Molecular diagnostic tests for AI index case

- ✓ **Is it bird flu ?**
  - ➢ Type A specific TaqMan
- ✓ **Is it H5 or H7 ?**
  - ➢ Subtype H5 and H7 specific TaqMan
- ✓ **Is it HP or LP strain ?**
  - ➢ PCR & sequencing of HA cleavage region
- ✓ **(Is it something else ? What?)**
  - ➢ TaqMan array (NDV, WNV, vvIBDV, other?)

AI TaqMan technology transfer to state labs and evaluation of different instrument platforms

Instruments

- Applied Biosystems ABI 7700 Sequence Detection System
- ABI 7500 Fast plate system
- Corbett Research Rotor-Gene Instrument
- Biorad iCycler
- Roche Lightcycler
- Cepheid Smartcycler

Transfer of AI TaqMan assays to state labs

- Harmonized assay using SOP, primer & probe sequences and assay conditions provided by AAHL
- Positive and negative controls (inactivated virus in RLT) sent to state labs
- Labs to extract RNA from positive control and perform real-time PCR on serial diluted RNA to determine analytical sensitivity
- Coded (“blind”) samples of strong and weak positives and negatives send to state labs for proficiency testing

(Traditional diagnostics also may need to be adapted to the outbreak strain)

Paul Selleck

Influenza Virus HI titres

Serum	A/tern/Australia/75 H5N3	A chicken/Vietnam/8/2004 H5N1
Anti-H5N3	640	40
Anti-H5N1	640	5120

This data indicates that using an antigen in the HI test that is not matched to the outbreak strain will result in reduced sensitivity of the test

Molecular diagnostics for AI (cont)

A further diagnostic challenge is illustrated by the information in our slide of the evolution of the current H5N1, which shows considerable genetic reassortment.

Pandemic preparedness requires the capability to sequence the whole genome to detect reassortments that may lead to a virus with different properties fro virulence or infectivity in poultry, humans or other animals

Avian influenza virus genetics

AI viruses have genomes with 8 segments

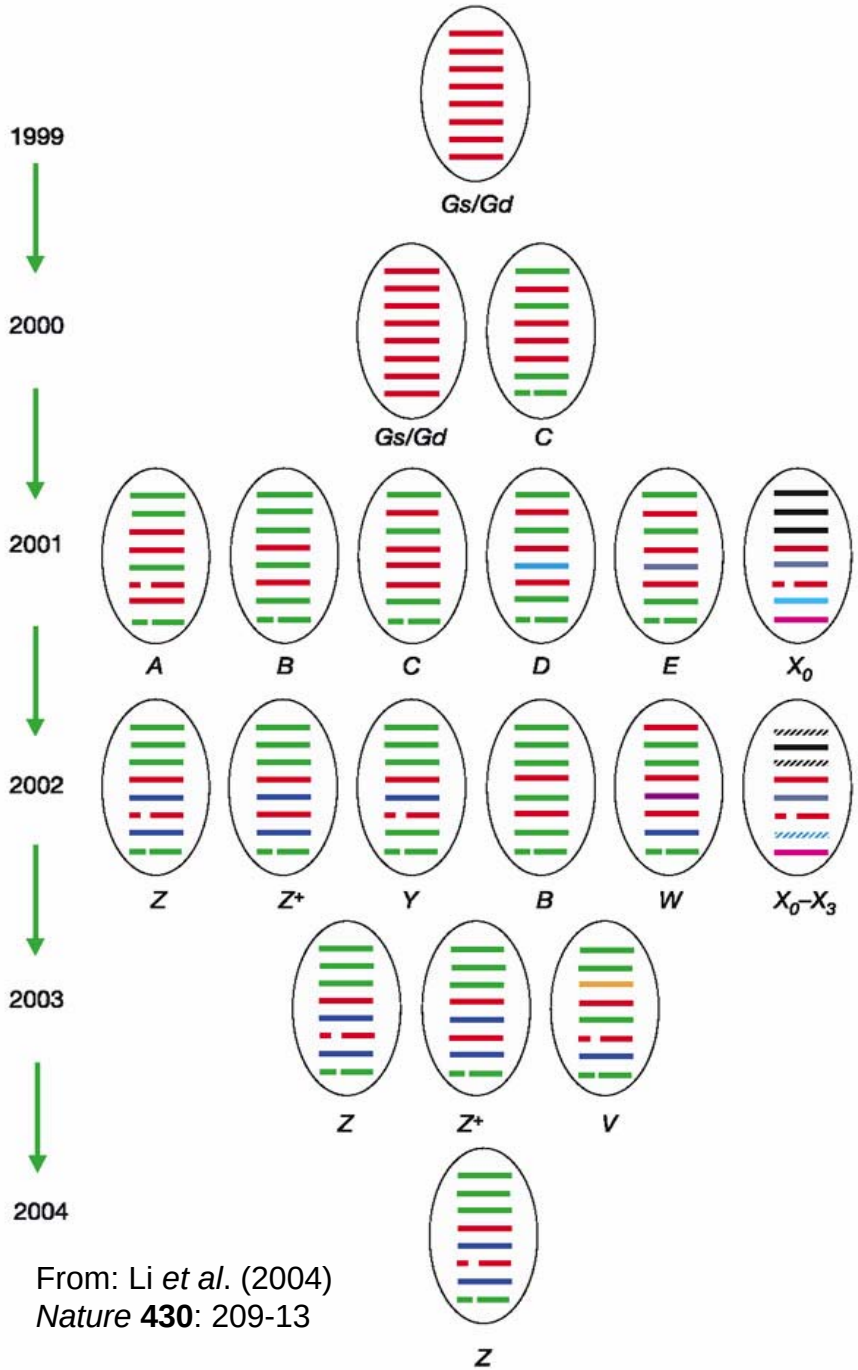
3 mechanisms for genetic evolution:

