

Avian Influenza:
A New Emerging Infectious Disease

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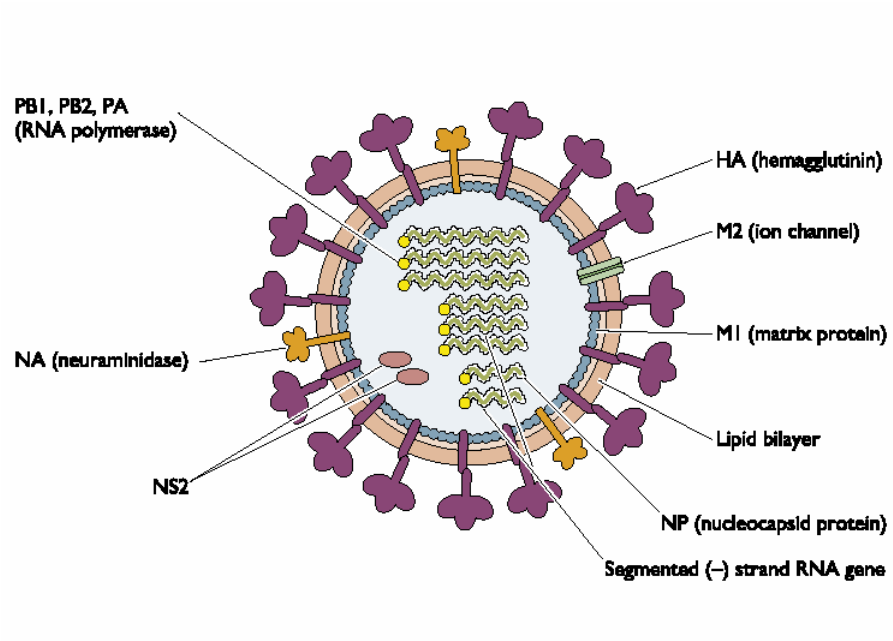
Aspects to be covered:

- The viruses
- The disease
- The emergence of H5N1
- Laboratory Diagnosis
- Real Time PCR
- R&D responses to the Asian epidemic
  - Vaccine evaluation
  - Point of sampling diagnostics evaluation

The Virus(es)

Influenza virus



Type

- Type A, B or C based on matrix (MA) and non-structural (NS) proteins

Subtype

- H1 - H16 based on HA protein
- N1 - N9 based on NA protein

Molecular pathotype

- HPAI or LPAI strains
- Molecular pathotype based on HA cleavage region of H5 or H7 strains

Sequence analysis for pathotyping

Molecular Basis of Pathogenicity: Proteolytic Activation of H

Inactive Precursor H_o (76 kD)

Activated H_1 (47 kD) H_2 (29 kD)

Molecular pathotyping of H5N1 strain (HA cleavage)

A/Chicken/Vietnam/8/2004 (H5N1) HPAI

```
CCT CAA AGA GAG AGA AGA AGA AAA AAG AGA GGA TTA TTT
Pro Gln Arg Glu Arg Arg Arg Lys Lys Arg*Gly Leu Phe
P   Q   R   E   R   R   R   K   K   R  *G   L   F
```

A/Tern/Australia/75 (H5N3) LPAI

```
CCC CAA AGG GAG ACA --- --- --- --- AGA GGT CTA TTT
Pro Gln Arg Glu Thr --- --- --- --- Arg*Gly Leu Phe
P   Q   R   E   T   -   -   -   -   R  *G   L   F
```

Molecular pathotype based on HA cleavage site

Isolate	Cleavage sequence
Non-pathogenic H5 subtypes	
A/chicken/Mexico/31381/94	P Q R E – – – – T R ↓ G
A/chicken/Pennsylvania/1/83 (CHO+)	P Q K K – – – – K R ↓ G
A/duck/Singapore/645/97	P Q R E – – – – T R ↓ G
Pathogenic H5 subtypes	
A/chicken/Pueblo/94	P Q R K R K – – T R ↓ G
A/chicken/Queretaro/20/95	P Q R K R K R K T R ↓ G
A/chicken/Pennsylvania/1370/83 (CHO-)	P Q K K – – – – K R ↓ G
A/chicken/Hong Kong/990/97	P Q R E R R R K K R ↓ G
A/Hong Kong/156/97- (human)	P Q R E T R R K K R ↓ G
A/Hong Kong/486/97- (human)	P Q R E R R R K K R ↓ G
Non-pathogenic H7 subtypes	
A/tern/Potsdam/79	P E I P K – – – – G R ↓ G
A/duck/Victoria/76	P E I P K – – – – K R ↓ G
Pathogenic H7 subtypes	
A/chicken/Leipzig/79	P E I P K K K – – G R↓ G
A/goose/Leipzig/137/79	P E I P K R K – – G R ↓ G
A/goose/Leipzig/187/79	P E I P K K K K – G R ↓ G
A/goose/Leipzig/192/79	P E I P K K K K K G R ↓ G
A/chicken/Victoria/76	P E I P K K K E – K R ↓ G

- Glycosylation of sites adjacent to the cleavage site can affect HA activation
- Loss of glycosylation presumably allows easier access of proteases and increased spread

From Zambon Rev Med Virol 11:227

High Pathogenicity Avian Influenza (HPAI) in Australia

Previous outbreaks of high-pathogenicity avian influenza (HPAI) in Australia all caused by viruses of H7 subtype

- **1997 NSW H7N4**
- **1994 Qld H7N3**
- **1992 Vic H7N3**
- **1985 Vic H7N7**
- **1976 Vic H7N7**

No cases of HP H5 in Australia

The Disease

Clinical Signs

- Very high mortality rate (almost 100%)
- Wattle and comb : swollen and cyanotic
- Sero-mucous nasal discharges and hypersalivation
- Feet - petechiae
- Diarrhoea
- Depression
- Softened egg shells

Depression & Diarrhoea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sudden high mortality

Swollen head and nasal discharges


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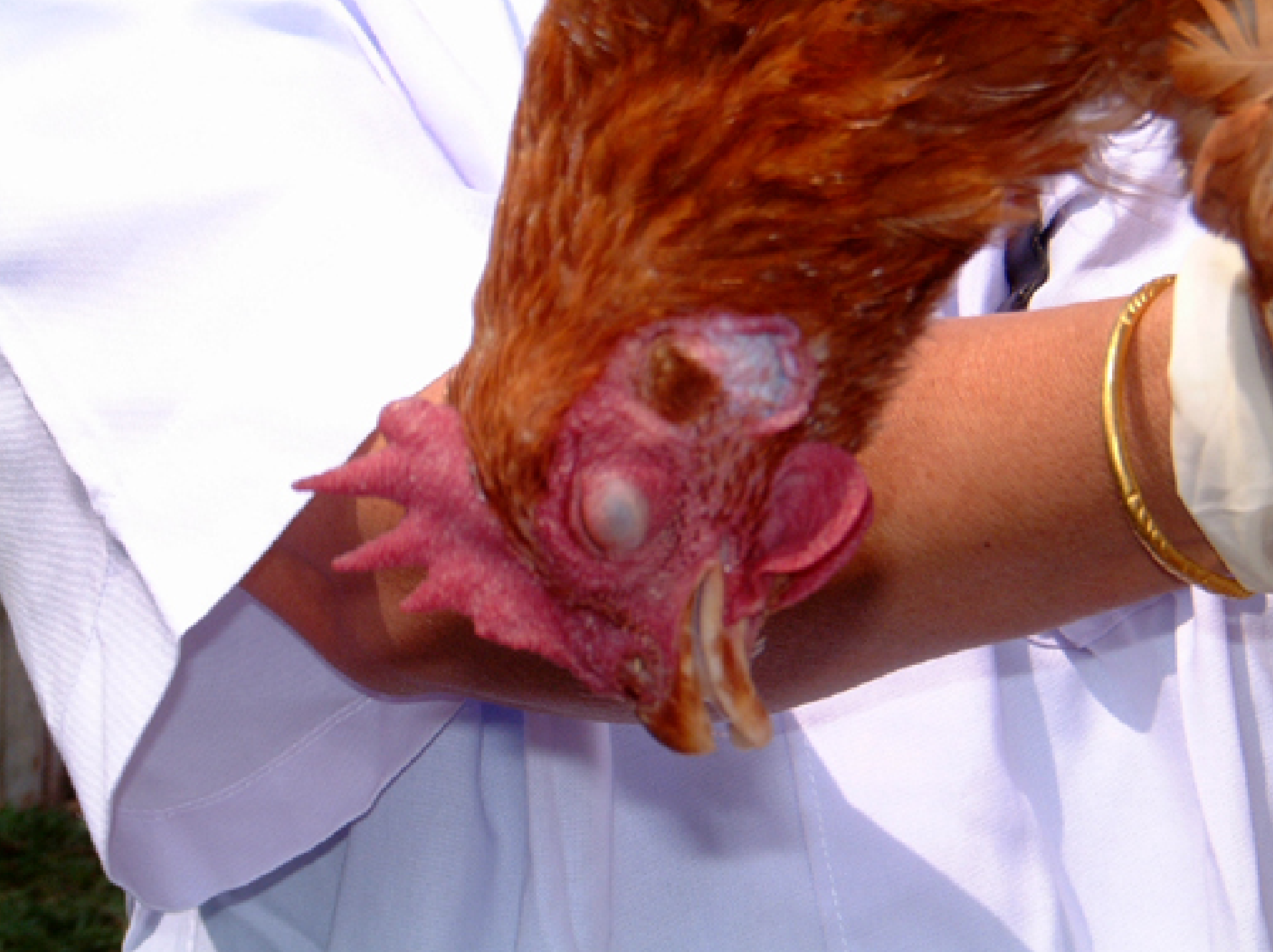
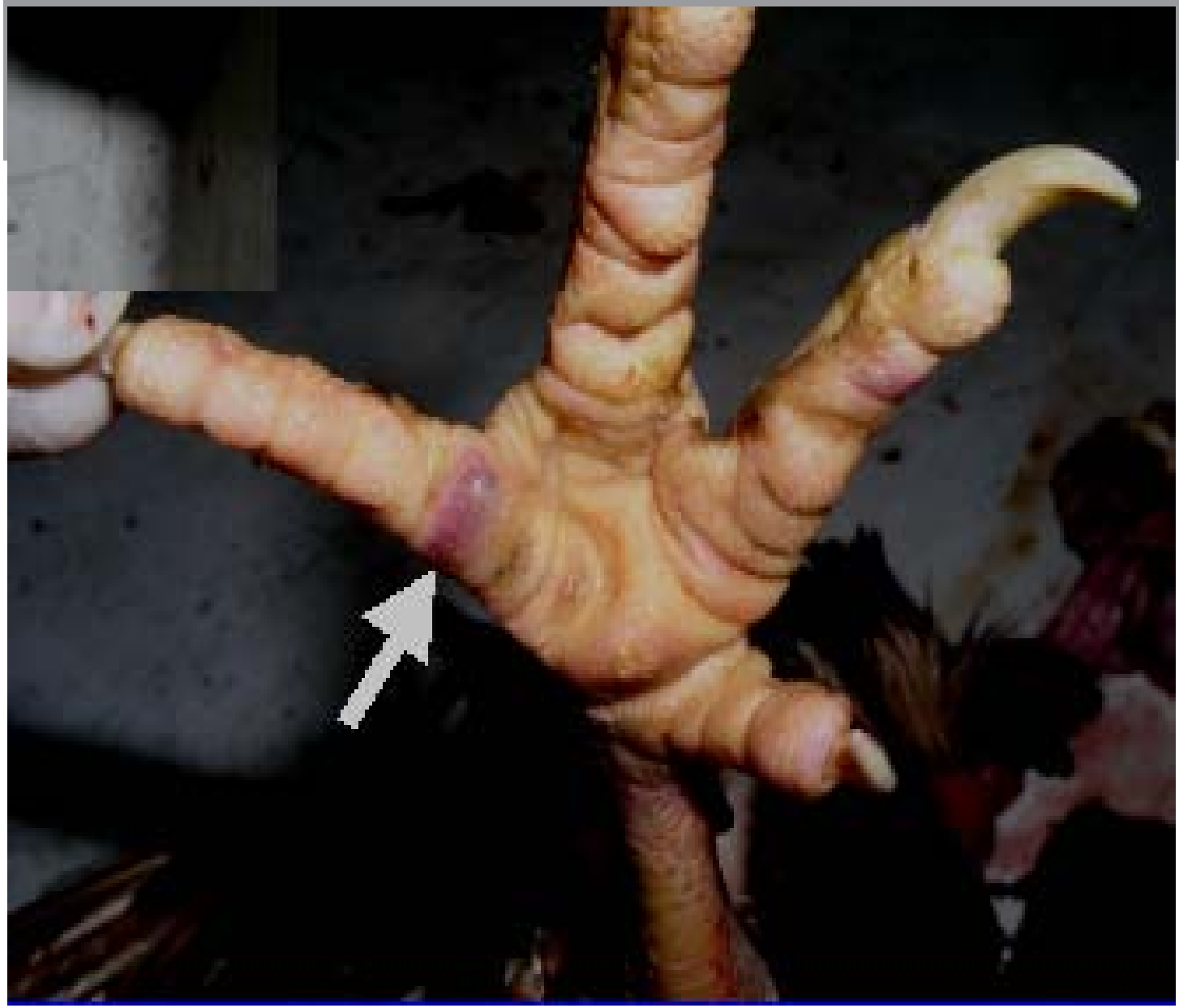
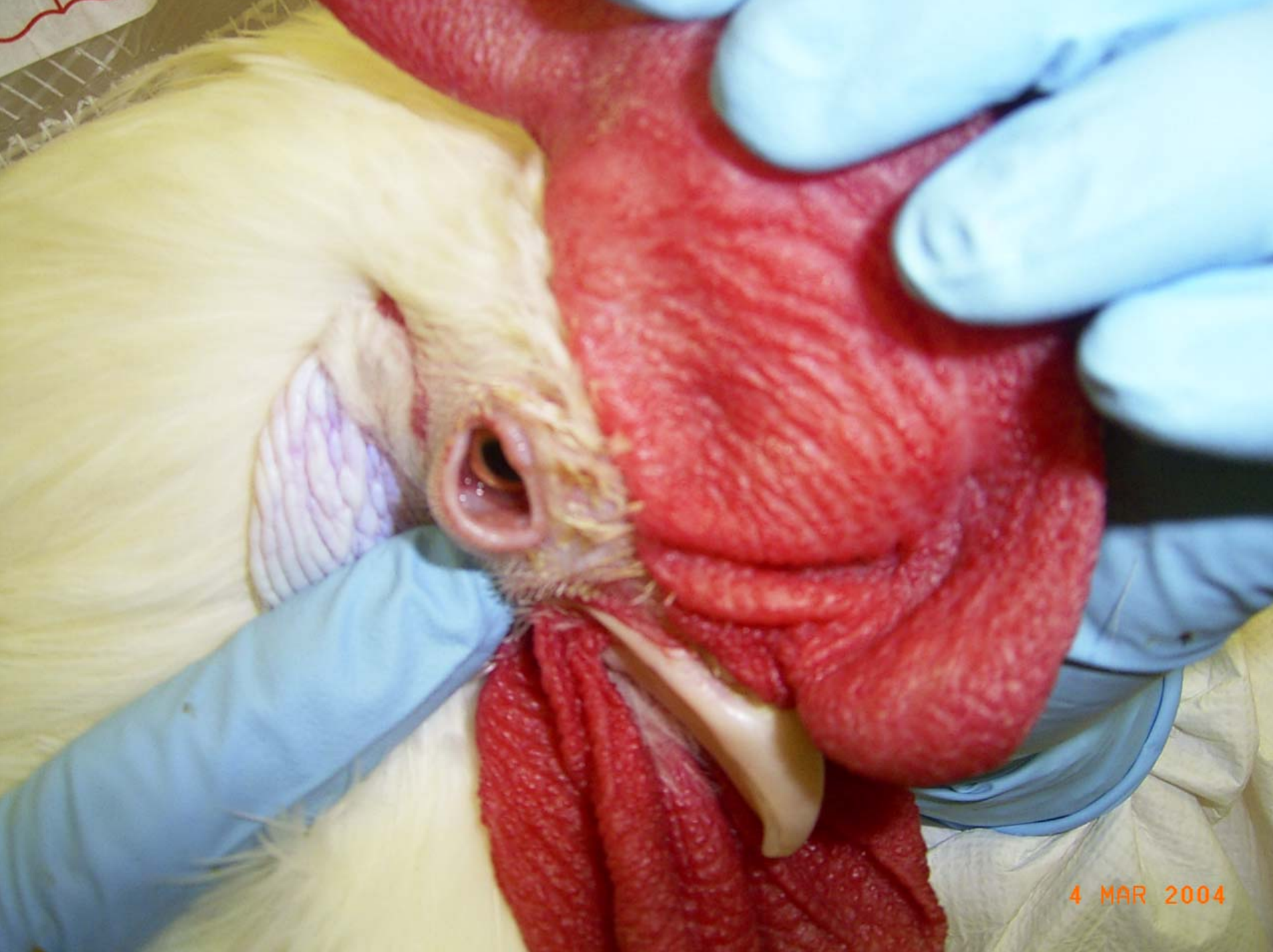




