# *Avian Metapneumovirus* circulation in Italian broiler farms

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**ABSTRACT** With increasing frequency, *avian Metapneumovirus* (aMPV) is reported to induce respiratory signs in chickens. An adequate knowledge of current aMPV prevalence among Italian broilers is lacking, with little information available on its economical and health impact on the poultry industry. In order to collect preliminary data on the epidemiological context of aMPV in broiler flocks, a survey was performed in areas of Northern Italy with high poultry density from 2014 to 2016. Upper respiratory tract swabs were collected and processed by A and B subtype-specific multiplex real-time reverse transcription PCR (RT-PCR). Samples were also screened for infectious bronchitis virus (IBV) by generic RT-PCR and sequencing. Productive data and respiratory signs were detailed where possible. The high prevalence of aMPV was confirmed in broilers older than 26 d and also attested in IBV-negative farms. All aMPV detections belonged to subtype B. Italian strain genetic variability was evaluated by the partial attachment (G) gene sequencing of selected strains and compared with contemporary turkey strains and previously published aMPV references, revealing no host specificity and the progressive evolution of this virus in Italy.

**Key words:** *avian Metapneumovirus*, Italy, broiler, epidemiology, vaccination

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## INTRODUCTION

*Avian Metapneumovirus* (**aMPV**) is the type species of the genus *Metapneumovirus*, classified in the recently status-elevated family *Pneumoviridae* within the order *Mononegavirales* (International Committee on Taxonomy of Viruses, 2015); 4 subtypes (A, B, C and D) were defined, based on genetic differences (Juhasz and Easton, 1994; Bäyon-Auboyer et al., 2000; Seal, 2000). The infection causes Turkey Rhinotracheitis (**TRT**) in turkeys, also targeting the upper respiratory tract in chickens and other avian species (Gough et al., 1988; Catelli et al., 2001; Cecchinato et al., 2016). In layers and breeders, aMPV can cause a drop in egg production and poor eggshell quality. These conditions imply severe economic losses in unprotected birds, especially when secondary pathogens are present.

After its first appearance in South Africa at the end of the 1970s, aMPV has been reported worldwide except for Australia, where it has not yet been detected (Bell and Alexander, 1990). Its quick and broad diffusion has been ascribed to many factors, some determined by the virus biology itself, others related to its transmission routes, such as national and international trade, or migratory wild birds, potentially carrying and spreading the virus from infected farms to naive ones (Turpin et al., 2008). aMPV genetic variability reflects on several practical aspects influencing evolutionary dynamics (de Graaf et al., 2008; Franzo et al., 2015), epidemiology (Catelli et al., 2004), and the efficacy of the adopted control strategies (Catelli et al., 2006; Cecchinato et al., 2012). Indeed, all of these changes can be due to natural or vaccine-induced immunity escape, or they can actually help this phenomenon (Catelli et al., 2010; Cecchinato et al., 2010). In Italy, aMPV infection is mainly sustained by subtype B and particularly affects turkey flocks (Cecchinato et al., 2013a; Listorti et al., 2014), where vaccination is commonly adopted. Nonetheless, the outbreaks of respiratory disease ascribable to aMPV are currently increasing among chickens (Cecchinato et al., 2013a). The high turnover and present unvaccinated status of the Italian broiler population could represent a favorable niche for aMPV persistence and adaptation to the new host, potentially leading to the emergence of more virulent phenotypes.


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Despite this, the precise prevalence of aMPV in broilers in Italy is still undetermined. Therefore, the infection consequences reflecting on productivity and animal health have not yet been well defined. In order to encourage the initial description of the rapidly changing epidemiology of aMPV in broiler flocks, a survey was performed from 2014 to 2016 in Northern Italy, where more than 65% of the whole Italian poultry production is located (Capua and Marangon, 2000). Moreover, the potential improvement of an extension of aMPV control strategies to broiler farms was investigated in a field vaccination trial.

## MATERIALS AND METHODS

### Longitudinal Studies

***Farms*** Forty-six commercial broiler farms, distributed over the high poultry density region of Northern Italy, were monitored through dedicated longitudinal studies or one-off samplings, from 2014 to 2016. Chickens were sampled by oropharyngeal or tracheal swabbing and 313 pooled samples were collected. Survey data were recorded on the collection date, farm location, bird age, respiratory sign presence, and infectious bronchitis virus (**IBV**) vaccination programs. In Italy, the most widely adopted protocol for IBV control in broilers is based on the coupled administration of Mass and 793b or QX-based vaccines, mainly at the hatchery (Franzo et al., 2016). No aMPV vaccination strategy is currently adopted in broilers.

