Istituto Zooprofilattico
Sperimentale delle Venezie

# Updates on molecular detection methods

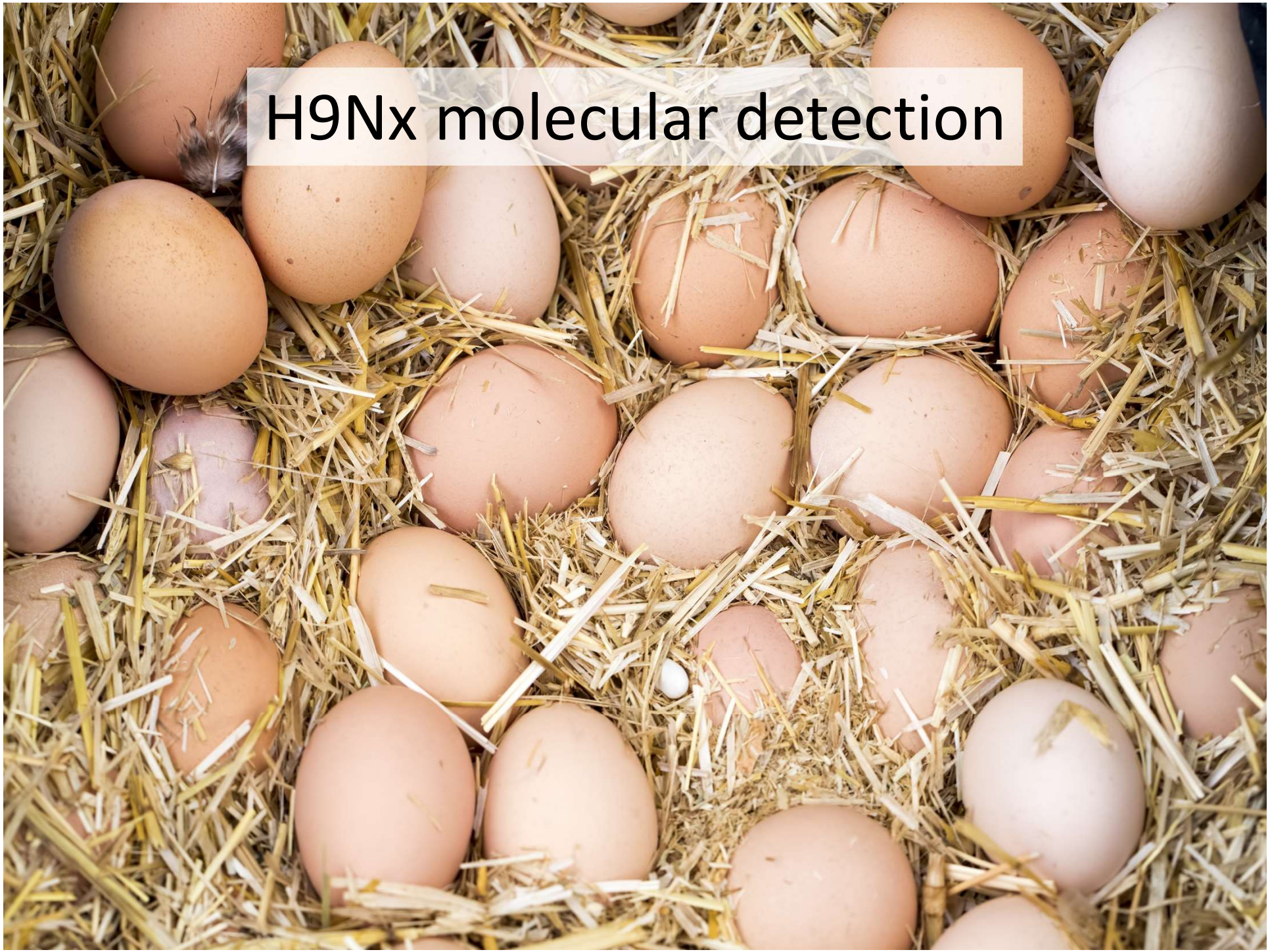
**Valentina Maria Panzarin**

EURL AI-ND, Development & Validation Group

27th Annual Meeting of the NRLs for AI and ND of European union member states

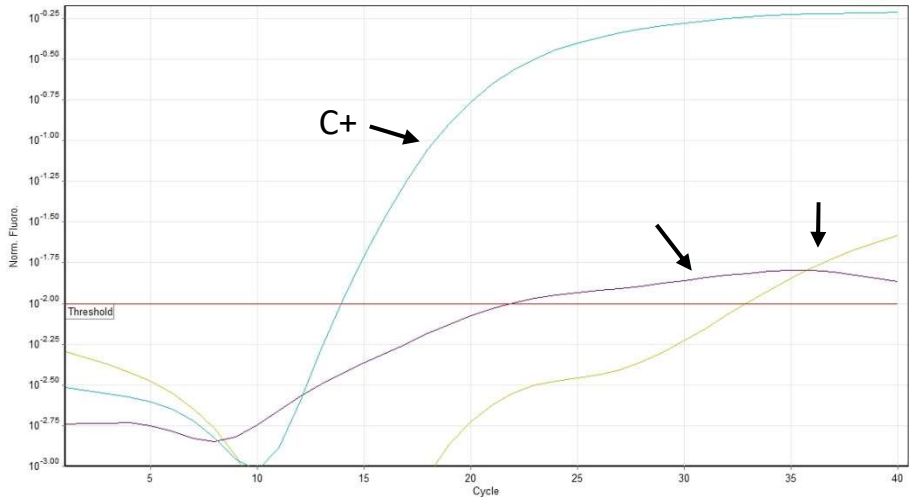
Virtual meeting, 6 – 7th October 2021

# H9Nx molecular detection



# Failures in detecting recent H9N2

No- or sub-optimal detection of strains from Africa and the Middle East with rRT-PCR from Monne et al., 2008 (SOP VIR 014)




| Test | Application % | Success rate % M04 |
|---|---|---|
| Monne-2008 | 48 | **81 (13/16)** |
| Hoffmann-2016 | 33 | **73 (8/11)** |
| In house | 3 | 100 |
| Other | 9 | 100 |

**Total: 81 (25/31)**

Data from Valastro

**7. Characteristics of the method**

This method was validated at the IZSVe according to the ISO/IEC 17025, employing AIV samples, selected avian viruses and bacteria available at the IZSVe repository, as well as materials, equipment and procedures as described above. The validation dossier is accessible upon request by contacting eurl.ai.nd.secretariat@izsvenezie.it

The method usually yields positive results for samples with Ct values < 35 by M-gene real time RT-PCR. However, sensitivity can be strain-dependent and might be affected by poor RNA quality, low viral load and the presence of PCR inhibitors. A sub-optimal amplification was observed for recent strains from Africa and the Middle East. The use of a degenerate probe (i.e. 5'-FAM-TTC TGG GCY ATG TCC AAT GG-TAMRA-3') might increase assay performances and allow an improved recognition of these strains. However, the degenerate probe lacks full validation.

