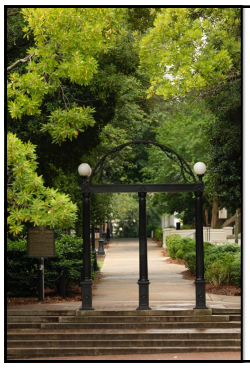

Wings of Worry: Avian Influenza Viruses

Daniel R. Perez, PhD

Poultry Diagnostic and Research Center,
Department of Population Health,
College of Veterinary Medicine,
University of Georgia

1

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UNIVERSITY OF GEORGIA

Flu lab (present)
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Teresa Mejias
Dikshya Regmi
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Lei He
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Flu lab (past members)
Flavio Cargnin Faccin

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Roberto Palomares
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John Hopkins U.
Sabra Klein et al

U. Rochester
Andrea Sant et al

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James Stevens

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Huw Davies et al

U. Oklahoma
Laura-Isobel McCall

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Anice Lowen
Katia Koelle
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Caswell Eidson endowment funds

2

Wings of Worry: Avian Influenza Viruses

- Bird Flu
- Zoonotic transmission of avian origin FLUAVs
- Modified live attenuated vaccines (MLVs) for mass vaccination strategies against avian influenza, including H5N1 HPAIV
- Aerosol exposure system – focus on MLV delivery
- Discussion of Jersey cows experimentally infected with H5N1 HPAIV
- Conclusions and finals thoughts

3

International Committee on Taxonomy of Viruses

Realm: *Riboviria*
Kingdom: *Orthornavirae*
Phylum: *Negarnaviricota*
Subphylum: *Polyploviricotina*
Class: *Insthoviricetes*
Order: *Articulavirales*

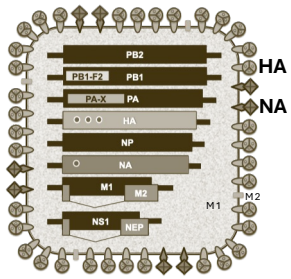
Digitally-colorized negative-stained transmission electron micrograph of avian influenza viruses. Credit CDC/F.A. Murphy

- Family: *Orthomyxoviridae*
- **Genus: *Alphainfluenzavirus* – type A - FLUAV**
- Genus: *Betainfluenzavirus* – type B - FLUBV
- Genus: *Deltainfluenzavirus* – type D - FLUDV
- Genus: *Gammainfluenzavirus* – type C - FLUCV
- Genus: *Isavirus*
- Genus: *Mykissvirus*
- Genus: *Quaranjavirus*
- Genus: *Sardinovirus*
- Genus: *Thogotovirus*

- **Lipid bilayer envelope derived from host plasma membrane**
- **Pleomorphic particles with a diameter in the range 80–120 nm**
- **RNA genome, segmented, negative sense, RNA-dependent RNA polymerase complex**

4

Influenza viruses are constantly changing



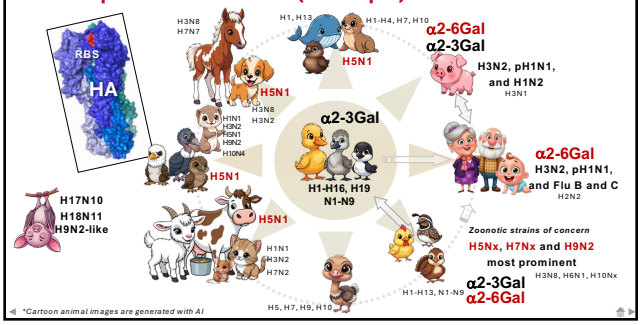
- **Hemagglutinin (HA) and Neuraminidase (NA)**
- Surface glycoproteins
- Divided into subtypes
- 17 HA subtypes (avian)
- 9 NA subtypes (avian)
- 153 theoretical combinations
- H17, H18, H9-like, N10, N11, N2-like (fruit bats)

- **Error Prone Polymerase**
- Point Mutations --- Changes on surface proteins (HA) leads to *Antigenic drift* and Evasion of immune responses

- **Segmented Genome = Reassortment**
- Reassortment leads to *Antigenic Shift* = Exchange of HA subtype
  1918 H1N1, 1957 H2N2, 1968 H3N2, 1977 H1N1, 2009 pH1N1

5

Flu Can Spread Between Animals (and People!)