***aMPV Detection and Characterization*** Ten dried swabs per shed were pooled in each sample and RNA was extracted using High Pure RNA Isolation Kit (Roche**,** Mannheim, Germany), following the manufacturer's instructions. aMPV presence was investigated by multiplex real-time reverse transcription polymerase chain reaction (**RT-PCR**) (Cecchinato et al., 2013), allowing for the simultaneous detection and differentiation between A and B subtypes.

***Infectious Bronchitis Virus Detection and Genotyping*** The 313 samples tested for aMPV were also tested for IBV using a general RT-PCR targeting a hypervariable region of the S1 gene as described by Cavanagh et al. (1999), with minor modifications. Positive samples were Sanger sequenced with the same primer pair at Macrogen Europe (Amsterdam, The Netherlands) and the obtained sequences were analyzed to distinguish IBV field strains from the vaccine strains applied in the farms, as reported by Franzo et al. (2014).

***Sequencing of aMPV Attachment (G) Protein Gene*** Sixteen aMPV positive samples (6 from 2014; 5 from 2015; 5 from 2016) were selected to achieve a minimum coverage of the examined area and period. In order to evaluate the possible host specificity of the circulating strains, 2 turkey farms near some of the chosen broiler farms were also sampled in 2014, where turkeys were showing aMPV related clinical signs and swabs were processed as described previously.

From all of the 18 selected samples, the partial G gene was amplified and sequenced in accordance with Cecchinato et al. (2010) at Macrogen Europe (Amsterdam, The Netherlands). Sequences were analyzed using Bioedit software (Hall, 2011) and then aligned using Clustal W (Larkin et al., 2007) against other Italian and foreign subtype B sequences (Cecchinato et al., 2010). Phylogenetic analysis was carried out with the Neighbor joining algorithm implemented in MEGA6 software (Tamura et al., 2013). Bootstrap values were obtained with 1,000 replicates. Branches with bootstrapping values >70 were considered significant.

### Vaccination Field Trial

In 2015, 2 broiler farms (6 sheds) were selected on geographical bases, in an area where aMPV was considered an endemic pathogen. The 2 farms had previously reported respiratory problems caused by aMPV. Therefore, the introduction of a vaccination strategy and consequent longitudinal monitoring were planned. Twelve-day-old chickens were vaccinated by coarse spray vaccination with an aMPV subtype B-based vaccine (VCO3 strain) and sampled by oropharyngeal swabbing, starting from 11 d post vaccination and then weekly tested (18, 25, 32 d after vaccination). The samples were initially tested by A and B subtype-specific multiplex real time RT-PCR (Cecchinato et al., 2013) and, if positive, the partial G gene was amplified, sequenced, and analyzed to distinguish field from vaccine strains, as previously described.

## RESULTS AND DISCUSSION

Eighty samples (25.6%), originating from 27 out of 46 sampled broiler farms (58.7%), were aMPV-positive (Table 1). aMPV subtype B was the only detected subtype, underlining its high prevalence in Italy and the endemic nature of aMPV infection in Italian broilers, particularly in densely poultry-populated areas where turkey and broiler production coexists closely. The persistence of aMPV was precisely supported by the detection of identical strains (MF543418, MF543419 from Farm 30, MF543408, MF543421 from Farm 39) in the same farm and by their recurrence, even for 3 consecutive production cycles (Farm 3, 17, 30, and 39) (Table 1). Indeed, this phenomenon could be the consequence of farm management choices, such as a too short intercycle period or the application of incorrect cleaning and disinfection procedures between cycles. Another possible route for introduction could reside in the 2-way diffusion between chicken and turkey farms, also suggested by the close similarity among broiler and turkey sequences (Figure 1). aMPV was detected mainly in birds older than 26 d (95%) (Figure 2), usually in association with respiratory signs (68.8%). Interestingly, respiratory cases appeared frequently in aMPV-positive samples (37.6%), suggesting its direct role in the