Subcutaneous petechiae and swollen feet

Subcutaneous petechiae feet


4 MAR 2004

PATHOLOGICAL EXAMINATION

Gross Pathological Features

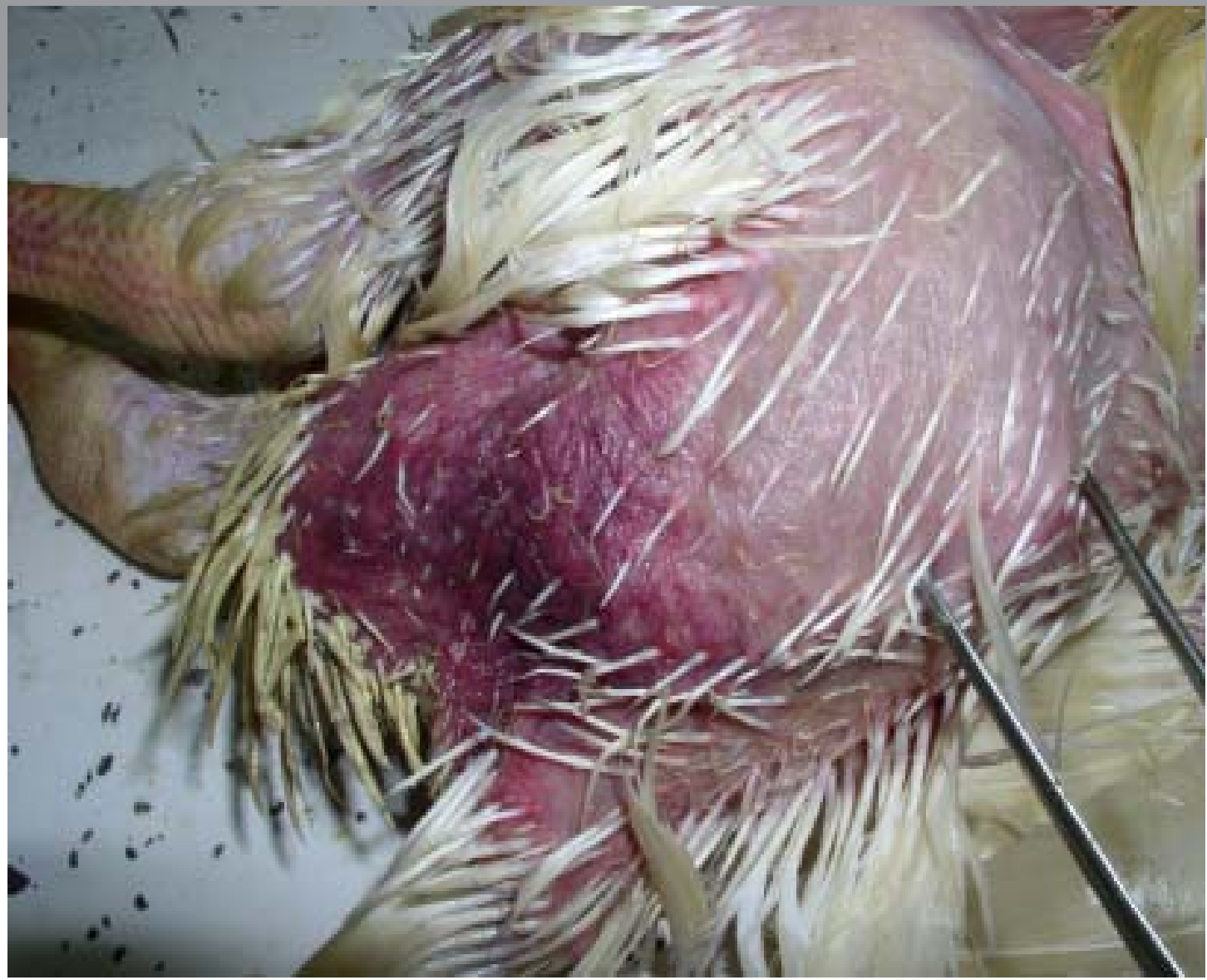
- **Wattle and comb: petechiae, cyanosis**
- **Subcutaneous tissues of feet : petechiae**
- **Thigh & chest muscle: haemorrhages**
- **Trachea: hyperaemia**
- **Proventriculus: oedema**

Gross Pathological Features

- **Epicardium & myocardium: petechiae**
- **Lung: congestion, haemorrhages**
- **Liver: very fragile, necrosis, haemorrhages**
- **Spleen: swollen**
- **Ovary: haemorrhages, congestion, necrosis**

