Original Article
Detection of Avian Metapneumovirus Subtypes A and B in Moroccan Broiler Farms

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ABSTRACT

Background: Avian metapneumovirus (aMPV) is a widespread infectious respiratory pathogen affecting turkeys and chickens, with co-predominance of the subtypes A and B.

Objectives: There is no official reports in Morocco about the subtypes of aMPV circulating. Hence, using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) subtypes-specific A and B, we aimed at detecting and identifying the potential subtype(s) circulating.

Methods: We conducted a longitudinal study on three broiler flocks that were strictly not vaccinated against aMPV and were located in two different geographical regions. We studied two flocks that expressed typical swollen head syndrome (SHS) and sampled them once. Furthermore, we sampled dead birds of one flock confirmed seropositive from a previous study. A total of 118 swabs pooled in 24 samples were subjected to RNA extraction and amplified using a triplex RT-PCR for specific detection of aMPV subtypes A and B. Additionally, serum samples were taken at slaughtering age to cross-check the molecular results. A total of 84 sera were analyzed with a commercial indirect enzyme-linked immunosorbent assay (ELISA) kit to detect and titer antibodies against the two subtypes.

Results: Avian metapneumovirus was detected by qRT-PCR in all flocks. About 87.50% of the samples were positive for aMPV-B, and 16.67% for aMPV-A and aMPV-B simultaneously. All flocks showed seropositivity, confirming the molecular findings.

Conclusion: The present investigation is the first molecular study in Morocco to elucidate the circulation of aMPV-A and aMPV-B in broiler farms in Morocco with a dominance of aMPV-B and the possibility of co-presence of both subtypes.

Keywords: Avian metapneumovirus, Broilers, Morocco, RT-PCR, Subtypes

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Introduction

Avian metapneumovirus (aMPV) is a virus that causes respiratory and reproductive distress in chickens and turkeys. This condition leads to impaired performance and increased mortality, particularly when accompanied by secondary infections. While the virus has traditionally been downplayed in broilers, recent field investigations have highlighted the direct implication of aMPV in respiratory problems (Al-Hasan et al., 2022; Nguyen et al., 2021; Franzo et al., 2017; Tucciarone et al., 2018).

Previously, aMPV was classified into four subtypes (aMPV-A to D) based on variations in the glycoprotein (G), which is responsible for surface attachment, and the antigenic differences between strains (Cook & Cavanagh, 2002). However, discovering two divergent viruses in a monk parakeet and a gull has raised the possibility of new subtype candidates (Canuti et al., 2019; Retallack et al., 2019).

Although subtype B is more prevalent than subtype A (Mernizi et al., 2023a), both aMPV-A and aMPV-B are widespread except in Australia and Canada (Suarez et al., 2019). In the United States, only subtype C has been reported in turkeys, without evidence of spread in broilers per se (Cha et al., 2013). The presence of aMPV-C has also been confirmed in Asia (Kwon et al., 2010) and recently in wild birds in Italy, but it belongs to a different lineage known as the Eurasian sub-lineage (Graziosi et al. 2022; Tucciarone et al., 2022; Toquin et al., 2006). On the other hand, subtype D has only been detected in turkeys in France (Bäyon-Auboyer et al., 2000).

Diagnosing aMPV infection based solely on clinical signs is irrelevant due to the non-pathognomonic symptoms, especially in broilers. Isolating the virus is time-consuming, labor-intensive, and requires a viable virus. Therefore, routine detection of aMPV can be achieved through serology, molecular tests, or a combination of both, depending on the timing of the sample (Lemaitre et al., 2018). Serological tests such as ELISA detect antibodies produced after infection (Rautenschlein et al., 2011), but the results are delayed by at least two weeks for seroconversion (Guionie et al., 2007). Molecular techniques such as polymerase chain reaction (PCR), which detect viral genetic material, are preferred during the infectious phase and provide sensitive, specific, and rapid results (Franzo et al., 2014). They can also differentiate between subtypes using specific gene-sequence-based real-time like quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) (Cook & Cavanagh, 2002; Guionie et al., 2007).

