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A novel array of real-time RT-PCR assays for the rapid pathotyping of type I avian paramyxovirus (APMV-1)

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ABSTRACT

Newcastle disease (ND) caused by virulent avian paramyxovirus type I (APMV-1) is a WOAH and EU listed disease affecting poultry worldwide. ND exhibits different clinical manifestations that may either be neurological, respiratory and/or gastrointestinal, accompanied by high mortality. In contrast, mild or subclinical forms are generally caused by lentogenic APMV-1 and are not subject to notification. The rapid discrimination of virulent and avirulent viruses is paramount to limit the spread of virulent APMV-1. The appropriateness of molecular methods for APMV-1 pathotyping is often hampered by the high genetic variability of these viruses that affects sensitivity and inclusivity. This work presents a new array of real-time RT-PCR (RT-qPCR) assays that enable the identification of virulent and avirulent viruses in dual mode, i.e., through pathotype-specific probes and subsequent Sanger sequencing of the amplification product. Validation was performed according to the WOAH recommendations. Performance indicators on sensitivity, specificity, repeatability and reproducibility yielded favourable results. Reproducibility highlighted the need for assays optimization whenever major changes are made to the procedure. Overall, the new RT-qPCRs showed its ability to detect and pathotype all tested APMV-1 genotypes and its suitability for routine use in clinical samples.

1. Introduction

Avian paramyxoviruses type 1 (APMV-1) of the *genus* Avian orthoavulavirus 1 (AOaV-1) (ICTV, 2017) have been reported in over 240 domestic and wild bird species worldwide (Barbezange and Jestin, 2003; Jindal et al., 2009; Hoque et al., 2012; Snoeck et al., 2013; Dodovski et al., 2017; Napp et al., 2017; Absalón et al., 2019; Hicks et al., 2019; Mansour et al., 2021; Steensels et al., 2021; Mngumi et al., 2022; Nooruzzaman et al., 2022; Sun et al., 2022; Wang et al., 2022; Goraichuk et al., 2023) and display considerable variation in their pathogenicity, i.e., from subclinical infections to acute disease with high mortality, depending on the strain and host factors (Suarez et al., 2020). The severity of clinical signs triggered by viral intracerebral inoculation of 1-day-old SPF chickens (intracerebral pathogenicity index, ICPI) is used as a starting ground to define a harmonized categorization of APMV-1 pathotype as velogenic (i.e., highly virulent strains, viscerotropic or neurotropic), mesogenic (viruses of intermediate virulence producing respiratory and nervous disorders) or lentogenic (i.e., avirulent and low pathogenic viruses causing mild/subclinical signs) (WOAH, 2021). Velogenic and mesogenic APMV-1 strains are formally recognized as causative agents of Newcastle disease (ND), a World Organisation for Animal Health (WOAH) and European Union (EU) listed disease which has become a global economic burden given i) the high mortality in affected poultry and ii) the impact of control measures

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applied to curtail the spread of the infection. As an alternative to *in vivo* pathogenicity testing, the WOAH and the EU have accepted sequencing of the cleavage site (CS) of the fusion protein gene precursor (F0) for ND case definition (Commission Delegated Regulation EU, 2020/689, 2019; WOAH, 2022a). As a matter of fact, the presence of multiple basic amino acids that permit fusion protein cleavability by ubiquitous host proteases (furin-like) and subsequent systemic infection is a predictor of high pathogenicity in chickens. Contrarily, avirulent and low pathogenic viruses are characterized by monobasic CS that limits their intra-host spread (Toyoda et al., 1987; Pritzer et al., 1990; Alexander, 2000; WOAH, 2021).

The variety of virulence profiles of APMV-1 is accompanied by great genetic diversity, most likely resulting from the extensive viral circulation in poultry and wild birds and from vaccine-induced immune pressure (Ramey et al., 2017). In 2019, the joint effort of a network of experts led to a unified classification of APMV-1 in 2 classes and 21 genotypes, based on the phylogeny of the complete fusion protein gene (F). Class I consists of genotype 1, and three subgenotypes mostly referable to lentogenic viruses detected in wild birds. Class II envisages the highest genetic variability comprising velogenic, mesogenic and lentogenic viruses of 20 genotypes (I-XIV, XVI-XXI) detected in domestic and wild birds, as well as a plethora of subgenotypes and variants with different hierarchical levels (Dimitrov et al., 2019; Twabela et al., 2021). Genotype VI shows the highest genetic diversity and includes the widespread APMV-1 pigeon variant (PPMV-1) (Akhtar et al., 2016; Napp et al., 2017; He et al., 2018; Zhan et al., 2021; Rogers et al., 2021; Ramsubeik et al., 2023). Most of the live vaccine strains used worldwide for the immunization of flocks also derive from class II viruses, more specifically from lentogenic and mesogenic viruses of genotypes I-III (Hu et al., 2022; Mayers et al., 2017).

The rapid recognition of a ND case is of utmost importance so that health authorities may timely establish control measures to prevent the spread of the disease. By far, diagnosticians prefer using molecular methods rather than *in vivo* pathogenicity tests, both for ethical reasons related to animal welfare and for a rapid reporting of laboratory results. However, the sensitivity of such methods is hampered by the high genetic variability among different APMV-1 strains. This complexity is well represented by the variety of assays for APMV-1 characterization ($n \geq 15$) reported by laboratories taking part to the annual proficiency test organized by the EURL for avian influenza (AI) and ND, that highlights the lack of a universal test for APMV-1 pathotyping (data source: EURL Proficiency test AQUA IN 2022 for Avian Influenza and Newcastle Disease, Final Report). The majority of the reported protocols are based on RT-PCR followed by Sanger sequencing of the amplification product, which significantly increases time-to-results. To improve sensitivity, an isolation step in embryonated SPF chicken eggs prior genome amplification might also be performed, further rising turnaround time.

