Poultry Science Journal
ISSN: 2345-6604 (Print), 2345-6566 (Online) http://psj.gau.ac.ir
DOI:10.22069/psj.2022.20165.1814

Avian Metapneumovirus Review: A Focus on Broilers

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Poultry Science Journal 2023, 11(1): 1-17

Keywords
Broiler
Vaccination
Epidemiology
Avian Metapneumovirus
Swollen head syndrome

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Article history
Received: April 30, 2022
Revised: August 07, 2022
Accepted: August 23, 2022

Abstract

Avian Metapneumovirus infection first emerged in South African turkeys, followed by respiratory problems in chickens with a *swollen head syndrome*. The etiological agent is a Metapneumovirus in the *Pneumoviridae* family and the first *pneumovirus* identified in avian species. This virus causes respiratory and reproductive affections that are worsened in the presence of other pathogens. The present review summarized the current knowledge about the virus's properties and spread, its different subtypes, and the immunological and pathological mechanisms, especially in the broilers. The diagnostic methods are based on serology and essentially ELISA to show and titer antibodies following infection in naïve birds. Molecular tools such as PCR aim to detect and subtype avian Metapneumovirus genetic material. Besides biosecurity, prevention relies mostly on good management and vaccination.

Introduction

The avian Metapneumovirus-induced disease is a concerning threat to poultry production. Even though the higher consideration of turkeys, more personal and anecdotal communications started to point out the avian Metapneumovirus and its impact on broilers (Franzo *et al.*, 2017). A constant rise in the number of aMPV citations was noticed over time. With a yearly increase of citations of 2.4% from the first publication, aMPV is considered an important emerging pathogen, as much as other avian viruses since the Avian Influenza virus has, for instance, a mean percentage of 2.6% (Bertran *et al.*, 2020). In this regard, it was relevant to review the existing literature on aMPV, providing synthesized and up-to-date knowledge about the virus and the induced disease, focusing on the particularities of such infection in broilers.

Origins

At the end of the 1970s, a new and acute respiratory affection appeared in turkeys in South Africa, showing nasal and ocular discharge besides slight sinusitis on the infraorbital sinuses, described as Turkey Rhinotracheitis (Suarez *et al.*, 2019).

During the same decade, an unknown respiratory disease was reported in chickens in South Africa, causing respiratory symptoms and facial edema. It was called SHS, which stands for *Swollen Head Syndrome*. The illness was about respiratory signs of the upper tract alongside swollen heads expressed in a few flocks (Alexander, 1990, cited by Suarez *et al.*, 2019). The disease was thought to be caused by a bacterium (*E. coli*) or a mixed infection of avian Coronavirus (Georgiades *et al.*, 2001). The detection of "Turkey Rhinotracheitis Virus" (TRTV) antibodies in affected birds (Pattison *et al.*, 1989, cited by Al-

Please cite this article as Amine Mernizi, Hicham Fathi, Juan Luis Criado, Mohammed Bouslikhane, Abdeljelil Ghram, Mohammed Mouahid & Saadia Nassik. 2023. Avian Metapneumovirus Review: A Focus on Broilers. Poult. Sci. J. 11(1): 1-17.

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Ankari *et al.*, 2001) and the isolation of TRTV-like viral particles in chickens suffering from *swollen head syndrome* led to believe that TRTV could be the causative agent of SHS (O'Brien, 1985; Lister *et al.*, 1986; Jones *et al.*, 1987, cited by Georgiades *et al.*, 2001). Since then, aMPV or related syndromes have been described worldwide, affecting breeders, layers, and broilers (Franzo *et al.*, 2020).

Etiology

Classification

When the disease appeared in Europe, it was suggested that the virus belonged to the *Paramyxoviridae* family according to different properties. Still, since it did not haemagglutinate red blood cells, the virus was classified in the subfamily of *Pneumovirinae*, which lacks haemagglutinin and neuraminidase glycoproteins (Lamb *et al.*, 2005). Because that virus was the first and the only *pneumovirus* described to affect birds, it used to be identified as avian pneumovirus (Cook, 2000).

Although the virus shows some characteristics of a *pneumovirus*, it differs from the mammalian virus at the molecular level (Cook and Cavanagh, 2002) in that it doesn't encode nonstructural proteins NS1 and NS2 (Broor and Bharaj, 2007). Therefore, it was classified as a new genus, the *Metapneumovirus.* It is assumed that avian and human viruses share a common host derived from bats (Amarasinghe *et al.*, 2017).

Morphology

This virus is pleomorphic. It can be found in two forms: spherical or filamentous. The diameter of the spherical particles varies a lot within a range from 80 to 200 nm, reaching even 500 nm. Its second geometrical form has a diameter between 80 and 100nm and a length of 1,000nm (Lamb *et al.*, 2005; Kaboudi and Lachheb, 2021).

Structure

aMPV has a genome made of a single strain, non-segmented RNA molecule, with negative polarity (Broor and Bharaj, 2007; Kamal, 2008; Amarasinghe *et al.*, 2017; MacLachlan and Dubovi, 2017; Kaboudi and Lachheb, 2021). This genome, composed of eight viral protein genes, is arranged following the order 3′-N-PM-F-M2-SH-G-L-5′ with a leader and a trailer, respectively, at 3′ and 5′ ends (Figure 1).