**Table 1.** aMPV-positive samples from longitudinal studies and one-off samplings with the associated information.

| Farm | Cycle | Shed | Age (Days) | Collection date (mm/aaaa) | IBV strain characterization | Respiratory signs | Sample internal ID/ Accession Number |
|---|---|---|---|---|---|---|---|
| **1** | - | 1 | 39 | 06/2015 | Vaccine strain | x | 320 |
| **3*** | 1 | 1 | 13 | 07/2014 | Vaccine strain | x | 16 |
| | | 2 | | | Vaccine strain | x | 17 |
| | | 1 | 20 | | Vaccine strain | - | 33 |
| | | 2 | | | Vaccine strain | - | 34 |
| | | 1 | 27 | | Vaccine strain | x | 35 |
| | | 2 | | | Vaccine strain | x | 36 |
| | 2 | 2 | 34 | 10/2014 | Vaccine strain | - | MF543402 |
| | 3 | 1 | 38 | 01/2015 | Vaccine strain | x | 165 |
| | | 2 | | | Vaccine strain | x | 166 |
| **4** | - | 1 | 36 | 11/2015 | Vaccine strain | x | MF543406 |
| | | 2 | | | Vaccine strain | x | 377 |
| **8** | - | 1 | 47 | 10/2015 | Vaccine strain | x | 362 |
| | | 2 | | | Vaccine strain | x | 366 |
| | | 3 | | | Q1 | x | 370 |
| **10** | - | 2 | 35 | 03/2016 | Vaccine strain | x | MF543411 |
| | | 3 | | | QX | x | 510 |
| **11** | - | 2 | 39 | 03/2016 | QX | x | 535 |
| **12** | - | 5 | 45 | 08/2015 | Vaccine strain | x | MF543405 |
| | | 6 | | | Vaccine strain | x | 337 |
| | | 7 | | | Vaccine strain | x | 341 |
| **13** | - | 5 | 32 | 09/2015 | Vaccine strain | - | 342 |
| **15** | - | 1 | 36 | 10/2014 | Vaccine strain | - | 106 |
| | | 2 | | | Vaccine strain | - | 107 |
| | | 2 | 44 | | Vaccine strain | - | 108 |
| | | 3 | | | Vaccine strain | - | 109 |
| **17*** | 1 | 3 | 26 | 07/2014 | Vaccine strain | - | 31 |
| | | 3 | 40 | 08/2014 | Vaccine strain | - | 38 |
| | | 3 | 49 | | Vaccine strain | x | 43 |
| | | 2 | 50 | | Vaccine strain | - | 44 |
| | 2 | 3 | 45 | 10/2014 | Vaccine strain | - | MF543420 |
| | | 4 | | | Vaccine strain | - | 93 |
| | 4 | 3 | 36 | 03/2015 | Vaccine strain | - | 223 |
| | - | 3 | 54 | 04/2015 | Vaccine strain | x | 247 |
| **18** | - | 1 | 50 | 07/2015 | QX | x | 330 |
| | | 2 | | | Vaccine strain | x | 331 |
| **19** | - | 1 | 49 | 01/2016 | QX | x | 444 |
| | | 2 | | | Vaccine strain | x | 445 |
| **21** | - | 2 | 38 | 01/2016 | Vaccine strain | x | MF543409 |
| **24** | - | 1 | 38 | 05/2016 | QX | x | 611 |
| | | 3 | | | Vaccine strain | x | 612 |
| **26** | - | 2 | 42 | 02/2016 | Vaccine strain | x | MF543410 |
| **27** | - | n.r. | 42 | 10/2015 | Vaccine strain | x | 372 |
| **29** | - | n.r. | 40 | 10/2015 | Vaccine strain | x | 398 |
| **30*** | 1 | 1 | 30 | 10/2014 | Vaccine strain | - | 83 |
| | | 2 | | | Vaccine strain | - | 85 |
| | | 1 | 38 | | Vaccine strain | - | MF543418 |
| | | 2 | | | Vaccine strain | - | 87 |
| | | 3 | | | Vaccine strain | - | 90 |
| | 2 | 1 | 30 | 11/2014 | Vaccine strain | - | MF543419 |
| **32** | - | 2 | 41 | 10/2015 | Vaccine strain | x | 359 |
| | | 5 | | | Vaccine strain | x | 360 |

