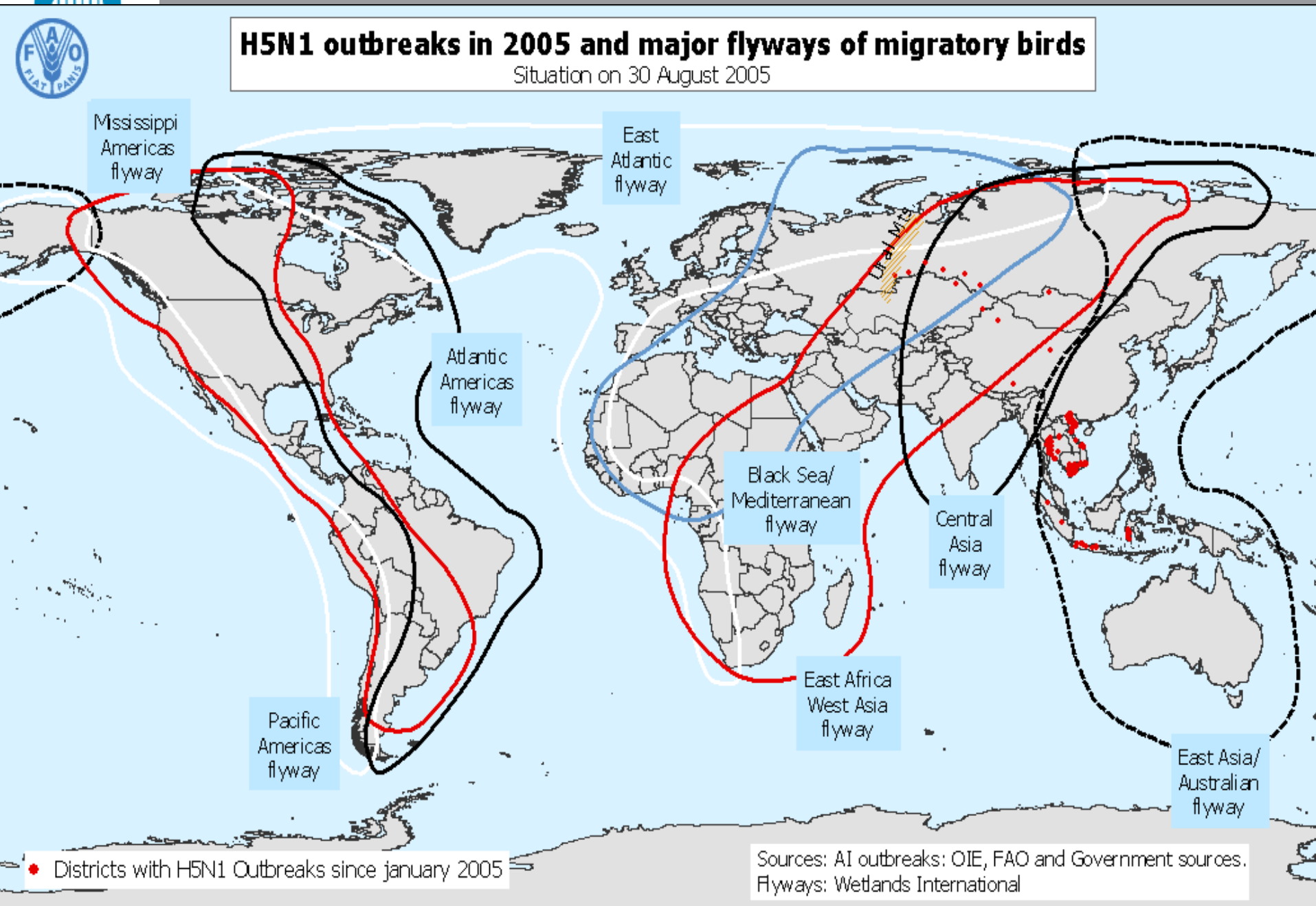

H5N1 outbreaks in 2005 and major flyways of migratory birds
Situation on 30 August 2005
Mississippi Americas flyway
East Atlantic flyway
Ural Mts
Atlantic Americas flyway
Black Sea/ Mediterranean flyway
Central Asia flyway
East Africa West Asia flyway
Pacific Americas flyway
East Asia/ Australian flyway
Districts with H5N1 Outbreaks since january 2005
Sources: AI outbreaks: OIE, FAO and Government sources.
Flyways: Wetlands International