1. Point mutations and deletions
2. Intergenic recombination between segments
3. **Genetic reassortment during dual infection of a cell**

(Hence the evolution of H5N1 viruses as illustrated)

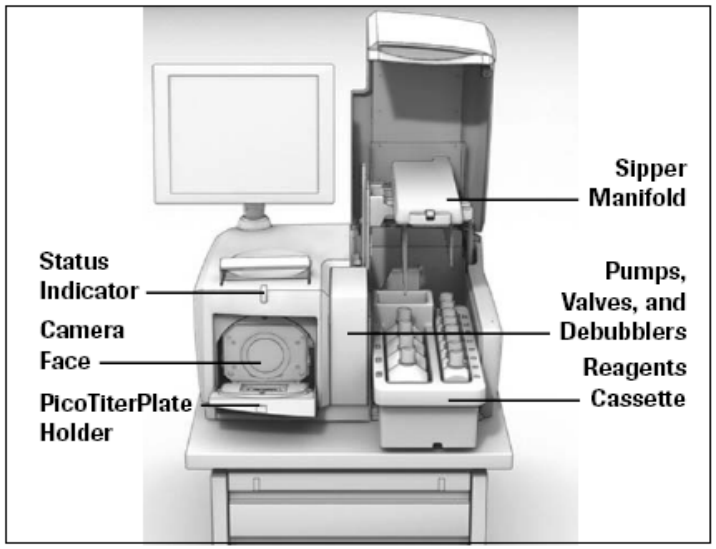
Diagnostic challenges

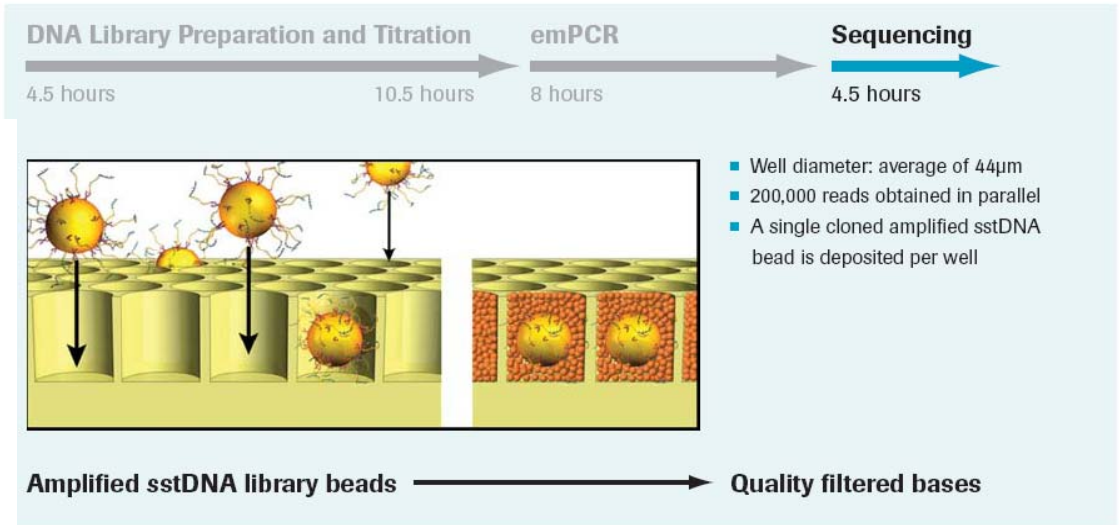
- Primer/probe design
- **Whole genome sequencing**