**Table 1.** *Continued.*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **33** | - | n.r. | 36 | 02/2016 | QX | x | 504 |
| **34** | - | 1 | 44 | 01/2015 | Vaccine strain | x | MF543403 |
| **35** | - | 1 | 43 | 04/2016 | Vaccine strain | x | MF543413 |
| | | 2 | | | QX | x | 557 |
| | | 3 | | | QX | x | 558 |
| **36** | - | 1 | 34 | 04/2015 | Vaccine strain | x | MF543404 |
| | | 2 | | | Vaccine strain | x | 249 |
| | | 3 | | | Vaccine strain | x | 250 |
| | | 4 | | | Vaccine strain | x | 251 |
| **37** | - | 1 | 47 | 03/2016 | QX | x | MF543412 |
| **39*** | 1 | 1 | 42 | 08/2014 | Vaccine strain | x | 40 |
| | | 2 | | | Vaccine strain | x | MF543408 |
| | | 1 | 47 | | Vaccine strain | - | 45 |
| | | 2 | | | Vaccine strain | - | 46 |
| | 2 | 2 | 45 | 10/2014 | Vaccine strain | - | MF543421 |
| | 4 | 1 | 37 | 03/2015 | Vaccine strain | - | 220 |
| | | 2 | | | Vaccine strain | - | 221 |
| | 5 | 1 | 34 | 05/2015 | Vaccine strain | x | 255 |
| | | 2 | | | Vaccine strain | x | 256 |
| **44** | 1 | 2 | 32 | 10/2015 | Vaccine strain | x | MF543407 |
| | | 3 | | | Vaccine strain | x | 396 |
| | | 4 | | | Vaccine strain | x | 397 |
| | 2 | 4 | 49 | 12/2015 | QX | x | 405 |
| | - | 2 | 50 | 03/2016 | Vaccine strain | x | 530 |
| | | 3 | | | Vaccine strain | x | 531 |
| | | 4 | | | Vaccine strain | x | 532 |
| **46** | 1 | 2 | 43 | 02/2015 | Vaccine strain | x | 192 |
| | 5 | 3 | 44 | 11/2015 | Vaccine strain | x | 380 |

Accession numbers instead of sample IDs are reported when the sequence was obtained.
x, presence of respiratory signs; –, absence of respiratory signs.
*Farms with aMPV persistence in subsequent cycles.

observed symptomatology. This finding is particularly relevant because aMPV has been considered a minor pathogen in broilers for a long time. In 91.7% of the samples, IBV vaccine strains were identified, in line with the vaccination protocol adopted in the respective farms. Almost all IBV field strain-positive samples originated from farms/sheds displaying overt respiratory symptoms and the majority belonged to the QX genotype (24 out of 26, 92.3%), whereas the remaining 2 were classified as Q1 strains (7.7%). Even if present, aMPV and field IBV coinfections were only occasionally found (3.5%), and clearly in these cases, the clinical signs were more likely to be sustained by IBV infection. The new epidemiological picture described herein could be due to a progressive adaptation of the virus persistently circulating in the new host population. G gene phylogenetic analysis from the selected strains, identified in broilers from 2014 to 2016, revealed the progressive and continuous evolution of this virus in Italy (Figure 1), when compared with the local strains. In fact, the viruses clustering together in the same Italian cluster, show heterogeneity, as testified by the presence of smaller subclusters (Figure 1). Nevertheless the substantial identity among sequences from broiler and turkey farms, revealed by the comparison of the partial G gene of these strains (MF543402, MF543408, MF543420, MF543421) with 2 strains from turkeys (MF543422, MF543423) (Figure 1) from the same period, suggests the absence of host-specific adaptation determinants, at least in the considered region. Thus, even if additional and more extensive studies are necessary to confirm this hypothesis, other factors are likely shaping aMPV epidemiology. Differences in animal management and the presence of additional stressing agents or concomitant immune-suppressive infections could have affected the infectious pressure or the host susceptibility. In this sense, the progressive selection of new broiler genetic lines, possibly more susceptible to aMPV infection, could be a relevant factor as reported for other livestock diseases. Interestingly, the observed low prevalence of IBV infection is ascribable to efficient control strategies (Franzo et al., 2016) and may also play a role in reducing the field strain circulation. IBV vaccines could have altered the competitive equilibrium between IBV and aMPV (Cavanagh et al., 1999; Cook et al., 2001), favoring the latter indeed. These findings lead to the main role of chickens in the maintenance and circulation of aMPV, especially in this area where both species are closely reared. Consequently, the implementation of a prevention strategy in broiler farming that is similar to the one currently adopted in turkeys, appears essential and more than just useful.