*Cartoon animal images are generated with AI

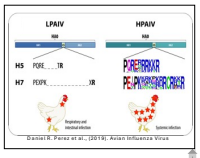
6

Avian Influenza (Bird Flu): A Threat to Birds and The Economy

- Originally termed fowl plague in 1878
- Results in significant economic losses due to disease/deaths and culling to control infection
- Isolates are classified based on pathogenicity in domestic chickens (terminology adopted in 1981)

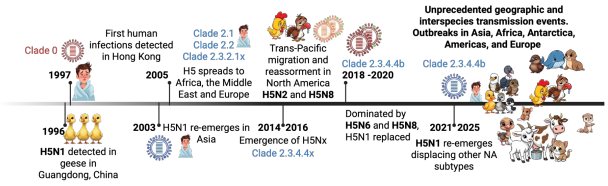
- **Low pathogenic avian influenza (LPAI) viruses:**
- Most isolates are low pathogenic – H9N2 LPAI viruses are the most prominent
- Associated with outbreaks of varying intensity in domestic birds

- **Highly pathogenic avian influenza (HPAI) viruses:**
- Circulation of LPAI viruses that evolve into HPAI viruses
  - *Restricted to H5 and H7 subtypes*
  - *HA with polybasic cleavage site*
  - *Intravenous Pathogenicity Index test of ≥1.2 (75% mortality)*



7

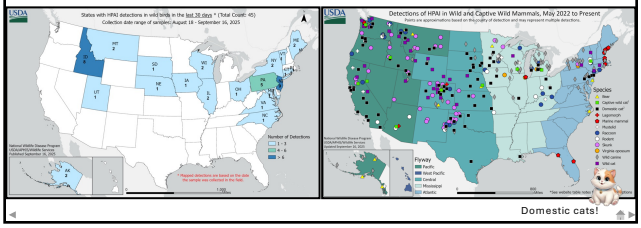
H5N1 highly pathogenic avian influenza virus: Two-way transmission between poultry and wild birds



8

HPAIV (H5N1 2.3.4.4b) Surveillance & Impact: Wild Birds / Mammals

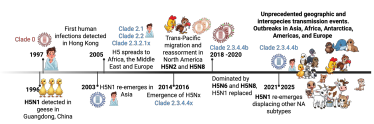
Since Dec. 2021
- 14,369 detections in wild birds across all 50 states
- 647 reports of infections in mammals
- Growing number of wild mammals (skunks, raccoons, cougars, bobcats, harbor seals)
- Migration to Central and South America (~October '22), sub-Antarctica (October '23), mainland Antarctica (February '24)



Domestic cats!

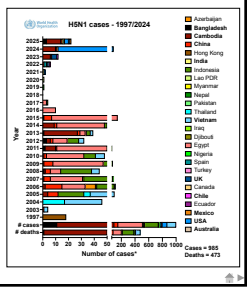
9

H5N1 highly pathogenic avian influenza virus: Two-way transmission between poultry and wild birds



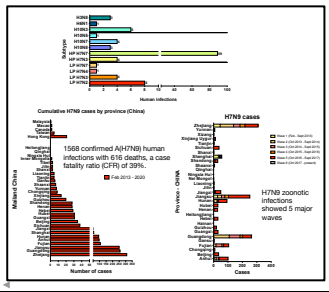
Significant impact on poultry/egg production in the US

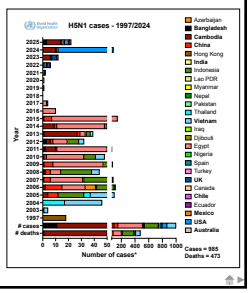
- **Current HPAI outbreak** (Feb. 2022 - present)
  - 174,830,000 US birds affected flocks across all 50 states
    - 1,709 flocks across (788 commercial, 921 backyard)
- **Previous HPAI outbreak** (2014 - 2015)
  - >50,000,000 US birds affected flocks across 15 states
    - 232 (211 commercial, 21 backyard)
  - Estimated **$3 billion dollars** in agricultural-related damages



10

Zoonotic Infections on the Rise: A Consequence of Poultry-Adapted Avian Flu



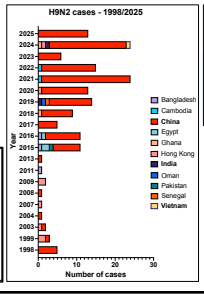


11

H9N2 LPAIVs: Endemic, Zoonotic, and Associated with Significant Economic Losses in Poultry