From: Li *et al*. (2004) *Nature* **430**: 209-13

Molecular Diagnostics: New technology for Rapid genome sequencing – not just a toy





Single person in a day: 200,000 independent sequencing reactions → 2,000,000 bases

Article: *Nature* advance online publication; published online 31 July 2005 | doi: 10.1038/nature03959

Genome sequencing in microfabricated high-density picolitre reactors

New technology will be adopted in reference laboratories

- Sequence more than 20 million bases in 4-5 hrs
- DNA library preparation- for RNA viruses need to add extra step of cDNA
- Use to identify quasi-species
- Potential applications to
  - find new viruses

Some examples of R&D responses to the Asian epidemic

Biosafety in the lab is of absolute importance

H5N1 Titration Study in Ducks: A/muscovy duck/Vietnam/453/2004

Deb Middleton

5 X 5 commercial ducks

6 weeks old

-ve by cELISA to AI antibody (all H types)

-ve to H5 by HI

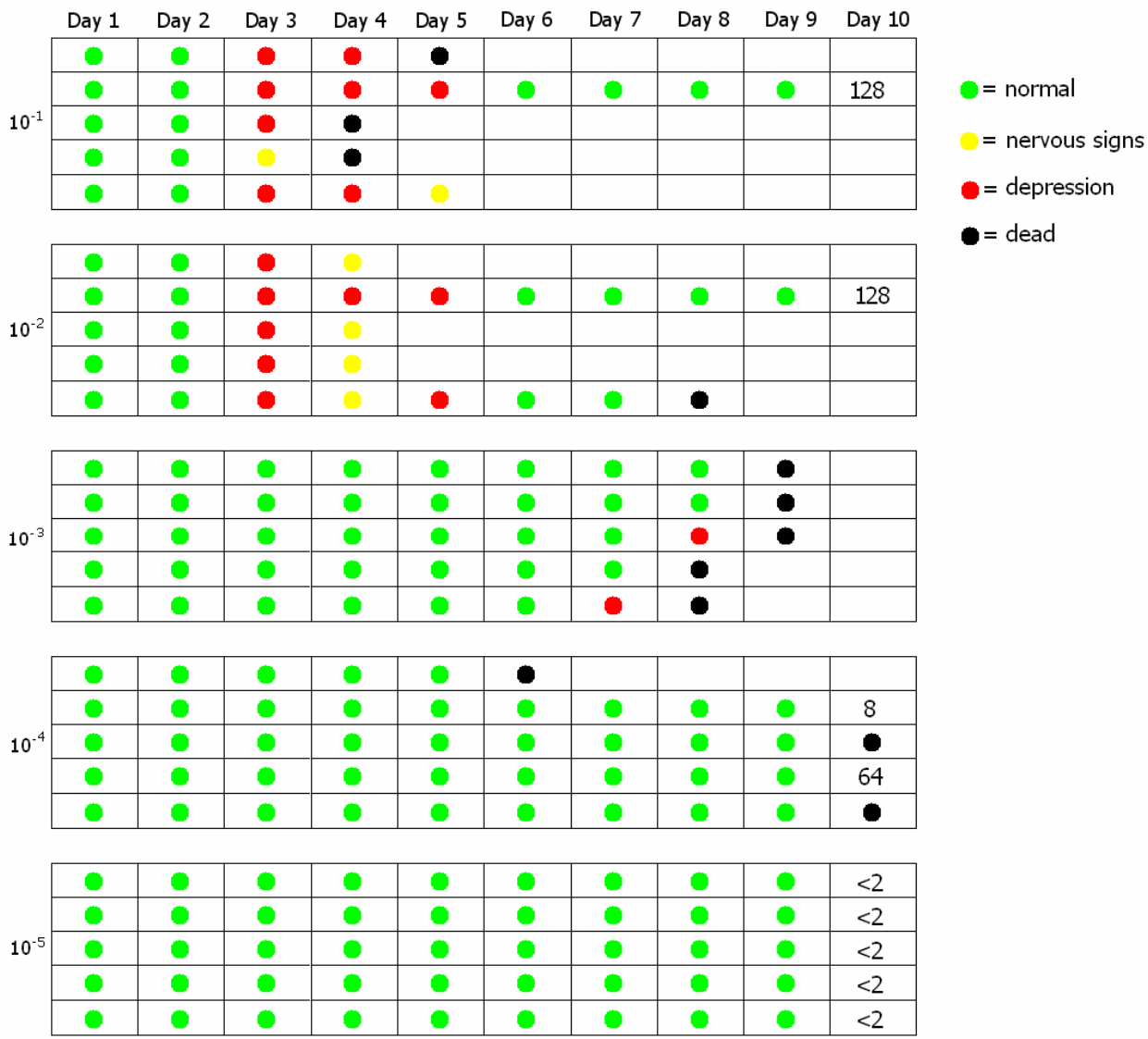
10^{-1} to 10^{-5} infected AF

I/N; I/Oral; I/Ocular

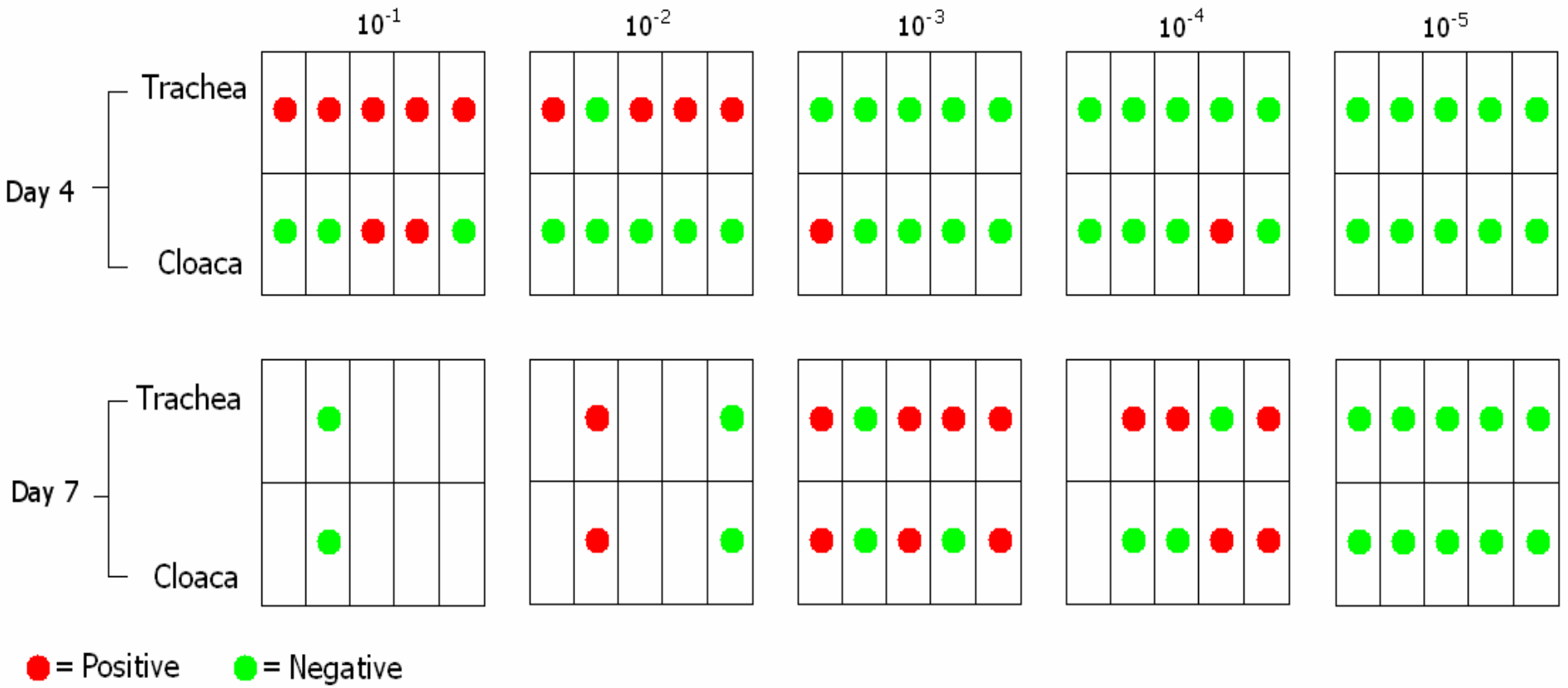
Observed for 10 days

- Clinical signs
- Virus excretion
  - Days 2, 3, 4, 7
- Antibody
  - Day 10

Clinical observations – titration study



Virus isolation – titration study



Calculation of challenge dose

An infected bird

- Exhibits clinical illness and/or
- Viral detection in tracheal or cloacal swabs on day 4 or day 7
- Titration end-point of $10^{3.2}EID_{50}$

Recommended 10^{-3} AF

- $10^{4.7}EID_{50}$ (equivalent to $10^{1.5}DID_{50}$)
- Over 30 duck infectious $doses_{50}$