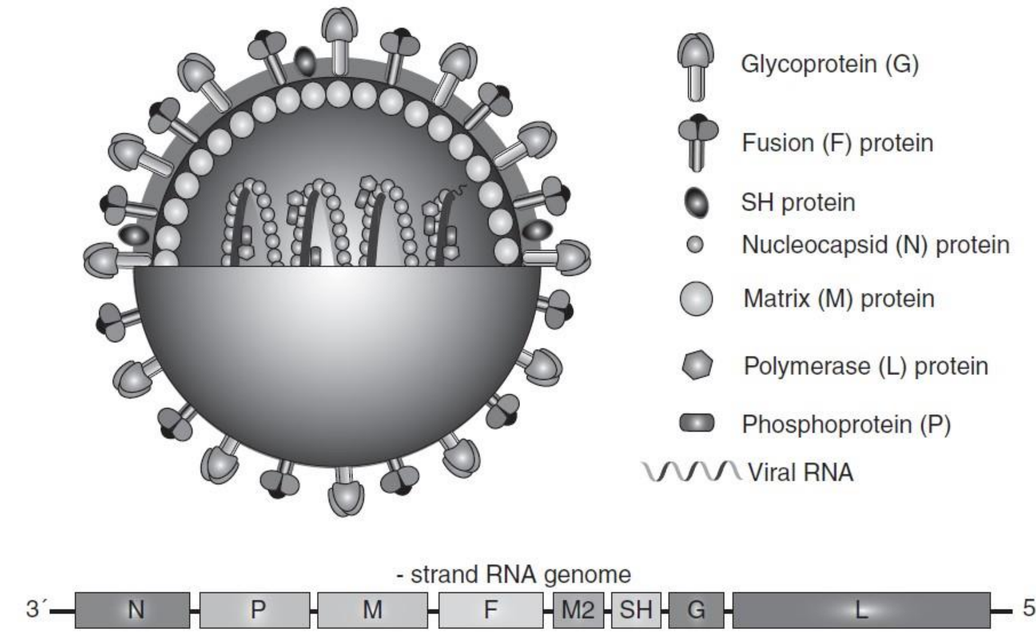

Figure 1. Schematic structure and genomic organization of avian Metapneumoviruses (Rautenschlein *et al.*, 2013)

The polypeptides G, F, N, and M, are particularly important. For instance, the glycoprotein G is responsible for attaching to the host cell and such a process has not yet been identified clearly for aMPV (Suarez *et al.*, 2019). The glycoprotein G also shows higher genetic heterogeneity compared to other polypeptides. This genetic variation allowed differentiating vaccine strains from field ones, by analyzing the G gene (Catelli *et al.*, 2010; Lupini *et al.*, 2011; Mescolini *et al.*, 2020). In addition, glycoprotein G is relevant in the case of molecular and epidemiological investigations and triggers opportunities for intensive sequencing and characterization of strain (Franzo *et al.*, 2020). It should be pointed out that since the G protein might contain a region with a high similarity between strains (Nguyen *et al.*, 2021), investigations such as the aforementioned differentiation could be in

association with genes of hydrophobic protein SH (Catelli *et al*., 2010) or fusion protein (Arafa *et al*., 2015).

Studies on the functions of glycoprotein F support the comparison of different aMPV subtypes regarding their pathogenicity and host-tropism spectrum (Yun *et al*., 2015). This glycoprotein upholds the virions' cell fusion and penetration process, cleaved in F1 and F2 proteins. It also permits fusion between infected and adjacent cells, making the transmission of the virus from one cell to another possible. Unlike other viruses with F protein, such as the avian Orthoavulavirus-1, aMPV has neither haemagglutinin nor neuraminidase activities. Thus, the absence of these two glycoproteins could explain the lower mortalities caused by the aMPV virus. It should be pointed out that the two proteins G and F regulate the adhesion and penetration of the virus into the host cell, representing a key factor of viral pathogenesis, and act as principal antigens, inducing hence a protective immunity (Govindarajan *et al*., 2004; Hu *et al*., 2011; Hu *et al*., 2017).

They are probable targets of the host immune system because of their location on the virus surface, particularly G, and their immunologic importance supported by T cell epitopes on this protein (Cecchinato *et al*., 2010). Notwithstanding, extensive studies investigating these proteins' interaction with the host receptors, and the immune response are still lacking (Naylor *et al*., 2010). The nucleoprotein N is the viral capsid's main constituent, which coats and protects the nucleic acid forming the nucleocapsid. The P and L proteins are also considered part of the nucleocapsid (Collins and Karron, 2013). They play a key role in viral replication (Leyrat *et al*., 2013), whereas the M "matrix" protein stabilizes and envelopes the virion.

Physical and chemical properties

The aMPV shares several common physical-chemical properties with the *Paramyxoviridae* family, except for some differences. In this regard, it is quite interesting to compare the specificities of the aMPV with the avian Orthoavulavirus-1 (Newcastle Disease Virus) (Suarez *et al*., 2019) (Table 1).

Table 1. Comparison of physicochemical properties of avian Metapneumovirus and avian Orthoavulavirus-1

Properties	Avian Metapneumovirus	Avian orthoavulavirus-1
Sensitivity to solvents	Lipids (ether, chloroform)	Phenols, ammoniums, etc.
Temperature inactivation in 30 minutes	56°C	60°C
pH stability	3 to 9	3 and above
Survival at ambient or room temperature	7 days of drying	Stability in organic matter
Flotation density in sucrose gradient	1.21 gram/mL	1.07 – 1.14 gram/mL (Dolganiuc *et al*., 2003)
Neuraminidase activity	No	Yes
Haemagglutination activity	No	Yes

Subtypes

Depending on both nucleotide and deduced amino acid sequence data, with particular emphasis on the changes in the genetic sequence of glycoprotein G, the aMPV can be classified into four antigenically different subtypes, named A, B, C, and D (Cook and Cavanagh, 2002).

The aMPV-A, B, and D, by sharing 38% aminopeptide identity of glycoprotein G, group together, and their other viral proteins – except SH – remain conserved (Eterradossi *et al*., 2015). On the other hand, comparing sequences, genomic organization, codon usage bias, and phylogenetic location of the aMPV-C and hMPV (human Metapneumovirus) subgroup A allowed the proposition of a close relationship between hMPV and the avian subtype C (Govindarajan *et al*., 2004; Brown *et al*., 2014; cited by Suarez *et al*., 2019). Thus, the Metapneumoviruses were subclassified into type-I Metapneumoviruses with aMPV-A, B, and D members and into type-II Metapneumoviruses with aMPV-C genetic sub-lineages and the hMPV. The subtype C showed no significant serological relationship with aMPV-A and aMPV-B. It is poorly neutralized by subtype A or B monospecific antisera but not by monoclonal antibodies that differentiate subtype A and B, suggesting that this subtype could represent a second and distinct aMPV serotype (Alvarez *et al*., 2003).