In order to evaluate the potential benefit of aMPV vaccination in broilers, a field trial was performed in an area where aMPV had already proven to be a

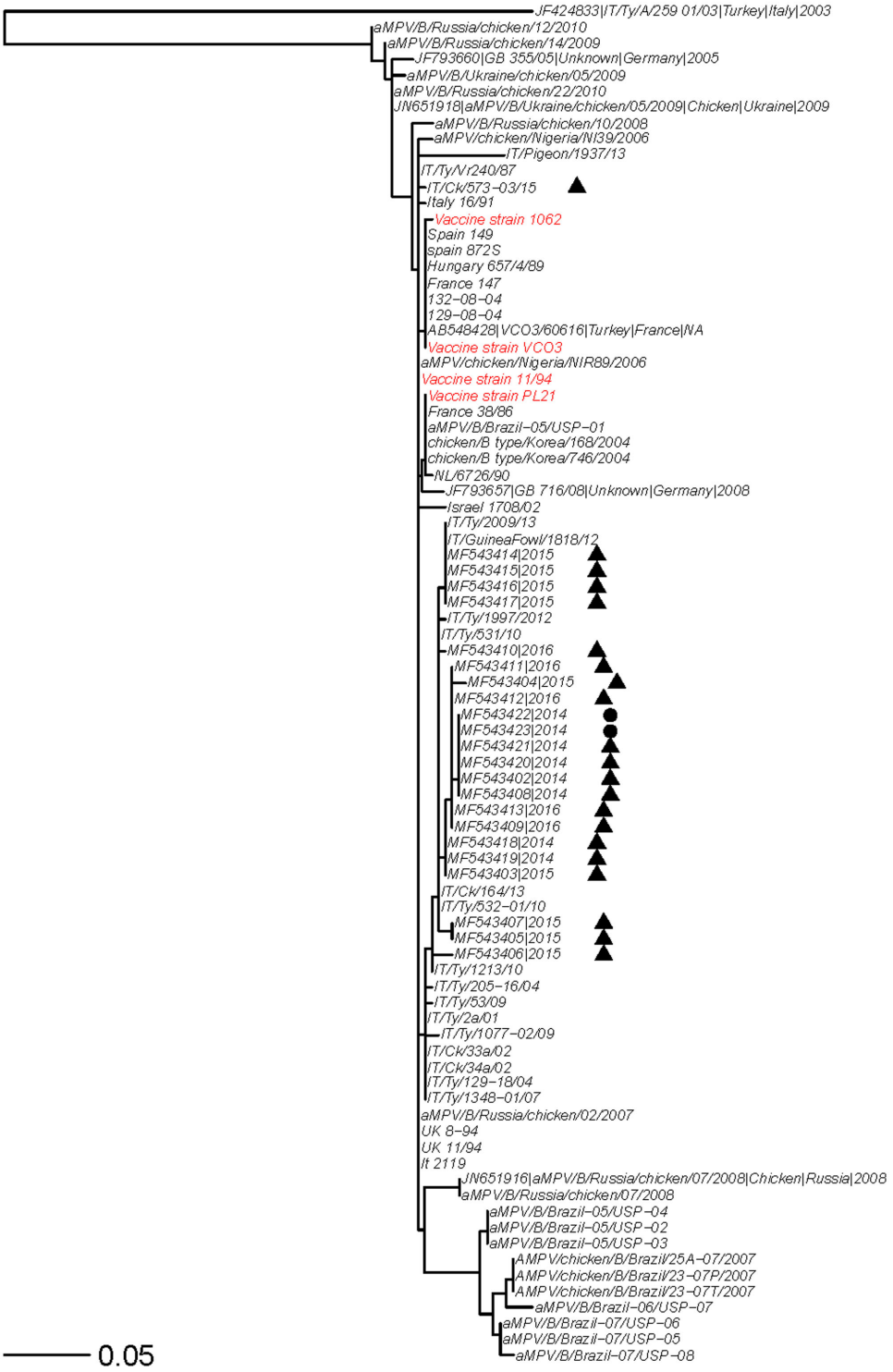


**Figure 1.** Phylogenetic tree based on partial G gene sequences of freely available aMPV and Italian sequences. Vaccine strains are in red. Sequences obtained in this study (from longitudinal studies and aMPV vaccination trial in broiler farms) are marked by a black triangle and turkey sequences are marked by a black circle.

health and productivity issue for both turkeys and chickens. The 2 selected farms had previously experienced aMPV-related problems in the absence of other pathogens (data not shown), and were therefore chosen to verify the efficacy of aMPV vaccination. As a demonstration of aMPV endemic presence, 3 out of 4 sheds tested positive for subtype B field strains in Farm 1 (Table 2), appearing identical to each other (Table 2, Figure 1) but phylogenetically distant from the vaccine used. In one shed of Farm 2, a vaccine-related strain was detected right after the vaccination and again at 36 d old (25 d after vaccination). The results of the strain characterization highlight, on the one hand the field strain circulation, and on the other the persistence of