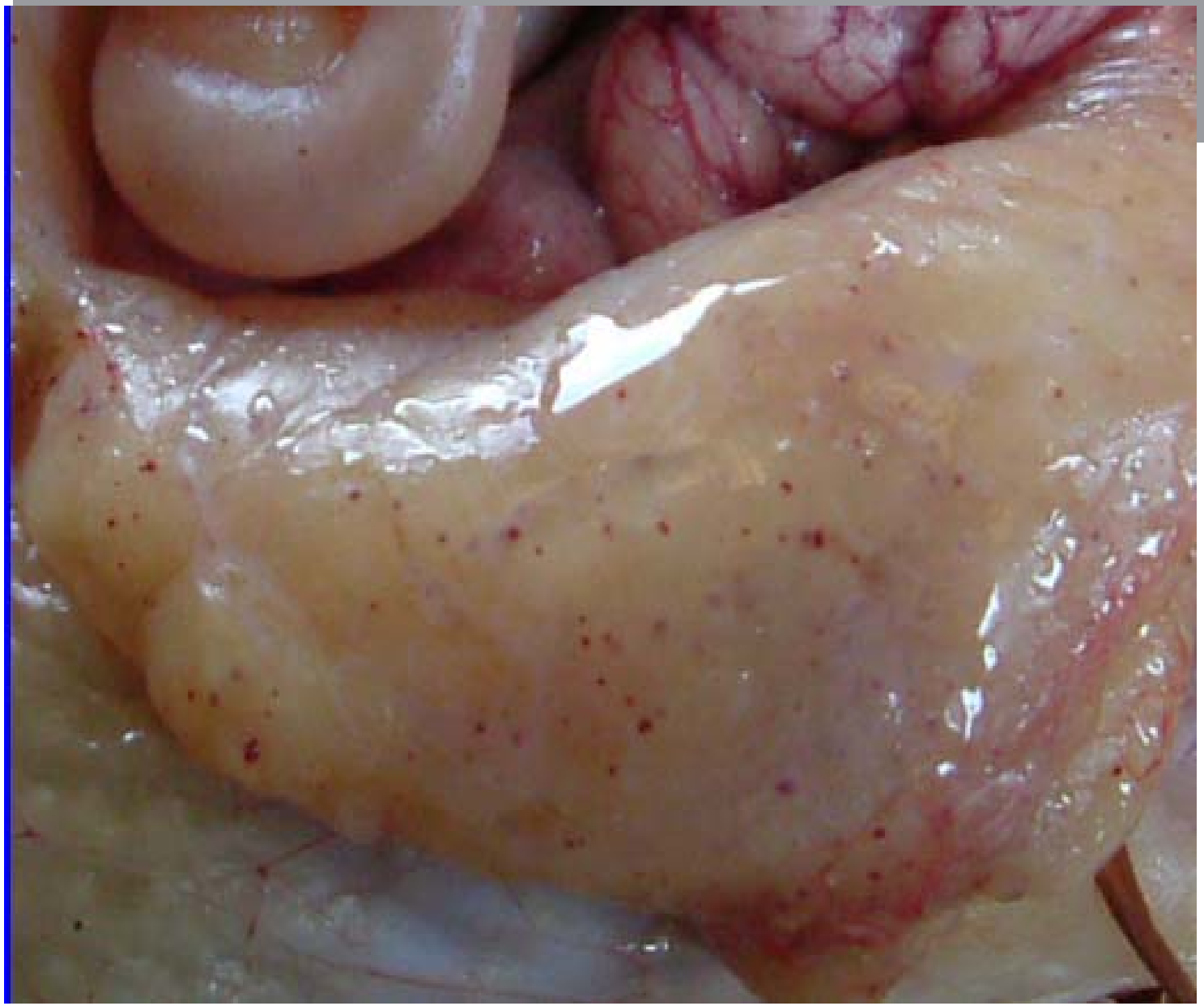
Cyanotic comb and wattle


3 MAR 2004



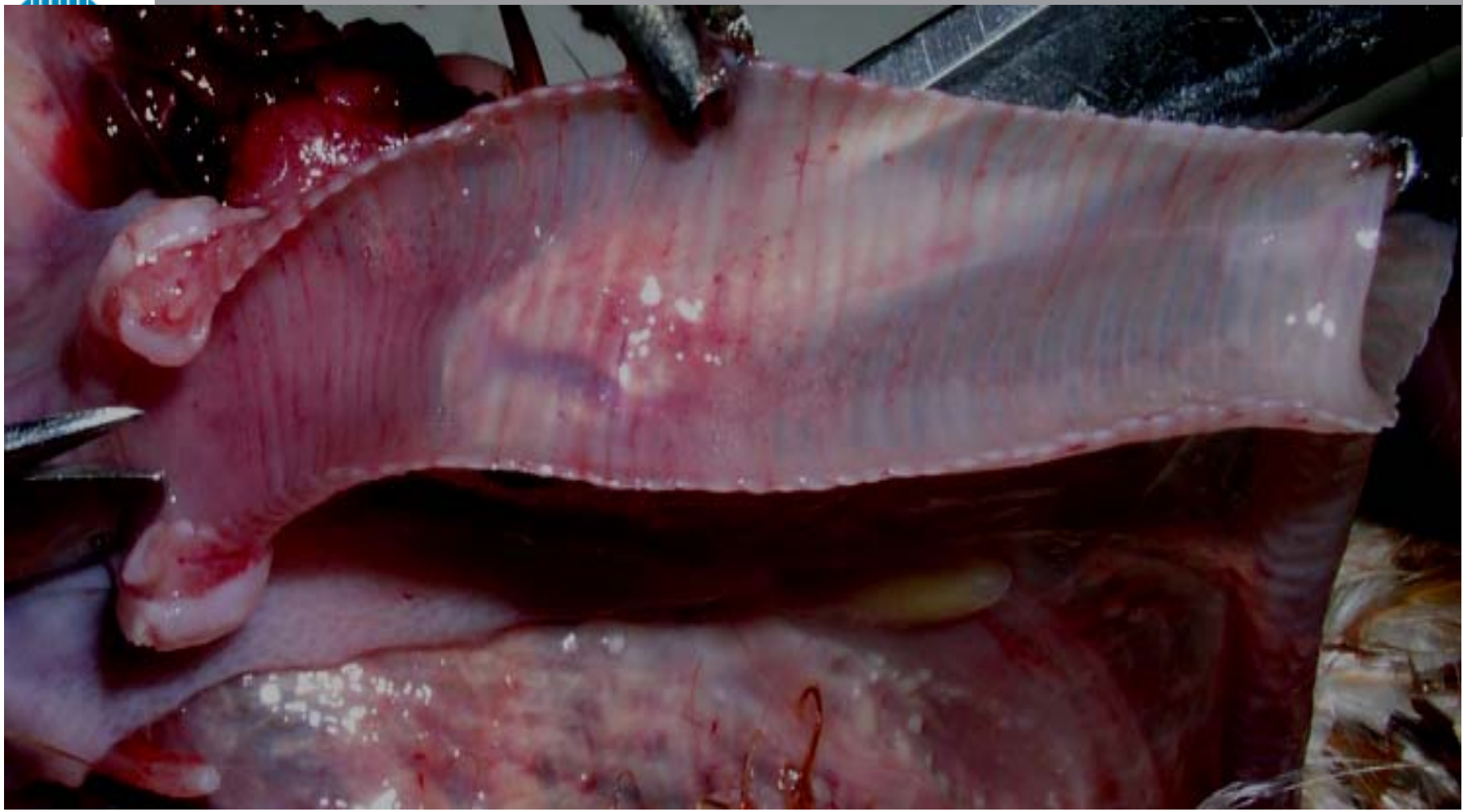
Subcutaneous haemorrhages in chest and feet