Real-time RT-PCR (RT-qPCR) based on hydrolysis probes would enable faster APMV-1 pathotyping with a higher level of sensitivity and specificity, although their design is challenged by the identification of APMV-1 genome regions discriminative of virulent and avirulent viruses and, at the same time, conserved among genotypes to guarantee assay inclusivity and sensitivity. In the past, several attempts were made to accomplish this ambitious goal (Wise et al., 2004; Kim et al., 2006; Fuller et al., 2009), but the routine use of these methods on newly emerged variants highlighted poor performance. Sabra et al., 2017 modified the assay developed by Kim et al., 2006 to improve detection of pigeon-derived virulent APMV-1 of genotypes VI and XXI from Egypt, Pakistan, South Korea, Ukraine and Bulgaria. More recently, Bhande et al., 2023 have modified the F gene RT-qPCR by Wise et al., 2004 to detect current APMV-1, using a multi sequence alignment (MSA) of genotypes V, VI, VII, XIII, XIX and XXI. These methods base their pathotyping strategy on the detection of virulent strains only and do not foresee a crosscheck with the simultaneous use of oligonucleotide sets targeting lentogenic viruses. This approach may have potential limitations and lead to false negatives, considering the observation of mismatches of these oligonucleotides sets against targeted genotypes currently circulating in Europe (i.e., VI, VII, XIII, XXI) and the poor coverage with respect to untargeted genotypes that might be introduced by migratory birds (Hicks et al., 2019). In addition, as virulent-specific assays do not foresee the detection of lentogenic and avirulent strains in the sample (including live vaccines), the probability of diagnostic dropouts of such pathotype-specific assays increases as a result of the limited replication and lower viral load of virulent strains in infected or vaccinated birds.

This study intends to address the need for a rapid and updated pathotyping system for APMV-1 by developing an array of three RT-qPCRs capable of distinguishing virulent and avirulent viruses, putatively of any current genotype. Discrimination is first accomplished with pathotype specific probes that match the CS sequence of virulent (i.e., velogenic and mesogenic APMV-1 with polybasic CS, hereafter referred as “Vir”) and avirulent (i.e., lentogenic and low pathogenic APMV-1 with monobasic CS, hereafter referred as “Avir”) viruses. For further downstream confirmation, amplification products can undergo Sanger sequencing to determine the F protein CS sequence.

2. Material and methods

2.1. Assays design

The nucleotide sequence of the F gene of APMV-1 strains detected worldwide in different bird species was downloaded from the Virus Pathogen Resource (ViPR) (Pickett et al., 2012) on 4th July 2019.

MAFFT version 7 (Multiple Alignment with Fast Fourier Transform) online server set with default parameters (Katoh et al., 2019; Kuraku et al., 2013) was employed to align sequences against class I and class II pilot datasets developed by Dimitrov et al., 2019. Geneious Prime 2020.1.2 (https://www.geneious.com) (Biomatters Ltd., Auckland, New Zealand) was used to visualize the MSAs and remove low quality scores yielding datasets of 284 (class I) and 1489 (class II) sequences.

Maximum likelihood phylogenetic trees were generated in IQTREE v1.6.12 (Nguyen et al., 2015) performing an ultrafast bootstrap resampling analysis (1000 replications) (Hoang et al., 2018) and visualized in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Monophyletic groups within tree topologies were used as a guide to identify conserved regions among different genotypes encompassing the F gene CS.

The Entropy-One online tool freely accessible at the HIV Sequence Database (https://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html) was used to calculate Shannon entropy $H(i)$ within genotype and amongst different genotypes, in order to assess the level of nucleotide variation within the identified target regions.

The find-duplicates option available in Geneious Prime 2020.1.2 (Biomatters Ltd., Auckland, New Zealand) was employed to collapse the MSAs. Unique sequences were used to develop three independent oligonucleotides sets (i.e., A, B, C) with pathotype-specific probes, to be employed in simultaneous RT-qPCR reactions to cover the entire APMV-1 variability. In detail, SET A targets virulent and avirulent class II viruses of genotypes I-IV, IX-XI; SET B matches virulent and avirulent class II viruses of genotypes V-VIII, XII-XXI; SET C identifies class I lentogenic APMV-1 (Table 1). PCR products length (149–218 bp) covering APMV-1 CS permits Sanger sequencing to further confirm pathotype assignment. Physical properties of primers and probes were determined with the IDT OligoAnalyzer online tool (https://eu.idtdna.com) (Leuven, Belgium). Finally, their specificity was cross-checked against lentogenic, mesogenic and velogenic APMV-1 comprised in the datasets with Geneious Prime 2020.1.2 (Biomatters Ltd., Auckland, New Zealand).

Upon completion of the validation process, *in silico* specificity was reassessed on 24th October 2022, using unpublished sequences newly made available at the IZSVe during routine diagnostic activities.

Table 1
Primers and pathotype-specific probes for class I and class II APMV-1 pathotyping by RT-qPCR.

Set	Oligonucleotide	Sequence 5'→ 3'	Nt. position in F gene
A	Class II A for	CTC ACC CCY CTT GGT GA	274–457[a]
	Class II A rev	GGA GRA TGT TGG CAG CAT T	
	Class II A avir MGB probe	FAM-CCT ATA AGG CGY CCC TGT YTC-MGB	
	Class II A vir MGB probe	CY5-CCT AYA AAG CGT YTC TGY CTC C-MGB	
B	Class II B for	GAR GCA TAY AAC AGA ACA	244–392[b]
	Class II B rev	GTY GCA ACC CCR AGA GCT A	
	Class II B avir MGB probe	FAM-GAR ACA GGG ACG YCT TAT AGG-MGB	
	Class II B vir MGBa probe	CY5-ARA CGC TTY ATA GGT GC-MGB	
	Class II B vir MGBb probe	CY5-AAR CGY TTT RTA GGT GC -MGB	
C	Class I C for	CMG GGA CAA TTA TCA TCA A	173–390[c]
	Class I C rev	GGC TAC ACC TAA TGC GA	
	Class I C MGB probe	FAM-CAG GAG CGK TTG RTA GG-MGB	

[a] Nucleotide positions refer to strain LaSota (II) with ViPR accession number AF077761.
[b] Nucleotide positions refer to strain PPMV-1/02VIR1875/turtledove/Italy/2002 (VI) with ViPR accession number KU377529.
[c] Nucleotide positions refer to strain DE-R49/99 (class 1) with ViPR accession number DQ097393.

