DETECTION OF AVIAN INFLUENZA IN HUMANS: MOLECULAR DIAGNOSTIC APPROACHES

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Outline

- Overview of Avian Influenza
- Molecular diagnostics for Avian Influenza

“This presentation has no financial or commercial affiliations with any company and does not provide any benefits to any organization”

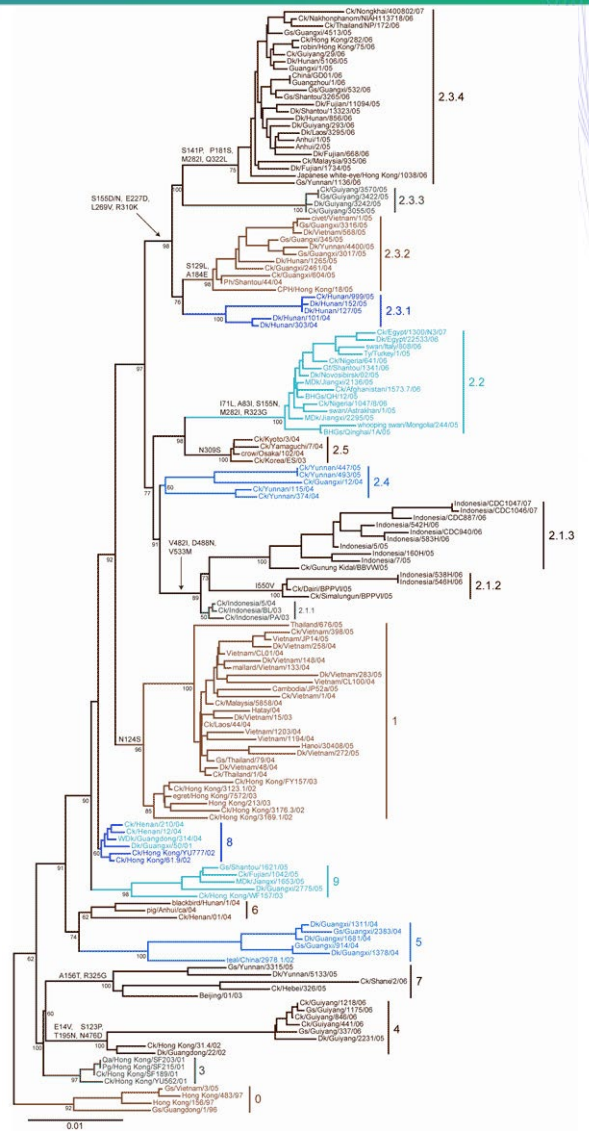
Overview of Avian influenza virus

Avian influenza viruses (AIVs)

- An infectious viral disease of birds caused by **type A influenza viruses**
- Classified by HA (hemagglutinin) and NA (neuraminidase)
- HA cleavage site: ***PQRERRRKR/G*** – marker of high pathogenicity
- HPAI strains, such as H5N1 and H7N9, can cause severe disease in poultry and pose a zoonotic risk to humans