Subcutaneous haemorrhages in chest and feet

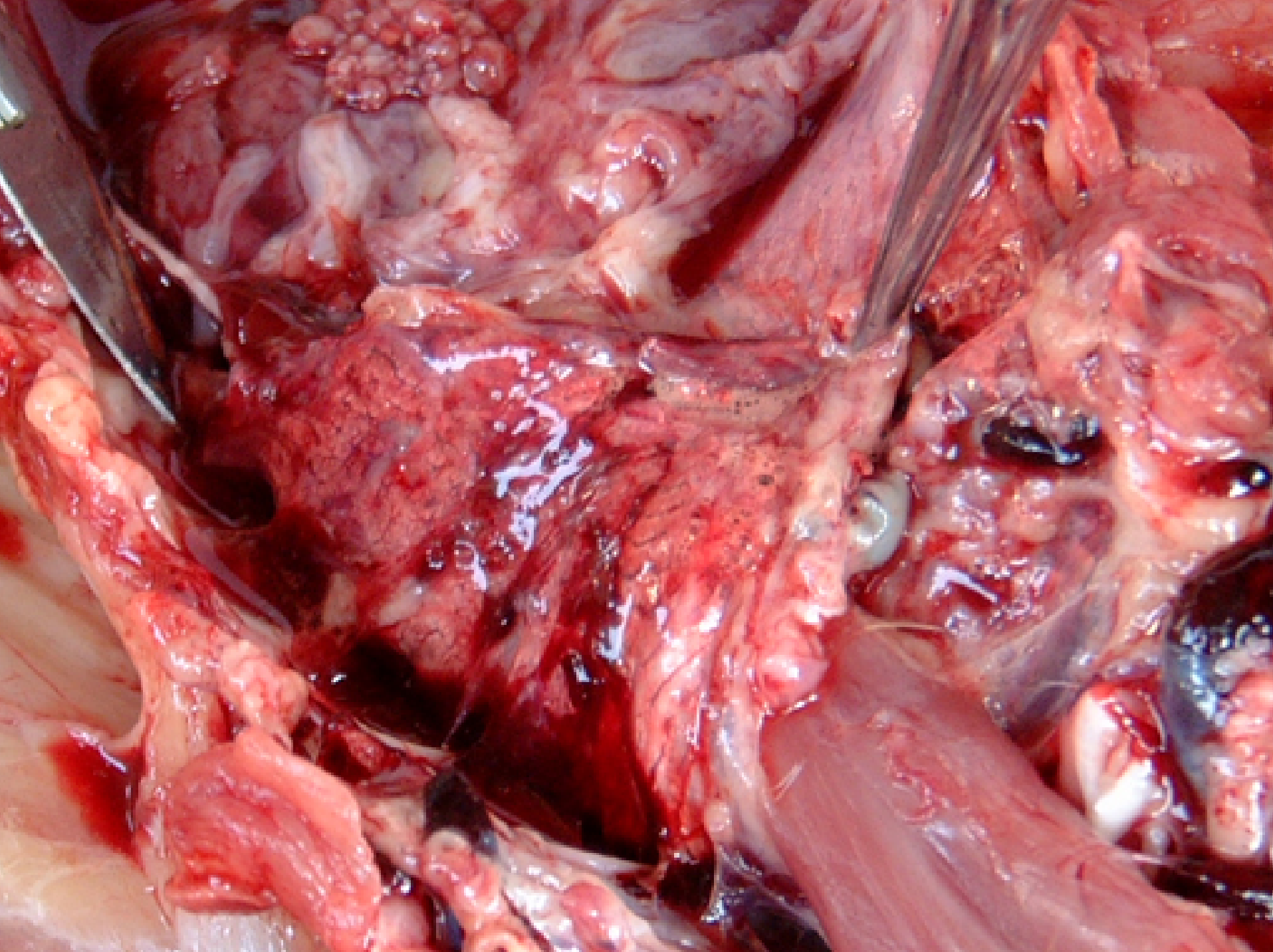


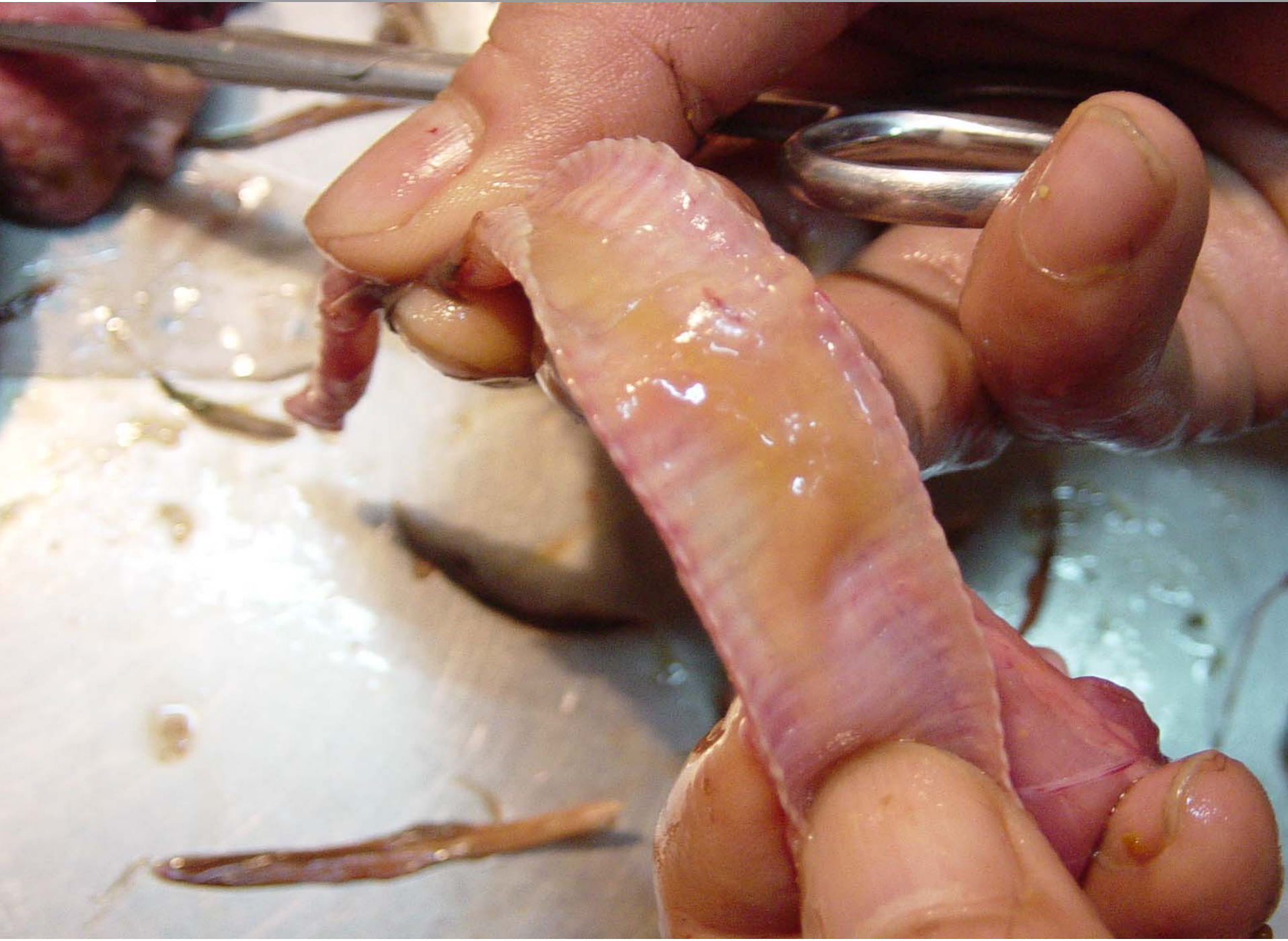
Petechiae in peritoneum

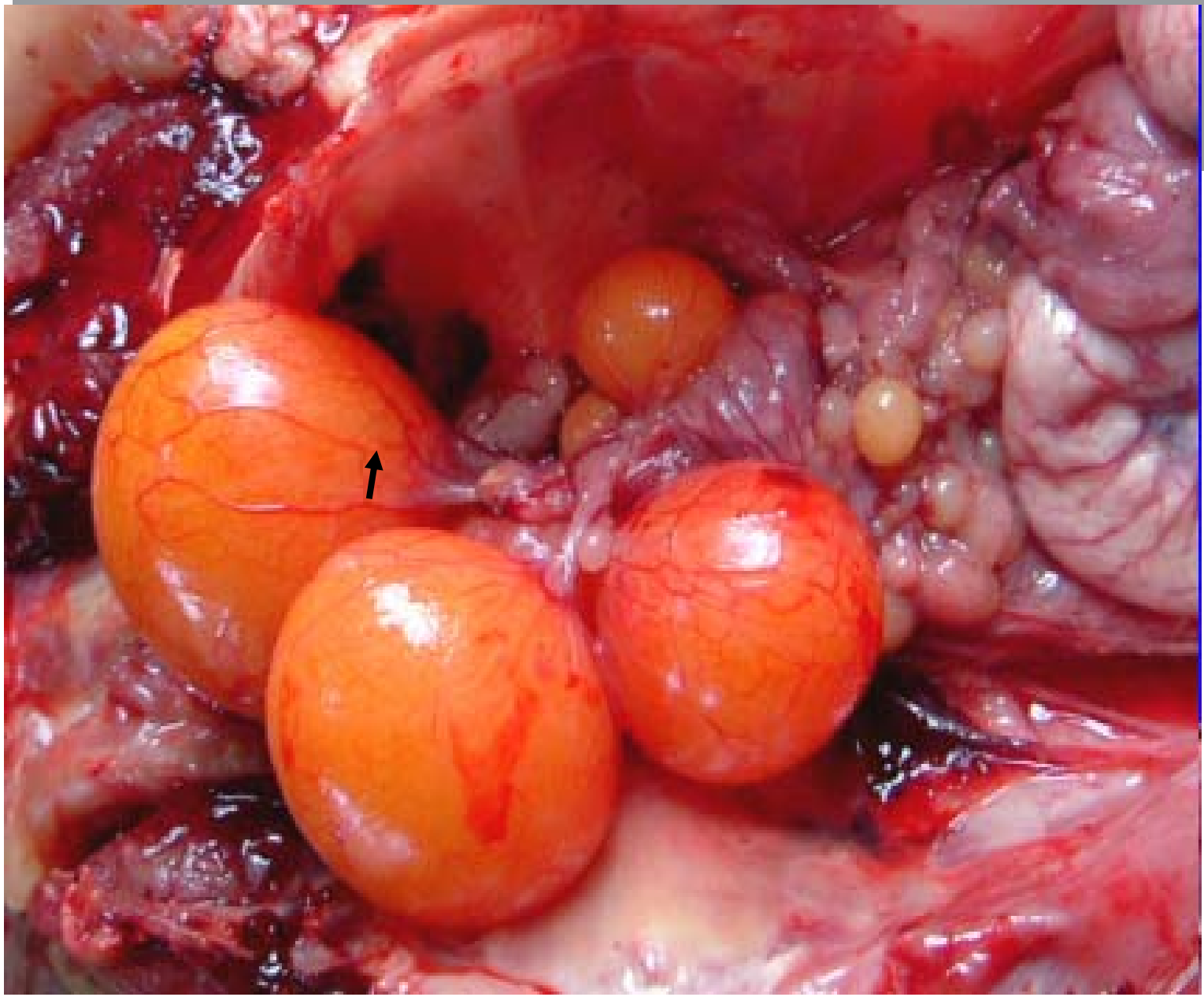
Petechiae in thigh muscle



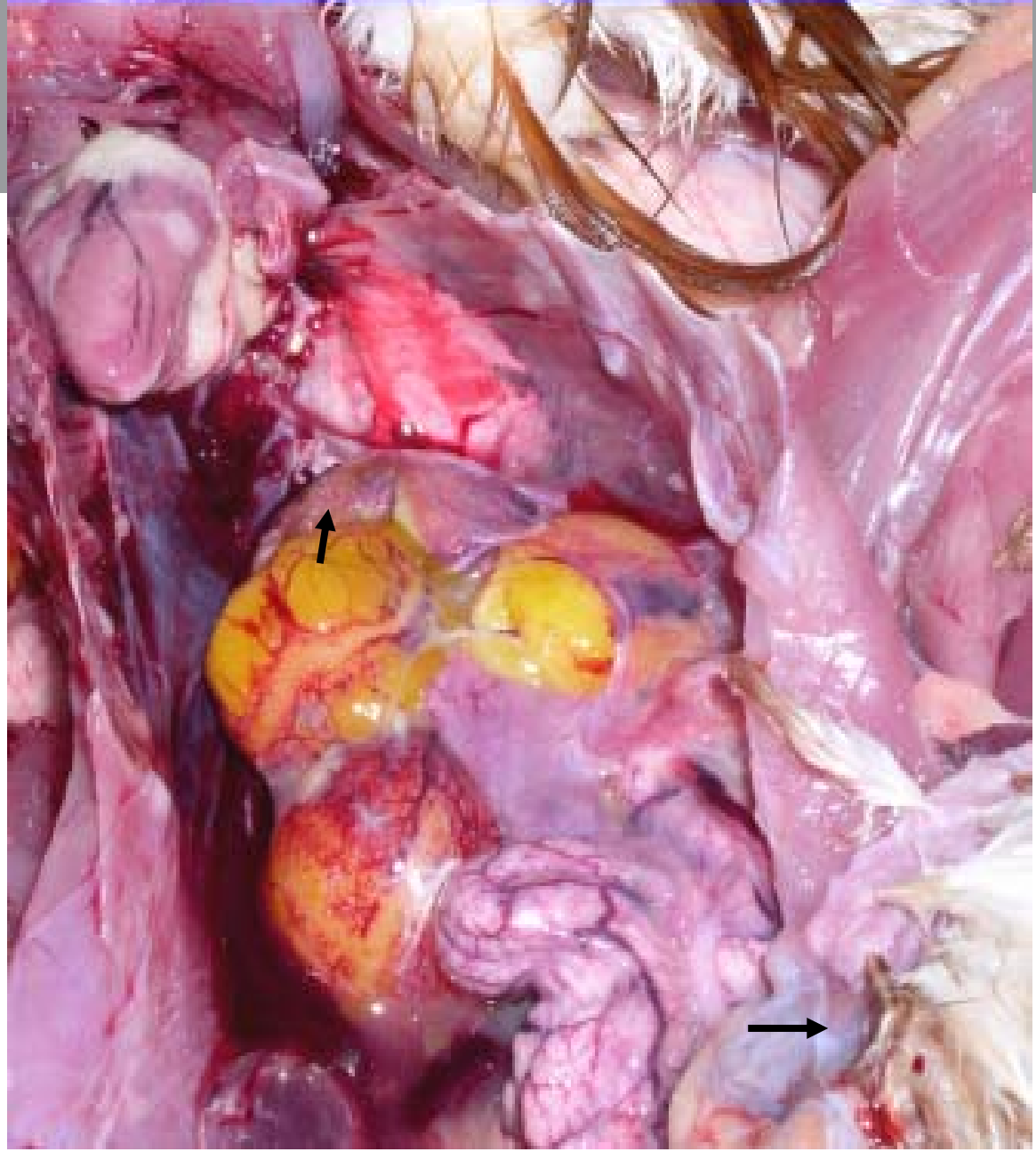
Hyperaemic upper tracheal mucosa