Still, the aMPV-C does not show any ability to induce disease naturally in chickens since it has never spread in broiler flocks in the USA (Cha *et al*., 2013). However, some severe respiratory infections were observed in broiler farms in China and associated with the subtype C (Wei *et al*., 2013), even though available data, based on only the M genus, couldn't precisely indicate the genetic lineage involved (Eterradossi *et al*., 2015).

Although the subtype D was only detected once in turkeys, in France, in the 1980s (Bayon-Auboyer *et al*., 2000), discovering the presence of aMPV-D many years ago raised the possibility that this subtype might have a more widespread distribution and that it

would still be present in France (Cook and Cavanagh, 2002).

Surprisingly, seroconversion was observed in inoculated chickens, demonstrating, for the first time, that chickens are susceptible to aMPV-D infection. Nevertheless, in experimental conditions, the replication of subtype D seemed to be limited because of the lack of viral RNA detection and virus isolation from the trachea. These findings indicated that aMPV-D could have a different target organ for its replication in chickens (Brown *et al*., 2019).

The detection of the two subtypes, namely C and D, pointed out that more subtypes of aMPV might be identified in the future. Two distinct recently isolated aMPV from a monk parakeet and an aquatic wild bird were proposed as new aMPV subtypes (Retallack *et al*., 2019; Canuti *et al*., 2019).

In fact, it was suggested that the RT-PCR methods could not be sensitive enough and successfully identified "new" aMPV subtypes. Thus, a multi-diagnostic approach remains a must to detect further subtypes of aMPV (Cook and Cavanagh, 2002).

Epidemiology

Geographic Distribution

As mentioned, aMPV and related syndromes are present in most countries where intensive poultry industries are underway. The aMPV was first reported in South Africa and later in the other parts of the world. Molecular tools allowed further detection, in the different production types of *Gallus gallus,* in countries like Mexico (Rivera-Benitez *et al*., 2014), Turkey (Bayraktar *et al*., 2018), and various geographic areas across Africa (Sid *et al*., 2015; Hutton *et al*., 2017; Bakre *et al*., 2020; Mernizi *et al*., 2022).

Asia has recorded a high number of detection of aMPV in different parts of the continent, whether in the Gulf area (Al-Ankari *et al*., 2004; Al-Shekaili *et al*., 2015; Al-Hasan *et al*., 2022), the Middle East (Banet-Noach *et al*., 2005; Gharaibeh and Algharaibeh, 2007; Seifi and Boroomand, 2015), the South (Ali *et al*., 2019), Association of Southeast Asian Nations (Lim *et al*., 2009; Nguyen *et al*., 2021) or even the Far East (Mase *et al*., 2003; Kwon *et al*., 2010; Sun *et al*., 2014). It is also a continent showing the simultaneous circulation of aMPV-A and AMPV-B (Mase *et al*., 2003; Banet-Noach *et al*., 2005), or even multiple subgroups' presence, involving subtypes A, B, and C, in China (Owoade *et al*., 2008; Wei *et al*., 2013; Yu *et al*., 2018) and Korea (Lee *et al*., 2007; Kwon *et al*., 2010).

In Canada, no reports or suspicions of aMPV infection were stated, even though serological evidence of subtype-C in some turkey hen's farms experiencing egg drop syndrome (Jardine *et al*., 2018).

The only subtype reported in the USA is the aMPV-C. Studies were conducted to understand the sudden emergence and sporadic occurrence of the disease in the country, speculating on wild birds' role in the viral transmission in several regions of the USA and possibly in Central and Southern America (Turpin *et al*., 2008). Thus, aMPV has a worldwide distribution, except in Australia, where the virus has not yet been detected.

In Europe, recent phylogenetic studies on different aMPV subtype B sequences collected across the continent showed that field strains tend to cluster according to their geographic origin (with few exceptions only). Interestingly, the subtype B viruses have continued to evolve since their first appearance in the eighties (Mescolini *et al*., 2020).

Subtypes Predominances

Subtype B has been shown to have a more global predominance than subtype A, as described below (Figure 2).

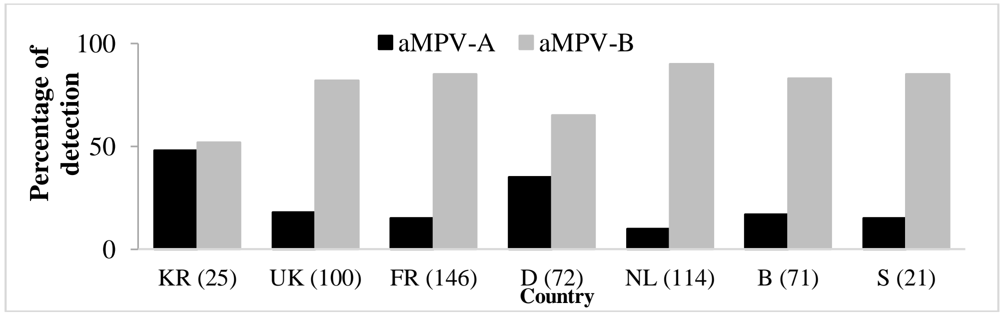

Figure 2. Detection of aMPV-A and aMPV-B subtypes using RT-PCR in Korea (KR), United Kingdom (UK), France (FR), Germany (D), Netherlands (NL), Belgium (B), and Spain (S) in chickens and turkeys (Jones and Worthington, unpublished data). The numbers between parenthesis correspond to the total of samples tested per country.