2.2. Experimental settings and optimization of RT-qPCR assays

Pulmonary tissue was obtained from SPF chickens, placed in phosphate buffered saline with antibiotics and antimycotics (10,000 IU/ml penicillin, 10 mg/ml streptomycin, 0.25 mg/ml gentamycin, and 5000 IU/ml nystatin) (PBSa) in a ratio 1:4 w/v, and homogenized with a stainless steel bead for 3 min at 30 Hz using a TissueLyser II (Qiagen, Hilden, Germany). Supernatant was collected after centrifugation for 2 min at 15000 × *g*, and artificially contaminated with representative APMV-1 viruses (SET A: APMV-1/turkey/Italy/14VIR7742/2014 (II); SET B: PPMV/peacock/Italy/13VIR1895/2013 (VI.2.1.1.1); SET C: APMV-1/chicken/Bulgaria/11VIR1897/2011 (class I)) at medium and low concentration. Total nucleic acids were purified from spiked samples using the QIAsymphony DSP Virus/Pathogen Midi kit (Qiagen, Hilden, Germany) on a QIAsymphony SP instrument (Qiagen, Hilden, Germany) (sample volume 300 μl; custom protocol). All the lysates were added with the Intype IC-RNA (Indical Bioscience GmbH, Leipzig, Germany) as foreseen by the upstream APMV-1 screening assay in place at the IZSVe (Sutton et al., 2019).

The performance of different RT-qPCR kits (i.e., AgPath-ID One-Step RT-PCR Reagents, Applied Biosystems, Waltham, MA, USA; GoTaq 1-Step RT-qPCR System, Promega Corporation, Madison, WI, USA; QuantiNova Multiplex RT-PCR Kit, (Qiagen, Hilden, Germany); QuantiTect Multiplex RT-PCR Kit, (Qiagen, Hilden, Germany); TaqMan Fast Virus 1-Step Master Mix, Applied Biosystems, Waltham, MA, USA; SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase, Invitrogen, Walthman, MA, USA) was preliminarily evaluated using the above material. The TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Waltham, MA, USA) exhibited higher sensitivity and lower quantification cycle (Cq) values and was thus selected for subsequent optimization steps. A multi-parametric grid encompassing different combinations of testing conditions (i.e., annealing temperature, magnesium concentration and oligonucleotides concentration) was developed to determine optimal settings for each assay, as assessed by clear amplification plots with the lowest Cq values. Samples were analyzed in duplicate under the different parameters combinations employing 5 μl template in a 25 μl reaction mix. Runs were performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) equipped with VeriFlex blocks allowing different temperature zones, with a ramp/rate of 1.6 °C/s. Data were analyzed using the Design & Analysis Software Version 2.6.2 (Applied Biosystems, Waltham, MA, USA), with auto adjustment of the baseline and single threshold manually set at 0.04 ΔRN. Table 2 outlines optimal RT-qPCR conditions for each assay. PCR products were subject to electrophoresis using the QIAxcel Advanced system (Qiagen, Hilden, Germany) to verify their size. Amplicons pre-diluted 1:5 v/v with molecular grade water were sequenced with a 3130xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). The above protocol was employed for the entire validation process, except otherwise specified.

2.3. Analytical specificity (ASp)

A wide panel of virulent and avirulent APMV-1 isolates representing different genotypes of class I and II ($n = 66$) and previously characterized by the IZSVe and SVA, was tested to assess the inclusivity of the RT-qPCR array and to verify the correct pathotype definition. The simultaneous testing of all the samples with the three assays allowed a thorough evaluation of their capacity to discriminate between virulent and avirulent viruses. Exclusivity was verified by testing non-target bacteria and viruses of avian species, including different APMV subtypes and avian influenza viruses (AIV). The absence of cross reactivity with sample matrix components was checked by testing APMV-1 negative specimens from birds (swabs, tissue homogenates and PBSa). All samples were tested in duplicate (Supplementary Table S1 – sheet ASp) as reported in paragraph 2.2. Alternatively, samples were processed with the IndiMag Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany) on a Maelstrom 9600 Nucleic Acid Extractor (TANBead Taiwan Advanced Nanotech Inc., Taoyuan City, Taiwan) and run on a 7500 Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA) with a ramp/rate of 3.5 °C/s. For these samples, Sanger sequencing of the RT-qPCR products was not performed.

2.4. Limit of detection (LOD) and repeatability

Lung homogenate prepared as described previously was used to serially dilute titrated APMV-1 isolates representing different genotypes ($n = 10$) with diverse levels of match against the oligonucleotide sets developed in the study (Table 3). Spiked samples were analyzed with the respective RT-qPCR assay, based on the targeted genotype, employing the experimental conditions reported in paragraph 2.2. Each dilution

Table 2
Optimal experimental settings for the RT-qPCRs array. Primers and probes concentration refers to each oligonucleotide employed in the reaction.

Set	Oligonucleotides concentration	Thermal cycle
A	1 μM primers, 300 nM probes	50 °C 5 min; 95 °C 20 s; 45 × [95 °C 30 s, 60 °C 45 s]
B	1 μM primers, 250 nM probes	50 °C 5 min; 95 °C 20 s; 45 × [95 °C 30 s, 55 °C 45 s]
C	900 nM primers, 250 nM probe	

Table 3
Analytical sensitivity and repeatability of different APMV-1 genotypes spiked in lung homogenates. The LOD is expressed as EID_{50}/100 µl. The amplification efficiency %*E* and the correlation coefficient are also reported. For each strain, intra- and inter-assay repeatability was expressed as percent coefficient of variation of Cq values. Vir = velogenic and mesogenic APMV-1 with polybasic CS; Avir = lentogenic and low pathogenic APMV-1 with monobasic CS. n.a. = not available; * = vaccine strain.

