29th Annual Meeting

of the National Reference Laboratories for Avian Influenza and Newcastle Disease of European Union Member States

Istituto Zooprofilattico Sperimentale delle Venezie

Parma
2-3 October 2023

An update on molecular detection methods

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Development and validation of an array of RT-qPCR for the molecular pathotyping of APMV-1

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Diagnostic workflow for APMV-1 at EURL

APMV-1 screening
SOP 151 (Sutton)

Negative

Positive

In vivo pathotyping
of **index case**
SOP 007 (ICPI)

ICPI ≥ 0,7 virulent

Molecular pathotyping
SOP 1002 (Kant)

Negative

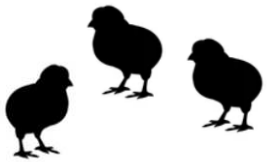
Molecular pathotyping
SOP 063 (De Battisti)

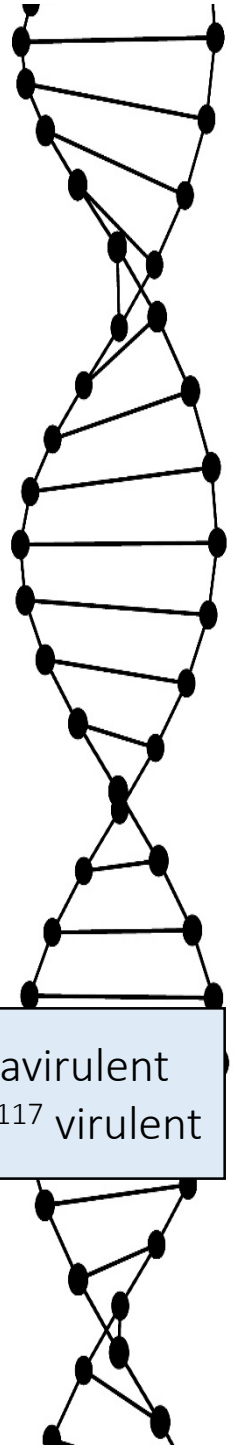
Negative

Positive

Positive

^{112}G-R-Q/K-K-R-F^{116} L^{117} avirulent
^{112}R/K-R-Q/K/R-K/R-R^{116} F^{117} virulent