The subtype B, unlike the subtype A, tended to be more detected over time (Chacon *et al*., 2011). According to a comparative study of both subtypes (Aung *et al*., 2008), it is stated that one of the causes of such a pattern in chickens is the longer persistence of subtype B in tissues than subtype A (Table 2).

Table 2. Detection of the genetic material of aMPV-A and aMPV-B with RT-PCR in different organs following an experimental challenge at 3, 6, and 10-11 days post-infection (PI) (modified from Aung *et al.,* 2008)

Organs	Days post-infection (PI)		
	3 PI	6 PI	10-11 PI
Nasal turbinates	■	■	
Harderian Gland	■	■	
Trachea	▒	■	▒
Lung		▒	
Spleen		▒	
Bursa Cloacalis		▒	

■ Detection of both aMPV-A and aMPV-B
▒ Detection of aMPV-B only.

Another explanation might be, surprisingly, the incapacity of chickens, in experimental conditions, to transmit subtype A horizontally to naïve birds (Brown *et al*., 2019).

Transmission

Even though direct contact transmission through airborne can bring viral particles into straight contact with the host cells, closeness with ill birds and physical contact seems to be, experimentally, a must to reproduce the disease in the case of aMPV (Eterradossi *et al*., 2015).

There is no evidence of vertical transmission of aMPV, even if the virus can infect the reproductive tract (Suarez *et al*., 2019). However, it should be emphasized that this mode of contamination was reported in ducks, suggesting that the semen is a source of the virus (Eterradossi *et al*., 2015). Moreover, aMPV and Infectious Bronchitis virus have been detected both in cockerels' testes issued from a flock with reduced fertility (Villarreal *et al*., 2007). Still, in this case, the role of aMPV is not investigated enough. Bearing that aMPV can disseminate in the environment, it was indicated that farms in proximity, particularly turkeys, may be exposed to the circulating virus and subsequently develop the disease (Lupini *et al*., 2011).

Natural Hosts and the Role of Wild Birds in Virus Spread

Turkeys are considered the main viral host species involved in aMPV spread in countries historically known for rearing a high population of birds. However, relevant strain exchanges between turkeys and broilers have been demonstrated, making unlikely the preeminent role of turkeys in the aMPV epidemiology (Franzo *et al*., 2020). On the other hand, since the aMPV has been confirmed to have a direct role in inducing respiratory signs in broilers (Tucciarone *et al*., 2018):

- It could be suggested that chickens might play a role in the circulation and transmission of aMPV.
- It would also reject the qualification of aMPV as a minor respiratory pathogen in broilers.

Besides description of *Gallus gallus* species and turkeys as the main host species of aMPV, serological evidence of aMPV infection has been described in pheasants (Catelli *et al*., 2001), Guinea fowls (Cecchinato *et al*., 2018), and aMPV-C has been reported in Muscovy ducks (Sun *et al*., 2014).

The role of wild birds in virus transmission was previously stated (Turpin *et al*., 2008). In this regard, Anseriformes, Columbiformes, Falconiformes, Psittaciformes, and Waterfowls are among the migratory birds involved in such virus spread. Although commercial exchanges of live poultry or the creation of "single markets," like in Europe, might have allowed viral transmission over long distances (Franzo *et al*., 2020), the flyways of wild birds could explain such widespread aMPV over the world. Hence, the limit of aMPV replication in these bird species should be further investigated as it could support the hypothesis that such birds may represent a natural reservoir of virus infection (Rizotto *et al*., 2019).

Besides, poultry houses are attractive for wild birds for the available feed and shelters. Thus, birds like pigeons and sparrows could actively participate in aMPV transmission among commercial poultry flocks in endemic areas. Because of their lower susceptibility to aMPV infection, these birds act as mechanical vectors rather than natural reservoirs/hosts (Gharaibeh and Shamoun, 2012). Interestingly, identical subtype viruses have been detected in Brazil, the UK, China, and Nigeria, where intercontinental movements of migratory birds seem unlikely (Owoade *et al*., 2008).

Pathogenesis

The aMPV infects the upper respiratory tract, like the nasal turbinate and trachea, and to a minor extent, the lungs and the air sacs, which are affected particularly when intercurrent infections are involved and exacerbated, prolonging the respiratory disease (Al-Ankari *et al*., 2001). Besides, the ability of the virus to replicate in the reproductive system has been confirmed. Although the role of aMPV is not clear enough when found in the oviduct, it is currently often assumed, in field investigations, and based on

suitable diagnostic tools, that a drop in egg production could be associated with aMPV infection, even without clear evidence (Hess *et al.*, 2004).

Thus, the virus targets epithelial cells of both respiratory and genital tracts (Suarez *et al.*, 2019). Twenty-four hours after infection, the virus can be detected in the nasal cavity and the trachea. The maximum viral quantity is obtained between three and five days post-infection.

aMPV in turkeys was isolated from the nasal cavity for up to 14 days, and its viral genome was detected for up to 17 days (Alexander and Jones, 2008). In broilers, the replication of aMPV is very similar. However, the virus recovered only a few days after experimental inoculation. In that case, the virus presence was limited to nasal and sinus tissues, trachea, and occasionally lungs with small amounts. Hence, this particularity reflected the short-lived and localized damage of aMPV in chickens (Catelli *et al*., 1998).

Because the virus is associated with the deformation of cilia and ciliated epithelial cells of both nasal turbinates and trachea, as mentioned before, it may result in the loss of cilia (Hartmann *et al.*, 2015) and the increase of susceptibility of the epithelium to secondary pathogens (Suarez *et al.*, 2019). Macrophages may contribute to the spread of aMPV from the respiratory tract to other peripheral tissues (Suarez *et al.*, 2019). Immunosuppressive properties of some aMPV strains, such as the interference with innate immune response *in vitro* (Hartmann *et al.*, 2015) or the reduction of vaccine efficacy and vaccination responses (Chary *et al.*, 2002a), have also been described. When birds are infected with aMPV only, antibody production and waning of respiratory signs coincide with the clearance of the virus infection (Suarez *et al*., 2019).