Any modification to this SOP by third party laboratories should be supported by proper validation data assessing that the method is still fit-for-purpose.

From: SOP VIR 014
https://www.izsvenezie.com/documents/reference-laboratories/avian-influenza/diagnostic-protocols/

Wide use of the rRT-PCR from Monne et al., (2008) across Europe and worldwide..

# Time for a more extensive update of Monne-2008 assay

## Purposes of the assay

**Demonstration of freedom** from H9 in a **population** and in **single animals**, **confirmation** of H9 **diagnosis** or **suspect** in Europe and in endemic regions

## Need of a sensitive and specific test

Literature review and inventory of available protocols (2008 onward)

*N* = 6

H9Nx HA sequences download, curation, alignment (MSA)

≈ 5K sequences (2015-2021) from Eurasia/Africa, avian and mammals

MSA oligos mapping of designated protocols, MSA editing for primers/probe concatenation

Entropy calculation of the concatamers and selection of candidate assays

Identification of unique haplotypes, MSA stratification

Monne-2008:

Identification and scoring of the most variable sites for oligos update

Haplotypes prioritization and oligos update for the assay «H9-new 2021»

«H9-new 2021» assay *in vitro* optimization

Analytical validation

(OIE standards) and evaluation of IC use

Comparison of "H9-new 2021" against Monne-2008 and Saito-2019

Technical modifications for assay implementation (extraction and amplification reagents)

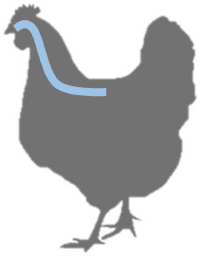
Diagnostic validation

(OIE standards)