Clade Evolution and Geographic Spread

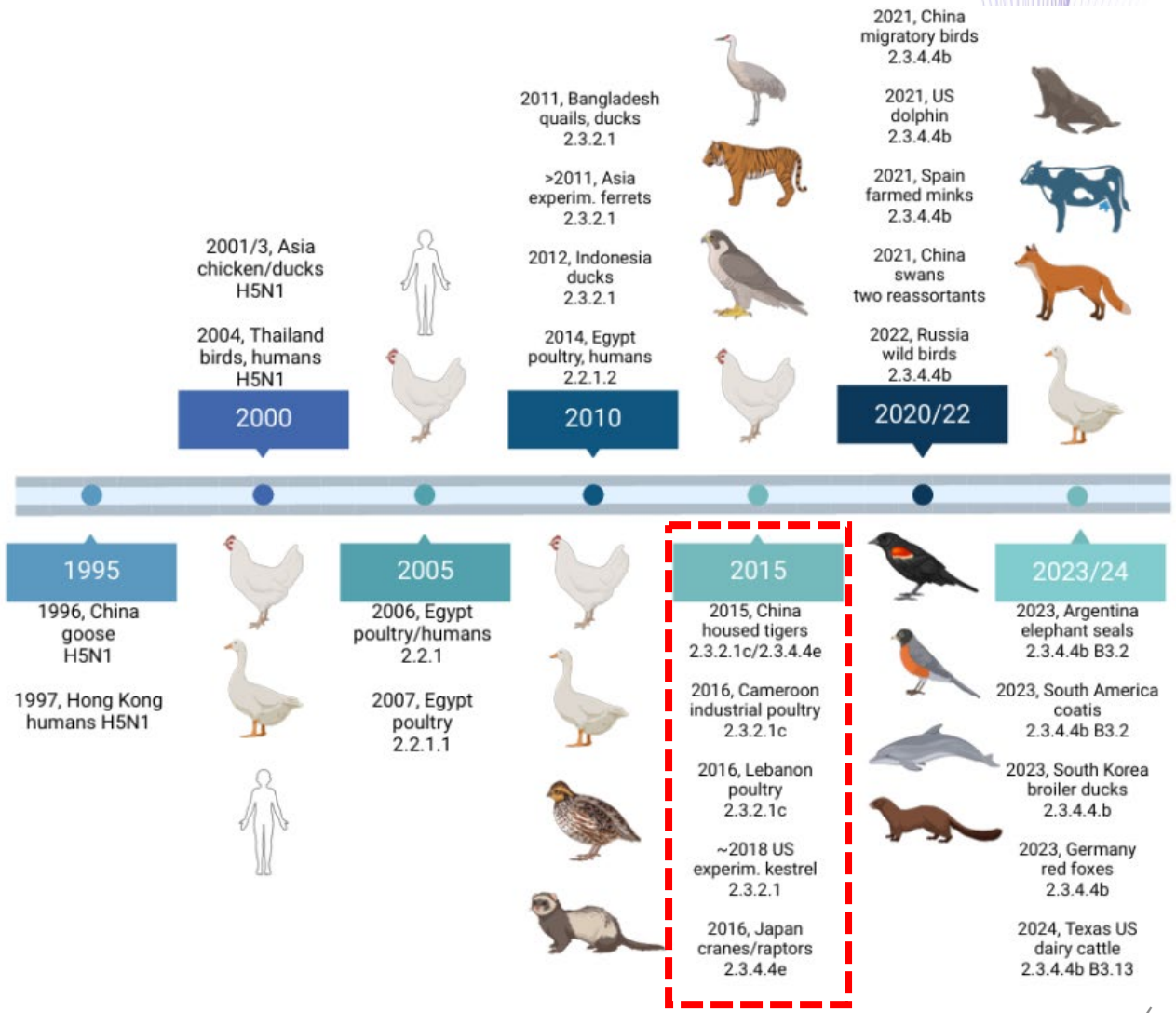
- WHO/OIE/FAO classify H5 viruses into genetic clades based on HA gene
- 10 unique first-order numbered clades (0-9)
- Clade 2 showed a level of diversity (clades 2.1–2.5)



Emerg Infect Dis. 2008;14(7). https://doi.org/10.3201/eid1407.071681

Clade Evolution and Geographic Spread

- Clade 2.3.4.4 emerged in 2014; 2.3.4.4b dominant in 2020s
- Each clade has different host range, virulence, and geographic distribution



International Journal of Health Sciences, 9(1), 89–112

Epidemiology of Avian Influenza in Southeast

- Endemic circulation in countries like Vietnam, Indonesia, Cambodia, and Thailand
- Clade 2.3.4.4b H5N1 and H5N6 are predominant in recent years
- Seasonal patterns linked to migratory birds and live bird markets

University Hospital Preparedness for Avian Influenza virus Outbreak: Molecular Laboratory Perspective

Role of Molecular laboratory: perspecti

- Biosafety containment
- Diagnostic capacity enhancement
- Data integration and communication
- Collaborative network

1.Biosafety Measures

- BSL-2+ or BSL-3 protocols:
  - Secure sample handling, with inactivation before RNA extraction
  - Use of sealed, automated extraction systems to minimize aerosol risk

2. Diagnostic Capacity Enhancement

- **Real-time RT-PCR**
  - Gold standard for rapid, sensitive detection
  - Recommended by WHO and CDC for H5/H7 detection
  - Detection of matrix (M) gene for type A identification
  - Subtyping with HA/NA gene-specific primers
- **Next-generation sequencing (NGS)**
  - Full genome characterization
  - Useful for identifying novel or reassortant strains

Key Parameters for Selecting Molecular Microbiology Tests in Clinical Laboratories

- Analytical performance
- Clinical performance
- Target coverage and panel design (single VS multiplex)
- Regulatory and validation status
- Workflow and laboratory compatibility (TAT, platform, contamination control)
- Cost and sustainability: RLU

Reagents and Kits

- **Commercial Real-Time PCR Kits**
  - Influenza virus panel (A/B, H1/H3, H5)
  - Point of care test (Influenza A/B/RSV/COVID)
- **Next generation sequencing**
  - Universal primer for influenza virus

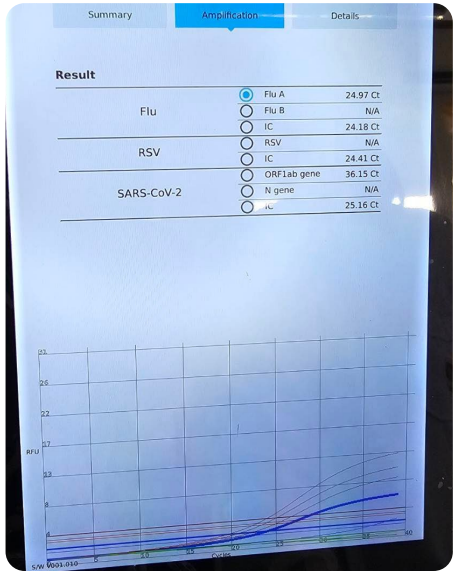
Inactivation influenza virus (H5N1)