Strain spiked in lung homogenate	Genotype	Set	LoD EID_{50}/100 µl	%*E*	R^2	%CV
APMV-1 Ulster*	I	A (Avir)	$10^{1,5}$	95.67	0.996	n.a.
APMV-1 V4 like*	I	A (Avir)	$10^{1,5}$	98.50	0.986	≤ 4.2
APMV-1 VG/GA*	I.1.1	A (Avir)	$10^{1,83}$	105.10	0.982	n.a.
APMV-1 B1*	II	A (Avir)	$10^{1,5}$	103.68	0.982	n.a.
APMV-1 LaSota*	II	A (Avir)	$10^{2,83}$	108.30	0.981	n.a.
APMV-1 Herts	IV	A (Vir)	$10^{1,62}$	95.24	0.889	≤ 4.4
APMV-1/chicken/California/18–016505–1/2018	V.1	A (Vir)	$10^{4,62}$	80.83	0.872	≤ 6.5
PPMV-1/pigeon/Italy/19vir8321/2019	VI.2.1.1.2.2	B (Vir)	10^{1}	100.60	0.892	≤ 6.7
APMV-1/bassette chicken/Belgium/4096/2018	VII	B (Vir)	$10^{1,62}$	108.41	0.972	n.a.
APMV-1/chicken/rus/Krasnodar/9.1/2019	VII 1.1	B (Vir)	$10^{2,62}$	92.81	0.996	n.a.
APMV-1/Macedonia/20VIR1984–1/2020	VII.2	B (Vir)	$10^{1,5}$	108.30	0.996	n.a.
APMV-1/chicken/Nigeria/4TACK15–18T_21RS744–46/2020	XIV.2	B (Vir)	$10^{2.62}$	80.15	0.960	n.a.
APMV-1/chicken/Camerun/3490–168/2008	XVII	B (Vir)	$10^{2,83}$	90.65	0.969	n.a.
PPMV-1/pigeon/Luxembourg/18175752/2018	XXI.1.1	B (Vir)	$10^{2,62}$	88.50	0.942	n.a.
APMV-1/avian/Bulgaria/11VIR1897/2011	1	C (Avir)	$10^{1,7}$	81.07	0.990	≤ 2.9

was tested in triplicate in order to determine the LOD (i.e., the highest dilution testing positive by RT-qPCR and yielding the CS sequence). To compare the sensitivity of the pathotyping RT-qPCRs with that of a screening method, the assay by Sutton et al., 2019 was performed in parallel.

Repeatability was tested for APMV-1 showing different analytical sensitivity in lung homogenate and representing either virulent and avirulent strains. In detail, dilutions corresponding to high (i.e., the LOD + 1 $\log_{10}$) and low (i.e., the LOD) viral titres were analyzed in triplicate on three different days by two operators. Repeatability was expressed as the percent coefficient of variation (%CV) of the Cq values recorded within and between days. Only higher dilutions were subject to Sanger sequencing, as described above.

2.5. Diagnostic performance

Clinical samples with known reactivity as per previous testing performed by the IZSVe and SVA were used to evaluate the diagnostic performance of the RT-qPCRs array. The panel comprised 59 APMV-1 samples of different genotypes originating from Europe, Asia, Africa and the Middle East and representing a variety of avian species and matrices, as well as 67 APMV-1 negative samples from experimentally challenged chickens (Bortolami et al., 2022; Zamperin et al., 2021) and wild birds (Supplementary Table S1 – sheet DP). Samples were tested with the three RT-qPCR assays followed by Sanger sequencing as described above, in parallel with the screening protocol by Sutton et al., 2019.

2.6. Reproducibility

To assess the reproducibility of the RT-qPCR assays and their capacity to yield consistent results, an inter-laboratory exercise was organized involving the IZSVe, SVA and FLI. For this purpose, a panel of 11 blind samples (both virulent and avirulent) prepared in PrimeStore MTM (Longhorn Vaccines and Diagnostics, Bethesda, MD, USA) were provided (Supplementary Table S1 – sheet ILR) together with the necessary amplification reagents. The quality standards foreseen by the ISO/IEC 17043:2010 were applied for samples preparation. Each laboratory employed the nucleic acids extraction method (i.e., QIAsymphony DSP Virus/Pathogen Midi kit, Qiagen, Hilden, Germany; IndiMag Pathogen Kit, Indical Bioscience GmbH, Leipzig, Germany; QIAamp Viral RNA Mini Kit, Qiagen, Hilden, Germany) and the real-time platforms (i.e., QuantStudio 5 Real-Time PCR System, Applied Biosystems, Waltham, MA, USA with ramp/rate 1.6 °C/s; 7500 Fast Real-Time PCR System, ThermoFisher Scientific, Waltham, MA, USA with ramp/rate 3.5 or 1.6 °C/s; CFX96 Deep Well Real-Time PCR System, Biorad, Hercules, CA, USA with ramp/rate 3.2 °C/s) in place at their facilities. Fleiss' kappa was calculated to measure the agreement among the three laboratories according to the method proposed by (Falotico and Quatto, 2015) and implemented in the package raters (Quatto and Ripamonti, 2022) under the R environment (R Core Team, 2020). Kappa's confidence interval was calculated using the percentile bootstrap ($n = 1000$). Paired t-tests were conducted on the Cq values recorded by three laboratories to reveal significant differences in the mean values. The resulting p values were adjusted according to Holm's method (Holm, 1979).

To further verify the precision of the pathotyping protocols, the IZSVe tested an additional panel of blind lyophilized samples ($n = 14$) (Supplementary Table S1 – sheet EQA) provided by the FLI, as part of the German external quality assessment (EQA) program. Agreement with the expected results was assessed by Cohen's kappa.

For both the reproducibility exercises, Sanger sequencing of the amplification products was not performed.