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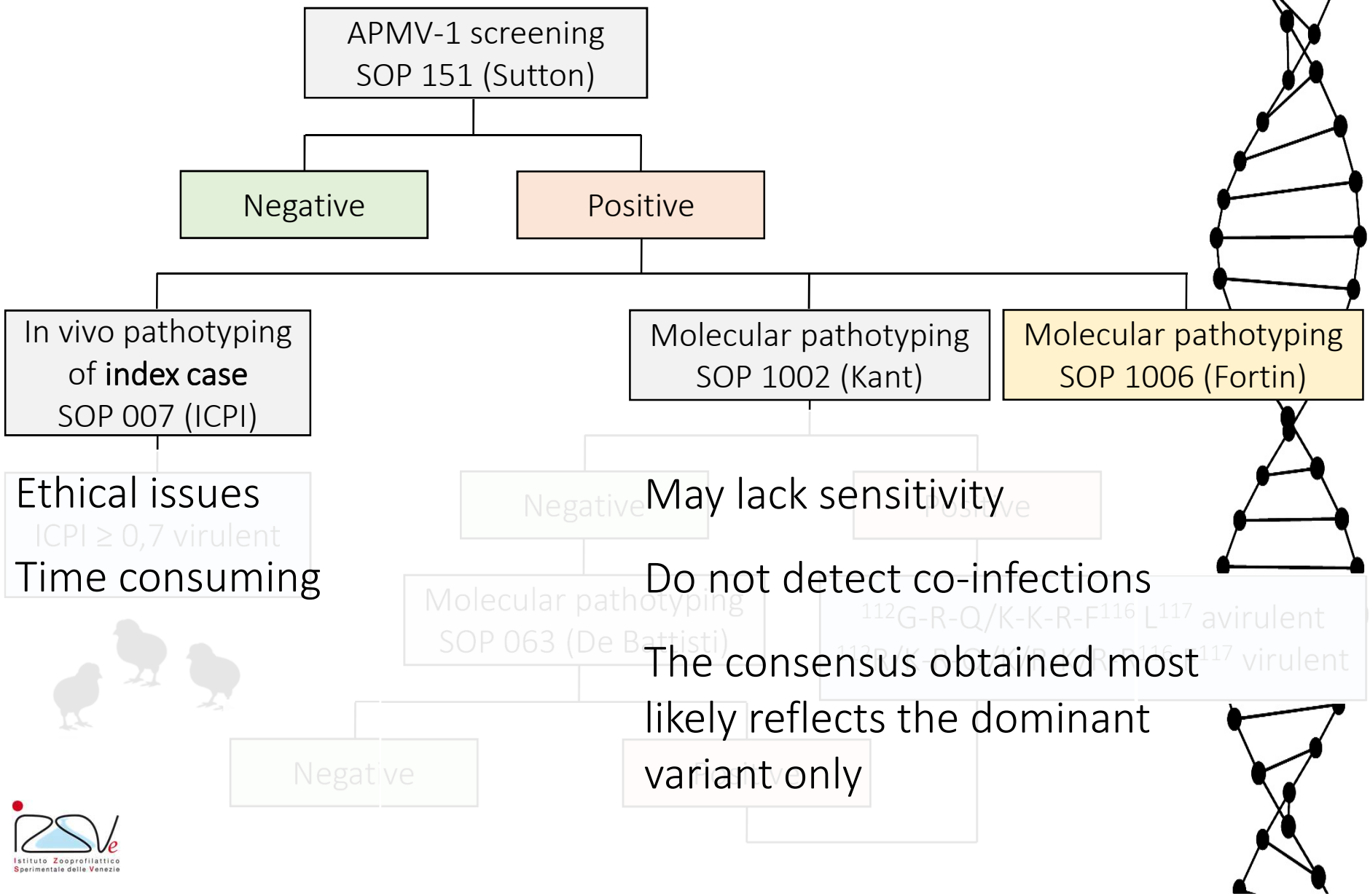
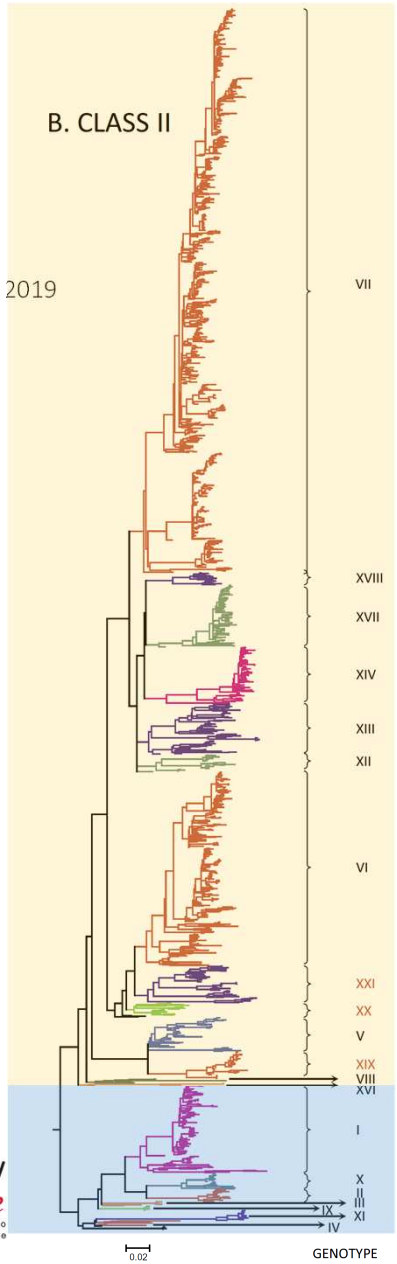
Diagnostic workflow for APMV-1 at EURL



