی استان سمنان به روش RT-PCR در خلال سال‌های ۱۳۹۵ تا ۱۳۹۹بوده است.

روش کار: نمونه‌گیری از نای، شوانا و سینوس جوجه‌های گوشتی متعلق به ۸۵ گله گوشتی انجام گرفت. تمام پرندگان بالاتر از سه هفته سن داشتند و هر پنج سوآپ به‌عنوان یک نمونه لحاظ شدند. ده سوآب (دو نمونه) از هر گله اخذ گردید و برای استخراج RNA و انجام تست RT-PCR به آزمایشگاه ارجاع داده شد.

نتایج: تعداد ۳۰ گله (۳۵/۳ ٪) از ۸۵ گله مورد آزمایش با استفاده از جفت پرایمر Nd/Nx از جهت وجود متاپنوموویروس پرندگان مثبت ارزیابی شدند. مضاف بر اینکه ۲۸ گله با استفاده از جفت پرایمر Ga/G12 به تحت تیپ B و دوگله به تحت تیپی غیر از تحت تیپ B و احتمالا تحت تیپ A، C و یا D آلوده بوده‌اند.

نتیجه‌گیری نهایی: با توجه به عدم واکسیناسیون متاپنوموویروس پرندگان در استان سمنان، این میزان از آلودگی نشان‌دهندۀ آلودگی با ویرس وحشی است و واکسیناسیون می‌تواند نقش موثری در کنترل استرس‌های تنفسی ناشی از متاپنوموویروس پرندگان داشته باشد.

واژه‌های کلیدی: متاپنوموویروس پرندگان، گله گوشتی، پرایمر Nd/Nx، RT-PCR، تحت تیپ B

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