In Morocco, official subtyping data is not available, as research is limited to national serological evidence and the identification of several risk factors associated with seropositivity (Mernizi et al., 2023b). Therefore, the present study aims to highlight the circulation of aMPV-A and aMPV-B in broiler flocks using a quantitative triplex RT-PCR targeting the G gene, which allows for differentiation between these two subtypes. These findings will be supported by confirmation through blood sera tested with ELISA.

Materials and Methods

Sampling protocol

The investigation was designed as a longitudinal study focusing on broilers that were strictly not vaccinated against aMPV. The study involved three flocks: 2-N38, 2-N39, and 2-TS416 (designated with internal codification), located in two areas in Morocco. From 3 to 5 weeks old, each flock was swabbed every 3 or 4 days, specifically from the trachea. On each occasion, ten birds were randomly chosen and sampled.

At the request of the responsible veterinarians, tracheal and turbinate swabs were collected from two neighboring flocks located in another geographic area. These flocks were over five weeks old and had reported cases of SHS (swollen head syndrome). A one-time sampling of ten randomly selected birds per flock was conducted seven days after the appearance of clinical signs.

Furthermore, molecular analysis was conducted to verify the aMPV serological positive results obtained from a previous study. Swabs were collected post-mortem from the preserved trachea and inner side of the skin of eight bird skull heads that exhibited typical swelling. These birds belonged to a flock that had already been tested and confirmed positive for aMPV using serology.

To confirm the detection of aMPV, a serology test was also performed for all flocks. Twenty sera were collected at the slaughter age for each flock in the longitudinal study, and 12 sera were sampled simultaneously with the swabs for the one-off sampling flocks. Although both types of samples were obtained from the same birds, as mentioned earlier, the collected tissues and serum were not paired or individually identified.

Fresh blood samples were collected from the brachial wing veins of the birds by puncturing the alar veins. The samples were then stored in sterile tubes and transported to the Avian Pathology Unit of the Hassan II Agronomic and Veterinary Institute in Rabat. Serum extraction and preservation in Eppendorf tubes at -20 °C were carried out for subsequent analysis using a commercial indirect ELISA kit capable of detecting and titrating antibodies against both subtypes A and B (CIVTEST AVI TRT®, HIPRA S.A. ELISA kit, Amer, Spain).

The mean titers, validity tests, and coefficients of variation were automatically calculated by flock and sample series using the HIPRASOFT® 5.0 software from HIPRA S.A. (Amer, Spain).

Table 1 provides an overview of the sampling protocol for the different flocks included in the study.

Samples preparation and RNA extraction

Following identification and date referencing, swabs were immediately placed in an icebox without any transport media and delivered to the Avian Pathology Unit of the Hassan II Agronomic and Veterinary Institute in Rabat. The samples were then stored at -20 °C until the extraction of RNA was performed.

The skull heads were stored at the Avian Pathology Unit of the Hassan II Agronomic and Veterinary Institute in Rabat, maintained at a temperature of -20 °C. The skin was preserved to increase the likelihood of detecting the genetic material of the aMPV.

For each flock, every five swabs (or four swabs in the case of post-mortem samples) were pooled together, resulting in a total of 24 pools.

The RNA extraction from the dry pools was carried out using the PureLinkTM Viral RNA/DNA Mini Kit® from Thermo Fisher Scientific (Waltham, Massachusetts, USA), following the manufacturer's instructions.

It is important to note that RNA ideally should be stored at -80 °C. However, due to the unavailability of equipment capable of reaching such low temperatures, the RNA samples were only stored at -20 °C.

Reverse-transcription and amplification

The RNA amplification was processed using a single-step triplex real-time reverse transcriptase PCR (RTRT-PCR) targeting the G gene. In this technique, the RNA sequences of aMPV subtype A and B, along with an en-

Table 1. Sampling protocol for the longitudinal study and the one-off sampling flock

Flock	Age (d)	Swabbed Organ	Swabs (No.)	Sera (No.)
2-N38	23	Trachea	10	20
	26		10	
	28		10	
	35		10	
2-N39	23	Trachea	10	20
	26		10	
	28		10	
	35		10	
2-TS416	26	Trachea	10	20
	31		10	
	35		10	
2-MK36	37	Trachea and nasal turbinate	10	12
2-MK37	42	Trachea and nasal turbinate	10	12
1-TS59*	36	Trachea; subcutis (heads)	08	-

*The samples belong to a seroepidemiological study performed in 2021.

dogenous control, were transcribed reverse and amplified in a single tube using specific primer pairs in the polymerase chain reaction.