Characteristics of Secondary Infection

Since the first report of disease, several bacteria have been isolated from positive cases of aMPV, such as *Avibacterium paragallinarum*, *Pasteurella multocida*, *Staphylococcus*, *Mycoplasma gallisepticum, Klebsiella,* and *E. coli*. Field investigations incriminate the presence of infectious pathogens, in particular the aMPV, and their contribution to animal infection and disease. Moreover, further agents are described and seem to be involved, such as bacteria *Proteus mirabilis* and *Pseudomonas aeruginosa* (Nasif *et al*., 2019), or even viruses, like Newcastle Disease virus (Shin *et al*., 2002) or variants of IBV (Tegegne *et al*., 2020). The latter is particularly interesting as it shows both viruses' ability to co-infect and replicate without interference. Utmost clinical cases are often complicated by bacterial infection. Therefore, it could explain why the aMPV is considered a minor pathogen agent in broilers.

Immunity

The following table reviews different immunological aspects, innate and adaptive, in aMPV infection (Table 3).

Clinical Signs

Even if respiratory problems characterize the symptomatology, the disease is usually less clearly defined in chickens, particularly broilers with few symptoms. Above all, the aMPV was believed to be always associated with SHS syndrome (*Swollen Head Syndrome)* (Cook, 2000). It was also thought that the infection may not always be associated with clinical symptoms. In fact, in broiler chickens, signs are just less evident compared to turkeys. Still, and particularly when exacerbated by complicating agents, respiratory distress may be severe in broilers (Hess *et al*., 2004).

Even though the widespread respiratory signs within a flock, usually affection and mortality rarely exceed 4% and 2%, respectively (Suarez *et al*., 2019). Depression, coughing, nasal exudates, and frothy eyes appear initially, followed by swelling of periorbital sinuses, typical of the *swollen head syndrome* (Aung *et al*., 2008). Prolapse of the nictating membrane is also reported as the first symptom in broilers before the previously described signs and the extension of the “swelling” to the infraorbital sinuses, submandibular region, and the neck (Eterradossi *et al*., 2015). Nervous signs such as torticollis, disorientation, or opisthotonos may also be present, presumably due to a suggested effect of the virus on the ear (Suarez *et al*., 2019).

It should be pointed out that no genetic relation between the disease and chickens has been reported so far, which means any breed of chickens can become ill indiscriminately. However, the age at the time of infection by aMPV potentially contributes, and increasing the dose of the challenging virus with growing age is necessary to reproduce clinical signs (Gharaibeh and Shamoun, 2012).

This finding is common in different parts of the world, reflected by higher seropositivity in old broiler flocks, especially during winter (Kwon *et al*., 2010; Seifi and Booromand, 2015; Ali *et al*., 2019). Besides, environmental and broiler farm conditions, including ventilation, stocking density, litter condition, and general hygiene, would impact the infection (Al-Ankari *et al*., 2004).

Table 3. Different mechanisms of immunity against aMPV infection

Immunity	Description of the mechanisms
Innate	
Physical barrier	Innate defense mechanisms are well-developed in birds. Physical barriers like eggshells, feathers, skin, epithelium, mucus, and ciliary movements in the respiratory tract, prevent pathogens from entering the body (Rautenschlein *et al*., 2019).
Non-specific	Innate immune mechanisms are activated after pathogen invasion. In the case of aMPV, interferon α, inducible nitric oxide synthase mRNA expression, and virus-induced apoptosis are particularly involved (Hartmann *et al.*, 2015). At the same time, NK cells may influence the acute phase of aMPV-infection (Rautenschlein *et al*., 2011).
Maternal antibodies	Maternally-derived antibodies (MDA) are naturally transferred to offspring through egg yolk. The antibodies are at the maximum level titers after hatching and decrease progressively to disappear in the second week of age. An experimental study demonstrated that even with high levels, maternal antibodies anti-aMPV present in both circulation and tracheal fluid couldn't protect against homologous aMPV challenge (Rubbenstroth and Rautenschlein, 2009).
Adaptive	
Mucosal	Mucosal immunity is short-lasting. In broilers, AMPV-specific IgA tends to decrease 10 to 11 days following infection, after detection in the nasal secretion, Harderian Gland, bile (Rautenschlein *et al*., 2011), and increased levels in the lacrimal fluid and tracheal washes (Ganapathy *et al*., 2005). It should be pointed out that high MDA may disturb IgA production in the upper respiratory tract (Smialek *et al*., 2016).
Cell-mediated	It is the most important mediated immunity in aMPV protection, particularly against respiratory infection (Suarez *et al*., 2019). T-cells can infiltrate the Harderian Gland and control local virus replication at the entry site (Rautenschlein *et al*., 2011). In addition, CD8+ cells accumulate in the Harderian Gland in broilers and contribute alongside NK cells to faster viral clearance and recovery after infection than in turkeys (Rautenschlein *et al*., 2011). After a T-cell-suppression model, experimental birds showed a tardy recovery from aMPV-induced clinical signs and slowed virus clearance, indicating cytotoxic T cells' role in aMPV-pathogenesis (Aung *et al*., 2008).
Humoral	Although humoral immunity has less significance in protecting against aMPV infection, high levels of antibodies may reduce the severity of the disease in chickens by limiting virus replication (Rubbenstroth and Rautenschlein, 2009). After field infection, significant titers of virus-neutralization antibodies are produced as early as five days post-infection (PI), and ELISA antibodies as soon as seven days PI (Jirjis *et al*., 2002). Clinical symptoms do not always accompany seroconversion in the case of chickens (Owoade *et al*., 2006). Still, broilers respond serologically in a comparable manner to turkeys to both subtypes, aMPV-A, and aMPV-B (Rautenschlein *et al*., 2011).