Laboratory Diagnosis

Sampling for Diagnosis

For avian influenza in general

Cloacal swabs + tissues

blood for serology from potential survivors

For H5N1 in particular

Tracheal swabs, cloacal swabs + tissues

blood for serology from potential survivors

Tissues of particular diagnostic interest:

proventriculus, pancreas, spleen, intestine, caecal tonsil

trachea, lung (brain, for NDV differential)

Diagnostic tests

Serology

Usually screen by C-ELISA for FLU A Abs – 1 day

HI test for H type Abs – 1 day

N type Abs can be detected by NI test – more complex

AGID test for FLU A Abs is no longer used, but still available

Diagnostic tests

Agent Detection:

Virus isolation – 2 to 4 days, 10 days for a negative

(on swabs or PM specimens)

Impression smears + IFAT – 3 to 4 hours

(on PM specimensP

Immunohistochemistry – 2 days

(on PM specimens)

PCR & gene sequencing – 2 to 3 days

(is being used on clinical specimens in SE Asia)

Real time PCR – 4 to 6 hours

(on swabs or PM specimens)

Diagnostic tests

Agent Characterization:

Detect the isolate in allantoic fluid by haemagglutination, direct rapid test or real time PCR

(sometimes assisted by EM and immuno-EM)

H typing, by HI

N typing, by neuraminidase inhibition

Molecular characterization

- **a single step with agent detection by real time PCR for H5 and H7**
- **PCR and sequencing for pathotyping, molecular epidemiology (H gene or selected other genes) or genotyping (whole genome sequencing)**

A/chicken/NSW/2/97 H7N4

Main Diagnostic Tools in 1997

Virus isolation in eggs

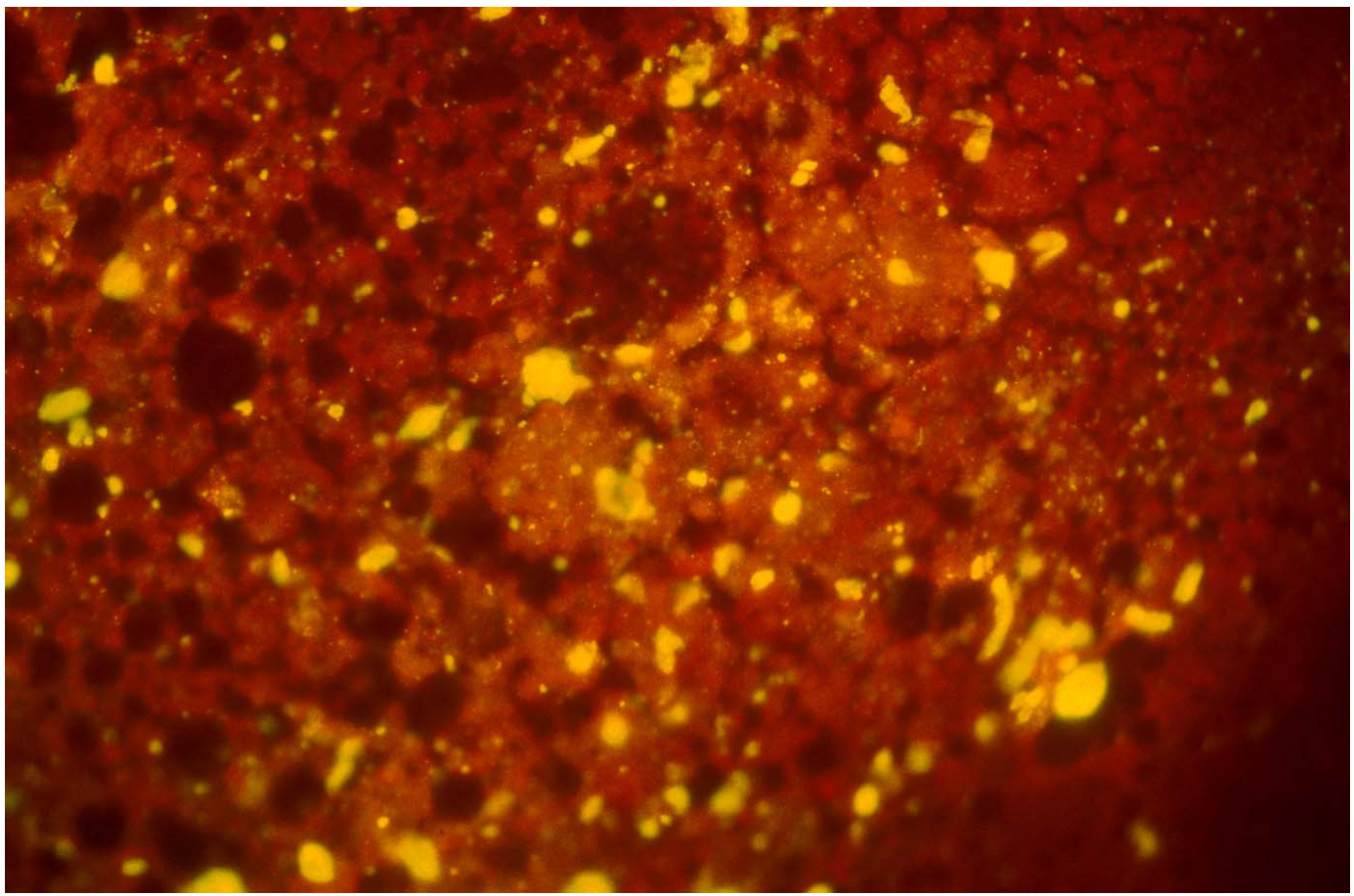
- H and N typing
- IVPI
- For a rapid test, the pancreatic impression smear