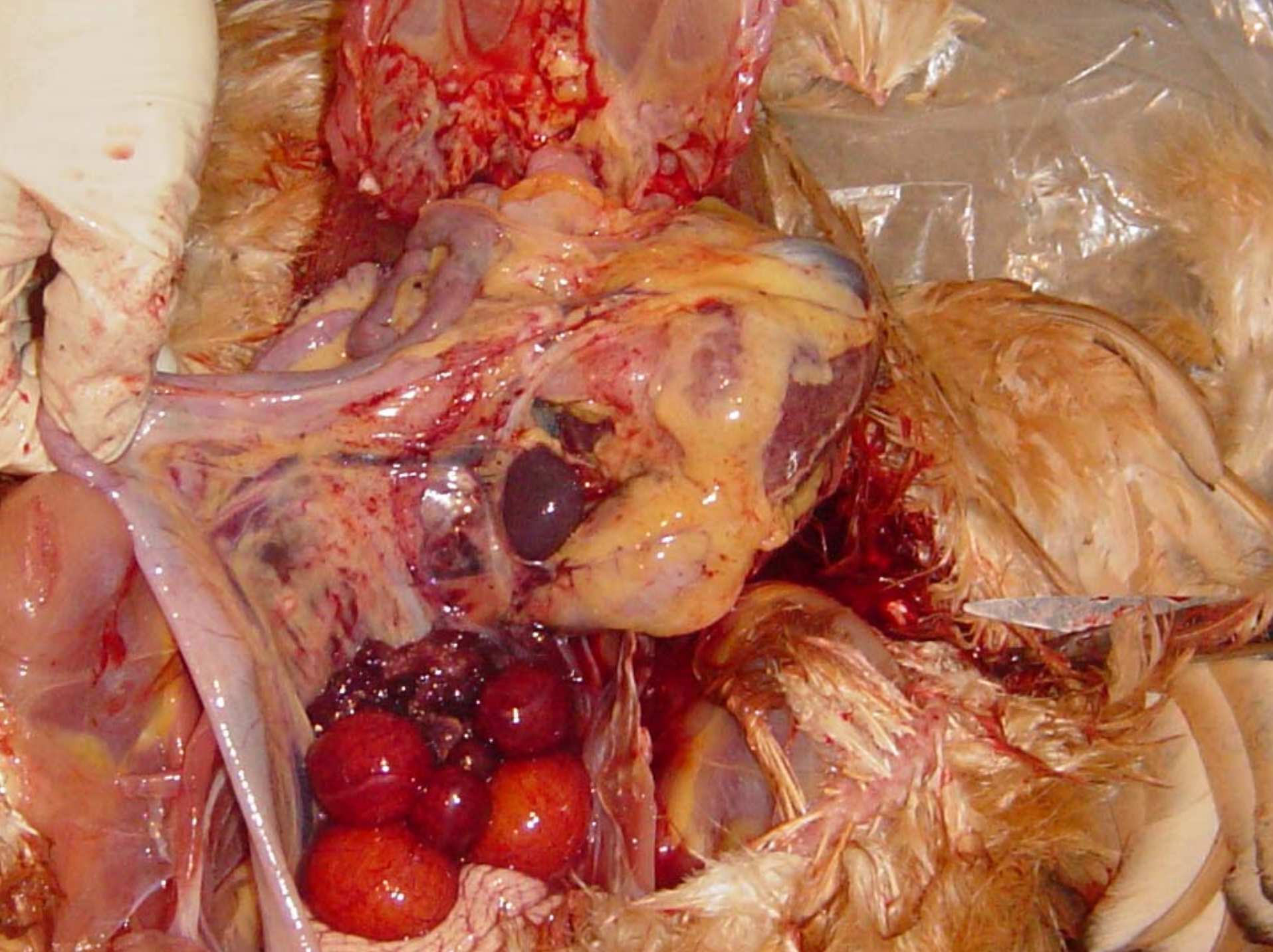




Congested ovary



Haemorrhagic ovary



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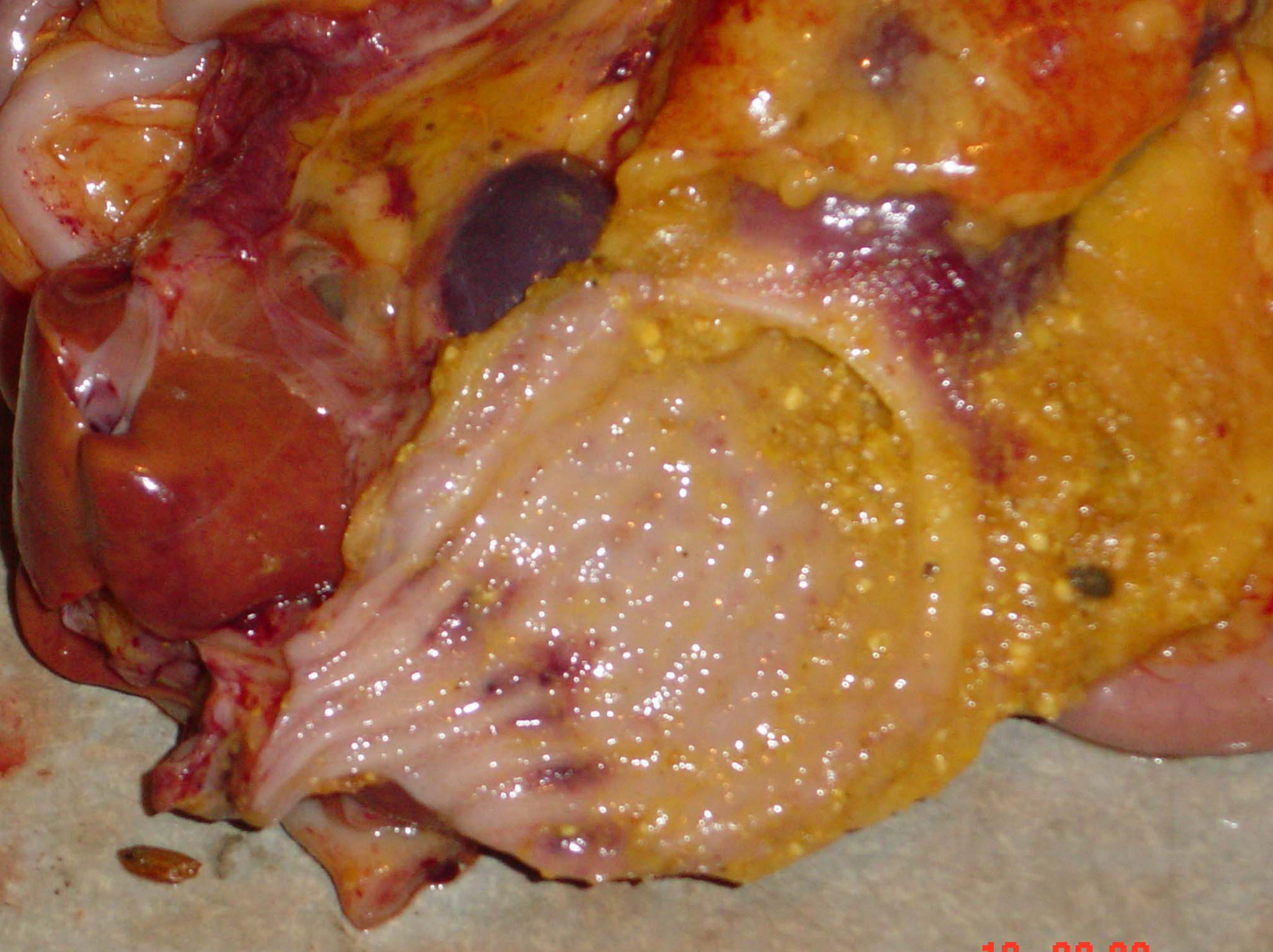
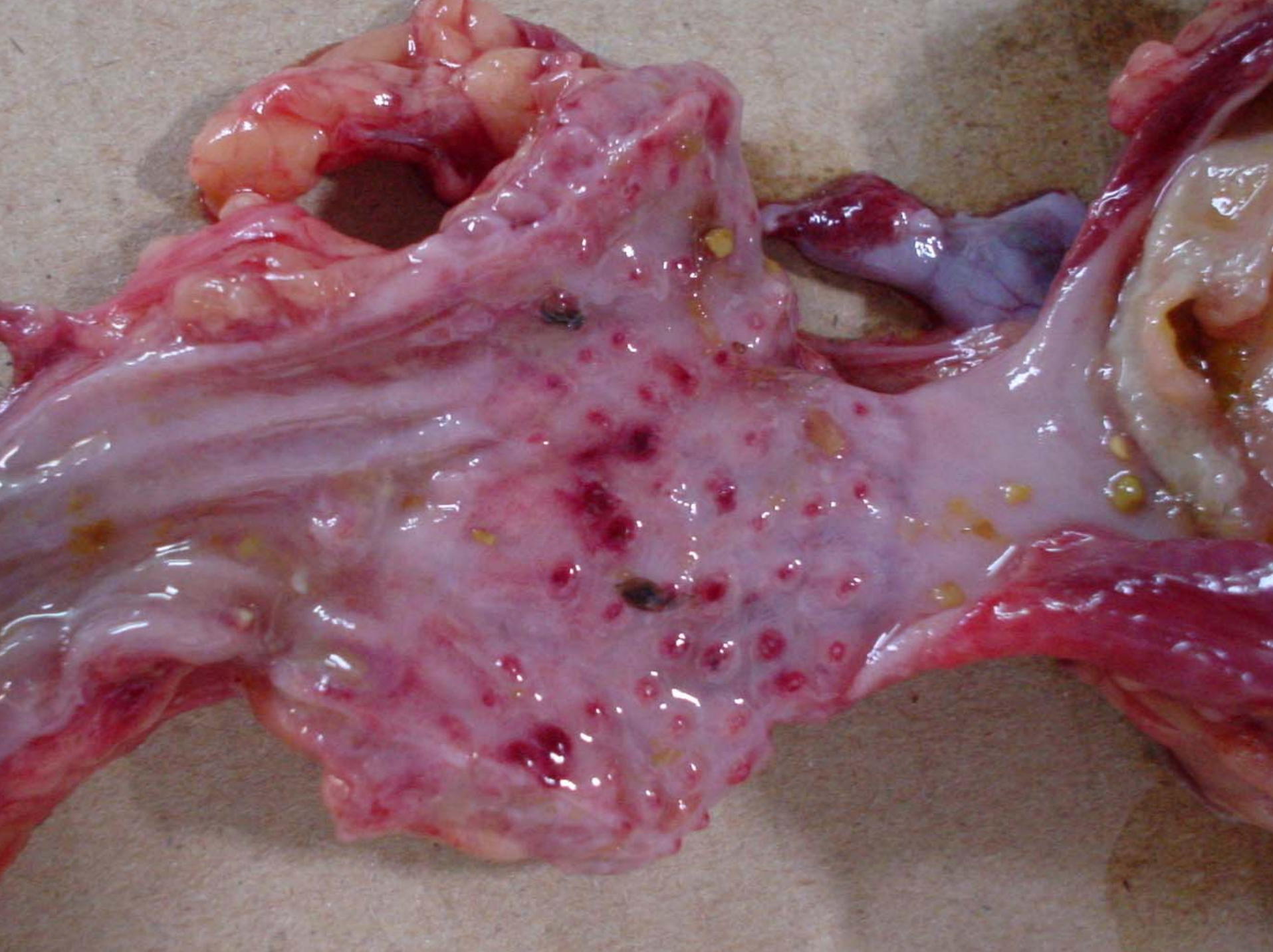
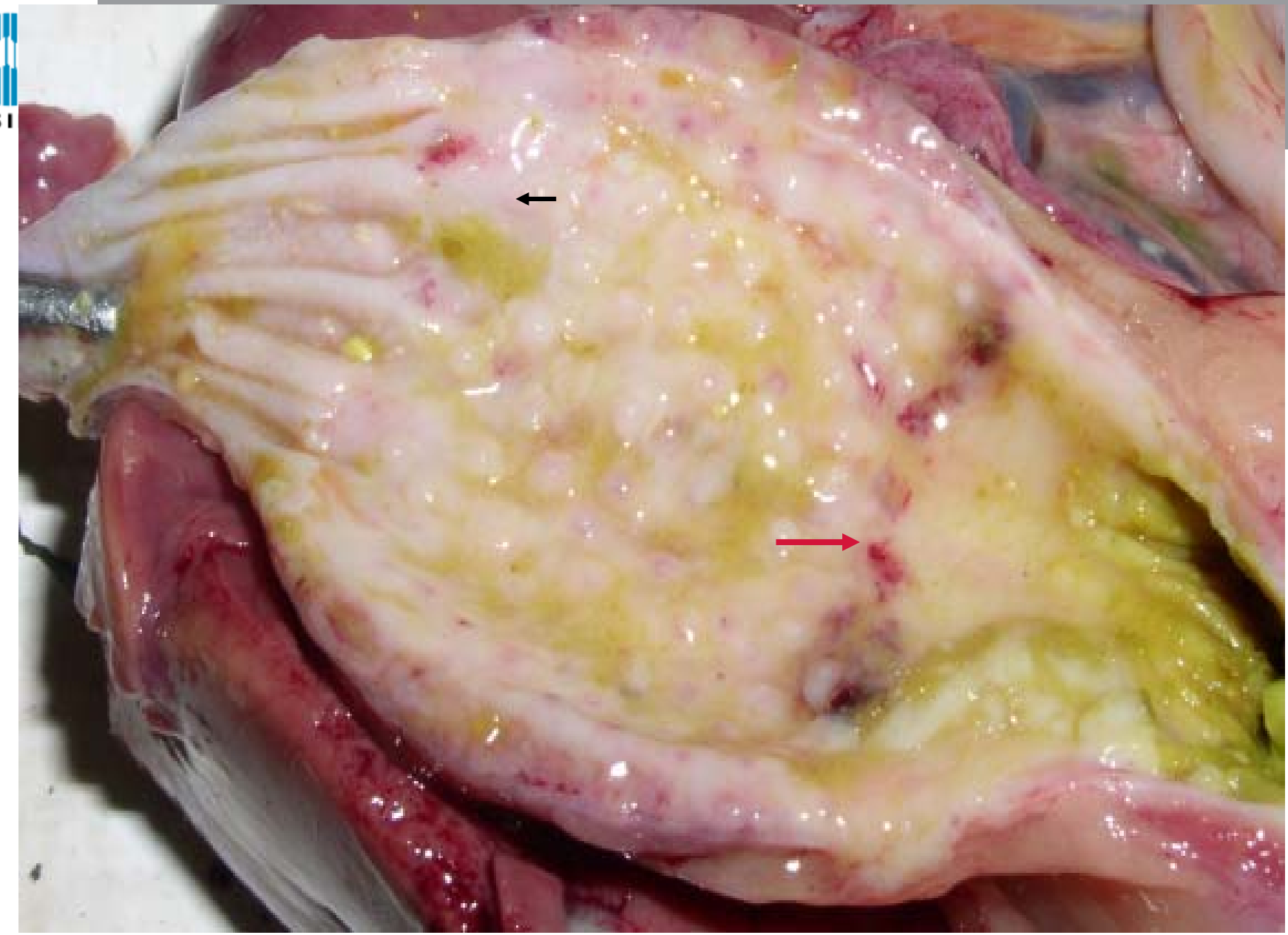
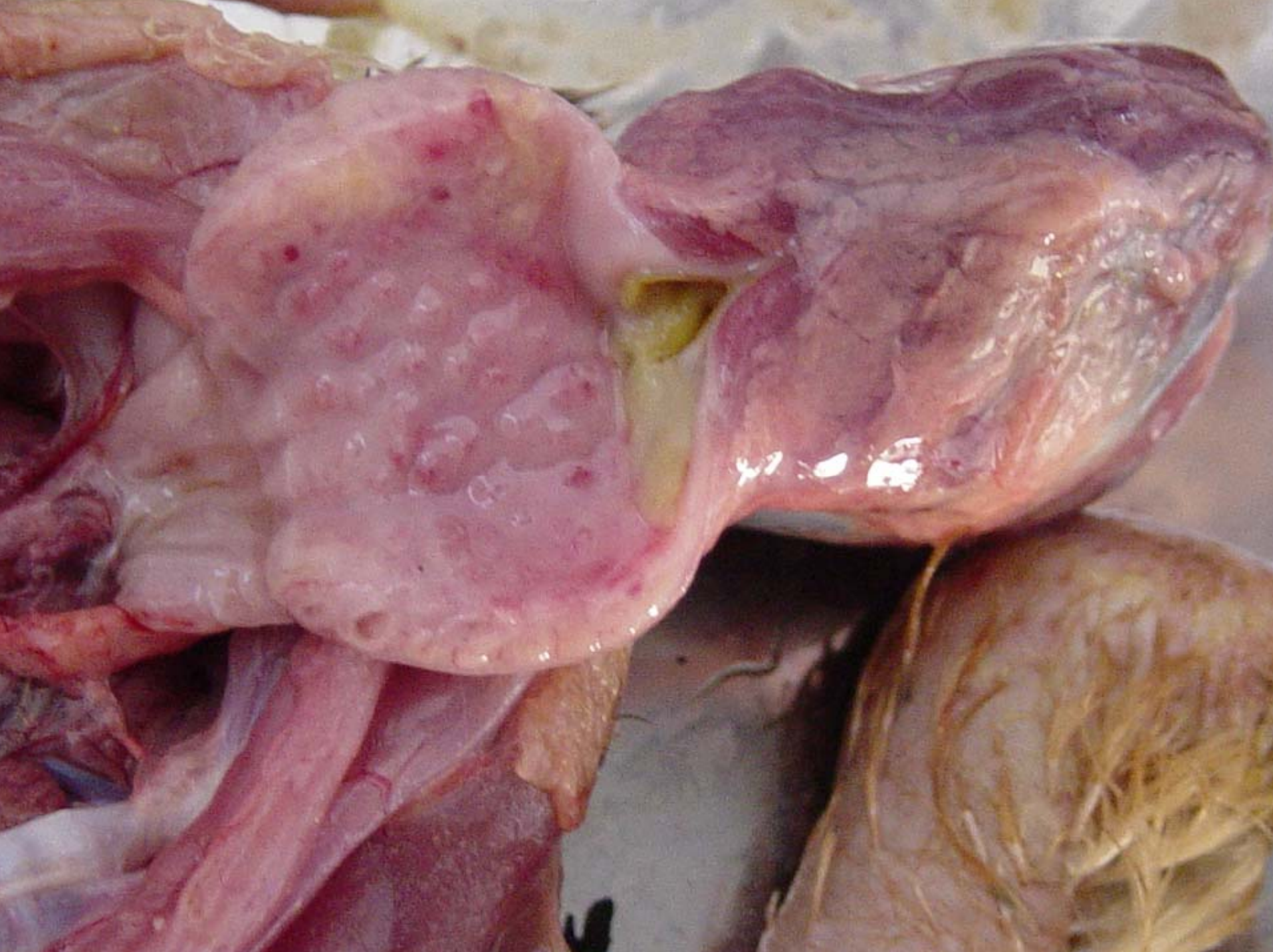