Istituto Zooprofilattico Sperimentale delle Venezie

# Analytical validation of «H9-new 2021» (OIE standards)

1- **Analytical sensitivity** (samples artificially spiked) close to 1 $EID_{50}$/100 µl for all the lineages and the sample matrices

| | **Tracheal swab**  | | | **Cloacal swab**  | | | **Oviduct (1:10)**  | | |
|---|---|---|---|---|---|---|---|---|---|
| | LoD (Ct) $EID_{50}$/100 µl | *E* (%) | $R^2$ | LoD (Ct) $EID_{50}$/100 µl | *E* (%) | $R^2$ | LoD (Ct) $EID_{50}$/100 µl | *E* (%) | $R^2$ |
| **Korean** | **1,51** (33,85) | 90,5 | 0,995 | **1,51** (33,48) | 96 | 0,997 | **1,51** (34,8) | 90,4 | 0,998 |
| **G1** | **3,16** (34,06) | 99,9 | 0,996 | **3,16** (35,06) | 94,8 | 0,998 | **3,16** (36,04) | 99,4 | 0,998 |
| **Y280** | **1,41** (33,78) | 95,6 | 0,998 | **1,41** (33,81) | 98 | 0,997 | **14,12** (32,01) | 97,8 | 0,996 |
| **Y280 + IC** | | | | **1,41** (33,53) | 97,24 | 0,998 | **14,12** (32,13) | 91 | 0,99 |

2- LoD is not affected by **internal control co-amplification** (IC-RNA, Indical Bioscience)

# Analytical validation of «H9-new 2021» (OIE standards)

Within run

| | LoD + 4 log | | LoD + 1 log | | LoD | |
|---|---|---|---|---|---|---|
| | CV | Agmt (%) | CV | Agmt (%) | CV | Agmt (%) |
| **Korean** | ≤ 0,01 | **≥ 98,64** | ≤ 0,01 | **≥ 98,83** | ≤ 0,03 | **≥ 97,24** |
| **G1** | ≤ 0,02 | **≥ 97,75** | ≤ 0,02 | **≥ 98,34** | ≤ 0,07 | **≥ 93,41** |

| | LoD + 4 log | | LoD + 1 log | | LoD | |
|---|---|---|---|---|---|---|
| | CV | Agmt (%) | CV | Agmt (%) | CV | Agmt (%) |
| **Korean** | ≤ 0,05 | **≥ 95,29** | ≤ 0,02 | **≥ 97,61** | ≤ 0,03 | **≥ 97,14** |
| **G1** | ≤ 0,01 | **≥ 99,23** | ≤ 0,02 | **≥ 98,27** | ≤ 0,04 | **≥ 96,17** |

3- **Repeatability** (samples artificially spiked)
Assessed on 3 independent replicates, by 2 technicians, in 3 different days

Dilutions comprise the **LoD**

(Almost) always, **above** the target value of **95% Agnt**

Between days

| | LoD + 4 log | | LoD + 1 log | | LoD | |
|---|---|---|---|---|---|---|
| | CV | Agmt (%) | CV | Agmt (%) | CV | Agmt (%) |
| **Korean** | ≤ 0,02 | **≥ 97,78** | ≤ 0,01 | **≥ 98,78** | ≤ 0,01 | **≥ 98,61** |
| **G1** | ≤ 0,02 | **≥ 98,07** | ≤ 0,03 | **≥ 97,43** | ≤ 0,04 | **≥ 96,49** |

| | LoD + 4 log | | LoD + 1 log | | LoD | |
|---|---|---|---|---|---|---|
| | CV | Agmt (%) | CV | Agmt (%) | CV | Agmt (%) |
| **Korean** | ≤ 0,04 | **≥ 96,41** | ≤ 0,02 | **≥ 98,17** | ≤ 0,02 | **≥ 97,79** |
| **G1** | ≤ 0,03 | **≥ 97,24** | ≤ 0,02 | **≥ 97,54** | ≤ 0,03 | **≥ 97,15** |

Istituto Zooprofilattico Sperimentale delle Venezie

# Analytical validation of «H9-new 2021» (OIE standards)

4- **Reproducibility** (still ongoing)..