No thought of AI being a zoonosis

A/chicken/NSW/2/97 H7N4

Rapid Diagnosis: 4 hours approx

Immunofluorescence on Pancreatic Impression Smears



Real Time PCR

Advantages of real-time PCR

- High sensitivity and specificity
- High throughput & automation
- Quantitative PCR over wide linear dynamic range
- Reduced risk of contamination
- Multiplex PCR possible

PCRs must be designed for the particular diagnostic purpose

Genetic variation among strains can affect performance:

- **Point mutations and deletions**
- **Intergenic recombination between segments**

Diagnostic challenge:

- **Primer/probe design**

Avian Influenza Virus Genetics

AI viruses have genomes with 8 segments

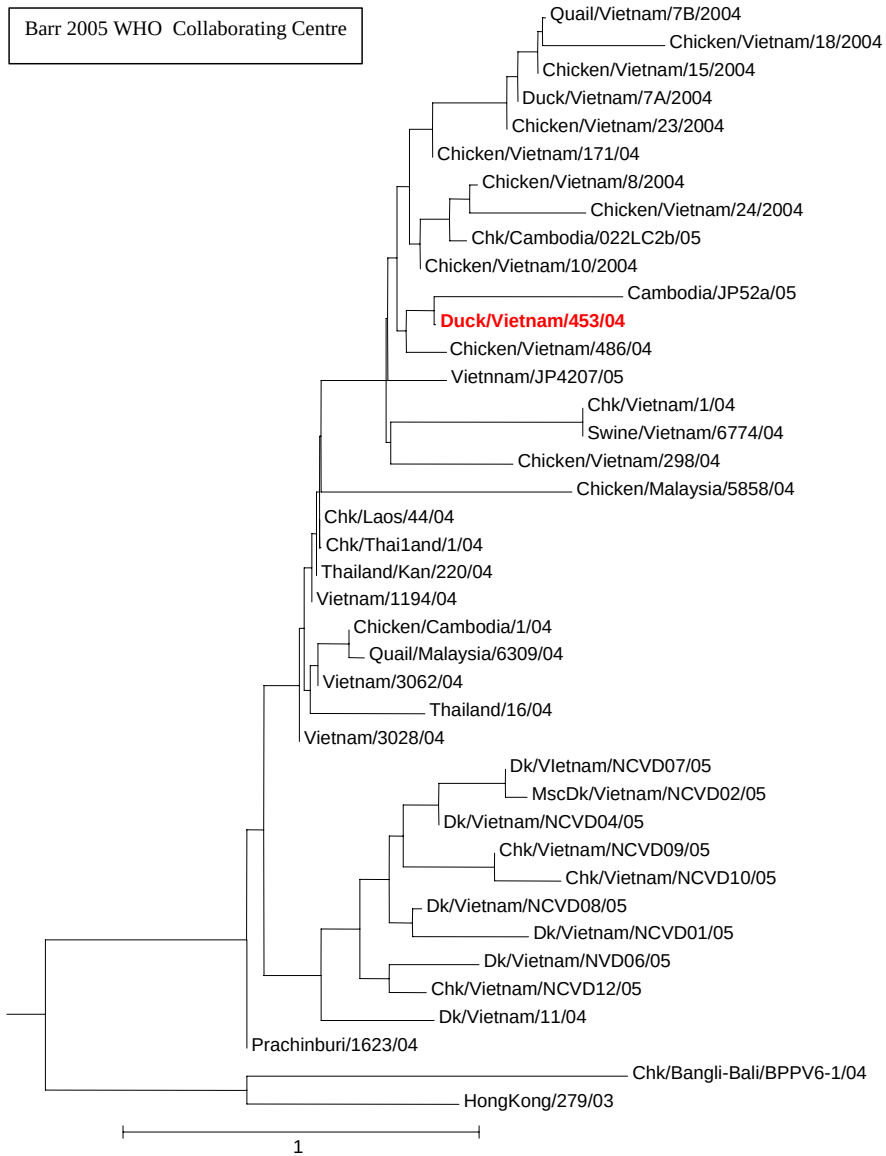
3 mechanisms for genetic evolution:

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



Therefore AAHL redesigned the H5 reagents to match the sequence of the H5N1 epidemic strain

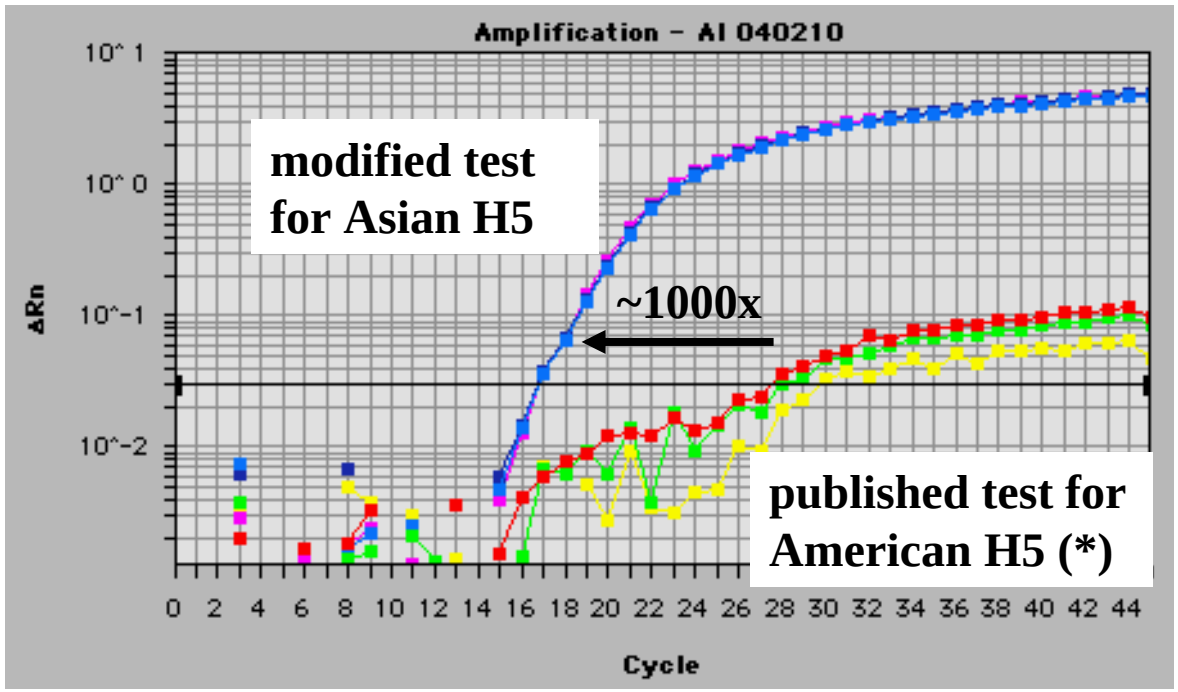
Increasing the sensitivity of both the H5 and the FLU A tests for the detection of the outbreak strain