Lesions

Macroscopic Lesions

The macroscopic lesions seen in broilers are mainly located in the head region. Oedematous lesions characteristics of the *swollen head syndrome* are not necessarily accompanied by significant respiratory damage, but following bacterial complications, the periocular subcutaneous edema may progress to fibrin-caseous swelling. Inflammatory lesions can extend in head tissues with blepharitis, conjunctivitis, possibly caseous otitis, maxillary arthritis, periostitis, or osteitis (Eterradossi *et al*., 2015). These findings were particularly reported in the first SHS cases, with birds showing nervous signs (Alexander and Jones, 2008). This caseous material can also be accumulated in the lower mandibular and wattles. Depending on the severity of bacterial complications, deep organs can be affected by airsacculitis and pneumonia, besides pericarditis and perihepatitis accompanied by splenomegaly (Aung *et al*., 2008).

Such observations are common in field outbreaks and usually associated with reports of birds' deaths, probably attributable to secondary infectious agents.

Ultramicroscopic Lesions

Ultrastructural studies of the upper respiratory epithelium damages in chickens have revealed intracytoplasmic eosinophilic inclusion bodies in the nasal turbinate and trachea (Aung *et al*., 2008), with ciliary malformations and progressive deciliation and

substitution of epithelial cells by non-ciliated cells (Aung *et al*., 2008; Hartmann *et al*., 2015).

Following an experimental aMPV mono-infection, damages to the nasal turbinate are almost always seen on the inner side of the turbinate spiral. Two days post-infection (PI), these damages were observed with a mild mononuclear cell infiltration edema in the lamina propria. At the same time, the entire mucosa was never affected, and inflammatory changes in sinuses and tracheas were less evident, with the same infiltration reported, starting from four days PI alongside congestion and thickening in the case of tracheal mucosa. At seven days PI, deciliation phenomena began to be observed in different tissues, mainly due to mononuclear infiltration, with complete recovery of nasal tissue being noticed at 18 days PI (Catelli *et al*., 1998). In the case of the *swollen head syndrome*, which involves bacterial infection, further histopathological changes are described, involving granulomatous inflammation of the subcutaneous cranial tissue, necrosis, and bacteria colonies.

Diagnostic

Clinical Diagnostic

The clinical signs can be helpful but not reliable as a diagnostic. In general, all the respiratory pathogens in the field are likely to induce similar symptoms, such as Mycoplasmosis, Avian Influenza, or Newcastle Disease.

Then, direct and indirect laboratory diagnostic techniques must be used for confirmation. To the best of our knowledge, cases of aMPV in broilers can have little presence of symptoms, which justifies screening tests from time to time for early disease detection and anticipated effective interventions, including (re-)adaptation of control strategies. Another critical point is that the utmost field cases observed are usually mixed respiratory infections, and marked synergism between aMPV and pathogens like *M. gallisepticum*, *O. rhinotracheale,* and *E. coli* has been demonstrated through higher mortality rates or exacerbated clinical symptoms (Marien *et al*., 2007; Rüger *et al*., 2021). Therefore, broiler investigations shouldn't always be mono-oriented but must cover a broad spectrum of pathogens.

Direct Diagnostic Methods

Direct diagnostic methods detect and identify the virus or the genetic material.

Sampling

Isolating or detecting aMPV is difficult in field conditions and, for some unexplained reasons, seems to be even harder in broilers than in turkeys (Ganapathy *et al*., 2005). Nevertheless, it must be kept in mind that the optimal timing for aMPV detection is before the onset of the first respiratory signs. It coincides with the period from day three until day five post-inoculation when birds eliminate the maximum of viruses, and the clinical signs are less evident. After that, the virus could often no longer be isolated as either small viral quantities remain in the bird (Figure 3) or other secondary agents are already present.

If obvious symptoms are observed in the field, it is recommended to sample birds with no clinical evidence from the same house or the neighboring one where signs of infection are not seen yet (Cook, 2000).

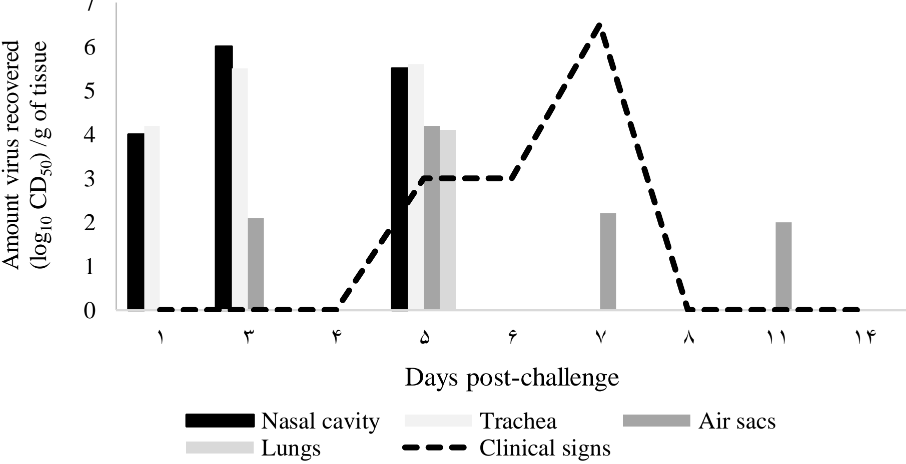

Figure 3. Timing for aMPV isolation from different organs according to incidence and evolution of clinical signs, following experimental infection (modified from Catelli *et al*., 1998)

Organs to be sampled in affected birds are the trachea and the lungs, and better chances of virus isolation are obtained when the sampling is from nasal and ocular discharges, turbinates' swabs, or scraps from infraorbital sinus tissues and contents (Aung *et al*., 2008).

Virus isolation can be carried out on tracheal organ cultures from chicken or turkey embryos used for the ciliostasis evaluation (Hartmann *et al*., 2015) or cultures like Vero cells inoculated with filtered supernatant of oropharyngeal swabs or nasal turbinates (Youn *et al*., 2021).