- Excercise with a territorial laboratory of the IZSVe → preliminary data on deployment
- PT Offlu (if H9Nx will be included)
- Labs possessing a wide H9 collection are welcome to participate

Contact us: vpanzarin@izsvenezie.it

Y280 is underrepresented

Test with *in vitro* transcribed RNA (on schedule)

5- **Analytical specificity**

**Inclusivity** for H9Nx only.

No cross-reaction (**exclusivity**) verified for:

- H1-16 AIV subtypes
- APMV 1-4; 6-9
- IBV
- NDV and IB vaccine strains
- APV, IBDV, CAV, GaHV, ILT
- West Nile virus
- *E. coli*
- *Staphylococcus spp*
- *Mycoplasma spp*

Istituto Zooprofilattico Sperimentale delle Venezie

# Diagnostic validation of «H9-new 2021» (OIE standards)

Estimates: 97% DSe & 98% DSp, with 5% error and 99% confidence

| Origin | Period | Matrice | Species | No. Positive |
|---|---|---|---|---|
| **Italy** | 2016-2021 | swabs, stool, isolates | chicken, turkey, mallard, teal, pheasant | **22** |
| **Africa** | 2017-2021 | swabs, organs, isolates, FTA | chicken, unspecified avian species | **26** |
| **Middle East** | 2019-2021 | FTA | chicken | **20** |
| **Asia** | 2021 | FTA | chicken | **6** |

**Total positive samples: 74**

| Origin | Period | Matrice | Species | No. Negative |
|---|---|---|---|---|
| **Italy** | 2018-2021 | swabs, organs | chicken, turkey, mallard, teal, pheasant, goose, quail, magpie, partridge, shoveler | **39:** 24 Neg AIV + 15 Pos |
| **Europe** | 2021 | swabs, organs, isolates | chicken, turkey, swan, sea gull | **14**: Pos H5, H3, H6 |

**Total negative samples: 53**

All the field samples tested as expected with the new assay

Provisional diagnostic cut-off:
Positive ≤ 35 Ct
Doubt > 35 Ct

**DSe = TP/(TP+FN)*100 = 100%**

**DSp = TN/(TN+FP)*100 = 100%**

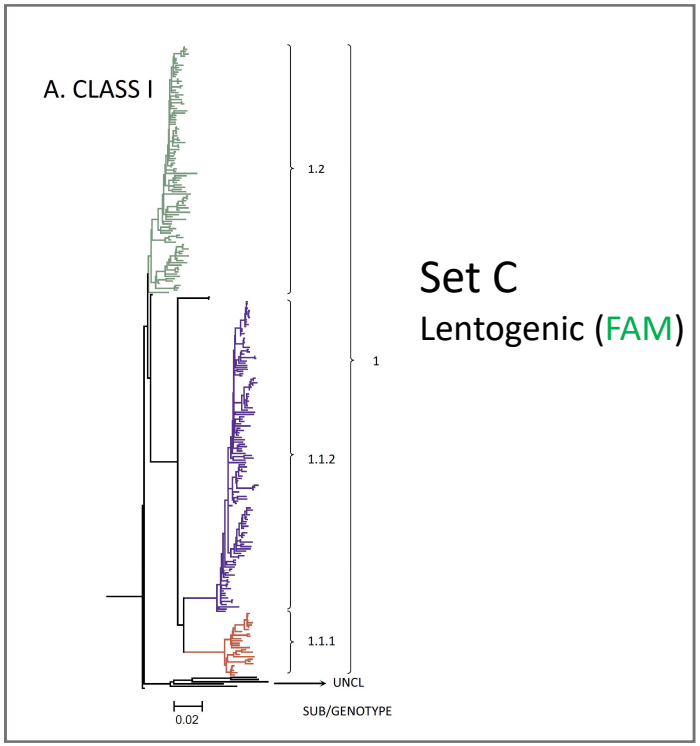
Istituto Zooprofilattico Sperimentale delle Venezie