To detect the amplified RNA of aMPV-A, aMPV-B, and the control's endogenous target gene (beta-actin), TaqMan probes labeled with fluorescent dyes (Fam, Cy5, and HEX) were utilized. The thermocycler measured the emitted fluorescence during the amplification process.

The endogenous control was based on detecting beta-actin, a "housekeeping" protein in the host cells from which the samples originated. The target beta-actin gene (endogenous RNA) was co-amplified (HEX channel) in each reaction. This control allowed for the assessment of sampling adequacy, sample storage and shipping, sample preparation, and the execution of the real-time RT-PCR.

A positive control was included to ensure the specificity and efficiency of reagents, the RT-PCR reaction, and the thermocycler. On the other hand, a negative control was used to exclude any contamination.

Following the manufacturer's instructions, the Kylt® aMPV A&B kit (AniCon Labor GmbH, Emstek, Germany) was employed for the amplification. The data obtained from the amplification was automatically processed using the ThermoCycler AriaMx® real-time PCR System software from Agilent Technologies (Santa Clara, California, USA).

Test evaluation

The analysis of the test began by conducting a validity check for the entire real-time RT-PCR series. This check ensured that the negative control samples for the FAM, HEX, and Cy5 channels were negative, while the positive control samples for these channels were positive with values greater than 15 and equal to or less than 35.

Additionally, internal control was used to validate each sample reaction and its real test result. The Cq value of the internal control channel (HEX) was compared to determine the validity of the sample reaction.

Finally, each sample's specific status of aMPV subtypes A and B was analyzed using the FAM and Cy5 channels, respectively.

Results

qRT-PCR

Table 2 presents the detection of aMPV in the different flocks and the number of positive results per pool.

The subtype B of aMPV was detected in all six flocks included in the study. It was found in 21 out of 24 pools, corresponding to 87.50% of the samples and indicating a high prevalence.

The proportion of pools testing positive for subtype A was 4 out of 24, corresponding to 16.67% of the samples. The subtype A of aMPV was always detected simultaneously with subtype B.

ELISA

The Figure 1 illustrates the results of the indirect ELISA test to detect and titer antibodies against aMPV subtypes A and B. The sera originating from the five flocks sampled were 2-N38, 2-N39, 2-MK37, 2-MK38, and 2-TS416.

Based on the cut-off value of the CIVTEST AVI TRT® kit, 54 out of the total 84 sera samples tested positive, indicating a seropositivity rate of 64.28%.

It is worth noting that all five flocks included in the study had geometric mean titers (GMT) above the cut-off value of 196. This means the antibody levels in all flocks were higher than the threshold considered for seropositivity. Therefore, all flocks demonstrated a seropositive status for aMPV, indicating previous exposure to the virus.

Discussion

Although aMPV infection has been known as the causative agent of SHS in broilers in Morocco for over 30 years, its role in respiratory problems as a primary agent has not been established. The present field investigation provided the first evidence of the circulation of aMPV in Moroccan broiler farms using RT-PCR and ELISA, confirming the presence of the virus and identifying its most important and prevalent subtypes.

The study revealed that subtype B of aMPV was detected in all flocks studied, indicating its widespread presence. In contrast, subtype A was only identified in two neighboring farms where clinical cases of SHS had been reported. These findings suggest that subtype B is the predominant circulating subtype, which is consistent with its high prevalence reported in North Africa and

Table 2. Detection of aMPV-A and aMPV-B by age from the longitudinal study and one-off samplings

Flock	Age (d)	Number of Pools	Positive Pools		
			aMPV-A	aMPV-B	aMPV-A & B
2-N38	23	02	00.02	02.02	00.02
	26	02	00.02	02.02	00.02
	28	02	00.02	02.02	00.02
2-N39	23	02	00.02	02.02	00.02
	26	02	00.02	02.02	00.02
	28	02	00.02	02.02	00.02
2-TS416	26	02	00.02	02.02	00.02
	31	02	00.02	02.02	00.02
	35	02	00.02	02.02	00.02
2-MK36	37	02	02.02	02.02	02.02
2-MK37	42	02	02.02	02.02	02.02
1-TS59	36	02	00.02	01.02	00.02
Total		24	04.24	21.24	04.24

the Mediterranean Basin (Lachheb et al., 2022; Sid et al., 2015; Franzo et al., 2017; Franzo et al., 2020; Tucciarone et al., 2017). Subtype B has been recognized for its high spreading capacity in the region.