H9N2 importance:

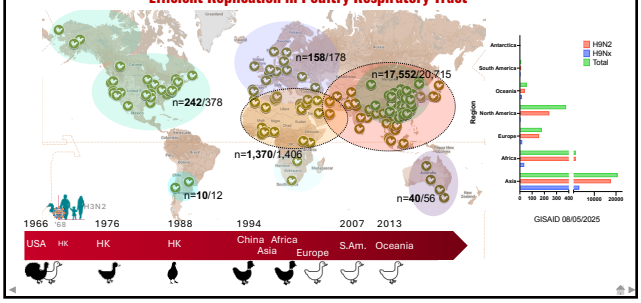
- Donors of internal gene segments for other zoonotic influenza strains of pandemic concern
- Zoonotic viruses themselves
- Human serological studies suggest high levels of exposure in endemic areas



Most recent human case: 5-year-old boy, Hunan province, China April 19, 2025

12

Importance of poultry adapted H9N2 LPAIVs: Widespread and Endemic Associated with Significant Economic Losses in Poultry Efficient Replication in Poultry Respiratory Tract



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Modified live virus (MLV) vaccines against H9 FLUAVs

- Prevention/control of avian influenza relies mostly on Whole Inactivated Virus + Adjuvant (WIV-adj) vaccines
- WIV-adj vaccines provide limited protection with high levels of humoral, but minimal or no cellular immunity.
- WIV-adj target the HA, the major target of neutralizing antibodies. Not suited for mass vaccination.
- HA's antigenic drift can make WIV-adj ineffective – Suboptimal vaccines can promote antigenic drift.
- ***Modified live attenuated virus (MLV) vaccines:***

Advantages

- **Mimic natural infection = Target multiple antigens**
- **Elicit mucosal, cell-mediated, and humoral immunity**
- **Mass vaccination**
  - **Drinking water,**
  - **Aerosol/Spray vaccination,**
  - **In ovo**

Disadvantages

- **Fear of reassortment**
- Like any other vaccine, response depends on immune status. Too attenuated = suboptimal protection
- May interfere with DIVA strategies
  - But is DIVA really needed? It depends!
  - Poultry for export vs internal consumption
  - Notifiable vs non-notifiable subtype

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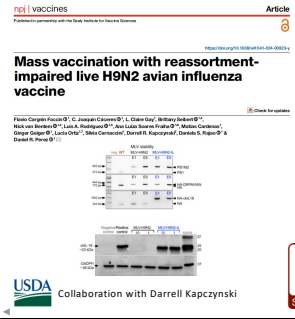
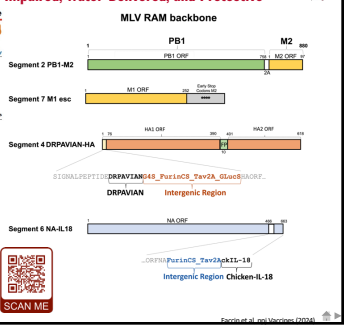
H9N2 MLV Vaccine: Reassortment-Impaired, Water-Delivered, and Protective





USDA Collaboration with Darrell Kapczynski

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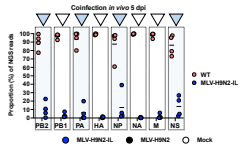
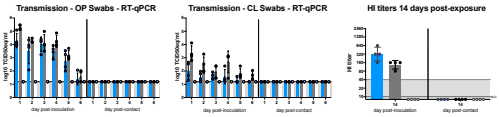
MLVs show fitness disadvantage compared to wild type H9N2 LPAIV infection in vivo

- 10MLV:1WT coinfections *in vivo* in chickens – no evidence of reassortment of modified gene segment
- No transmission to direct contacts - Presence of IL-18 leads to improved HI responses.