# AOAV-1 molecular pathotyping

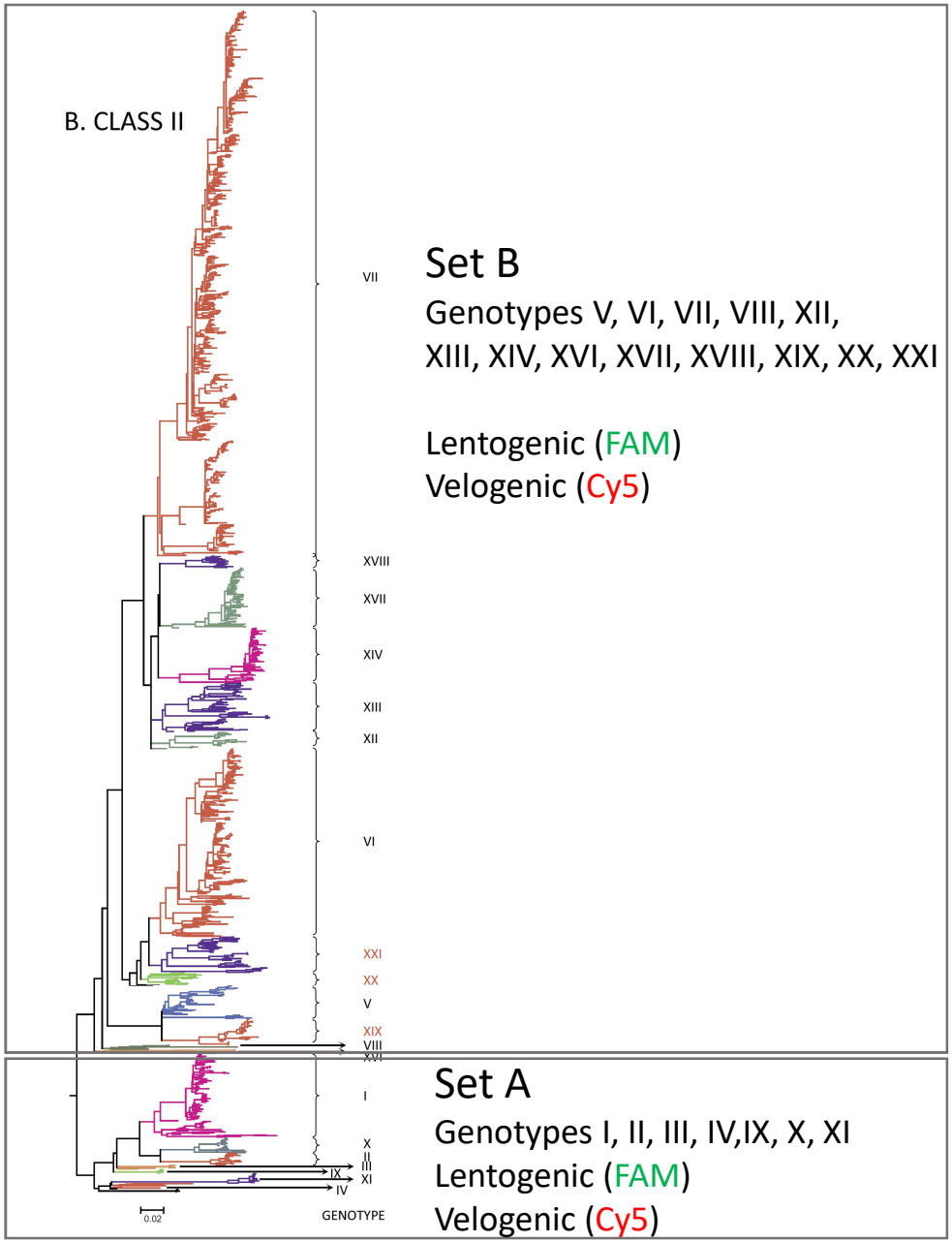
# AOAV-1 pathotyping: old story, new solutions?

Dual typing mode:

- Pathotype-specific probes
- Sanger sequencing of the F gene cleavage site (≈ 150-180 bp)




Set C
Lentogenic (FAM)




Set B
Genotypes V, VI, VII, VIII, XII, XIII, XIV, XVI, XVII, XVIII, XIX, XX, XXI

Lentogenic (FAM)
Velogenic (Cy5)