Our findings are consistent with previous reports that highlighted the dominance of aMPV subtype B over subtype A (Banet-Noach et al., 2005; Dos Santos et al., 2012) or non-B subtyped aMPV in general (Darebaghi et al., 2021). This finding further supports that subtype B is more widespread than subtype A.

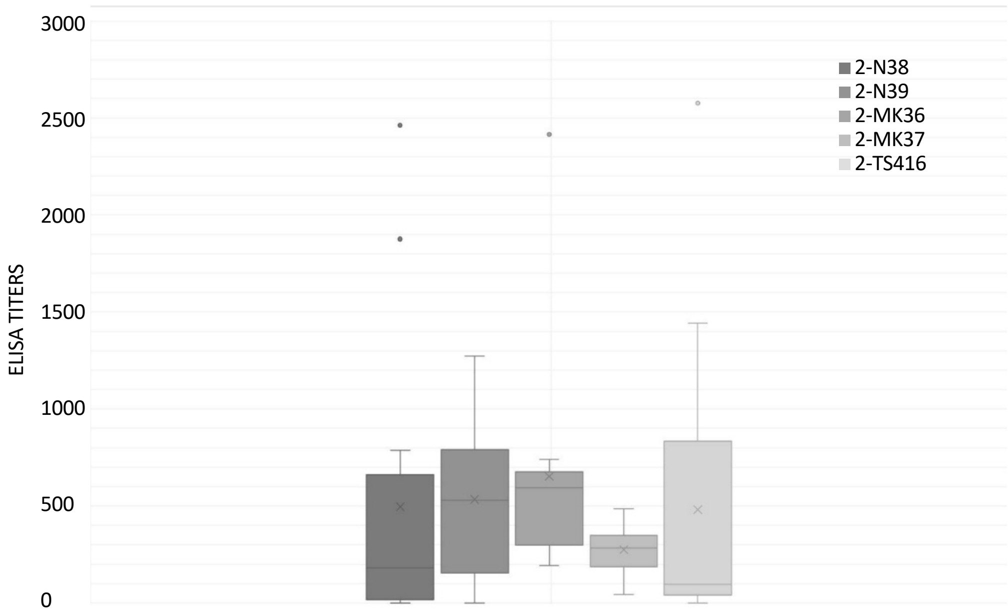

Figure 1. Detection and titration of antibodies against aMPV-A and aMPV-B by indirect ELISA in flocks (2-N38, 2-N39, and 2-7S416) and one-off sampling (2-MK37 and 2-MK38) using the CIVTEST AVI TRT®

Notes: Kit's cut-off=196, and GMT are represented by a cross mark "x."

The longitudinal study conducted in our investigation demonstrated the relevance of the results obtained. We could detect aMPV in flocks 2-N38 and 2-N39 starting from three weeks of age and onwards, even in the absence of respiratory symptoms. However, in the case of flock 2-TS416, respiratory symptoms were observed at five weeks of age, while the one-time sampled flocks, 2-MK36 and 2-MK37, exhibited clinical symptoms of swollen head syndrome. Notably, the sampling protocol for flock 2-TS416 was slightly delayed due to logistical issues and the unavailability of responsible individuals.

In field conditions, determining the exact onset time of infection and, thus, identifying the optimal sampling time can be challenging. All of Morocco's chicks usually come from breeders immunized against aMPV using live and killed vaccines. As a result, it is expected that flocks would not be exposed to field virus challenge earlier than three weeks of age, as the levels of maternal antibodies decline progressively and disappear only after two weeks from hatching (Rubbenstroth & Rautenschlein, 2009).

Furthermore, it is known that infected birds shed the highest quantity of virus from three to five days post-infection (Catelli et al., 1998). Considering these factors, the sample collection in our study was repeated within a short period, less than five days. This approach aimed to increase the likelihood of detecting the genetic material of aMPV during the active shedding phase.