- CDC has validated the following buffers for inactivation of Influenza A(H5N1):
  - TRIzol LS
  - 10% buffered formalin
  - Beta-Propiolactone (BPL)
  - Guanidinium-based Roche buffers
  - MagNA Pure 96 kit / MagNA Pure 96 External lysis buffer
  - MagNA Pure Compact / RNA isolation kit lysis buffer
  - MagNA Pure 96 Cellular RNA Large Volume Kit / MPLC RNA Isolation Tissue Lysis Buffer
  - Guanidinium-based Qiagen buffers
  - QIAamp Viral RNA mini kit / AVL buffer
  - QIAamp DSP Viral RNA mini kit / AVL buffer
  - Qiagen RNeasy RNA extraction kit / RLT buffer

https://www.aphl.org/programs/infectious_disease/influenza/Documents/HPAI_FAQ.pdf

Molecular Diagnostic Techniques for AI

- **Real time PCR**
  - Semi-automated system
    - Syndromic panel: include influenza virus (A/B/H1/H3/H5/)
    - Real time PCR (A/B/H5/H7)
  - Point of care test (Rapid PCR testing)(A/B/RSV/COVID)
  - **FDA-approved molecular test**: https://www.fda.gov/medical-devices/in-vitro-diagnostics/influenza-diagnostic-tests



Validation and verification

Table 12. Examples of Validation and Verification Parameters. Reprinted and modified from *Clinical Microbiology Newsletter*. Vol. 29, No. 12. Sloan LM. Real-time PCR in clinical microbiology: verification, validation, and contamination control. Pages 87-95. Copyright 2007. Used with permission from Elsevier.[93]

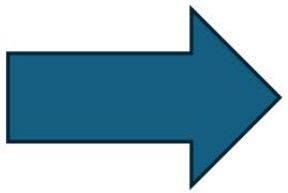
	Verification	Validation
Accuracy—Qualitative	50 positive specimens 50 negative, 10-day span	50 positive and 50 negative specimens, tested over 10 days
Accuracy—Quantitative	20 positive specimens in duplicate 50 negative specimens, three- to four-day span	40 specimens in duplicate and 50 negative specimens, tested over three to four days
Precision—Qualitative	Positive and negative specimen, 20-day span	Positive and negative specimen, 20-day span
Precision—Quantitative	Three replicates at two concentrations (within-run and between-day) (five days)	Two concentrations in duplicate run twice per day (20 days)
LoD	20 results at claimed LoD level	60 measurements to establish 20 results at established LoD to verify
Interfering substances	N/A	Evaluate possible genetic and chemical interference
Reference values	20 specimens per testing category	120 specimens from testing population

CLSI, MM19-A

Next generation sequencing

Modified some primers for use in whole genome sequencing

- Research project to monitor genetic diversity of influenza virus, RSV and SARS-CoV-2 over time



Siriraj Translational Microbial Genomics and Data Center
เพื่อพัฒนาและให้บริการทดสอบขั้นสูง สำหรับงานวิจัย และวินิจฉัยโรคติดเชื้อทางการแพทย์
(ภายใต้การสนับสนุนจากคณะแพทยศาสตร์ศิริราชพยาบาล)

Thailand Genomic Surveillance of Emerging Infectious Diseases Facilitating Rapid Response
พัฒนาระบบเฝ้าระวังโรคอุบัติใหม่ด้วยเทคโนโลยีจีโนมิกส์ พร้อมออกแบบชุดตรวจที่แม่นยำและทันสมัย
(ภายใต้การสนับสนุนจาก US CDC)

Impact of Genetic Change on Molecular Diagno

- Primer/probe mismatches from viral evolution affect RT-PCR sensitivity.
- Single nucleotide polymorphisms (SNPs) in target regions can:
  - Increase Ct values
  - Cause false negatives