Subtype H5 specific TaqMan test for Asian H5N1

Hans Heine

Test optimised for Asian lineage H5 compared with published test for American strains (*)

(*) (Spackman et al. (2002) J. Clin. Microbiol. 40: 3256-60)

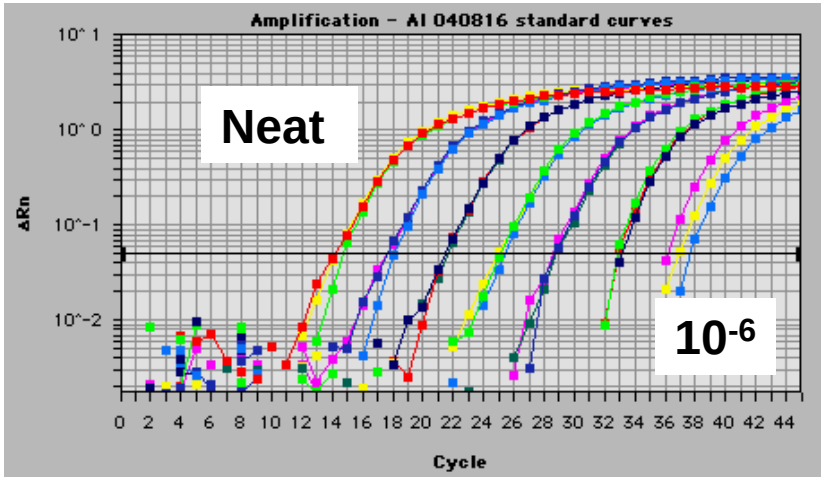


A/chicken/Viet Nam/8/2004 (H5N1)

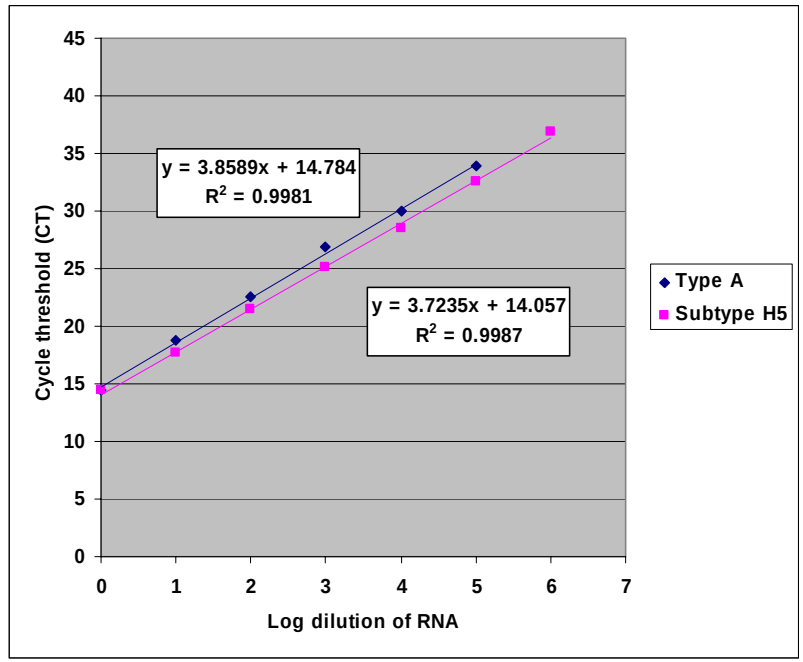
Conclusion:

~10^3 -fold increased analytical sensitivity for Asian H5N1

TaqMan standard curve



Log 10 dilutions of viral RNA A/chicken/Viet Nam/8/2004 (H5N1) were tested in triplicates using subtype H5 specific TaqMan® test (modified)