3. Results

3.1. Analytical performance

As part of the Stage 1 validation workflow set by the WOAH (2022b), specificity, sensitivity and repeatability were determined for the RT-qPCRs array developed in this study.

The assays proved to be specific, with no evidence of cross reaction against matrix components or non-target avian microorganisms. In contrast, all APMV-1 isolates of classes I and II representing genotypes 1, I, II, VI, VII, XIII, XIV, XVIII, XXI were detected and correctly pathotyped (Supplementary Table S1 – sheet ASp). A few class II isolates reacted both with SET A and SET B regardless of the assays target genotypes, but notably this did not lead to incorrect pathotyping. Being unavailable, additional genotypes could not be tested in this phase.

Analytical sensitivity was assessed for a variety of virulent or avirulent genotypes spiked in lung homogenates to mimic clinical samples, and all but one yielded a LOD ≤ $10^{2,83}$ EID_{50}/100 µl (Table 3). Efficiency and R^2 were within acceptable values (Bustin et al., 2009). All samples employed for analytical sensitivity were correctly pathotyped at any tested dilution. Overall, the LOD of the screening RT-qPCR run for comparison (Sutton et al., 2019) was lower by 1 log10, except for the VG/GA vaccine strain that was detected with a higher sensitivity by SET A (data not shown). Expectedly, only strain APMV-1/chicken/California/18–016505–1/2018 yielded a LOD of $10^{4,62}$ EID_{50}/100 µl. This sample represents genotype V.1 that is circulating

almost exclusively in North and Latin America (Absalón et al., 2019) and shows 1 mismatch at the hybridization region of the forward primer of SET A. An *in silico* verification of the impact of this mismatch resulted in a sharp drop of the primer's melting temperature that most likely affected assay sensitivity.

All the replicates and repeats analyzed for repeatability assessment tested positive and yielded the correct pathotype. At the highest virus concentration, the overall %CV intra- and inter-assay was ≥ 3.2 and 4.9, respectively. Under challenging conditions (i.e., at the LOD), repeatability was slightly lower (%CV between 2.9 and 6.7), although pathotyping was neither impaired by probes' recognition nor by Sanger sequencing of the amplification product.

3.2. Assays application on clinical samples

All APMV-1 positive specimens collected from poultry and wild birds were detected with the RT-qPCRs array and correctly pathotyped (Table 4; Supplementary Table S1 – sheet DP). The samples represent

Table 4
Diagnostic performance of the RT-qPCRs array on APMV-1 positive samples. For each specimen, the type of matrix, the genotype and the reactivity with the different oligonucleotide sets are reported, with specification of the pathotype interpretation (Vir = velogenic and mesogenic APMV-1 with polybasic CS; Avir = lentogenic and low pathogenic APMV-1 with monobasic CS) according to the reactive probe. The CS sequence (aa 113–118) obtained from the RT-qPCR product is also reported, when available.