Check for updates

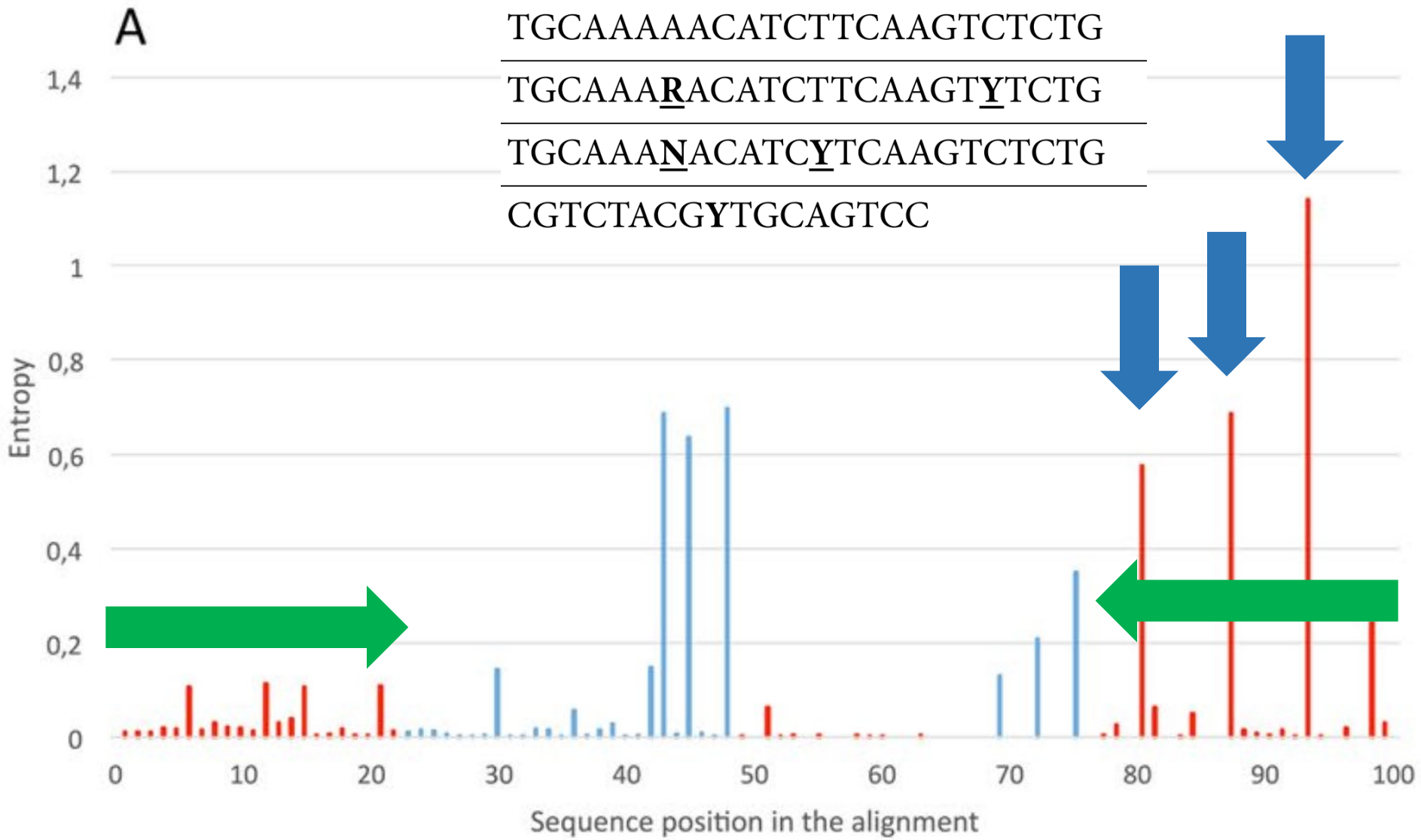
OPEN

Detection of avian influenza virus: a comparative study of the in silico and *in vitro* performances of current RT-qPCR assays

Andrea Laconi[1]✉, Andrea Fortin[1], Giulia Bedendo[1], Akihiro Shibata[2], Yoshihiro Sakoda[3], Joseph Adongo Awuni[4], Emilie Go-Maro[5], Abdelsatar Arafa[6], Ali Safar Maken Ali[7], Calogero Terregino[1] & Isabella Monne[1]✉

- ***In silico* Analysis**: Evaluated complementarity of primers and probes to the AIV matrix (M) gene using 4,088 sequences (2014 onward)

RV (5′→3′)
TGCAAAAACATCTTCAAGTCTCTG
TGCAAA**R**ACATCTTCAAGT**Y**TCTG
TGCAAA**N**ACATC**Y**TCAAGTCTCTG
CGTCTACG**Y**TGCAGTCC



Entropy plots **Products: 25-125**

Multiplex Dual-Target Reverse Transcription PCR for Subtyping Avian Influenza A(H5) Virus

Malaya K. Sahoo, Ingrid E.A. Morante, ChunHong Huang, Daniel Solis,
Fumiko Yamamoto, Uzoamaka C. Ohiri, Daniel Romero, Benjamin A. Pinsky

- Highly pathogenic avian influenza (HPAI) A(H5) viruses, especially clade 2.3.4.4b, pose a significant pandemic risk
- Development of an internally controlled, dual-target quantitative reverse transcription PCR (qRT-PCR) for specific detection and subtyping of influenza A(H5)

Emerg Infect Dis. 2024 Aug;30(8):1710-1713.