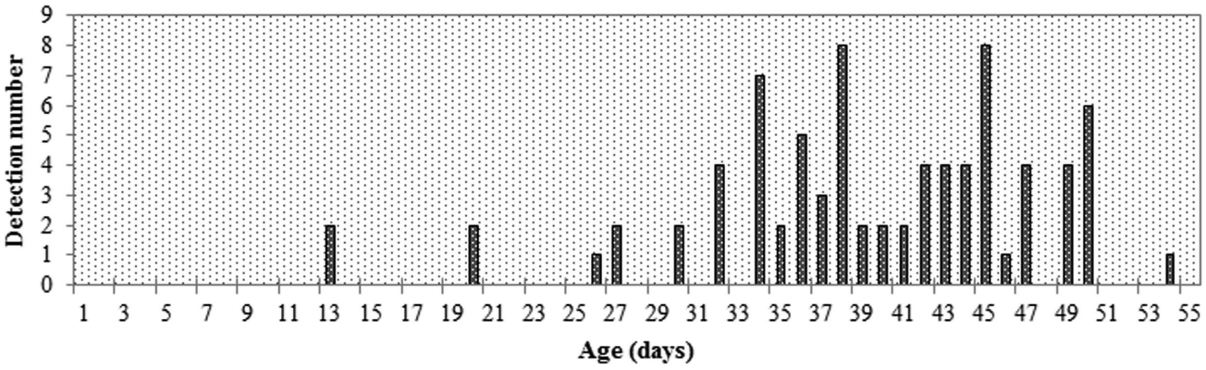


**Figure 2.** aMPV subtype B field strain detections in broilers at different ages from longitudinal studies and one-off samplings.

**Table 2.** Vaccination field trial: sampling points, results and accession numbers from multiplex real time RT-PCR and sequencing.

| Farm/Shed | Sampling point | Age (Days) | Days post vaccination | aMPV | Sequencing |
|---|---|---|---|---|---|
| Farm 1/Shed 1 | 1 | 22 | 11 | Negative | n.p.[a] |
| | 2 | 29 | 18 | Negative | n.p.[a] |
| | 3 | 36 | 25 | aMPV/B | Field strain MF543414 |
| | 4 | 43 | 32 | aMPV/B | Field strain (100% identity with MF543414) |
| Farm 1/Shed 2 | 1 | 22 | 11 | Negative | n.p.[a] |
| | 2 | 29 | 18 | Negative | n.p.[a] |
| | 3 | 36 | 25 | aMPV/B | Field strain (MF543416) |
| | 4 | 43 | 32 | aMPV/B | Field strain (MF543417) |
| Farm 1/Shed 3 | 1 | 22 | 11 | aMPV/B | Field strain MF543415 |
| | 2 | 29 | 18 | aMPV/B | Field strain (100% identity with MF543415) |
| | 3 | 36 | 25 | aMPV/B | Field strain (100% identity with MF543415) |
| Farm 1/Shed 4 | 1 | 22 | 11 | Negative | n.p.[a] |
| | 2 | 29 | 18 | Negative | n.p.[a] |
| | 3 | 36 | 25 | Negative | n.p.[a] |
| Farm 2/Shed 1 | 1 | 22 | 11 | Negative | n.p.[a] |
| | 2 | 29 | 18 | Negative | n.p.[a] |
| | 3 | 36 | 25 | Negative | n.p.[a] |
| | 4 | 43 | 32 | Negative | n.p.[a] |
| Farm 2/Shed 2 | 1 | 22 | 11 | aMPV/B | Vaccine strain (573–03/15) |
| | 2 | 29 | 18 | Negative | n.p.[a] |
| | 3 | 36 | 25 | aMPV/B | Vaccine strain (100% identity with 573–03/15) |
| | 4 | 43 | 32 | Negative | n.p.[a] |

[a]n.p., not performed.

the vaccine strain. Despite aMPV field strain detection, respiratory signs were not observed in any of the sheds, indicating the potential advantages of the vaccination in controlling aMPV infection in broilers, particularly in areas characterized by a high infection pressure and delicate balance among all the possible factors in the respiratory complex.

Finally, no aMPV subtype A strain was found, setting aside its circulation in Italy, where the previous detections were found to be of vaccine origin (Catelli et al., 2006; Lupini et al., 2011). The current study gives a comprehensive picture of field aMPV circulation in broiler farms, in the main poultry producing region of Italy and provides some preliminary results regarding the use of vaccination to control aMPV in broilers.

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