Nevertheless, identifying aMPV at such a young age could be linked to a general decreased immunocompetence predisposition, resulting from the intense genetic selection for rapid growth and high production rates (Nikbakht, 2022a; Nikbakht et al., 2022b).

An interesting finding in our study was the simultaneous co-circulation of aMPV-A and aMPV-B in the two sampled flocks: 2-MK36 and 2-MK37. Similar observations have been reported in Israel (Banet-Noach et al., 2005) and Brazil (Chacón et al., 2011). However, in our case, these two neighboring flocks belonged to an area known exclusively for broiler production, making it initially unlikely to find more than one subtype of aMPV, especially considering the absence of commercial turkeys or layers in the vicinity.

Additionally, it was surprising to detect the genetic material of aMPV one week after the recovery from SHS, assuming the virus should be rapidly cleared and bacterial complications would limit its detection. However, it has been demonstrated that convalescent flocks can experience reinfection by aMPV due to the continuous circulation of the virus within the flock or across the surrounding area (Al-Hasan et al., 2022).

In our study, the absence of an extended investigation involving other pathogens such as infectious bronchitis virus or *Mycoplasma gallisepticum*, known to be prevalent in respiratory complexes (Muofaq Khalaf & Jawad Ali, 2023; Motamed & Bashashati, 2022; Hajijafari Anaraki et al., 2022), assumed that the flocks investigated were aMPV mono-infected only. This plausible attribution may have contributed to the primordial lack of noticeable signs or complications during the visit or, paradoxically, the importance of aMPV as a major respiratory pathogen (Tucciarone et al., 2018) in the case of the flock 2-TS416 that showed respiratory distress. That same observation indicates a state of recovery within the flocks despite contradicting the reported occurrence of SHS in flocks 2-MK37 and 2-MK38, which typically involve bacterial secondary infections.

The serological analysis conducted in our study was highly relevant as it demonstrated positive antibody titers in flocks that were not vaccinated against aMPV and, subsequently, presumably exposed to field viruses. Remarkably, all flocks exhibited antibody levels higher than those typically observed in naïve birds or following vaccination. While titers are generally expected to be higher and more homogenous after infection, the possibility of immunosuppressive agents such as the Gumboro disease virus may have lowered the levels of anti-aMPV antibodies (Sharifi et al., 2022). This issue is particularly noteworthy because flocks 2-MK37 and 2-MK38 show symptoms consistent with clinical Gumboro cases despite the absence of overt signs or conclusive evidence during our visit.

Therefore, the positive serological results obtained in our study support and validate our molecular findings, considering the high national seroprevalence of aMPV in Morocco (Mernizi et al., 2022b). These findings further emphasize the importance of considering molecular and serological approaches to assess the presence and impact of aMPV in broilers comprehensively.

Conclusion

The present study provides valuable insights into the prevalence and co-circulation of aMPV subtypes in Moroccan broiler farms, highlighting the predominance of subtype B. The concurrent presence of subtypes A and B within the same flock is interesting. It emphasizes the need for further molecular characterization of the circulating subtypes in the country. There is limited information about the epidemiological situation of aMPV in Morocco compared to other regions of the world, including neighboring countries, and the origins of the field strains remain unclear.

Future research should focus on extending the molecular investigation and incorporating strain sequencing to address these knowledge gaps. By doing so, a more comprehensive understanding of the characteristics of aMPV isolates in Morocco can be gained, enabling the development of targeted strategies for controlling and preventing aMPV infections in broiler farms. This ongoing work will contribute to updating our understanding of aMPV epidemiology and inform the implementation of effective management measures in the poultry industry.

Ethical Considerations

Compliance with ethical guidelines

The present work was conducted before the implementation of the local Committee of Ethics of the Hassan II Agronomy and Veterinary Institute of Rabat. It, therefore, didn't undergo an evaluation from the aforementioned. The institution guarantees still that all animal procedures in the current work are in agreement with the Hassan II and Agronomy and Veterinary Institute of Rabat and the Moroccan Ministry of Agriculture recommendations, which are following international ethical standards (European Union Directive 2010/63/EU) legislation and animal research reporting of in vivo experiments (ARRIVE) guidelines.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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