Set up of the array of RT-qPCR targeting the F gene

Source:
Dimitrov et al., 2019

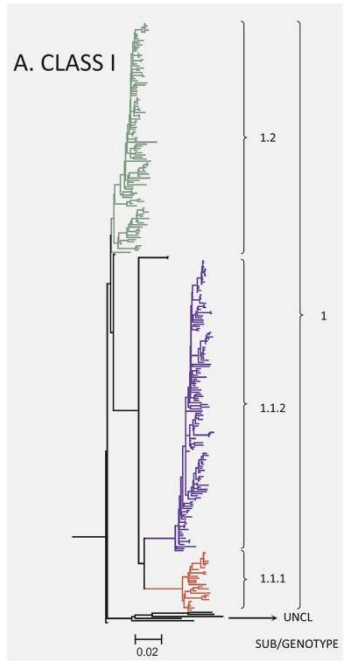


Array of RT-qPCR

SET A	SET B	SET C
common primers 1 probe avirulent 1 probe virulent	common primers 1 probe avirulent 2 probes virulent	primers 1 probe avirulent

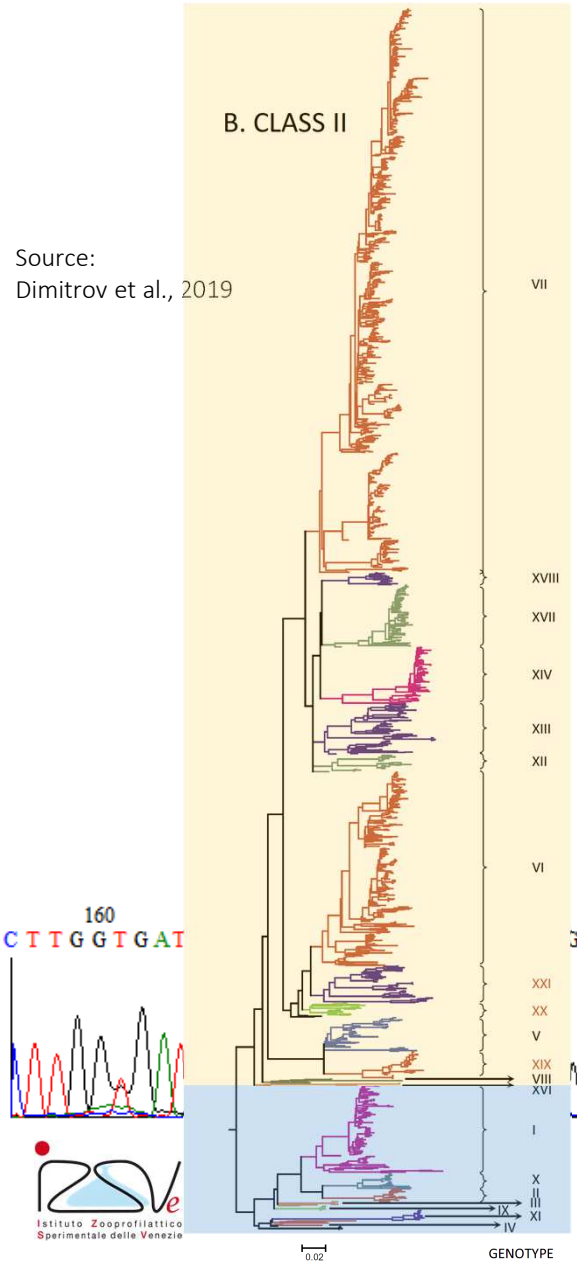
incl. PPMV-1

incl. vaccine strains



Pathotyping in dual mode