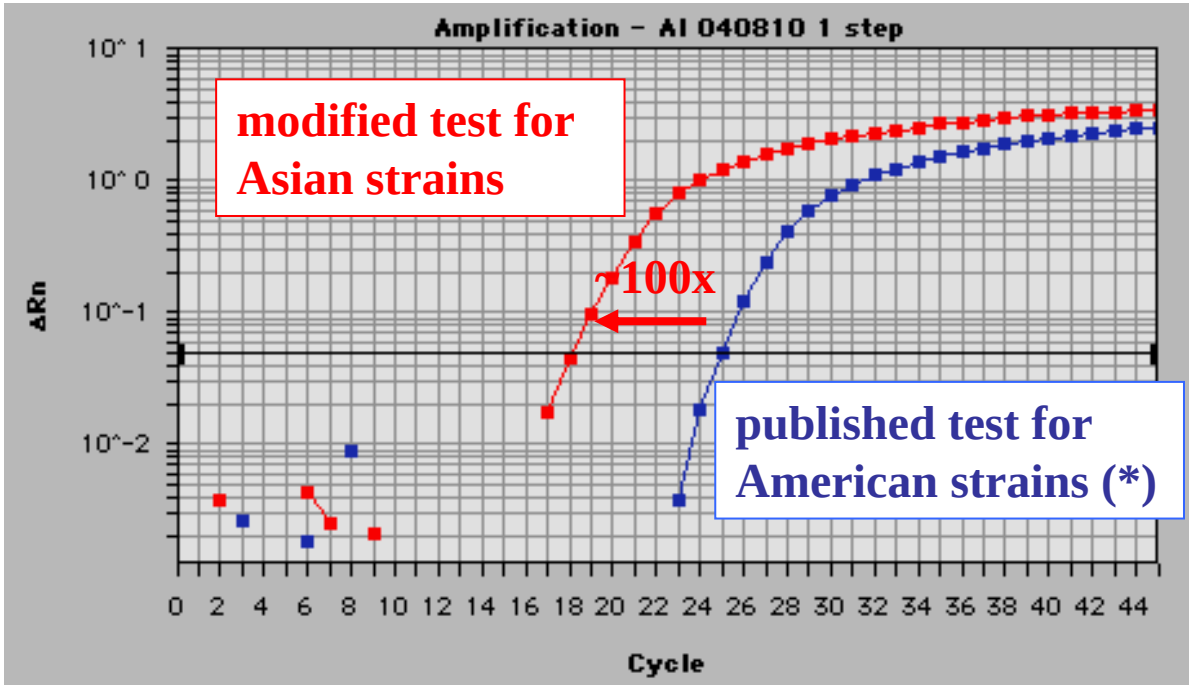
Conclusion:

Relative quantitation over ~10^6 -fold linear range;

Type A specific TaqMan test optimised for Asian strains

Importance of lineage for assay design,
- even for Type A !

Test optimised for Asian lineage compared with published test for American strains (*)
(*) (Spackman *et al*. (2002) *J. Clin. Microbiol*. **40**: 3256-60)



A/chicken/Viet Nam/8/2004 (H5N1)

Conclusion:

~ 10^2 -fold increased analytical sensitivity for Asian H5N1

Optimisation of Type A TaqMan for Asian H5 isolates

Comparison of cycle threshold (CT) values obtained using modified and published Type A-specific TaqMan assays (averages of triplicate reactions)

Virus isolate	Type A TaqMan (modified)	Type A TaqMan (published)
A/chicken/Vietnam/39/2004 H5N1	17.72 (± 0.27)	24.06 (± 0.30)
A/Shearwater/Aus/75 H5N3	18.19 (± 0.13)	18.00 (± 0.07)
A/chicken/NSW/1/97 H7N4	23.12 (± 0.11)	23.61 (± 0.14)