Molecular techniques

Real-time PCR usage is increasing in the diagnosis of infectious diseases. It is a sensitive, specific, and fast test suitable for diagnosing viral infections (Franzo *et al*., 2014).

The technique for aMPV molecular detection has better sensitivity than virus isolation (Eterradossi *et al*., 2015). It was developed using RT-PCR to extract viral RNA from affected bird tracheal samples and allowed the viral genome to be found for up to 17 days post-inoculation (Alexander and Jones, 2008). However, the RT-PCR fails to detect viral RNA of aMPV when the quantity of the virus is low. In this case, more sensitive protocols like nested PCR (Cook and Cavanagh, 2002), or real-time PCR (Cecchinato *et al*., 2012; Franzo *et al*., 2014), can be used to detect the virus.

Genetic differentiation subtypes

Subtype-specific RT-PCR can be a useful investigation tool. However, this approach can give misleading results in samples with more than one aMPV subtype since one subtype would remain undetected if the PCR test is not specific (Cook and Cavanagh, 2002).

Since only the nucleoprotein N is the more conserved gene amongst aMPV, it is possible to detect the four subtypes using an N gene sequence-based RT-PCR. In contrast, a G gene sequence-based test would differentiate the two subtypes, A and B only (Cook and Cavanagh, 2002).

To detect the American lineage of subtype C and distinguish it from A and B, RT-PCR must emphasize the M gene, which is very specific and sensitive (Shin *et al*., 2000). Another possibility is to use qRT-PCR targeting the G gene of aMPV-A, B, and D, and the SH gene of aMPV-C, leading to detection, identification, and even more, quantification of the four subtypes (Guionie *et al*., 2007).

Immunohistochemistry and in situ hybridization techniques

Immunohistochemistry (IHC) allows it to observe viral antigens in affected birds' nasal turbinate tissues as early as five days post-infection (Cha *et al*., 2013). In addition, such a technique provides the advantage of showing more details on the pathogenicity of the aMPV isolates. Still, in the respiratory tract, using sections of the trachea or turbinates and lung, the in situ hybridization allows the detection of aMPV genome between three and five days post-infection (Chary *et al*., 2002b).

As with immunohistochemistry, in-situ hybridization specificity and sensitivity offer quick and easy detection, identification, and quantification of the virus, making it an excellent alternative to more complex, multi-step protocols and time-consuming laboratory procedures for virus isolation (Hepp *et al*., 2021).

Indirect Diagnostic Methods

Indirect diagnostic methods show antibody production after microorganism contact as an immune response. Such techniques are useful in diagnostics, particularly in naïve flocks or when vaccination is not common because seroconversion follows virus or bacteria challenges. Thus, serological detection of aMPV in non-vaccinated broilers would greatly benefit.

Indirect immunofluorescence and sero-neutralization

The indirect immunofluorescence is sensitive and specific using aMPV-infected TOCs but can also be carried out in Vero (Cook and Cavanagh, 2002). However, this technique has limited use in the case of aMPV diagnostic. It is considered a complex manipulation, particularly for a large-scale sampling to analysis, where the ELISA is widely accepted, as per later description.

As with indirect immunofluorescence, the sero-neutralization can be carried out in different cells (Cook and Cavanagh, 2002), but it is less practical and less used, considered laborious and time costly, thus preferred for research purposes.

ELISA

The Enzyme-Linked Immunosorbent Assay, commonly known as ELISA, is a useful tool in screening and monitoring for the absence or presence of a disease challenge. It is quite interesting in the case of broilers facing exposure to a field aMPV.

Since their first use, different available commercial ELISA kits have been developed and are currently employed with variable results and specificities, depending on the antigen used and its geographical origin (Eterradossi *et al*., 1992; Mekkes and De Wit, 1998, cited by Xu *et al*., 2021). Also, for its sensitivity, it is reported that using a subtype as an ELISA antigen, which is different from that circulating, can reduce the chances of detecting false negatives (Gharaibeh and Algharaibeh, 2007). Lesser detection of vaccinal antibodies is also described if the aMPV strain used to prepare the coating antigen for the ELISA plates is heterologous (Eterradossi *et al.,* 1992, cited by Suarez *et al*., 2019). Nonetheless, cross-reactivity has been proved between the two virus subtypes, A and B (Suarez *et al*., 2019). A

recombinant nucleocapsid N-based ELISA was recently developed for aMPV subtypes A, B, and C and showed equal or better sensitivity than the whole virus-based ELISA. The nucleocapsid is the highest protein expressed in all aMPV and stimulates a substantial immune response in infected animals (Xu *et al*., 2021).

Sera from both acute and convalescent phases should be submitted for analysis (Suarez *et al*., 2019), as the two-step sampling would demonstrate the kinetics of the humoral response. Interestingly, since aMPV initially induces mild respiratory symptoms, often overlooked or not yet well manifested, antibodies can be detected even in samples considered too early for respiratory signs to wane (Brown *et al*., 2019). Although the ELISA test is widely accepted for monitoring broilers' protection, with a minimal cost regarding body weight produced, the benefit of such investment is often underestimated by the farmers, unfortunately.

Prevention

Farm Management

Best farm management procedures, such as reducing density, proper ventilation, and good general hygiene, help decrease the load of environmental microorganisms that cause secondary effects (Rautenschlein *et al*., 2011). Thanks to strict biosecurity, stamping out, and good management practices, aMPV subtype C infection in Colorado, United States, has already been eradicated (Suarez *et al*., 2019). However, because the subtype C case was the first emergence and the only example of successful eradication, it is important to consider vaccination in the preventive strategy alongside the abovementioned guidelines. In fact, vaccination plays a pivotal role in controlling avian infectious diseases and often matches expectations (Franzo *et al*., 2016).