Faccin et al, npj Vaccines (2024)

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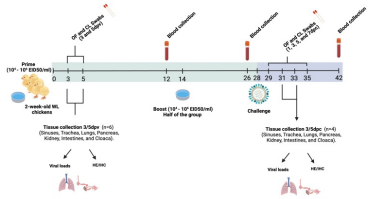
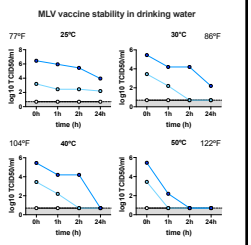
Potential field application – Drinking water vaccination with MLV-H9N2-IL



1. 10^4 EID_{50}/ml Prime-only
2. 10^5 EID_{50}/ml Prime-only
3. 10^6 EID_{50}/ml Prime-Boost
4. 10^5 EID_{50}/ml Prime-Boost
5. Mock-Challenge
6. Mock-Vaccination



MLV-H9N2-IL is stable during vaccine administration at different temperatures

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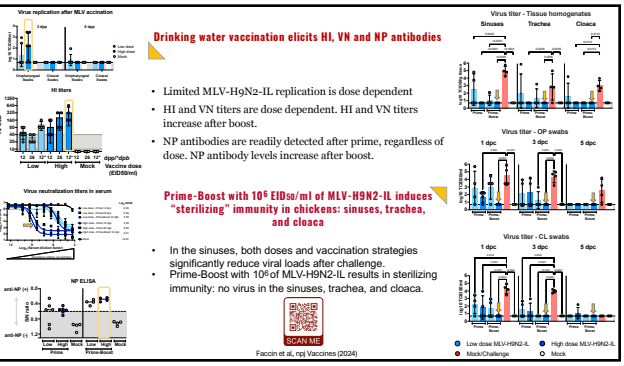

Drinking water vaccination elicits HI, VN and NP antibodies

- Limited MLV-H9N2-IL replication is dose dependent
- HI and VN titers are dose dependent. HI and VN titers increase after boost.
- NP antibodies are readily detected after prime, regardless of dose. NP antibody levels increase after boost.

Prime-Boost with 10^6 EID_{50}/ml of MLV-H9N2-IL induces "sterilizing" immunity in chickens: sinuses, trachea, and cloaca

- In the sinuses, both doses and vaccination strategies significantly reduce viral loads after challenge.
- Prime-Boost with 10^6 of MLV-H9N2-IL results in sterilizing immunity: no virus in the sinuses, trachea, and cloaca.

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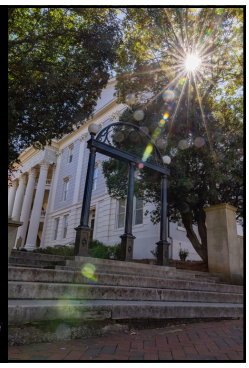
Sequential Aerosol and Oral Immunization with a Bivalent H9N2/H5N2 Vaccine Protects Against H5N1 and H9N2 Avian Influenza Challenges



Flavio Cargnin Faccin, L. Claire Gay, Dikshya Regmi, Robert Hoelz, Teresa D. Mejías, Darrell Kapczynski, Florian Krammer, and **Daniel R. Perez**

Submitted to npj Vaccines
08/18/2025

Poultry Diagnostic and Research Center
Department of Population Health
College of Veterinary Medicine

UNIVERSITY OF GEORGIA

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Journal of Virology

VIRUS-CELL INTERACTIONS
Month YYYY Volume XX Issue XX e00618-25
https://doi.org/10.1128/jvi.00618-25

Air-liquid interface model for influenza aerosol exposure *in vitro*

Brittany Seibert [1], C. Joaquin Caceres [1], L. Claire Gay [1], Nishit Shetty [2], Flavio Cargnin Faccin [1], Silvia Carnaccini [1], Matthew S. Walters [3], Linsey C. Marr [2], Anice C. Lowen [4], Daniela S. Rajao [1], Daniel R. Perez [1]

[1]Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, Georgia, USA
[2]Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, Virginia, USA
[3]Department of Internal Medicine, Pulmonary, Critical Care & Sleep Medicine Section, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA
[4]Department of Microbiology and Immunology, Emory University, Atlanta, Georgia, USA

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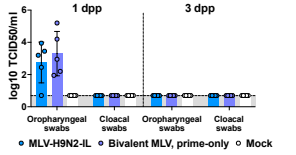
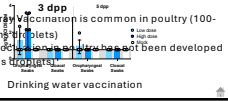
Aerosol administration results in limited MLV replication

Virus replication after aerosol MLV vaccination

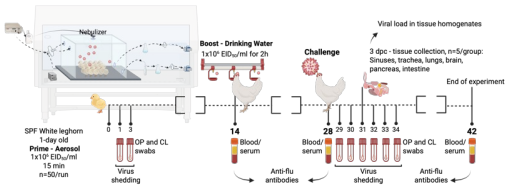


- Coarse Spray Vaccination is common in poultry (100-150 microns droplets)
- Aerosol Vaccination in poultry has not been developed (~4 microns droplets)