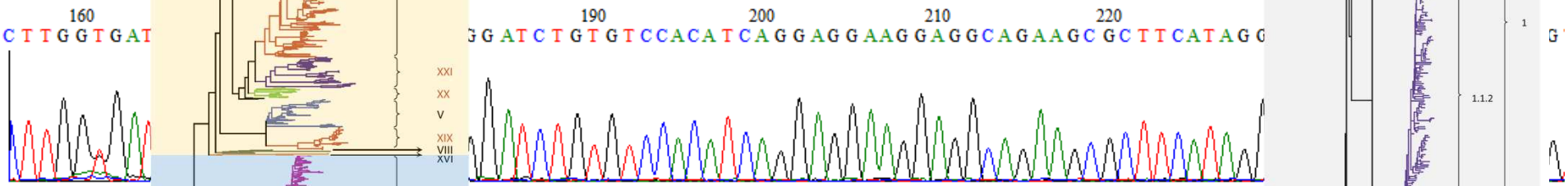
Source:
Dimitrov et al., 2019

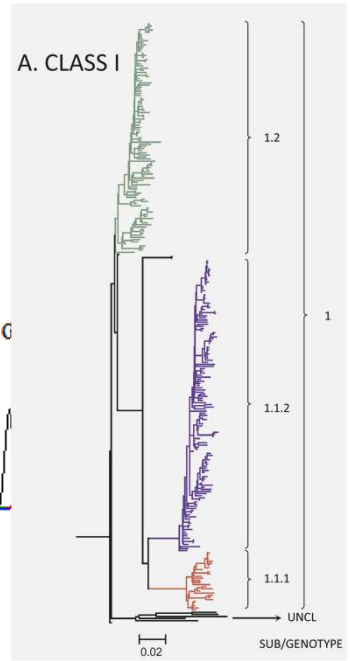


Array of RT-qPCR

SET A	SET B	SET C
common primers 1 probe avirulent 1 probe virulent	common primers 1 probe avirulent 2 probes virulent	primers 1 probe avirulent

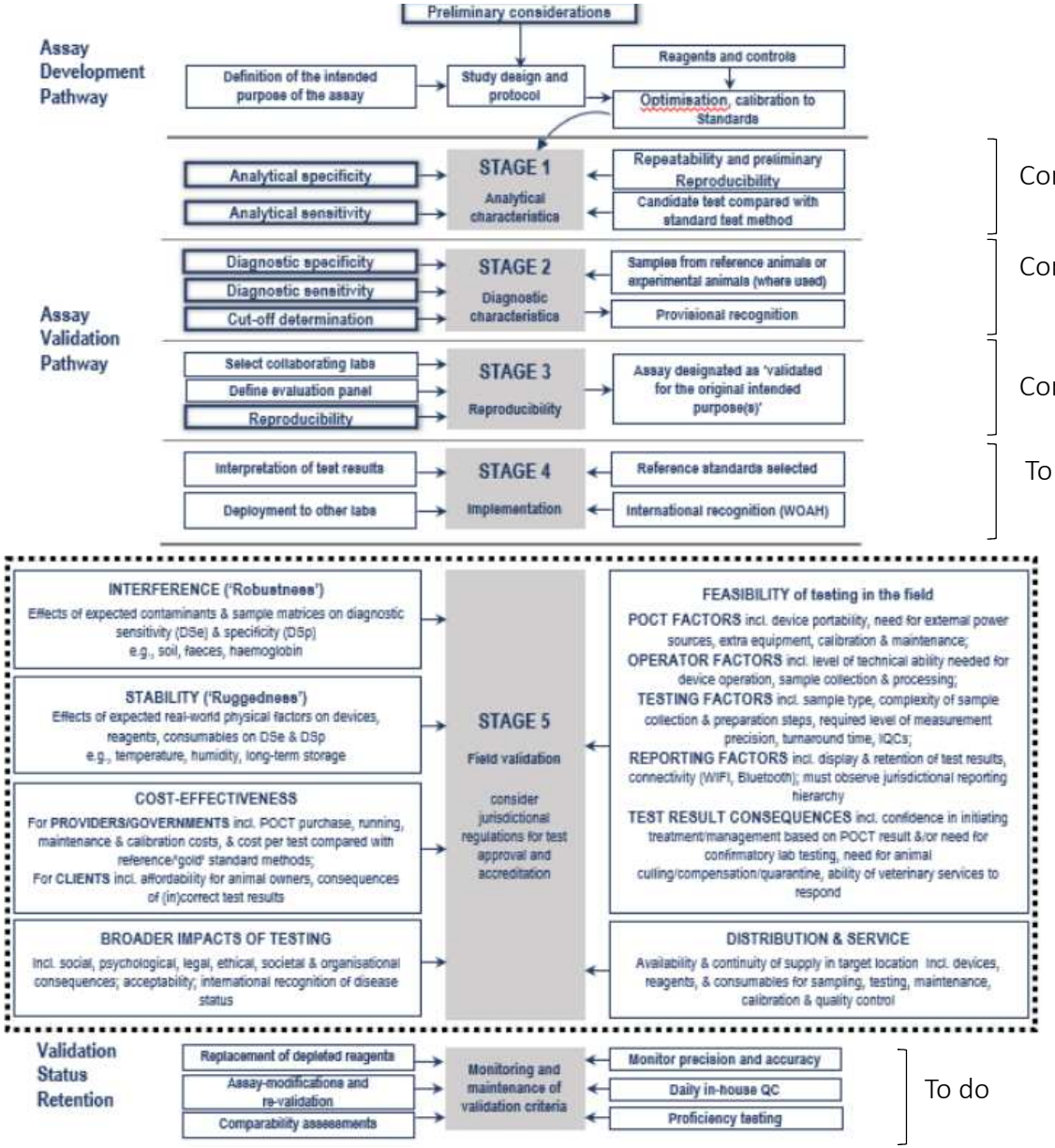
RT-qPCR product can undergo Sanger sequencing to obtain the **CS sequence**