Table 1. Primers and probes used in study of multiplex dual-target reverse transcription PCR for subtyping avian influenza A(H5) virus*

Target	Name	Sequence, 5′ → 3′	Final concentration
A(H5)	FluA_H5_v4_1F	TACCAGATACTGTCAATTTATTCAAC	400 nM
	FluA_H5_v4_1R	GTAACGACCCATTGGAGCACATCC	400 nM
	FluA_H5_v4_1Prb.FAM	FAM-CTGGCAATC/ZEN/ATG**R**T**R**GCTGGTCT-3IABkFQ	200 nM
	FluA_H5_v4_2F	TGGGTACCATCATAGCAATGAGCA	400 nM
	FluA_H5_v4_2R	AACTCCCTTCCAACTGCCTCAAA	400 nM
	FluA_H5_v4_2Prb.FAM	FAM-TGGGTACGC/ZEN/TGCGGACAAAGAATCCA-3IABkFQ	200 nM
A (M)	Pan-FluA-F	GACC**R**ATCCTGTCACCTCTGAC	400 nM
	Pan-FluA -R	AGGGCATT**Y**TGGACAAA**K**CGTCTA	400 nM
	Pan-FluA -prb_Q705	Q705-TGCAGTCCTCGCTCACTGGGCACG- BHQ-3	200 nM
Human RNase P	RNase P Fwd	AGATTTGGACCTGCGAGCG	100 nM
	RNase P Rev	GAGCGGCTGTCTCCACAAGT	100 nM
	RNase P Probe CF560	CF560-TTCTGACCTGAAGGCTCTGCGCG-BHQ-1	50 nM

- Two WHO-recommended H5 primer–probe sets targeting distinct HA gene regions were combined into a multiplex assay
- Primer-probe sets were optimized to cover recent clade 2.3.4.4b sequences with minimal mismatches (≥97% alignment)

Emerg Infect Dis. 2024 Aug;30(8):1710-1713.

Appendix Table 2. Alignment of Primer/Probe Set 1 to North American Influenza A(H5) Hemagglutinin (HA) Sequences available from GISAID January 1, 2022 to May 29, 2024.

	Forward Primer	Probe	Reverse Primer*		
Reference	TACCAGATACTGTCAATTTATTCAAC	CTGGCAATCATGATGGCTGGTCT	GGATGTGCTCCAATGGGTCGTTAC		
Set 1	TACCAGATACTGTCAATTTATTCAAC	CTGGCAATCATG**R**T**R**GCTGGTCT	GGATGTGCTCCAATGGGTCGTTAC	No.	%
	.....	.....	.....	5529	92.4
	.....C..	.....	.....	82	1.4
	.....T.....	.....	.....	58	1.0
	.....	.....	.....G.	44	0.7
	.....	.....G.....	.....	39	0.7
	.....	.....	.....T.....	36	0.6
	.....A.....	.....	.....	25	0.4
	..T.....	.....	.....	24	0.4
	.....	.....	.....A....	21	0.4
	.....	.....A.....	.....	13	0.2
	.....	.....T.....	.....	11	0.2
	.....	.....A.....	.....	8	0.1
	.....	.....T.....	.....	8	0.1
	.....	.....	.....A.....	8	0.1
	.....C.....	.....	.....	7	0.1
	.....	.....G.....	.....	6	0.1
	.....	.....C..	.....	6	0.1
	.....	.....G.....	.....	5	0.1
	.....	.....T.....	.....	5	0.1
	.....	.....	.....T.....	4	0.1
	.....	.....	.....T.....	4	0.1
	.....A.....	.....	.....	4	0.1
	.....	.....	.....T.....	4	0.1
	.....	T.....	.....	4	0.1
	.....T.....	.....T.....	.....	3	0.1

*The reverse primer sequence is presented as its reverse complement.
Sequences accessed May 29, 2024. Aligned sequences: 5987. GenBank: PP577943/GISAID: EPI3171488 (A/Texas/37/2024) is used as reference.
Primer and probe alignments are in-phase. Top 25 most abundant sequences are listed. Mixed bases are in bold.

5,975 out of 5,987 sequences (99.8%) had at most one mismatch

Appendix Table 3. Alignment of Primer/Probe Set 2 to North American Influenza A(H5) Hemagglutinin (HA) Sequences available from GISAID January 1, 2022 to May 29, 2024.