Vaccination

It is important to note that only oil-adjuvanted injectable vaccines were used to control the disease, particularly in long-life birds, and the serological techniques monitored their effectiveness. These vaccines are still the most appreciated, mainly in countries where registration is possible for such categories but not in nations where it is believed that introducing foreign live vaccine strains could result in new virulent viruses (Brown *et al*., 2014).

Live attenuated vaccines are applied for fattening species in areas with a high incidence of aMPV. The vaccination proved to decrease, for the same subtype of aMPV, the detection of the field virus at a younger age (Catelli *et al*., 2010). Live attenuated vaccines are well adopted in turkey production but not widely in broilers, largely due to the perceived poor cost/benefit impact (Bayraktar *et al*., 2018).

Only a low percentage of the broiler population is immunized in dense poultry-populated regions like in Europe (Franzo *et al*., 2020), where high turnover and unvaccinated flocks' status support the evidence of a favorable niche for aMPV persistence (Tucciarone *et al*., 2018).

Live vaccines stimulate humoral and local immunity in the respiratory tract. However, since the immunity following aMPV infection is cell-mediated (Jirjis *et al*., 2002), it is quite normal to find low and heterogeneous antibody titers following live vaccination after a single-dose administration or a primo-vaccination. Still, vaccines protect regardless of serological response (Ganapathy and Jones, 2007).

Cross-protection exists between subtypes A and B (Van De Zande *et al*., 2000), and aMPV-A or aMPV-B vaccines provide even protection against Colorado isolate subtype C (Cook *et al*., 1999, cited by Suarez *et al*., 2019). However, particular focus is accorded to subtype B, probably because of its high worldwide predominance and longer tissue persistence. Recent studies emphasized the homologous and heterologous protection conferred by subtype B derived-vaccine (Ball *et al*., 2022).

Besides, special attention should be given to vaccination techniques. Such vaccination targets all birds or a high percentage of the population to ensure flock protection. Thus, the hatchery administration is a key element for successful vaccination. To the best of our knowledge and based on field observations, vaccination in the hatchery shows the best expectation of quick onset of protection, thanks to the early and homogenous application.

A common misconception is that the goal of spraying a respiratory vaccine is to get chicks to inhale it. It is a means to recreate eyedrop on a mass scale, which is the best way of applying a respiratory vaccine. Another belief is that maternally-derived antibodies may interfere with and neutralize live vaccines, particularly in the first days of life. Still, it was demonstrated that there is no interference between antibodies and most of the available vaccines during the first week of age (Eterradossi *et al*., 2015).

Furthermore, among other interfering parameters, genetic improvements have been reducing the age of market broilers over the years (Schmidt, 2008). Then, it becomes more difficult to build a solid vaccination program during a shortened production period. Such evidence is true particularly against respiratory diseases, considering all revaccinations and joint administrations prioritizing Infectious Bronchitis and Newcastle Disease in endemic areas.

Although the first experiences describing interferences between IBV and aMPV vaccination, even if the latter has no adverse effect on protection against Infectious Bronchitis (Cook *et al*., 2001), several studies showed that protection against aMPV

is still possible at the earliest age of vaccination when co-administered with:

- Newcastle Disease vaccine alone (Ganapathy *et al*., 2006; 2014),
- Newcastle Disease and Infectious Bronchitis vaccines together (Awad *et al*., 2015) or
- Classical and variant Infectious Bronchitis vaccines simultaneously (Ball *et al*., 2019).

To conclude, spray vaccination in the hatchery presents the best results, better than farm coarse spray and much more than drinking water because of the poorer uniformities achieved and the least productive local immunity built up with this latter.

Whether some field strains are described to avoid vaccine-induced protection (Cecchinato *et al*., 2010; Catelli *et al*., 2010) or vaccine-derived viruses that have reverted to virulence (Lupini *et al*., 2011; Arafa *et al*., 2015), these findings have been related to two commercial vaccine strains only by far. Still, the live attenuated vaccines help to control the disease's severity and losses in production due to aMPV (Marango and Busani, 2007, cited by Ball *et al.*, 2022). Apart from that, current works on prevention strategies are interesting aMPV mutant vaccines using reverse genetics (Zhang *et al*., 2016), with alternative perspectives aiming at developing the *in-ovo* application (Worthington *et al*., 2003), recombinant vaccines (Hu *et al*., 2011), subunit vaccines (Tarpey *et al*., 2001), as well as virosome and DNA vaccines (Liman *et al*., 2007).

Conclusion

Despite the relevance of all research contributions and studies about aMPV, we are at an infancy level in understanding aMPV infection.

The lack of epidemiological data is related to the limited reports on aMPV distribution and virus characterization, originating from very few countries. Nevertheless, the rise in the number of publications from China in the last twenty years (Owoade *et al*., 2008; Wei *et al*., 2013; Sun *et al*., 2014), for example, demonstrated the importance of this virus as a primary respiratory agent and possibly, the continuous spreading and involvement of aMPV in the field. Our knowledge regarding the virus's behavior in mono-infected birds is still poor, and its isolation is difficult. The virus transmission modalities over distances are overlooked, and few serological results have been reliable since the first case evidence in the 1980s. Furthermore, most experimental models failed to reproduce the disease clinically under controlled conditions, especially in broilers. To the best of our knowledge, particularly in countries where aMPV status in broilers is still unknown or not clear enough, like Morocco, responding to questions unanswered like the preceding requires a good approach with the aim of giving a "big picture" description of the aMPV context, as a first step. In other words, studies should begin with serological explorations, proving, on the one hand, the circulation of aMPV in broiler chickens and defining, on the other hand, a quantitative appreciation of this circulation through seroprevalence. We also believe that such inputs need further investigations by veterinarians and poultry professionals to understand the possible risk factors associated with aMPV circulation and its induced disease in broilers.

Acknowledgments

For the review, E. Catelli, department of veterinary medical sciences, university of Bologna, Italy.

Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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