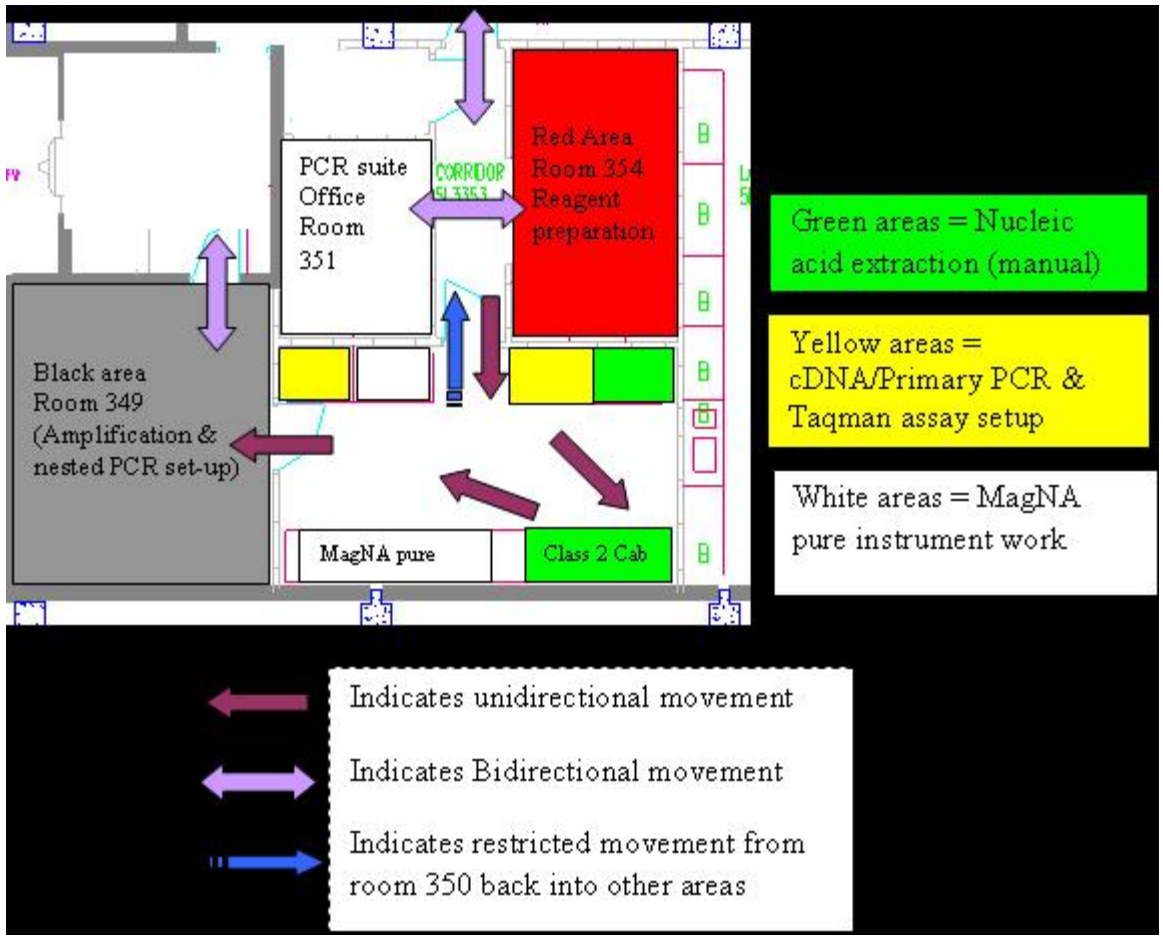
Conclusion:

Improved sensitivity for H5N1 isolates without negative effect on other strains

Because of it's analytical sensitivity in detecting the presence of genetic material exceptional care must be exercised to eliminate opportunities for trace cross contamination

Ensure the PM room is not a source of cross contamination!!

PCR suite layout and workflow (example)



Summary: Molecular tests for AI

AI TaqMan RT-PCR (3 tests)

- Specific for type A to detect all AI strains (including Asian)
- Specific for subtype H5 (Asian H5N1)
- Specific for subtype H7 (Australian)

TaqMan tests will detect H5 & H7 virus in mixed infections. Other TaqMan assays specific for NDV, West-Nile, IBDV can be performed simultaneously on same plate.

Conventional RT-PCR and DNA sequencing

- Molecular pathotyping of H5 & H7 by HA cleavage sequence