	Forward Primer	Probe	Reverse Primer*		
Reference	TGGGTACCATCATAGCAATGAGCA	TGGGTACGCTGCGGACAAAGAATCCA	TTTGAGGCAGTTGGAAGGGAGTT		
Set 2	TGGGTACCATCATAGCAATGAGCA	TGGGTACGCTGCGGACAAAGAATCCA	TTTGAGGCAGTTGGAAGGGAGTT	No.	%
	.....	.....	.....	5085	84.9
	.....	.....A.....	.....	209	3.5
	.....T.....	.....	.....	93	1.6
	...A.....	.....	.....	74	1.2
	.....	.....T.....	.....	48	0.8
	.....	.....	.....C.....	45	0.8
	.....C.....	.....A.....	.....	43	0.7
	.....	.....T.....	.....	41	0.7
	.....	...A.....	.....	32	0.5
	.....T.....	.....	.....	26	0.4
	.....	.....	.....A.....	17	0.3
	.....T.....	.....G.....	.....	15	0.3
	...T.....	.....	.....	15	0.3
	.....	.....T.....	.....	14	0.2
	.....	.....	.....A.....	13	0.2
	.....	.....	..C.....	12	0.2
	.....	.....	.....T.....	12	0.2
	.....	.....A.....G.....	.....	10	0.2
	.....C.....	.....	.....	9	0.2
	.....A.....	.....	.....	9	0.2
	.....	.....	.....A..	9	0.2
	.....	...T.....	.....	8	0.1
	.....	.....G.....	.....	8	0.1
	.....A..	.....	.....	8	0.1
	.....	.....	.....G.....	8	0.1

*The reverse primer sequence is presented as its reverse complement.
Sequences accessed May 29, 2024. Aligned sequences: 5990. GenBank: PP577943/GISAID: EPI3171488 (A/Texas/37/2024) is used as reference.
Primer and probe alignments are in-phase. Top 25 most abundant sequences are listed.

5,972 out of 5,990 sequences (97.7%) had at most one mismatch

Appendix Table 4. Alignment of Primer/Probe Set 1 to Avian Influenza A(H5) Sequences from Human Cases and Dairy Cows.

	Forward Primer	Probe	Reverse Primer*
Reference	TACCAGATACTGTCAATTTATTCAAC	CTGGCAATCATGATGGCTGGTCT	GGATGTGCTCCAATGGGTCGTTAC
Set 1	TACCAGATACTGTCAATTTATTCAAC	CTGGCAATCATGRTRGCTGGTCT	GGATGTGCTCCAATGGGTCGTTAC
Human: A/Texas/37/2024	.....	.....	.....
Human: A/Michigan/90/2024	.....	.....	.....
Human: A/Michigan/91/2024	.....	.....	.....
Human: A/Colorado/18/2022	.....	.....	.....
A/dairy cow/Texas/24_009367–003/2024	.....	.....G.....	.....
A/dairy cow/Texas/24_009367–005/2024	.....	.....G.....	.....
A/dairy cow/Texas/24_009367–009/2024	.....	.....G.....	.....
A/dairy cow/Texas/24_009367–010/2024	.....	.....G.....	.....
A/dairy cow/Texas/24_009367–013/2024	.....	.....G.....	.....
A/dairy cow/Texas/24_009367–014/2024	.....	.....G.....	.....
Other 190 sequences from dairy cows	.....	.....	.....

*The reverse primer sequence is presented as its reverse complement.
GenBank: PP577943/GISAID: EPI3171488 (A/Texas/37/2024) is used as reference. Mixed bases are in bold. Primer and probe alignments are in-phase.
Note: The 6 dairy cow sequences showing a mismatch with primer/probe set 1 do not contain any mismatches with primer/probe set 2.

- No primer–probe mismatches were identified in influenza A(H5) sequences from human cases in the United States (4 cases as of May 29, 2024)

Table 2. Avian influenza A(H5) virus genomic RNA used to evaluate inclusivity in study of multiplex dual-target reverse transcription PCR for subtyping avian influenza A(H5) virus*

Genome	Clade	GenBank accession no.	H5 Ct	M Ct	Ct difference
Kilbourne F181, A/duck/Singapore/645/1997 (H5N3)	EA-non-Gs/Gd	NA	18.3	17.7	0.6
A/quail/California/14-012546-1/2014 (H5N8)	Am-non-Gs/Gd	NA	22.9	19.0	3.9
A/mallard/Minnesota/16-041335-3/2016 (H5N2)	Am-non-Gs/Gd	MH546659	32.1	22.3	9.9
A/emperor goose/Alaska/17-004479-1/2016 (H5N2)	Am-non-Gs/Gd	MH546451	26.8	23.3	3.6
A/glaucous-winged gull/Alaska/16-041335-19/2016 (H5N2)	Am-non-Gs/Gd	MH546475	23.3	21.8	1.5
A/northern pintail/Alaska/16-041335-5/2016 (H5N2)	Am-non-Gs/Gd	MH546883	20.9	19.1	1.9

*Kilbourne F181 genomic RNA was obtained from BEI Resources (https://www.beiresources.org). Am-non-Gs/GD A(H5) genomic RNA was obtained from the US Department of Agriculture. The genomic RNA from these influenza A(H5) viruses was diluted 1:10 buffer AVE plus carrier RNA (QIAGEN, https://www.qiagen.com) and tested in duplicate. Ct values are means. Am-non-Gs/Gd, non-goose/GuangDong from the United States; Ct, cycle threshold; EA-non-Gs/Gd, Eurasian non-goose/GuangDong; M, matrix gene; NA, not available.