Sample	Source	Genotype	SET A	SET B	SET C	CS
APMV1_PPMV_pigeon_Sweden_SVA29_03_NGS46_2003	Intestine	VI	Vir	Vir	Neg	Not performed
APMV-1/dove/Italy/11VIR7110–9/2011	Intestine	VI	Vir	Vir	Neg	RRQKR*F
APMV-1/chicken/Bangladesh/11VIR1915–19/2011	Trachea	XIII	Neg	Vir	Neg	RRQKR*F
PPMV-1/pigeon/Italy/12VIR5499/2012	Stomach	VI.2.1.1.1	Vir	Vir	Neg	RRQKR*F
APMV-1/turkey/Italy/13VIR4184/2013	Trachea	I	Avir	Neg	Neg	Unsuccessful
APMV-1/chicken/Egypt/13VIR5009–21/2013	Lung/Trachea	II	Avir	Avir	Neg	GRQGR*L
PPMV-1/peacock/Italy/13VIR1895/2013	Organ	VI.2.1.1.2	Vir	Vir	Neg	RRQKR*F
APMV-1/chicken/Jordan/13VIR2720–2/2013	Organ	VII.1.1	Neg	Vir	Neg	RRQKR*F
APMV-1/chicken/Jordan/13VIR2720–4/2013	Organ	VII.1.1	Neg	Vir	Neg	RRQKR*F
APMV-1/chicken/Egypt/13VIR2962–116/2013	Trachea	VII.1.1	Neg	Vir	Neg	RRQKR*F
APMV-1/chicken/Ivory Coast/17RS804–26/2013	Organ	XVIII.2	Neg	Vir	Neg	RRQKR*F
PPMV-1/avian/Ethiopia/14VIR4296–4/2014	Intestine	VI.2.1.2	Neg	Vir	Neg	RRRKR*F
APMV-1/chicken/Italy/15VIR2663–3/2015	Lung	II	Avir	Neg	Neg	GRQGR*L
APMV-1/avian/Libya/15VIR5368/2015	FTA card	VII.2	Vir	Vir	Neg	RRQKR*F
APMV-1/avian/Libya/15VIR5371/2015	FTA card	VII.2	Neg	Vir	Neg	RRQKR*F
APMV-1/chicken/Nigeria/15VIR1737–3/2015	Organ	XIV.2	Neg	Vir	Neg	Unsuccessful
APMV-1/chicken/Nigeria/15VIR1737–4/2015	Organ	XIV.2	Neg	Vir	Neg	Unsuccessful
APMV-1/avian/Burkina Faso/15VIR2488–8/2015	Swab	XVIII	Neg	Vir	Neg	RRRKR*F
APMV-1/avian/Libya/15VIR5369/2015	Swab	XXI.1.1	Neg	Vir	Neg	KKRKR*F
APMV-1/turkey/Italy/16VIR3537/2016	Trachea	I.1	Avir	Neg	Neg	Unsuccessful
PPMV-1/pigeon/Italy/16VIR276–2/2016	Organ	VI.2.1.1.2	Vir	Vir	Neg	RRQKR*F
APMV-1/pigeon/Italy/16VIR1463–7/2016	Organ	VI	Neg	Vir	Neg	RRRKR*F
APMV-1/chicken/Sudan/16VIR4763–1/2016	Organ	VII.1.1	Neg	Vir	Neg	Unsuccessful
APMV-1/chicken/Sudan/16VIR4763–2/2016	Organ	VII.1.1	Neg	Vir	Neg	RRQKR*F
APMV-1/turkey/Italy/17VIR4141–1/2017	Trachea	I.1	Avir	Avir	Neg	GKQGR*L
APMV-1/turkey/Italy/17VIR7229–1/2017	Trachea	I.1	Avir	Avir	Neg	GKQGR*L
APMV-1/turkey/Italy/17VIR10741–2/2017	Trachea	I.1	Avir	Neg	Neg	GKQGR*L
APMV-1/turkey/Italy/17VIR10741–6/2017	Trachea	II	Avir	Neg	Neg	GKQGR*L
PPMV-1/pigeon/Italy/17VIR2719/2017	Organ	VI.2.1.1.2.2	Vir	Vir	Neg	RRQKR*F
PPMV-1/dove/Italy/17VIR7677–1/2017	Organ	VI.2.1.1.2.2	Vir	Vir	Neg	RRQKR*F
APMV-1/pigeon/Italy/17VIR7739/2017	Organ	VI	Neg	Vir	Neg	Unsuccessful
APMV-1/pigeon/Italy/17VIR8019/2017	Brain	VI	Vir	Vir	Neg	RRQKR*F
APMV-1/shoveler/Italy/18VIR1199/2018	Organ	I.2	Avir	Neg	Neg	Unsuccessful
APMV-1/Eurasian teal/Italy/18VIR1279–1/2018	Organ	I.2	Avir	Neg	Neg	Unsuccessful
PPMV-1/pigeon/Italy/18VIR9029–1/2018	Cloacal swab	VI.2.1.1.2	Vir	Vir	Neg	RRRKR*F
PPMV-1/pigeon/Italy/18VIR1275/2018	Organ	VI.2.1.1.2.2	Neg	Vir	Neg	RRQKR*F
PPMV-1/pigeon/Italy/18VIR3373–1/2018	Cloacal swab	VI.2.1.1.2.2	Vir	Vir	Neg	RRQKR*F
PPMV-1/pigeon/Italy/18VIR3373–3/2018	Cloacal swab	VI.2.1.1.2.2	Vir	Vir	Neg	RRQKR*F
PPMV-1/pigeon/Italy/18VIR9028–1/2018	Cloacal swab	VI.2.1.1.2.2	Vir	Vir	Neg	RRRKR*F
PPMV-1/pigeon/Italy/18VIR9028–2/2018	Cloacal swab	VI.2.1.1.2.2	Vir	Vir	Neg	RRRKR*F
PPMV-1/pigeon/Italy/18VIR9028–3/2018	Cloacal swab	VI.2.1.1.2.2	Vir	Vir	Neg	RRRKR*F
APMV-1/pigeon/Italy/18VIR7466–1/2018	Brain	VI	Neg	Vir	Neg	RRQKR*F
APMV-1/pigeon/Italy/18VIR7466–2/2018	Intestine	VI	Vir	Vir	Neg	RRQKR*F
APMV-1/avian/Congo/18VIR3696–2/2018	Cloacal swab	XIII	Neg	Vir	Neg	RRQKR*F
APMV-1/dove/Italy/19VIR8422–3/2019	Organ	XXI.2	Vir	Vir	Neg	RRQKR*F
PPMV-1/pigeon/Italy/20VIR3033–1/2020	Trachea	VI.2.1.1.2.2	Neg	Vir	Neg	RRQKR*F
APMV-1/pigeon/Cyprus/20VIR3543–3/2020	Intestine	VI.2.1.1.2.2	Neg	Vir	Neg	RRQKR*F
PPMV-1/pigeon/Italy/20VIR7088/2020	Organ	VI.2.1.1.2.2	Vir	Vir	Neg	RRQKR*F
APMV-1/chicken/North Macedonia/20VIR1984–1/2020	Lung	VII.2	Neg	Vir	Neg	RRQKR*F
APMV-1/pigeon/Cyprus/20VIR3543–9/2020	Intestine	XXI.1.1	Vir	Neg	Neg	RRQKR*F
APMV-1/dove/Italy/20VIR7750–9/2020	Organ	XXI.2	Vir	Vir	Neg	RRQKR*F
APMV-1/chicken/Italy/21VIR407–17/2021	Intestine	I.1.1	Avir	Avir	Neg	GKQGR*L
APMV-1/chicken/Italy/21VIR2608–16/2021	Organ	I.1.1	Avir	Avir	Neg	GKQGR*L
PPMV-1/avian/Italy/21VIR138–1/2021	Brain	VI.2.1.1.2	Vir	Vir	Neg	RRQKR*F
PPMV-1/avian/Italy/21VIR750–1/2021	Organ	VI.2.1.1.2.2	Vir	Vir	Neg	RRQKR*F
PPMV-1/dove/Italy/21VIR72–1/2021	Lung	VI.2.1.2.2	Vir	Vir	Neg	RRQKR*F
APMV-1/pigeon/Italy/21VIR1109–8/2021	Intestine	VI/I	Avir	Vir	Neg	RRQKR*F/GKQGR*L
APMV-1_chicken_Sweden_SVA230302SZ0005_M-2023	Cloacal swab	I	Avir	Avir	Neg	Not performed
APMV-1_PPMV_Pigeon_Sweden_SVA1469	Intestine	VI	Neg	Vir	Neg	Not performed