20

Optimizing MLV Vaccination: A Combined Aerosol-Oral Delivery Approach



Groups

1. **MLV-H9N2-IL, prime-boost**
2. **Bivalent (MLV-H9N2-IL, MLV-H5N2-IL), prime only**
3. **Bivalent (MLV-H9N2-IL, MLV-H5N2-IL), prime-boost**
4. **Bivalent WIV-adj (H9N2/H5N2), prime-boost**
5. **Mock-Vaccination – Challenge**
6. **Mock**

A/ck/Egypt/A15068/2018 (H9N2) – homologous
A/ck/Shanxi/BJ/2021 (H9N2) – drifted
A/ty/IN/3707-003/22 (H5N1) - homologous

H9N2 LPAIV @10^8 EID50/chicken
H5N1 HPAIV @50 ckLD50/chicken
Oculo-nasal-tracheal-cloacal routes
500 µL

Cargnin Faccin et al. submitted

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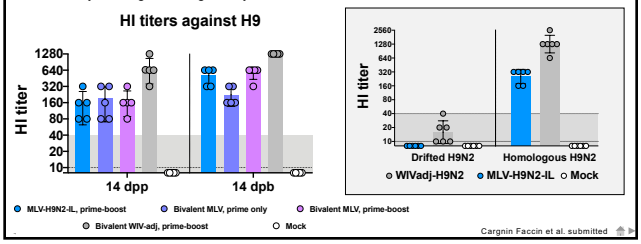
Aerosol/drinking water vaccination induced discernible HI responses against the homologous but not antigenically drifted H9 HA

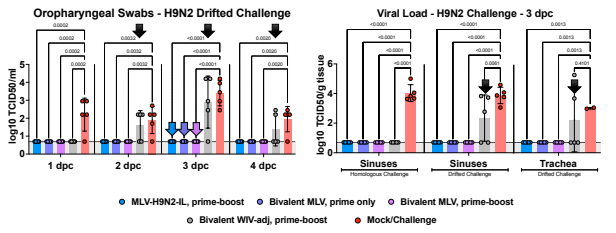
- Prime vaccination via aerosol induced HI titers against H9
- Boost via drinking water increased antibody titers against H9
- Antibody titers against antigenically drifted strain ≤40



Cargnin Faccin et al. submitted

22

Monovalent and bivalent MLVs provide protection against drifted H9N2 challenge in contrast to bivalent WIV-adj vaccine



Challenge with drifted H9N2 LPAIV @10^8 EID50/chicken
Oculo-nasal-tracheal-cloacal routes - 500 µL

Cargnin Faccin et al. submitted

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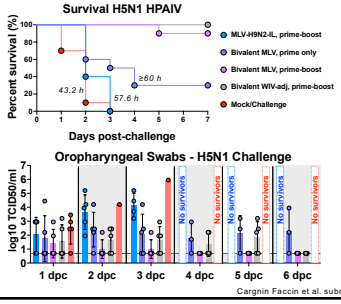
Bivalent MLV provides protection against H5N1 HPAIV

- Chickens were challenged with 50 ckLD$_{50}$ of H5N1 two weeks after boost (1.58 x 10^5 EID50/chicken)
- A bivalent vaccine administered through a prime-boost method resulted in a 90% protection rate and lower viral loads in comparison to other groups.



Cargnin Faccin et al. submitted

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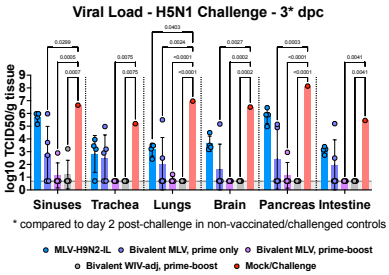
Bivalent MLV reduces H5N1 HPAIV replication in chickens

- A prime/boost bivalent MLV vaccine reduced virus titers in tissues after H5N1 HPAI challenge.