Validation pathway

Source:
WOAH Terrestrial Manual 2023



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Stage 1 – analytical validation

1. Analytical sensitivity

> 1log compared to Sutton 2019

2. Repeatability

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

3. Analytical specificity

Inclusivity verified for isolates representing genotypes 1, I, II, VI, VII, XIII, XIV, XVIII, XXI

No cross-reaction with matrix components or non-target avian viruses

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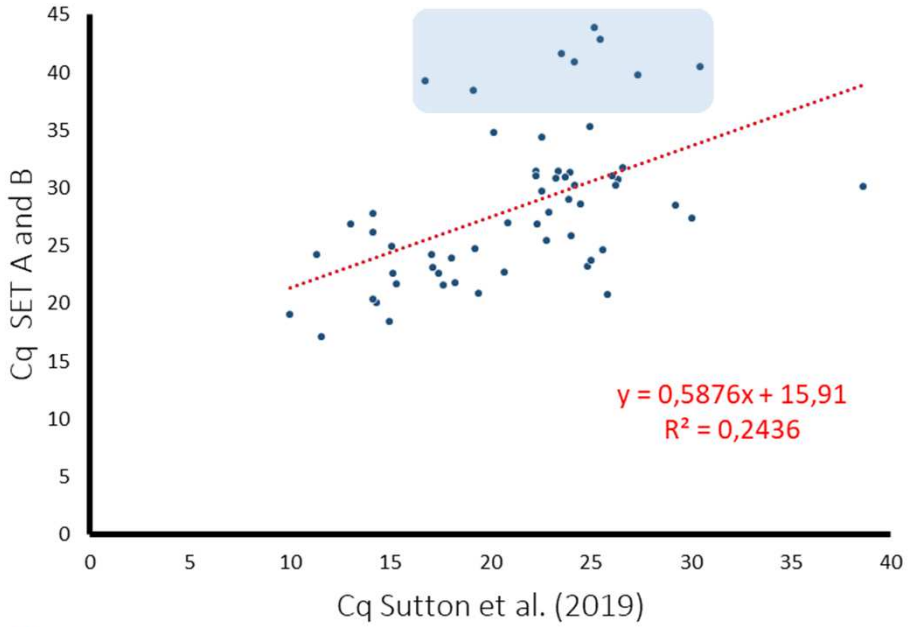
Stage 2 – diagnostic validation