genotypes I, II, VI, VII, XIII, XIV, XVIII and XXI from different countries in the European, Asian and African continents, thus confirming data previously obtained during analytical specificity assessment. Due to the lack of samples from the Americas and from Asian countries other than Jordan and Bangladesh, it was not possible to validate the RT-qPCR assays on the genotypes circulating in these areas. As observed during analytical validation, several samples cross reacted both with SET A and SET B while achieving correct pathotyping with both assays. A few cases showed high Cq values (≥ 40), although the assays led to correct virulent/avirulent discrimination by probes. The absence of a linear relationship with Cq values yielded by the screening protocol by Sutton et al., 2019 suggests that sensitivity of SET A and SET B appeared mostly strain-dependent and only in part determined by the viral load in the sample (Supplementary Table S1 – sheet DP). The amplification products of 8 samples out of 59 APMV-1 did not yield a clear sequence chromatogram. On the other hand, for 48 samples the CS sequence could also be obtained, further confirming the correct pathotype assignment by probes. Notably, 4 clinical samples of genotype VI that could not be directly pathotyped with the RT-PCR protocols developed by Kant et al., 1997 and De Battisti et al., 2013, were recognized by SET A and SET B (Supplementary Table S1 – sheet DP). Of these, 3 were successfully sequenced downstream the RT-qPCR.

No cross reaction was observed with APMV-1 negative samples, i.e., SPF chickens challenged with H9N2 or H7N1 as well as wild birds collected in Italy in 2022 (Supplementary Table S1 – sheet DP).

3.3. Reproducibility

The parallel testing of APMV-1 samples revealed an almost perfect agreement among laboratories (Fleiss kappa = 0.88; 95% CI: 0.64–1) (Nichols et al., 2010). Only one specimen was missed by Lab 2, while the remaining samples were detected and correctly pathotyped by all participants. Notably, Cq values varied significantly between laboratories. In detail, Lab 1 showed Cq values systematically lower than Lab 2 and Lab 3 (with $p < 0.001$) (Fig. 1; Supplementary Table S1 – sheet ILR). Lab 3 performed the exercise applying different ramp/rate during thermal cycling (i.e., 1.6 and 3.5 °C/s). Four out of 11 (4/11) samples yielded Cq > 40 and irregular amplification curves with the highest ramp/rate. Decreasing the speed of temperature change down to 1.6 °C/s resulted in an improved detection and lower Cq values.

All the samples included in the panel for the APMV-1 EQA program were detected and properly categorized as virulent or avirulent, yielding a Cohen's kappa of 1.

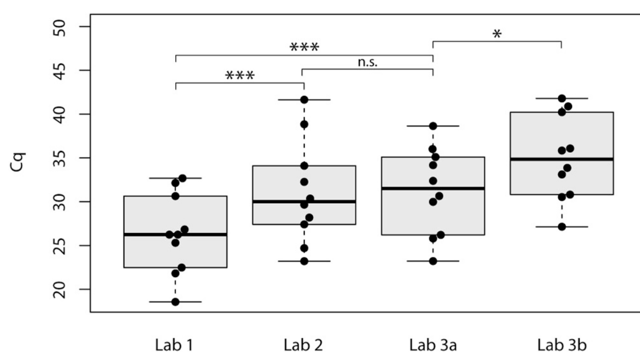

Fig. 1. Reproducibility study involving three different diagnostic laboratories (Lab1, Lab2, Lab3). Cq values are reported as dots along the *y*-axis. All laboratories employed the same batch of reagents and amplification conditions, while nucleic acids purification systems and real-time PCR instruments are reported in Supplementary Table S1 (sheet ILR) for each participant. Results obtained by Lab3 are reported for both the ramp/rates utilized: (a) = 1.6 °C/s and (b) = 3.5 °C/s. Asterisks indicate different levels of statistical significance of paired t-tests (*** = $p < 0.001$; * = $p < 0.05$); n.s. = not significant.

4. Discussion

For the initial assessment of APMV-1 pathotype, sequence determination of the cleavage site of the F protein gene is largely preferred over ICPI determination, as it reduces the use of live animals and costs, and shortens the time-to-results. However, this task is rather demanding for veterinary diagnosticians due the high genetic variability of APMV-1 that hampers effectiveness of existing molecular methods (Bhande et al., 2023; Sabra et al., 2017). Consequently, laboratories often decide to use multiple protocols to compensate for the lack of sensitivity and inclusivity of individual methods. In compliance with the WOAH recommendations (WOAH, 2021) this choice should carefully consider the genotypes that circulate locally, but also contemplate the possible introduction of viruses conveyed by wild birds. The constant threat for poultry species posed by *Columbiformes* as reservoirs of virulent PPMV-1 (Brown and Bevins, 2017; Hicks et al., 2019; Annaheim et al., 2022; Goraichuk et al., 2023) and the increasing number of ND outbreaks linked to the introduction of class II velogenic genotypes from wild birds (data source: https://www.izsvenezie.com/reference-laboratories/avian-influenza-newcastle-disease/workshops/) are issues of great concern, which would require the availability of widely inclusive molecular tools capable of distinguishing virulent and avirulent viruses with specificity.

The aim of this study was to develop and validate a reliable and widely inclusive array of RT-qPCRs for the determination of APMV-1 pathotype in clinical samples, in dual mode. Specific probes targeting the F gene CS and labeled with diverse dyes enable the discrimination of virulent and avirulent viruses. The size of the amplification product offers the chance to further confirm the pathotype assignment through Sanger sequencing and determination of the CS sequence. The design of the array consisting of one reaction for class I viruses, and two reactions for class II strains, allows the use of less degenerated oligonucleotides with higher affinity for the target region of different genotypes. However, mutations in primers and probes hybridization regions might result in a decrease of sensitivity, as demonstrated during LOD determination for strain APMV-1/chicken/California/18–016505–1/2018. Consistently with this observation, laboratory tests on clinical samples showed a certain variation in the biases of Cq values with respect to the screening RT-qPCR by Sutton et al., 2019, without however affecting the correct discrimination of virulent and avirulent viruses by pathotype-specific probes, even at high Cq. As a result, this prevented the identification of an unambiguous diagnostic cut-off value. Notably, the CS sequence was also determined for samples yielding high Cq values (i.e., 35 – 39). This is of great utility to confirm the pathotype for doubtful samples with a low viral load, and allows laboratories to perform pathotyping in dual mode or by RT-qPCR only, based on the equipment available at their facility and on the existing knowledge of the epidemiological situation and the reporting times required. As a matter of fact, remote areas where APMV-1 is endemic in poultry and domestic birds (Alexander et al., 2004) most often do not have sequencing facilities and could take advantage from this possibility. While leaving these considerations to end-users, the authors recommend Sanger sequencing of the array RT-qPCRs amplification products for samples with Cq ≥ 35; in absence of CS determination, such samples should be considered not typeable. Contrarily, for specimens with lower Cq values, CS sequencing downstream RT-qPCR can be optional.