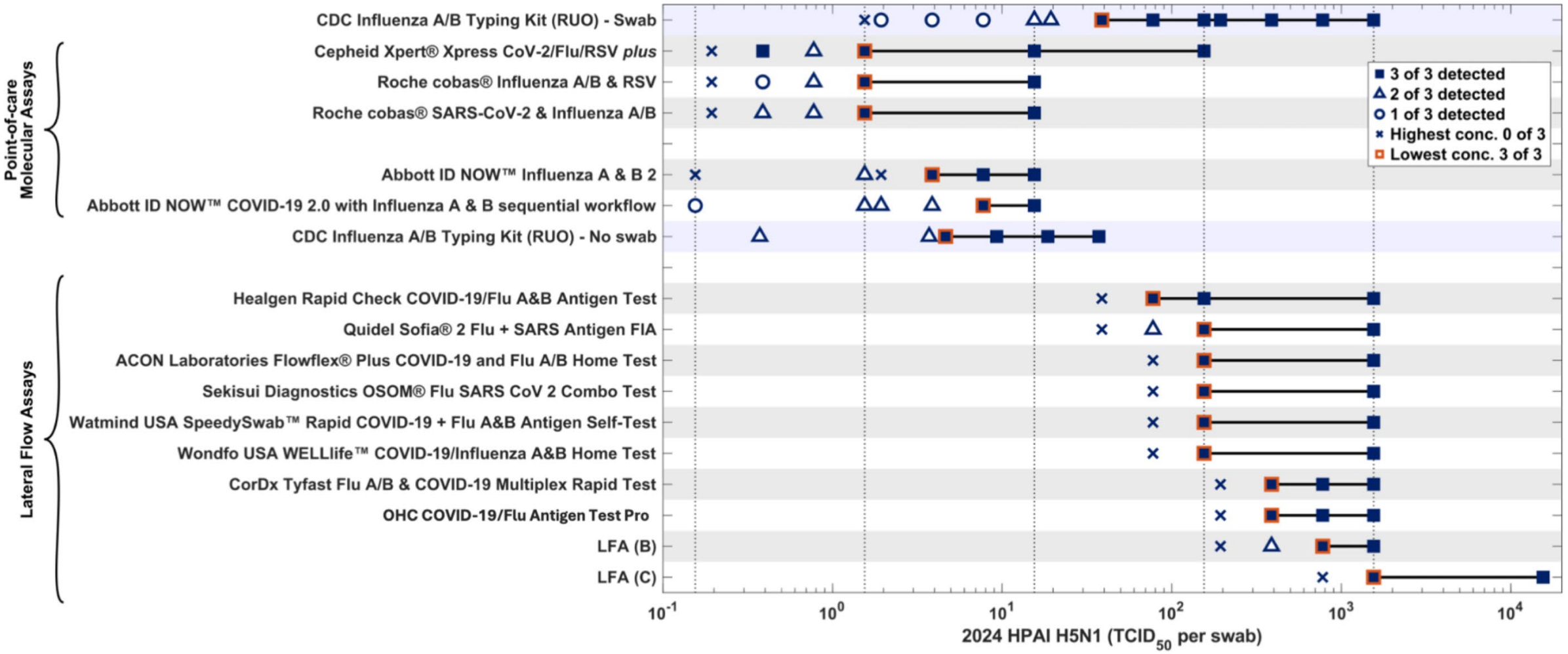
- Among the tested samples, A/mallard/Minnesota/16-041335-3/2016 (H5N2) showed the largest Ct gap, reflecting the highest count of mismatches (9) within primer–probe set 1

Multiplex Dual-Target Reverse Transcription PCR for Subtyping Avian Influenza A(H5) Virus

Malaya K. Sahoo, Ingrid E.A. Morante, ChunHong Huang, Daniel Solis,
Fumiko Yamamoto, Uzoamaka C. Ohiri, Daniel Romero, Benjamin A. Pinsky

- The 95% LLOD for the H5 target was 2.5 copies/µL (95% CI 1.8–5.3 copies/µL) for the clade and <0.5 copies/µL for the clade 2.3.4.4b ssDNA mix
- Clinical Validation: 97% detection of influenza A in positive respiratory samples

Performance of five POC molecular tests and 10 LFAs for detection of 2024 HPAI H5N1



doi: 10.1101/2025.04.15.25325613

Article

Development and Laboratory Validation of Rapid, Bird-Side Molecular Diagnostic Assays for Avian Influenza Virus Including Panzootic H5Nx

Matthew Coopersmith [1], Remco Dijkman [2], Maggie L. Bartlett [3], Richard Currie [4], Sander Schuurman [2] and Sjaak de Wit [2,5,*]

- To develop and validate Alveo Sense Poultry Avian Influenza Tests for detecting AIV (M gene) and subtypes H5, H7, and H9 in unprocessed oropharyngeal and cloacal samples