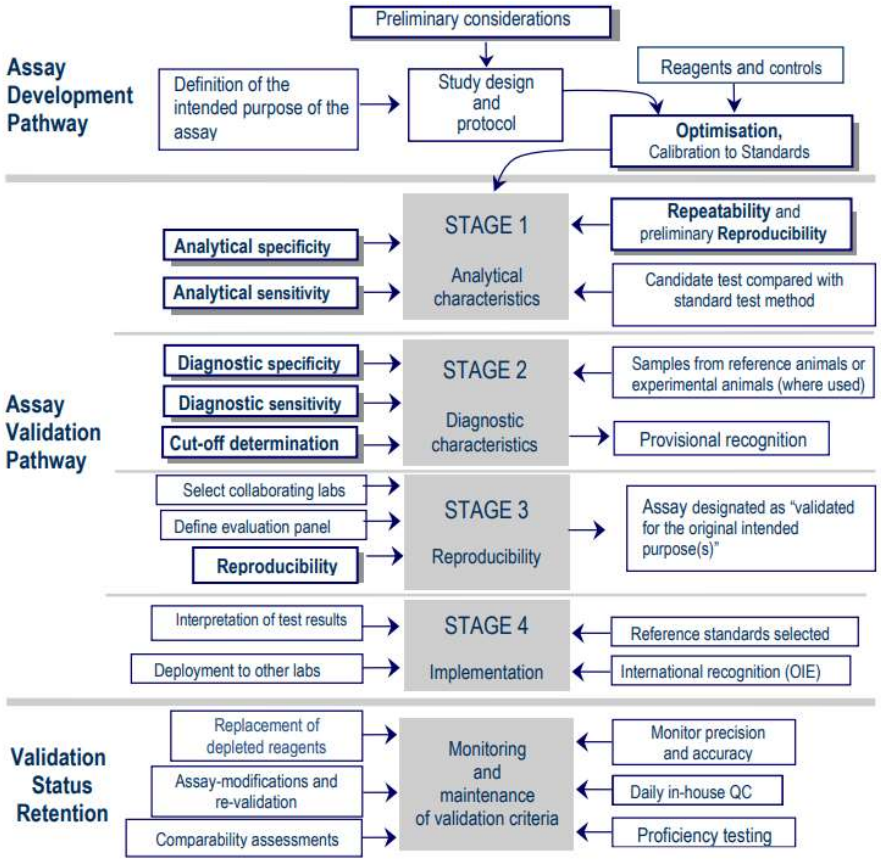
Set A
Genotypes I, II, III, IV,IX, X, XI
Lentogenic (FAM)
Velogenic (Cy5)

Dimitrov et al., 2019

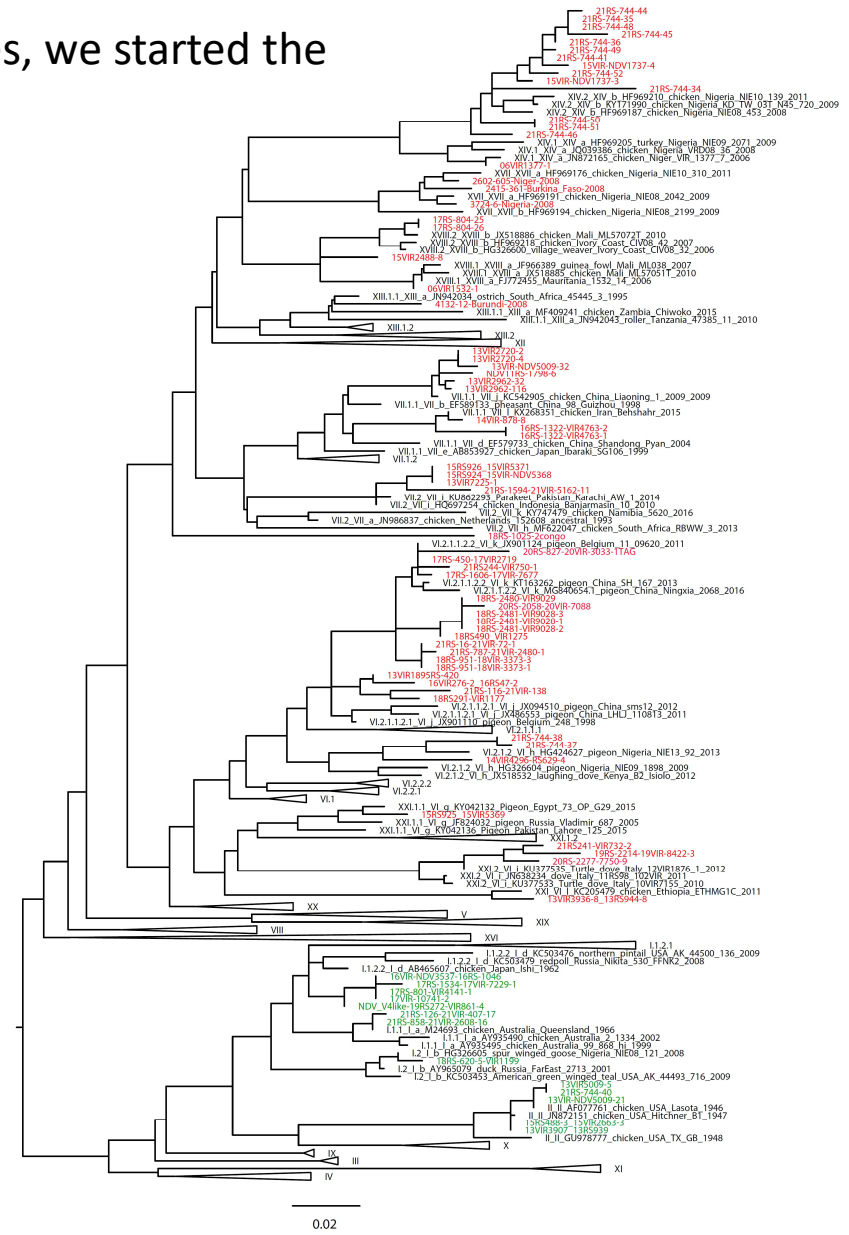
Istituto Zooprofilattico Sperimentale delle Venezie