* compared to day 2 post-challenge in non-vaccinated/challenged controls

MLV-H9N2-IL · Bivalent MLV, prime only · Bivalent MLV, prime-boost · Bivalent WIV-adj, prime-boost · Mock/Challenge

Cargnin Faccin et al. submitted

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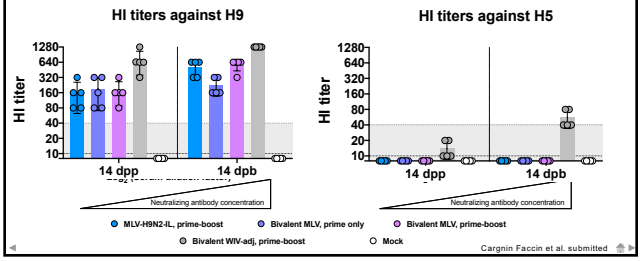
Aerosol/drinking water vaccination induced discernible antibody response against H9 but not against H5 FLUAVs

- Prime vaccination via aerosol induced neutralization antibody titers against H9
- However, neutralization antibody titers against H5 were low. Issue with H5 antigen, not MLV approach



MLV-H9N2-IL, prime-boost · Bivalent MLV, prime only · Bivalent MLV, prime-boost · Bivalent WIV-adj, prime-boost · Mock

Cargnin Faccin et al. submitted

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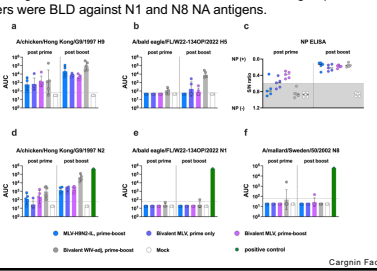
Aerosol/drinking water vaccination induced discernible antibody responses against H9 and N2 antigens but not against H5 FLUAVs

- Prime-boost vaccination induced antibodies against H9, N2, and NP as measured by ELISA
- ELISA antibody titers against H5 were below limit of detection in MLV groups
- ELISA antibody titers were BLD against N1 and N8 NA antigens.

Collaboration with
Robert Hoelzl
Dr. Florian Krammer

KRAMMER LAB
Mount Sinai



MLV-H9N2-IL, prime-boost · Bivalent MLV, prime only · Bivalent MLV, prime-boost · Bivalent WIV-adj, prime-boost · Mock · positive control

Cargnin Faccin et al. submitted

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Faster responses to avian influenza outbreaks with help from in-farm diagnostic tests

*AI generated images with Gemini

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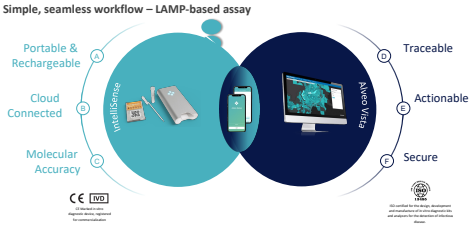
Alveo Sense Platform*

Simple, seamless workflow – LAMP-based assay



* **Disclaimer:** Neither the University of Georgia nor any member of the Perez Lab receives any form of compensation, financial or otherwise, from Alveo Technologies. The testing and evaluation of Alveo's diagnostic platform by personnel from the Perez Lab is conducted independently and is not a result of any compensatory agreement with Alveo Technologies.

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Alveo Sense Poultry Avian Influenza Test Type A H5 H9

ALVEO

Test Design:

Target	Individual Assays
Type A	2 unique regions of matrix gene for broad InfA detection
Subtype H5	Targeted clades of H5N1: • 2.3.4.4b (HPAI) • American non-specific lineage • Eurasian non-specific lineage
Subtype H9	Detect clinically relevant H9 strains

Limit of Detection:

Sample	Matrix Type	Equivalent PCR Ct GD TaqMan PCR
H5N2 (Am_nonGsGd)	Cloacal (Pool of 5)	33.5
	Oropharyngeal (Pool of 10)	33.0
H5N8 (2.3.4.4b)	Cloacal (Pool of 5)	30.6
	Oropharyngeal (Pool of 10)	31.9
H9N2 (G lineage)	Cloacal (Pool of 5)	31.6
	Oropharyngeal (Pool of 10)	31.6

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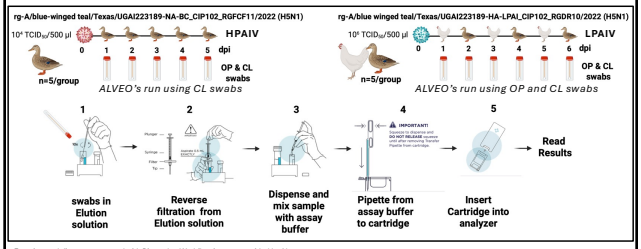
Experimental Flow using ALVEO's Detection System*



Experimental diagram generated with Biorender. Workflow images provided by Alveo

*Tests conducted as Research Use Only. Test components were not for sale.