Petechiae in proventriculus



The diagnoses at this stage:

The differential diagnosis includes :

- **ND**
- **AI**
- **IB**
- **ILT**
- **IBD**
- **EDS**
- **Fowl Cholera**

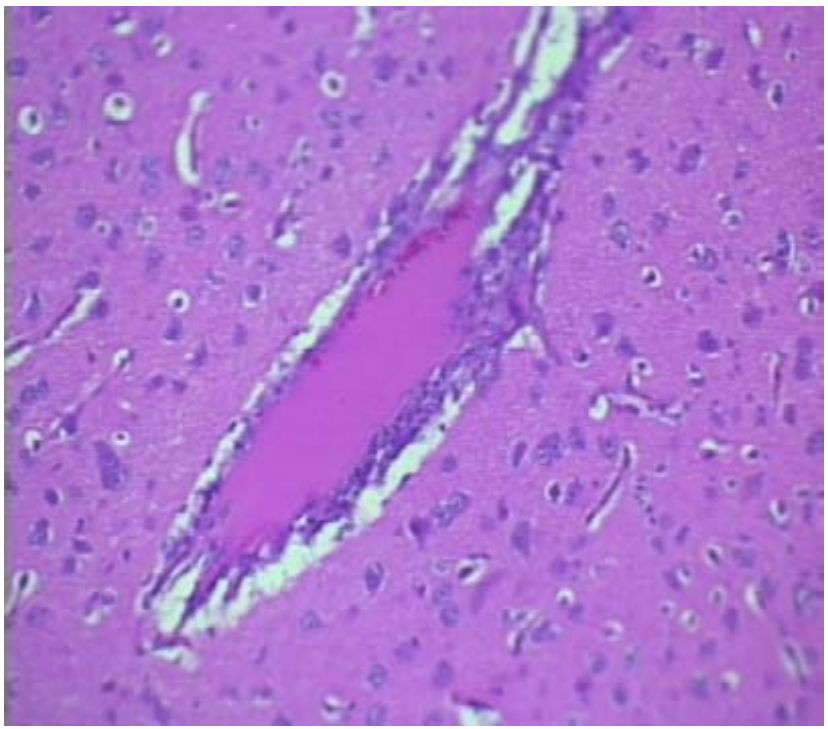
Histopathology

- **Brain: Encephalitis (lymphocyte infiltration, vasculitis, gliosis, myelin degeneration)**
- **Skeletal muscle: haemorrhages**
- **Epicardium & myocardium: haemorrhages**
- **Trachea: tracheitis, haemorrhages**
- **Lung: interstitial pneumonia, haemorrhages, congestion**

Histopathology

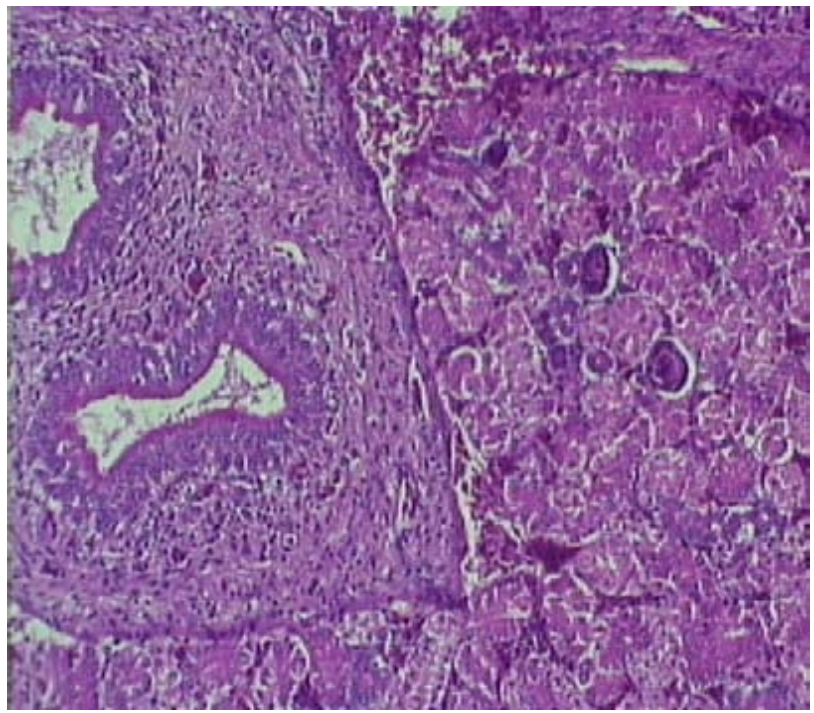
- **Proventriculus: proventriculitis**
- **Liver: hepatitis with necrosis & haemorrhages**
- **Spleen: congestion**
- **Kidney: congestion, nephritis, vasculitis**
- **Ovary: haemorrhages, fibrosis, necrosis**
- **Wattle & feet: oedema, haemorrhages**