POCT for avian influenza virus detect

- Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) combined with impedance-based detection
- Delivers results in ~45 minutes

CDC signs Alveo Technologies for fast, handheld test to detect avian flu in humans

By Dan Flynn on November 18, 2024

POCT for avian influenza virus detect

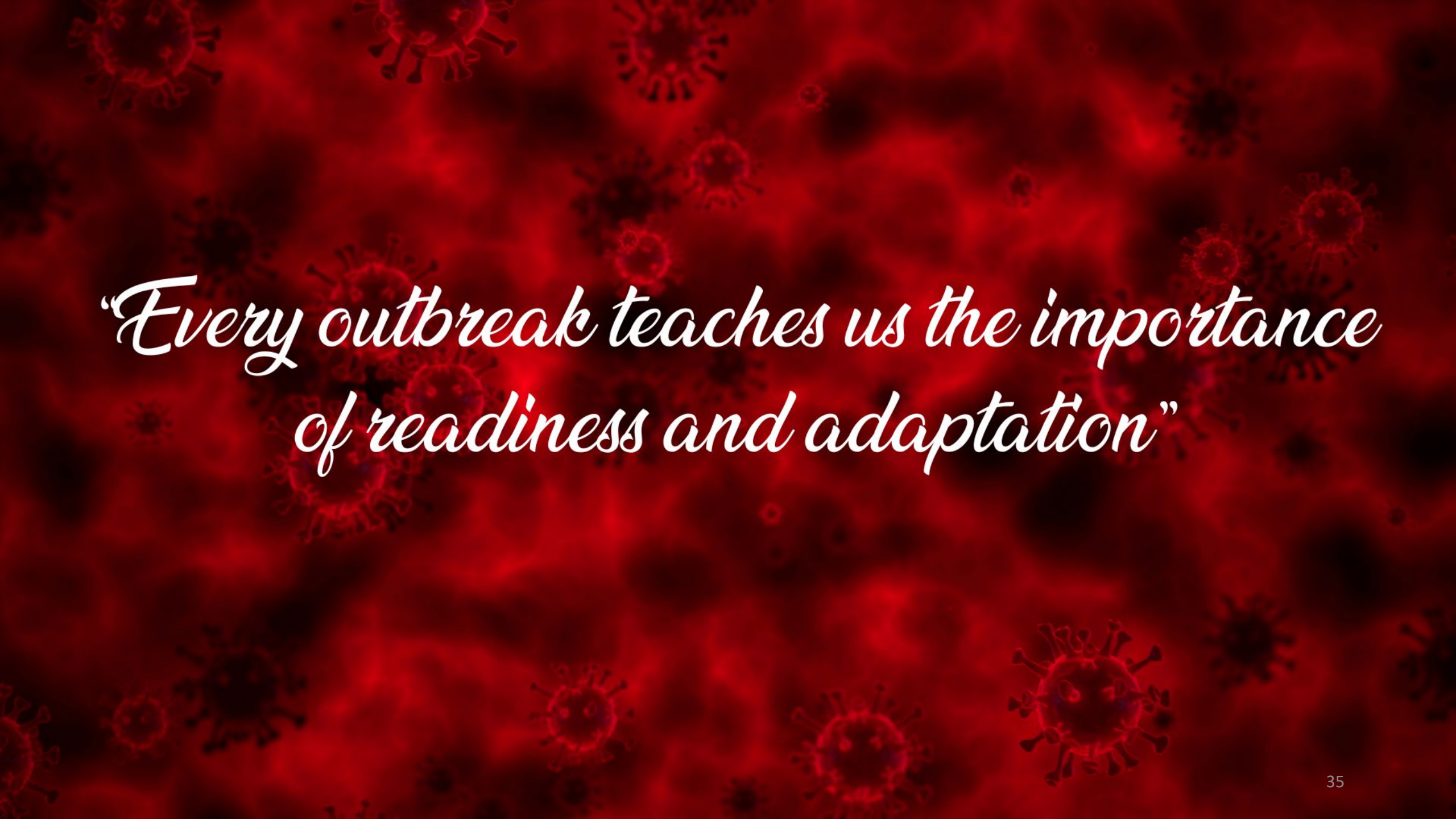
- High specificity (100%) with no cross-reactivity to non-AIV pathogens
- LoD95: Approximately Ct 30–33 (comparable to RT-PCR for moderate viral loads)
- Field testing: Successfully detected AIV in spiked and positive field samples
- Supports pooled testing (up to 5 cloacal or 10 oropharyngeal swabs)

3. Data Integration and Communication

- Real-time reporting
- Communication with the Department of Medical Sciences for outbreak coordination and confirmatory testing

4. Collaborative network

- Department of Medical Sciences, NIH (Thailand)
- Faculty of Engineering, Mahidol University
- Vidyasirimedhi Institute of Science and Technology (VISTEC)


"Every outbreak teaches us the importance of readiness and adaptation"