67 APMV-1 negative

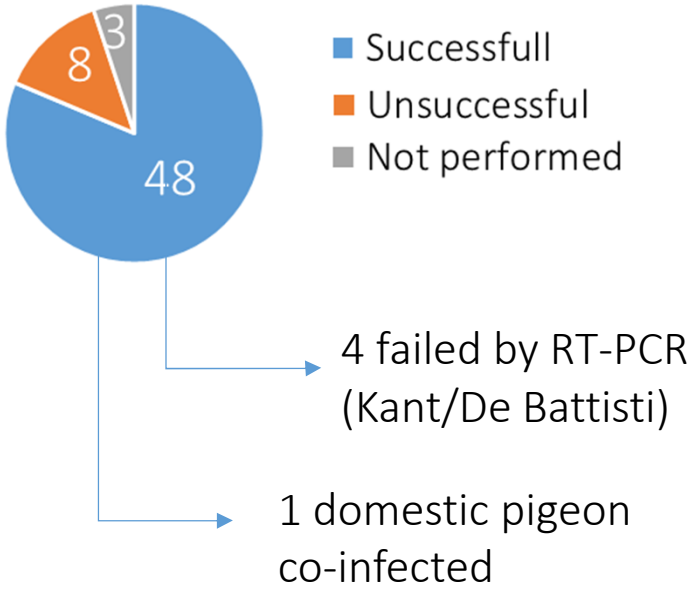
59 APMV-1 positive of genotypes I, II, VI, VII, XIII, XIV, XVIII, XXI

- Organs, swabs, FTA from Europe, Africa, Middle East, Asia
- Assessment in parallel with Sutton 2019 and Kant 1997

Pathotyping by vir/avir probes



Pathotyping by sequencing RT-qPCR products



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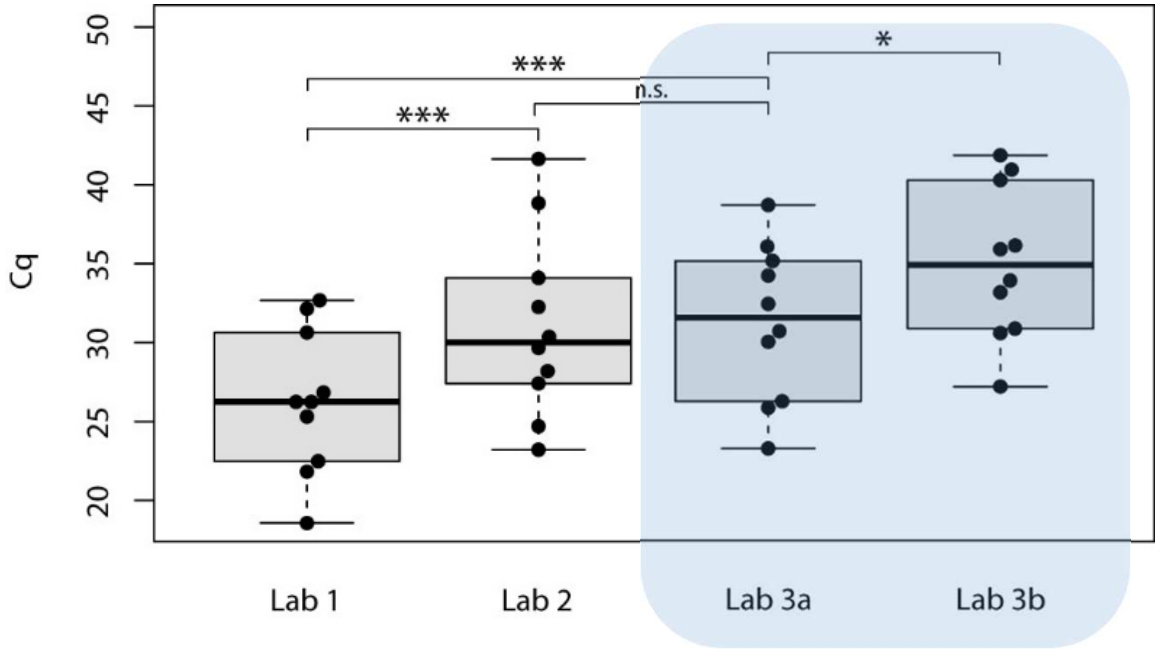
Stage 3 - reproducibility

FRIEDRICH-LOEFFLER-INSTITUT
FLI
Bundesforschungsinstitut für Tiergesundheit
Federal Research Institute for Animal Health