Vaccine/H5N1 challenge study

Control birds n=14

- PBS at day old and 3 weeks old

Poulvac i-AI H5N9, H7N1 n=15

- 1° vaccination at day old and booster at 3wo

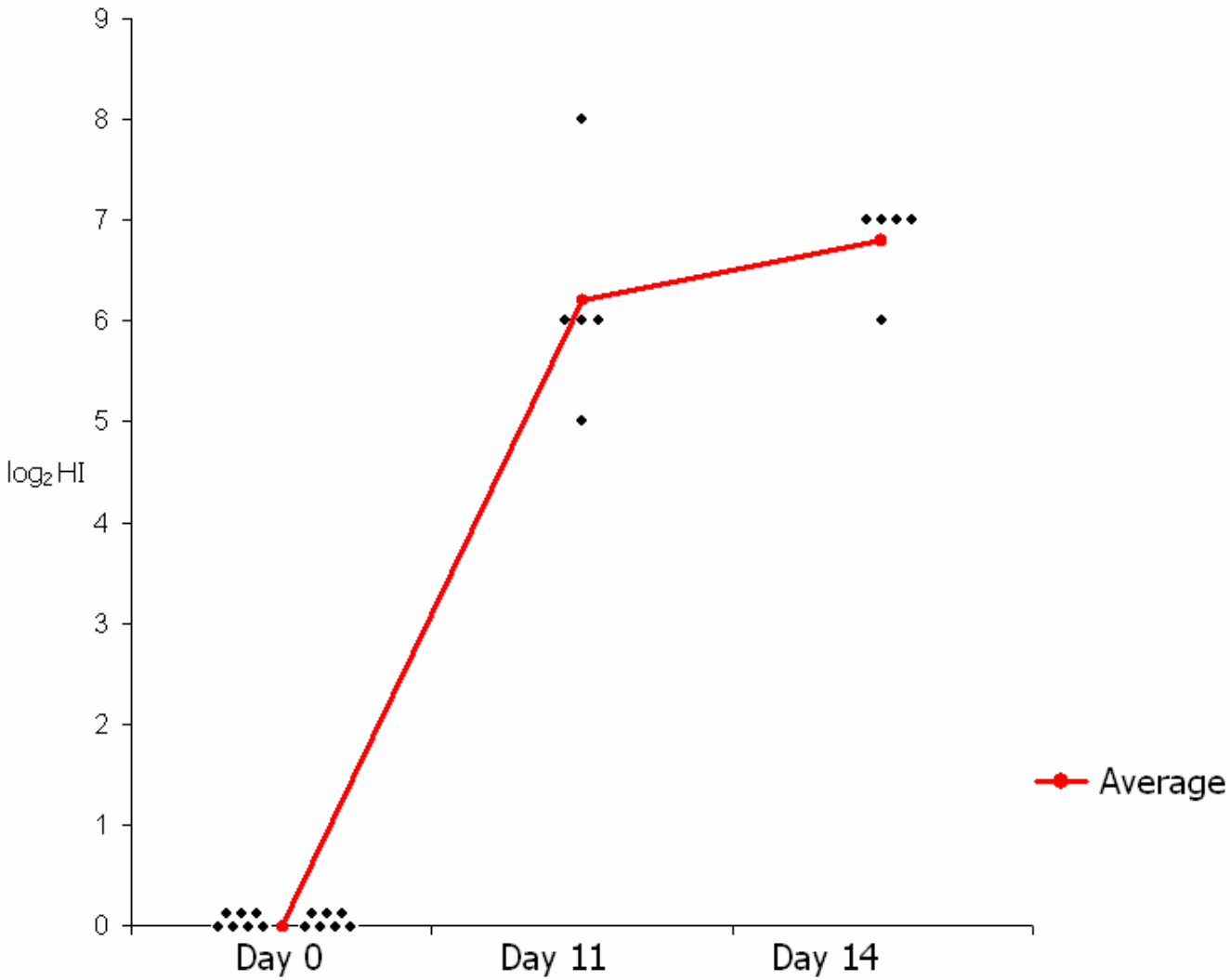
Poulvac i-AI H5N3 n=15

- 1° vaccination at day old and booster at 3wo

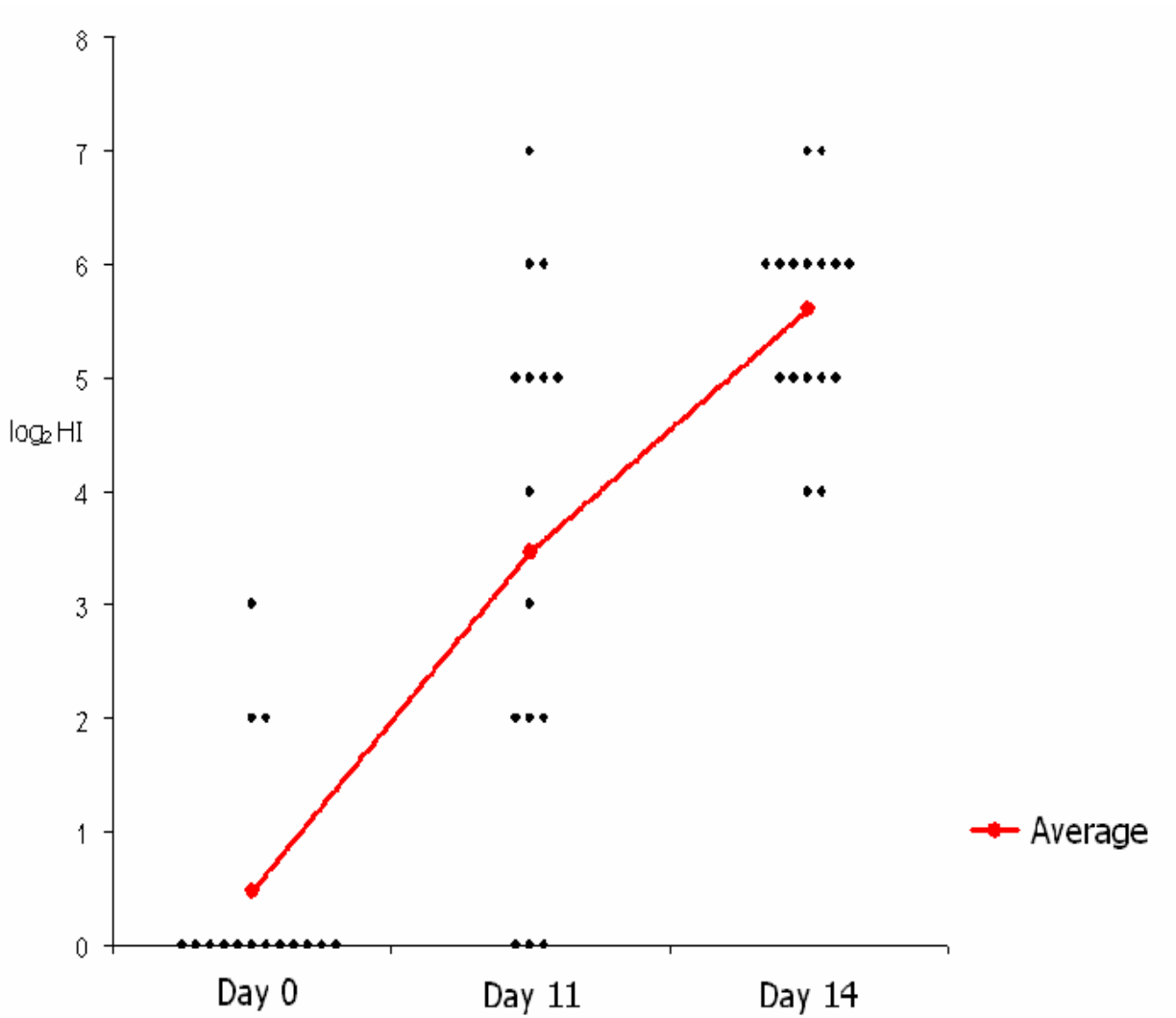
All birds challenged with H5N1 at 6wo

Clinical observations – vaccine study

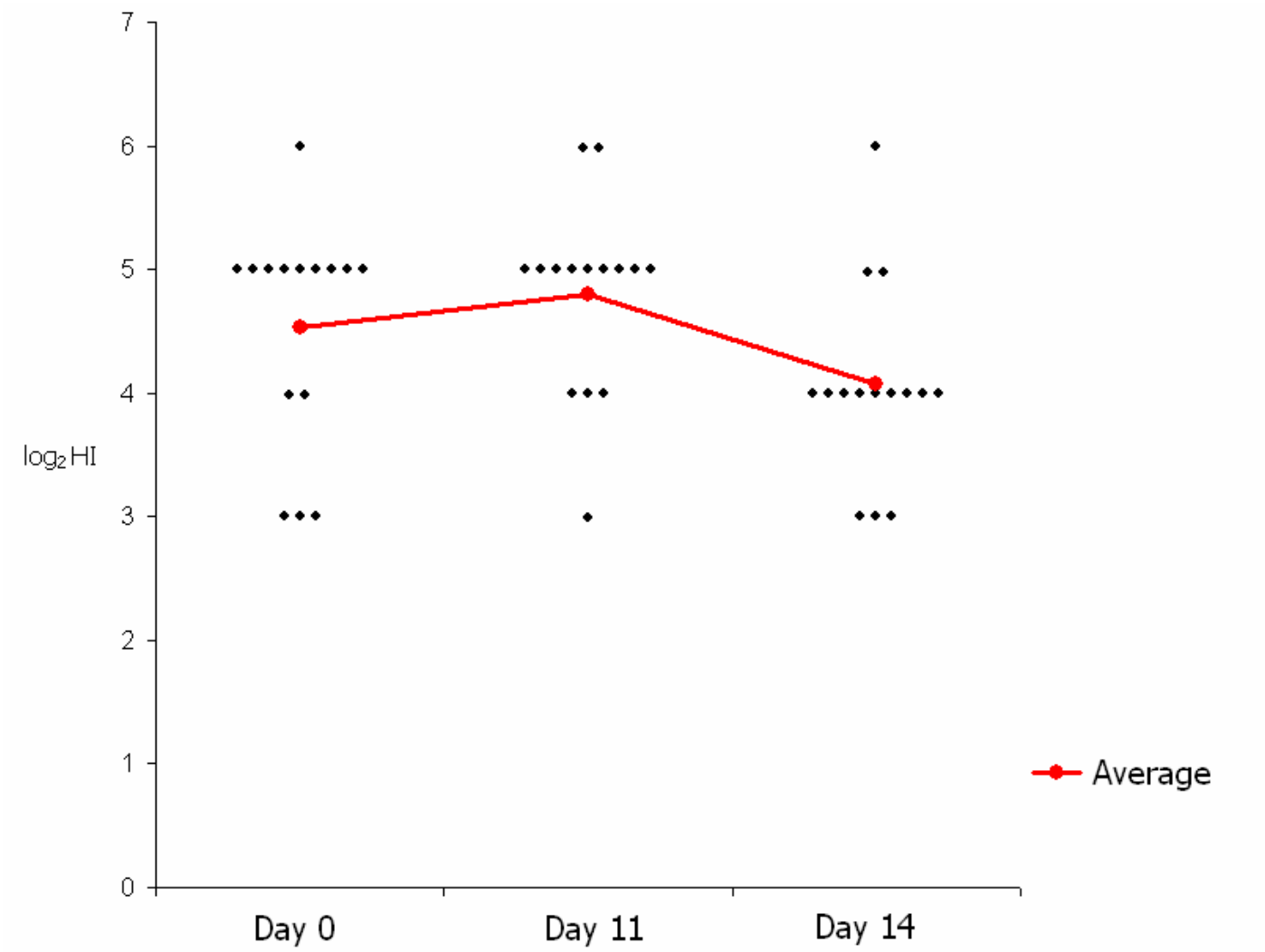
Serology - controls



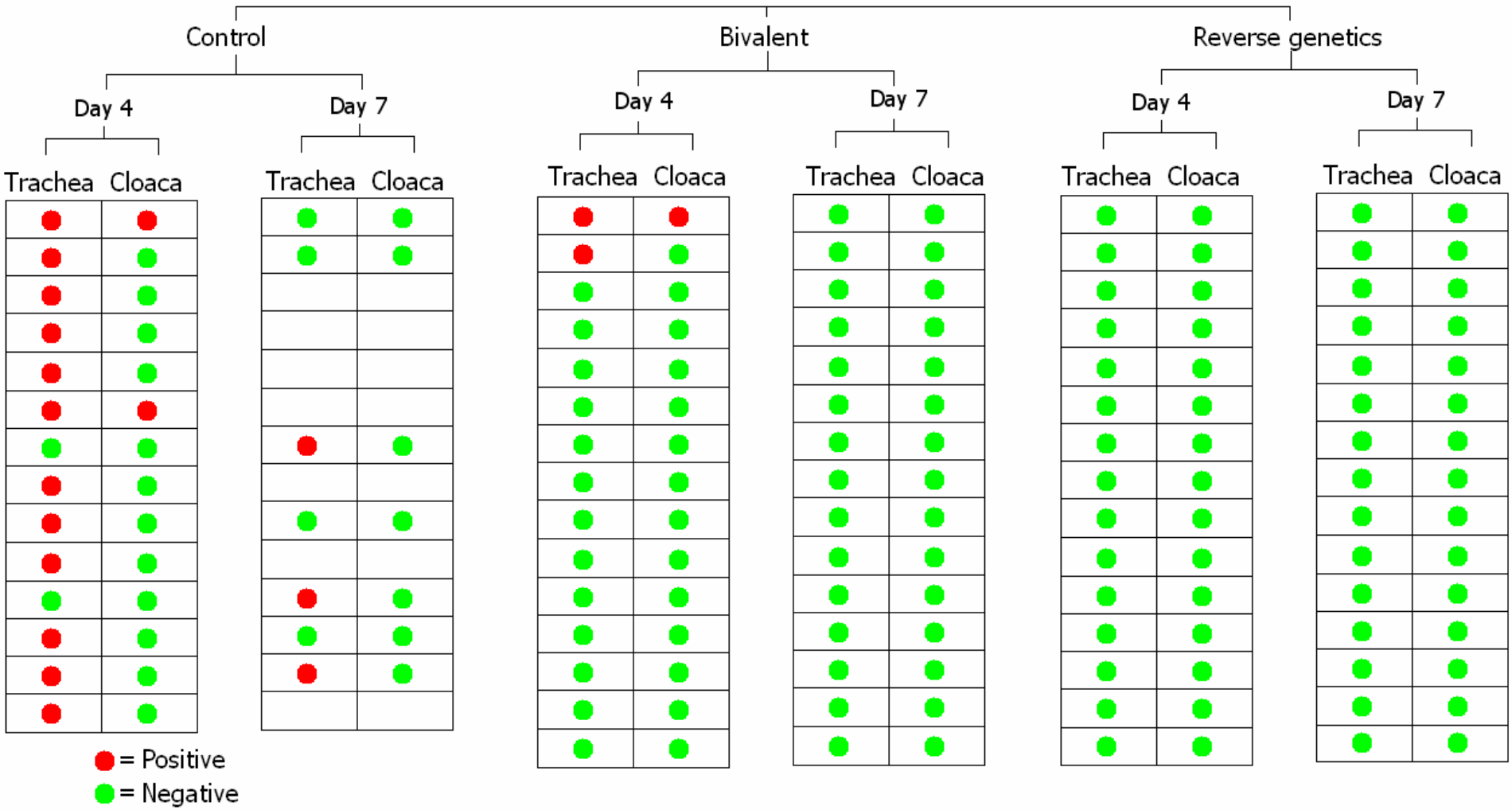
Serology – bivalent vaccine



Serology – H5N3 reverse genetics



Virus isolation – vaccine study



Summary

H5N9,H7N1 bivalent vaccine

- Protected ducks from H5N1 disease