Another key feature of the array of RT-qPCRs developed in this study is the possibility to detect co-infections of virulent and avirulent viruses. Sample APMV-1/pigeon/Italy/21VIR1109–8/2021 well exemplifies this casuistry. As a matter of fact, the application of the RT-qPCR assays revealed the presence of both mesogenic and lentogenic strains in the intestine of a domestic pigeon. The same sample produced no amplification product with the RT-PCR by De Battisti et al., 2013, while it tested positive by Kant et al., 1997 yielding a CS sequence typical of virulent PPMV-1. Upon isolation and re-testing with the protocol by De Battisti et al., 2013, the sample was successfully amplified and typed as

avirulent (vaccine strain of genotype I). Notably, the RT-qPCRs array applied to samples collected from SPF chickens subject to intracerebral inoculation, confirmed the co-presence of virulent and avirulent viruses. The deep investigation of this case highlights the potential of the RT-qPCRs array to detect co-infections in clinical samples without introducing biases. In contrast, APMV-1 pathotyping based on sequencing only, might yield inconsistent results with respect to ICPI, as the consensus sequence obtained rather frequently reflects the most abundant virus in the sample (Naguib et al., 2022). In addition, the RT-qPCRs array represents an attractive opportunity as DIVA (Differentiating Infected from Vaccinate Animals) strategy to monitor the circulation of virulent APMV-1 in vaccinated animals. Such a principle was employed by Moharam et al., 2019 who succeeded in detecting virulent APMV-1 in vaccinated flocks farmed in Egypt under low biosecurity standards, through the application of assays targeting genotype VII-b and LaSota clone 30.

During the validation process, the reproducibility of the RT-qPCR assays and their transferability to other laboratories were also evaluated through a collaborative study. Although the three participants correctly pathotyped all the detected samples, the bias of Cq values recorded among laboratories was indeed striking. The exercise envisaged the use of diverse nucleic acids systems and PCR platforms. It can be assumed that the different ramp/rates applied during thermal cycling might have affected the kinetics of the reaction, as previously demonstrated by other authors (Derendinger et al., 2018). Indeed, the comparison of laboratory data obtained by Lab3 with different ramp/rates confirms this hypothesis. In addition to ramp/rate, other major changes are known to influence molecular assays performance (e.g., nucleic acids system, amplification kit, temperature and duration of amplification steps, magnesium concentration, etc.). Thus, upon modification of the protocol herein described, it is recommended to optimize amplification settings based on the reagents and instruments employed and to verify the protocol under each laboratory specific conditions.

In short, the array of RT-qPCRs proposed in this study proved to be fit for APMV-1 molecular pathotyping both in the isolates and clinical samples herein analyzed. An evaluation of the array performance would be required also for genotypes not included in the study, although primers and probes were designed to maximize their coverage. However, as repeatedly remarked, the high genetic variability of APMV-1 is a threat for the effectiveness of molecular tools employed either for screening or typing, and the newly developed array of RT-qPCRs is not exempt from this issue. For this reason, in order to exploit its best potential, we recommend making a periodic *in silico* re-assessment of its performance as new sequences become available; in addition, laboratories are required to evaluate its use based on the genotypes and variants circulating in the target population. Importantly, molecular pathotyping may not always be predictive of the real pathogenic power of APMV-1 and PPMV-1 in poultry, thus *in vivo* tests should not be completely dismissed.

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CRediT authorship contribution statement

Conceptualization, **Isabella Monne** and **Valentina Panzarin**; Data curation, **Andrea Fortin**, **Viviana Valastro**, **Alessio Bortolami, Michele Gastaldelli** and **Valentina Panzarin**; Formal analysis, **Andrea Fortin**, **Siamak Zohari**, **Kristofer Andersson**, **Christian Grund**, **Marika Crimaudo**, **Viviana Valastro**, **Valeria D’Amico** and **Alessio Bortolami**; Funding acquisition, **Isabella Monne**, **Mattia Cecchinato** and **Calogero Terregino**; Investigation, **Andrea Fortin**, **Isabella Monne**, **Alessio Bortolami**, **Calogero Terregino** and **Valentina Panzarin**; Methodology, **Andrea Fortin**, **Andrea Laconi**, and **Valentina Panzarin**; Project administration, **Isabella Monne**, **Andrea Laconi** and **Valentina Panzarin**; Resources, **Siamak Zohari**, **Kristofer Andersson**, **Christian Grund**, **Isabella Monne**, **Viviana Valastro**, **Alessio Bortolami**, **Newcastle Disease Collaborating Diagnostic Group** and **Calogero Terregino**; Software, **Andrea Fortin**, **Andrea Laconi**, **Michele Gastaldelli** and **Valentina Panzarin**; Supervision, **Calogero Terregino** and **Valentina Panzarin**; Validation, **Andrea Fortin**, **Siamak Zohari**, **Kristofer Andersson**, **Christian Grund**, **Marika Crimaudo**, **Viviana Valastro**, **Valeria D’Amico**, **Maria Varotto** and **Alessio Bortolami**; Visualization, **Andrea Fortin** and **Valentina Panzarin**; Writing – original draft, **Andrea Fortin** and **Valentina Panzarin**; writing – review & editing, all the authors; all authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Newcastle Disease Collaborating Diagnostic Group

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Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2023.114813.

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