# Starting from the field..

After the generation of new African sequences, we started the validation pathway from stage 2..

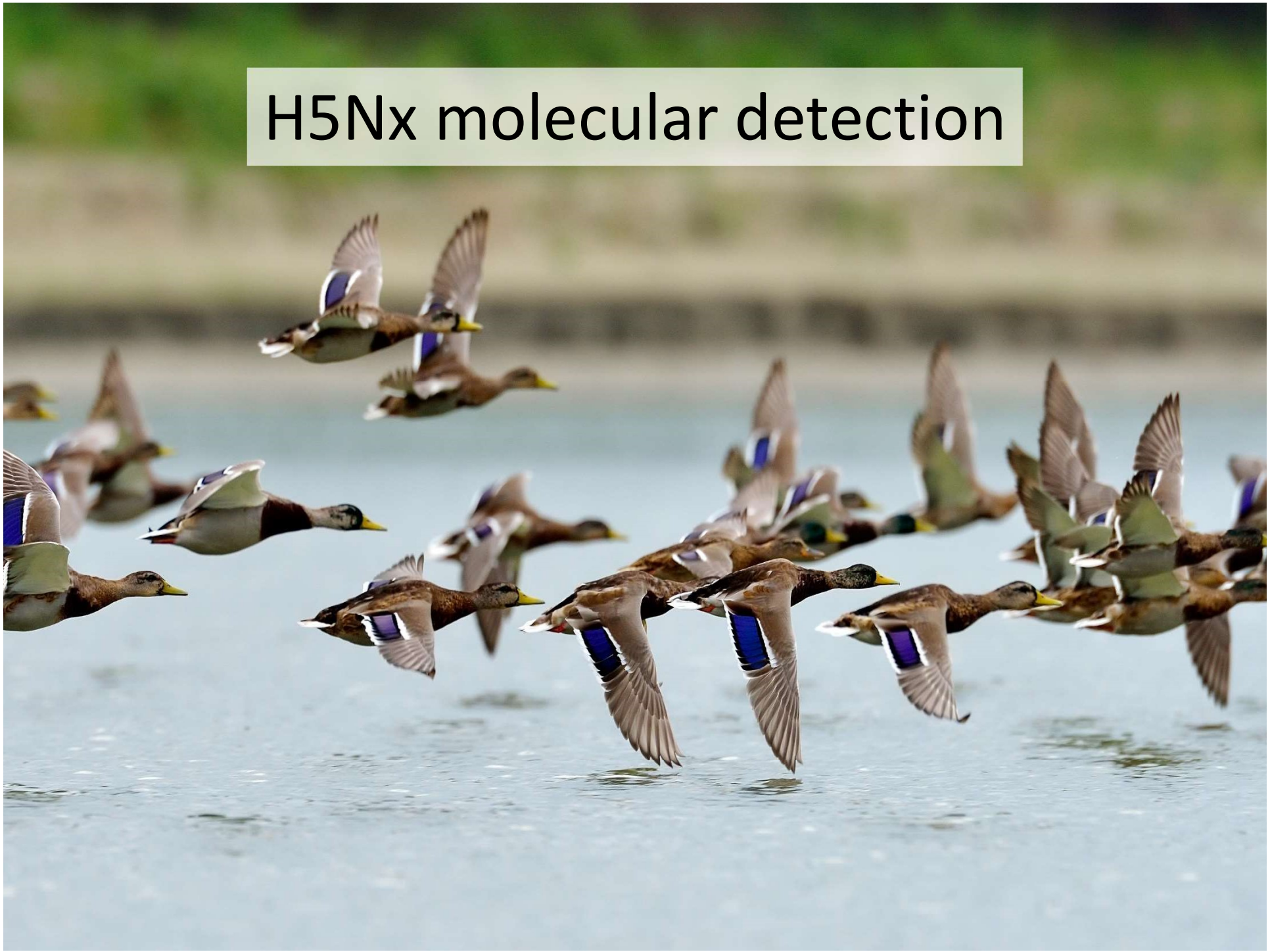



https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.06_VALIDATION.pdf



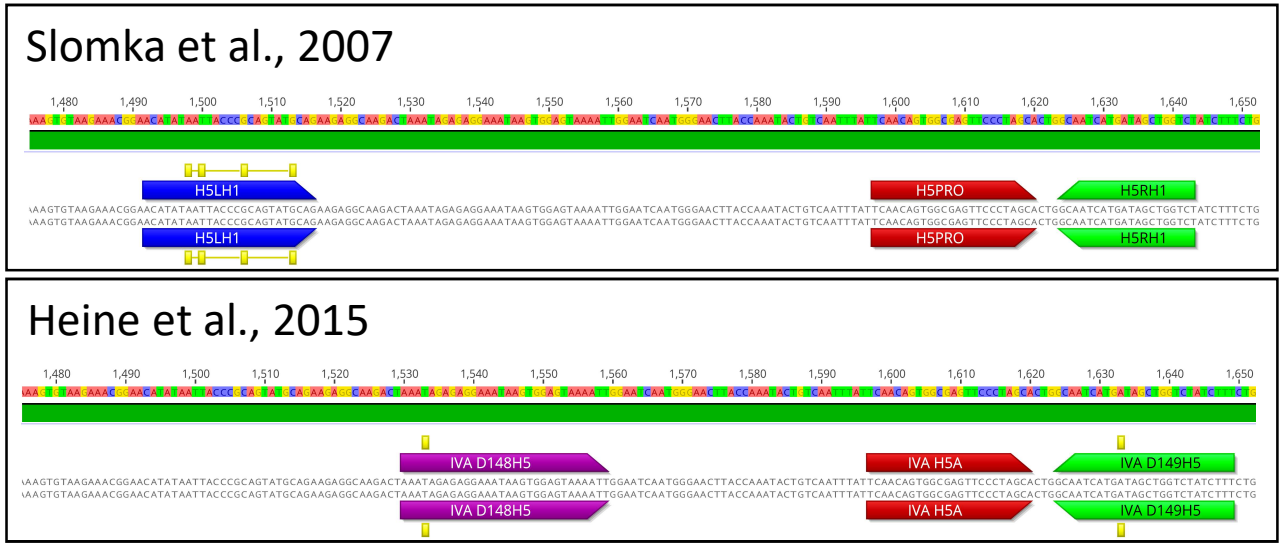

Istituto Zooprofilattico Sperimentale delle Venezie

# H5Nx molecular detection



# Failures in detecting Italian H5N1 LPAI

No- or sub-optimal detection of recent strains from wild duck with rRT-PCR from Slomka et al., 2007 (SOP VIR 143)




https://www.geneious.com

| | M-gene | H5 RT-PCR | H5 rRT-PCR | |
|---|---|---|---|---|
| | SOP VIR 018 | SOP VIR 125 | SOP VIR 143 | Heine-2015 mod |
| 21VIR6553-1 | 30,55 | LPAI | Negative | **22,27** |
| 21VIR6957-6 | 21,42 | LPAI | 38,57 | **22,82** |

New H5N3 LPAI with similar features were detected, phylogenetic analysis is ongoing
We'll keep the situation monitored…

# Final considerations and lessons learnt

Assays update has become a **bioinformatic matter**…

Share experience on how to select informative data, develope tools for managing big datasets, identify criteria to assure assays inclusivity

**Simplified validation** workflows are needed

To put in place effective methods to detect emerging variants and respond promptly to epidemics

Sometimes sequences are **too few…**

It remains crucial to share sequence data timely, to impede viruses from «escaping» our diagnostic tools

Please, share with us viruses and sequences!

# THANKS FROM THE EURL DEVELOPMENT & VALIDATION GROUP

We thank the EURL Labs of Diagnostic Virology, of Viral Genomics and Transcriptomics and of Experimental Animal Models for their support