SVA NATIONAL VETERINARY INSTITUTE

1. Reproducibility study with 3 participants: Fleiss kappa 0,88



Ramp/rate affects assay performance

Slower rates are recommended

2. EQA provided by the FLI (self assessment): Cohen kappa 1

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Recommendations for use of the new RT-qPCR array

Rapid method to discriminate virulent and avirulent APMV-1 in dual mode (probes and Sanger sequencing)

- **Optimization** is required if using different reagents/equipment
- Sanger can be **optional** in samples yielding **Cq ≤ 35** (cost-benefit considerations on time, costs, equipment)
- Sanger **needs** to be performed for samples yielding **Cq > 35**
- Potential as a **DIVA strategy**
- More validation data are required for strains from **Asia and America** and for **genotypes unavailable** at the EURL AI/ND
- Will this method replace SOP VIR 1002 and 063? **We don't know!**
- **Periodic reassessment** of oligonucleotides will be needed

All details available here

ELSEVIER

Journal of Virological Methods

Available online 16 September 2023, 114813

In Press, Journal Pre-proof What's this?

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

Andrea Fortin [a e], Andrea Laconi [b], Isabella Monne [a], Siamak Zohari [c], Kristofer Andersson [c], Christian Grund [d], Mattia Cecchinato [e], Marika Crimaudo [a], Viviana Valastro [a], Valeria D'Amico [a], Alessio Bortolami [a], Michele Gastaldelli [a], Maria Varotto [a], Newcastle Disease Collaborating Diagnostic Group [1], Calogero Terregino [a], Valentina Panzarin [a]

Soon, the procedure **SOP VIR 1006** also in the EURL website..

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Updates on diagnostic methods available in the EURL webpage

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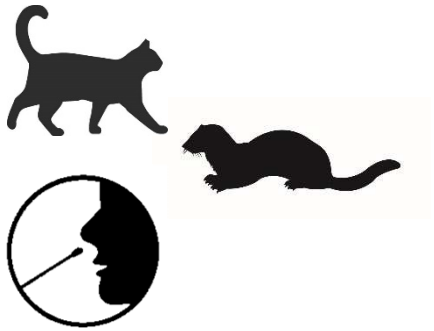
Updates on diagnostic methods

SOP VIR 1000 (sample preparation and NA extraction)
Validation of IndiMag Pathogen KF96 Cartridge (Indical Bioscience)

SOP VIR 151 (APMV-1 screening)
Unsuitability of the MagMax Pathogen RNA/DNA Kit (Applied Biosystems) for organs/tissues

SOPs VIR 018, 125, 143, 1004 (AIV screening and H5 detection)
Application extended to mammalian samples processing
Surveillance in human exposed to H5 HPAI



SOP VIR 1004 (AIV HA and NA subtyping)
Update of the oligonucleotides set for H13 detection
N2: RITA1 is recommended in place of RITA2
N6: issues with the RITA2 probe → James et al. is recommended instead
N5, N7, N8, N9: RITA2 as a first choice. James et al. provided as a second try

Updates on diagnostic methods

SOP VIR 1000 (sample preparation and NA extraction)
Validation of IndiMag Pathogen KF96 Cartridge (Indical Bioscience)

The information in this document is subject to change without notice.

Revision history

Revision	Date	Description
ed. 02	September 2023	• Application extended to mammalian samples processing • Interpretation of results • Removal of primer H13-F3 and update of H13 oligonucleotides concentration • Minor edits for clarity, style and consistency
ed. 01	July 2023	• Updated references • Updated oligonucleotides sets, reaction volume and cycling conditions • Inclusion of assays for H5 and H7 subtypes detection • Inclusion of the internal control (optional) for the H5 and H7 assays • Minor edits for style and consistency
ed. 00	April 2021	Initial release

ısystems) for organs/tissues

N6: issues with the RITA2 probe → James et al. is recommended instead
N5, N7, N8, N9: RITA2 as a first choice. James et al. provided as a second try

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Thanks for the attention, and for keep on sharing with us viruses, sequences and issues that help us improve diagnostic methods

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