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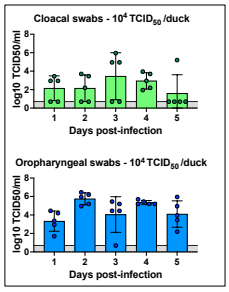
Alveo's signal corresponds with *HPAIV* H5N1 $TCID_{50}$ Titers



dpi	swabs	Type A	H5	H9
1	CL	+	+	-
3	CL	+	+	-
5	CL	+	+	-

Alveo's signal

*Tests conducted as Research Use Only.
**Test components were not for sale.

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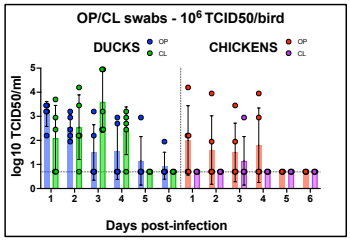
Alveo's signal corresponds with LPAIV H5N1 $TCID_{50}$ Titers



Species	dpi	swabs	Type A	H5	H9
Chicken	1	OP	+	+	-
		CL	inv	inv	inv
Duck	2	OP	-	+	-
		CL	+	-	-
Chicken	3	OP	+	+	-
		CL	-	-	-
Duck	4	OP	+	+	-
		CL	+	+	-
Chicken	5	OP	+	+	-
		CL	inv	inv	inv
Duck	6	OP	-	-	-
		CL	-	-	-

Alveo's signal

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Summary: MLVs to prevent and control avian influenza viruses

- MLVs offer an opportunity for mass vaccination – drinking water, aerosol, spray(?).
- MLVs are stable, immunogenic, do not transmit, and do not reassort (?)
- The inclusion of an immunomodulator (Flu-kine) enhanced the generation of neutralizing and non-neutralizing antibodies, suggesting a role in the host immune response.
- Aerosol vaccination results in limited MLV virus replication.
- Aerosol and drinking water vaccination elicits measurable serological antibody subtype specific responses against HA and NA. Opportunity for production of DIVA-compliant MLVs based on anti-NA responses? Anti-NP responses readily observed too.
- The bivalent MLV vaccine provides protection against homologous H5N1 HPAIV, despite negligible anti-H5 antibody responses.
- *The bivalent H9 MLV vaccine provides protection against (homologous) and drifted H9N2 FLUAV challenges (contrasting WIV-adj. vaccine).*

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Summary: Experimental use of ALVEO's portable diagnostic system

Intuitive, easy to operate, enables efficient result sharing.

Correctly identified 100% of expected results in ducks challenged with HPAIV-H5N1.

LPAIV-H5N1 Challenged Ducks

- Results aligned with viral load (based on $TCID_{50}$ measurements).
- OP positive for H5, CL positive for Type A at 2 DPI. These partial detections still represent actionable field results, as any positive signal would prompt confirmatory testing.

LPAIV-H5N1 Challenged Chickens

- Chickens exhibited different infection dynamics compared to ducks.
- OP pools performed as expected, confirmatory RT-PCR test underway on the OP pool from 5 DPI.
- CL pools produced two invalid results. The negative result at 3 DPI is likely attributable to low sample titer. The invalid result at 1 DPI may be due to a workflow deviation.
- **100% H9 exclusivity, no cross-reactivity.**

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Avian Flu Rapid Diagnostics Moving Forward:

- Current lack of globally harmonized data on how well rapid tests (specifically LFDs) perform across different AIV clades, geographies, and sample types.
- (Relatively) cheap, portable, and fast diagnostic tests that can be used for immediate detection in poultry farms, markets, and outbreak zones are coming along; however, ...
- Tests must be able to detect not only the dominant H5N1 clades but also other evolving and reassorting strains endemic in poultry and that pose a pandemic threat.
- Governments and response teams need transparent, evidence-based data to support their procurement and deployment decisions, as many current kits are unproven against avian variants.
- **RapidAIV**, a Global Collaborative to Harmonize and Validate Rapid Diagnostic Tests for Avian Influenza proposed by Erik Karlsson Director, National Influenza Center of Cambodia and WHO H5 Reference Laboratory, Coordinator, WHO Global COVID-19 Referral Laboratory, Institut Pasteur du Cambodge aims to assemble a collaborative network with common metrics to overcome the scattered and isolated small-scale assessments currently being conducted by individual labs.

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