- Serology data suggests infection occurred
- Shedding suppressed but not eliminated

H5N3 reverse genetics vaccine

- Protected ducks from H5N1 disease
- Serology data suggests infection did not occur
- No viral shedding detected

Point of Sampling Diagnostics

Kits for rapid antigen/agent detection potentially play an important role

Which kit to use – will OIE registration help?

We need to learn how to use the kits, issues of

- trained users (including sampling issues),
- Transport and storage issues
- validation of the diagnostic process
- Reporting issues – data capture and SOPs for response

Test kits evaluated in this study were:-

- Becton Dickenson Directigen Flu A & B,
- Biota Flu OIA,
- Anigen Rapid AIV Ag Test Kit,
- Anigen Rapid H5AIV Test Kit,
- Synbiotics Influenza Type A Antigen Test Kit

Point of Sampling Diagnostics

Analytical sensitivity

Dilutions of a stock virus (A/chicken/Vietnam/8/04 H5N1 with a titre of 108.1 EID50/ml) were made and 100 ul of each dilution added to the test kit sample buffer.

Dilution	Directigen	Flu OIA	Anigen AIV	Anigen H5	Synbiotics
1:5	4+	4+	4+	Neg	4+
1:10	2+	2+	2+	Neg	4+
1:50	Neg	Neg	?	Neg	3+
1:100	Neg	Neg	Neg	Neg	1+
1:500	Neg	Neg	Neg	Neg	Neg
1:1000	Neg	Neg	Neg	Neg	Neg

Point of Sampling Diagnostics

Tissues from H5N1 infected chickens

Ten percent suspensions of eight tissues from experimentally infected birds were tested by the addition of 100 ul to the test kit sample buffer.

- All tissues were strongly positive in all kits.

Cloacal swabs from H5N1 infected chickens

- The Directigen, Flu OIA and Anigen AIV kits all detected 3 of 4 swabs from experimentally infected chickens as strongly positive, with the 4th swab being weakly positive.
- The Anigen H5 failed to detect any of the swabs as positive.
- The Synbiotics kit has not been evaluated on swabs at this time.

All kits will be evaluated on tracheal swabs when clinical material is available.

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Thank You

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