Pathological changes - vasculitis



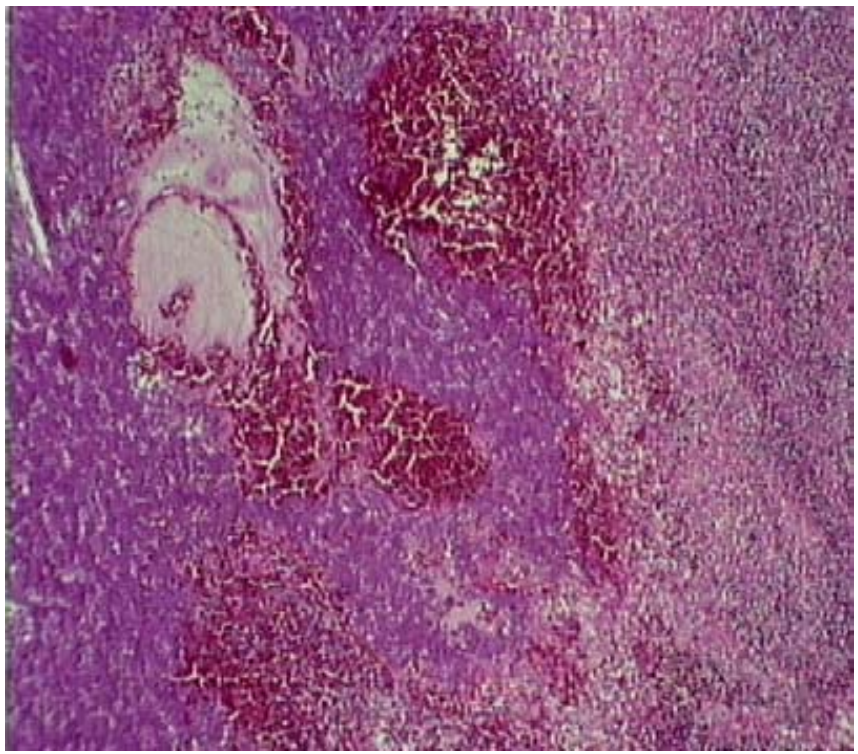
Brain : vaculitis,

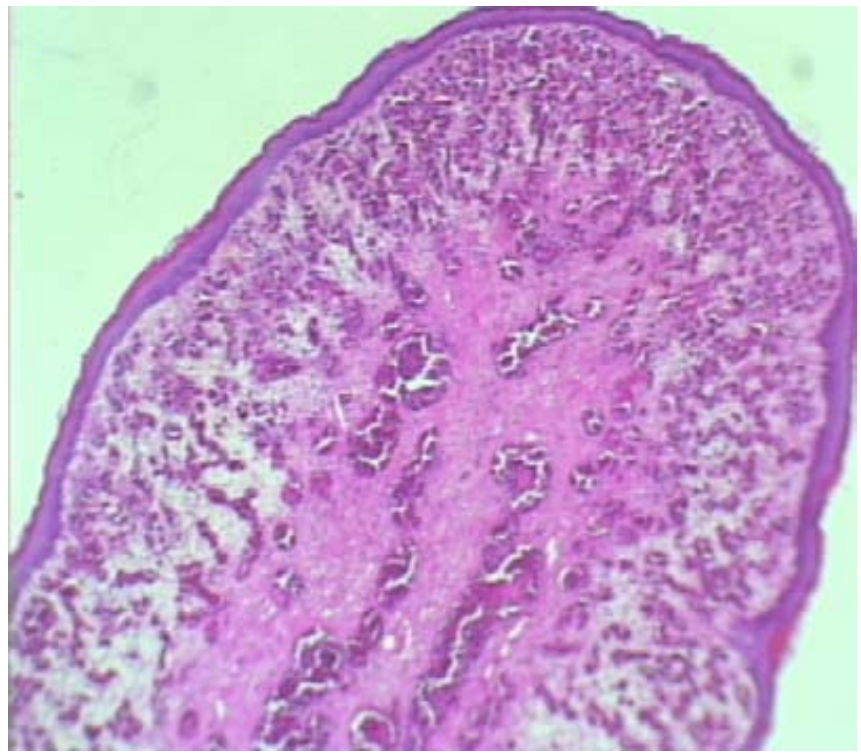
H&E, x 63



Kidney : vasculitis,

H&E, x 63

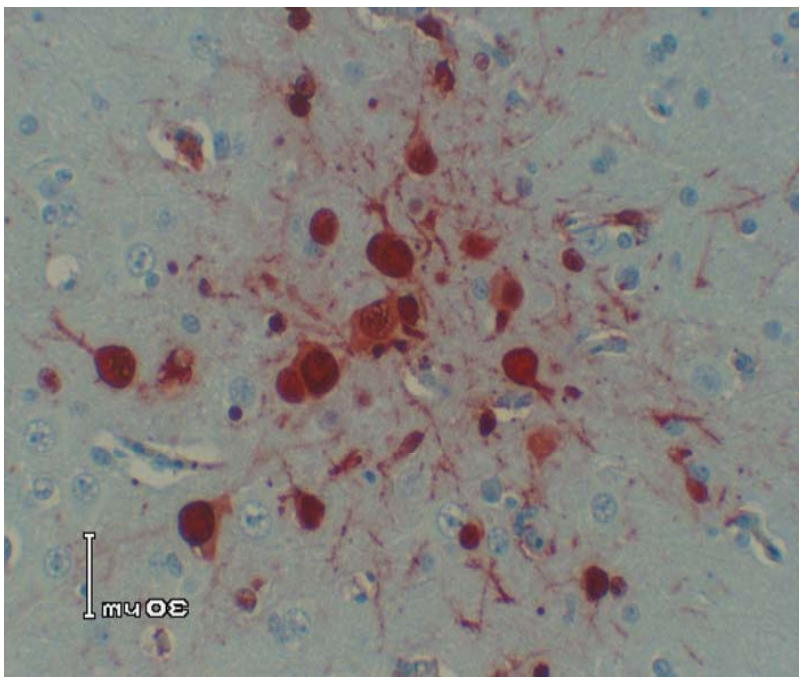




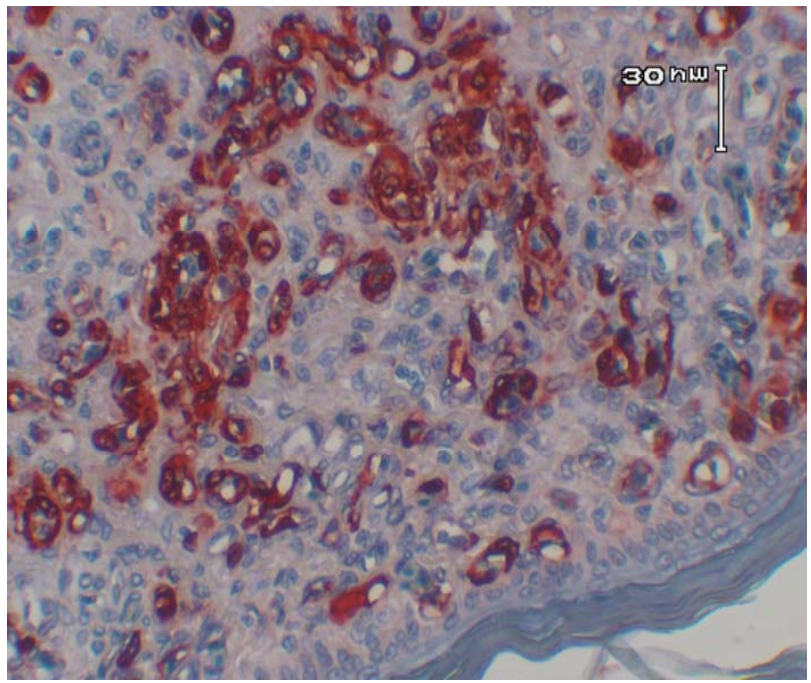
Liver : hemorrhages & necrosis
H&E, x 63

Wattle : hemorrhages, H&E, x 25

Localisation of Antigen by Immunohistochemical Staining



AI Antigen in Brain



AI Antigen in Wattle

Avian Influenza

The origin of the H5N1 pandemic of poultry

AI virus genetics:

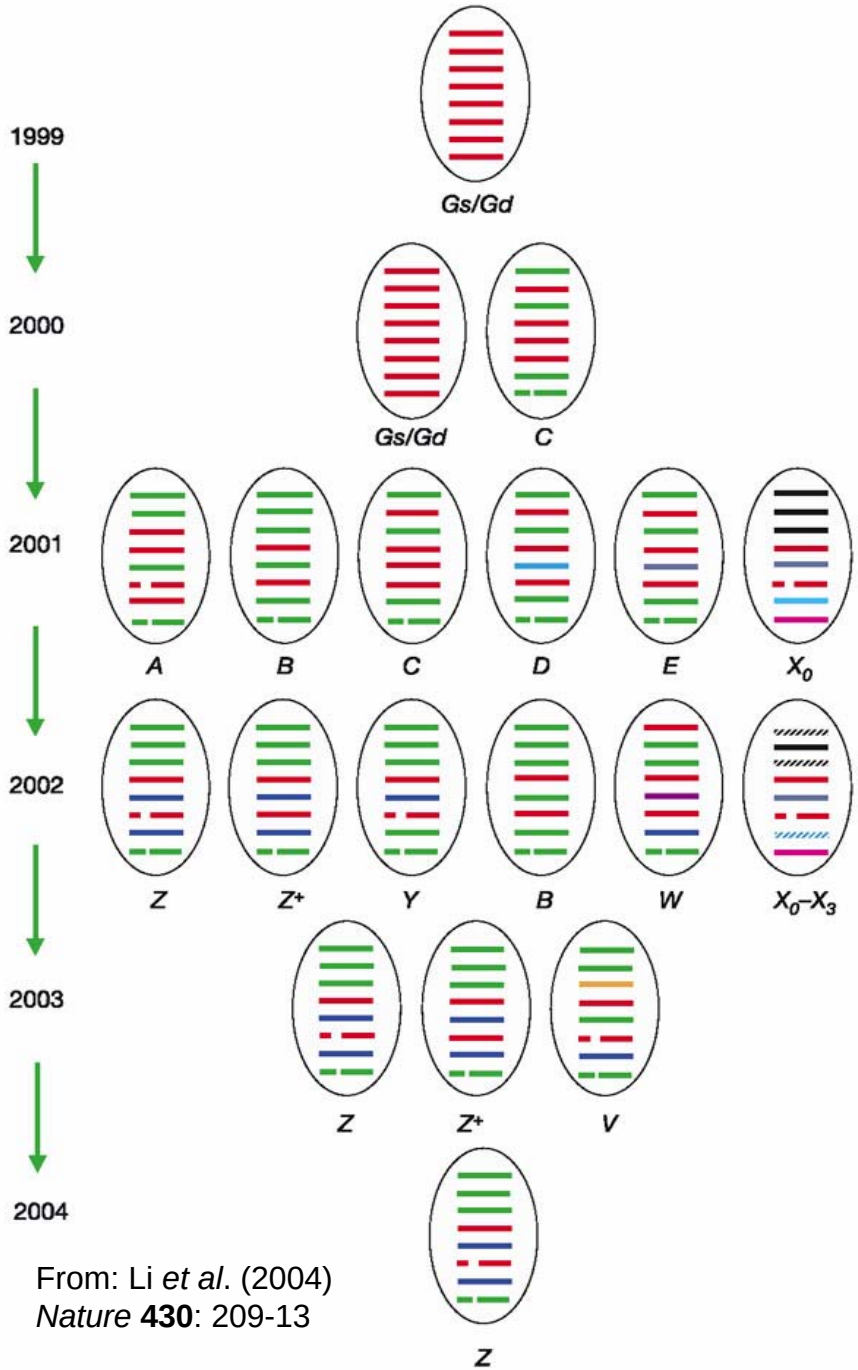
Not all H5N1s are the

AI viruses have genomes with 8 segments

There are 3 mechanisms for genetic evolution:

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

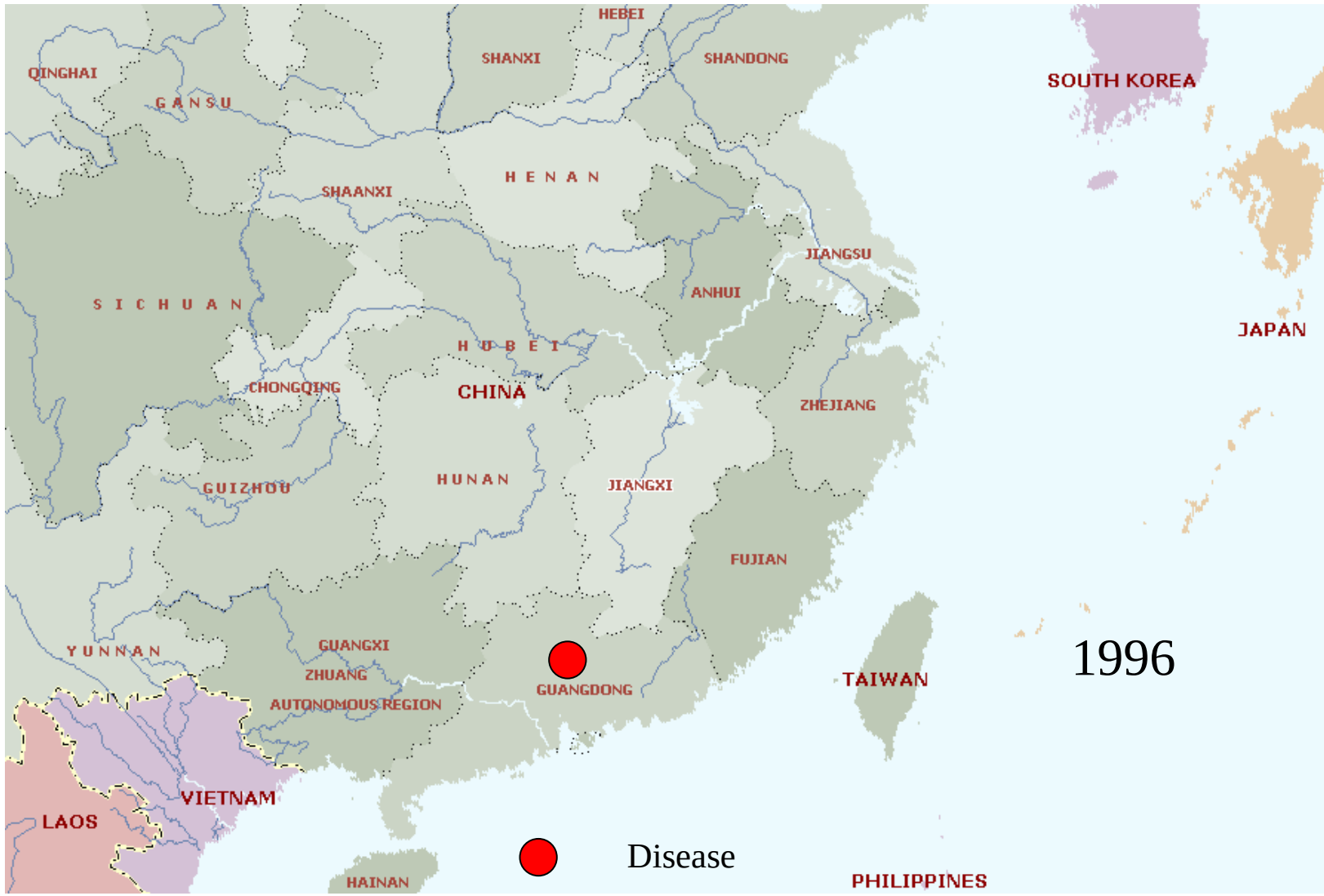
(Hence the evolution of H5N1 viruses as illustrated)



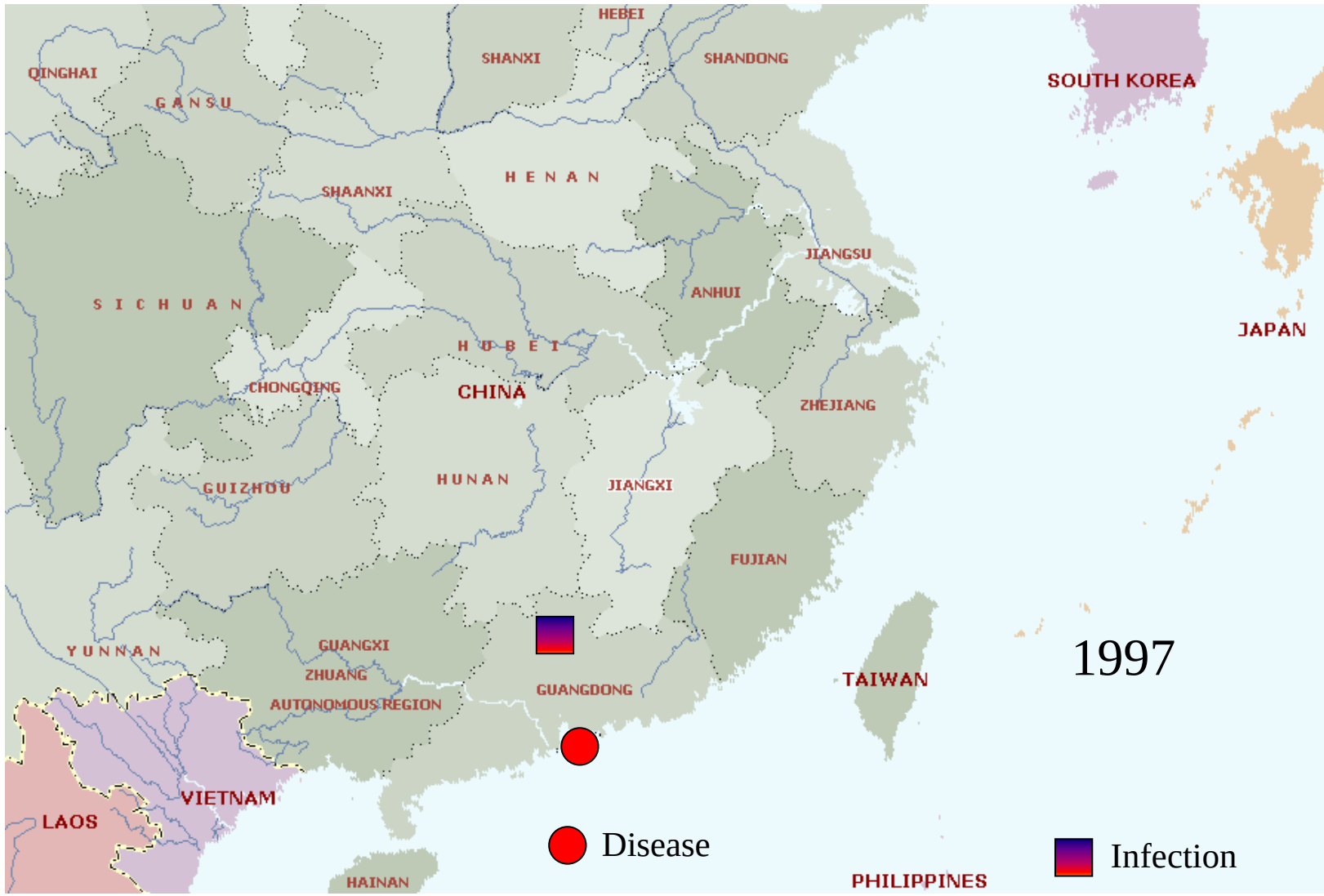
From: Li *et al*. (2004) *Nature* **430**: 209-13

H5N1 HPAI in Asia

Les Simms

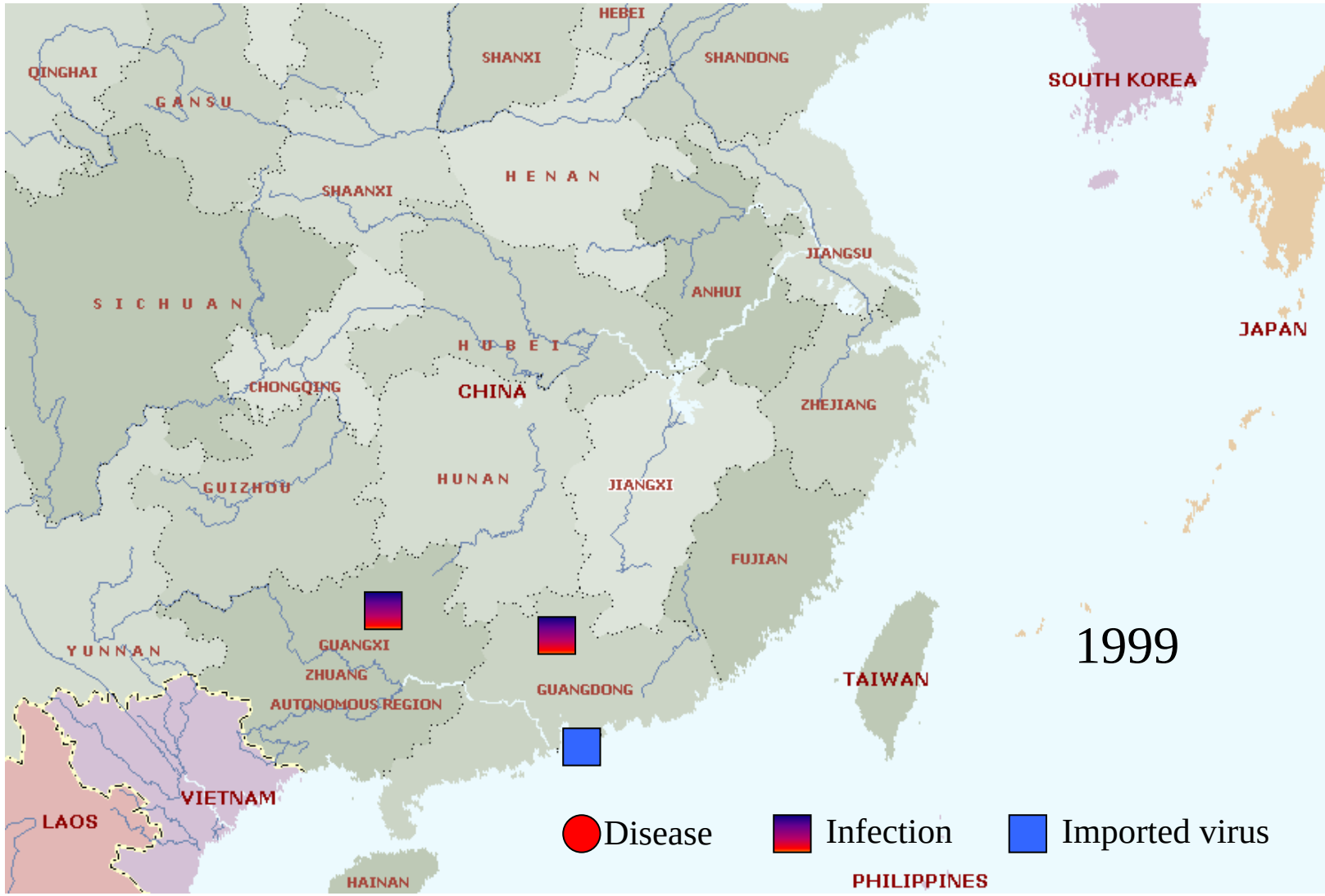


H5N1 HPAI in Asia



H5N1 HPAI in Asia

H5N1 HPAI in Asia



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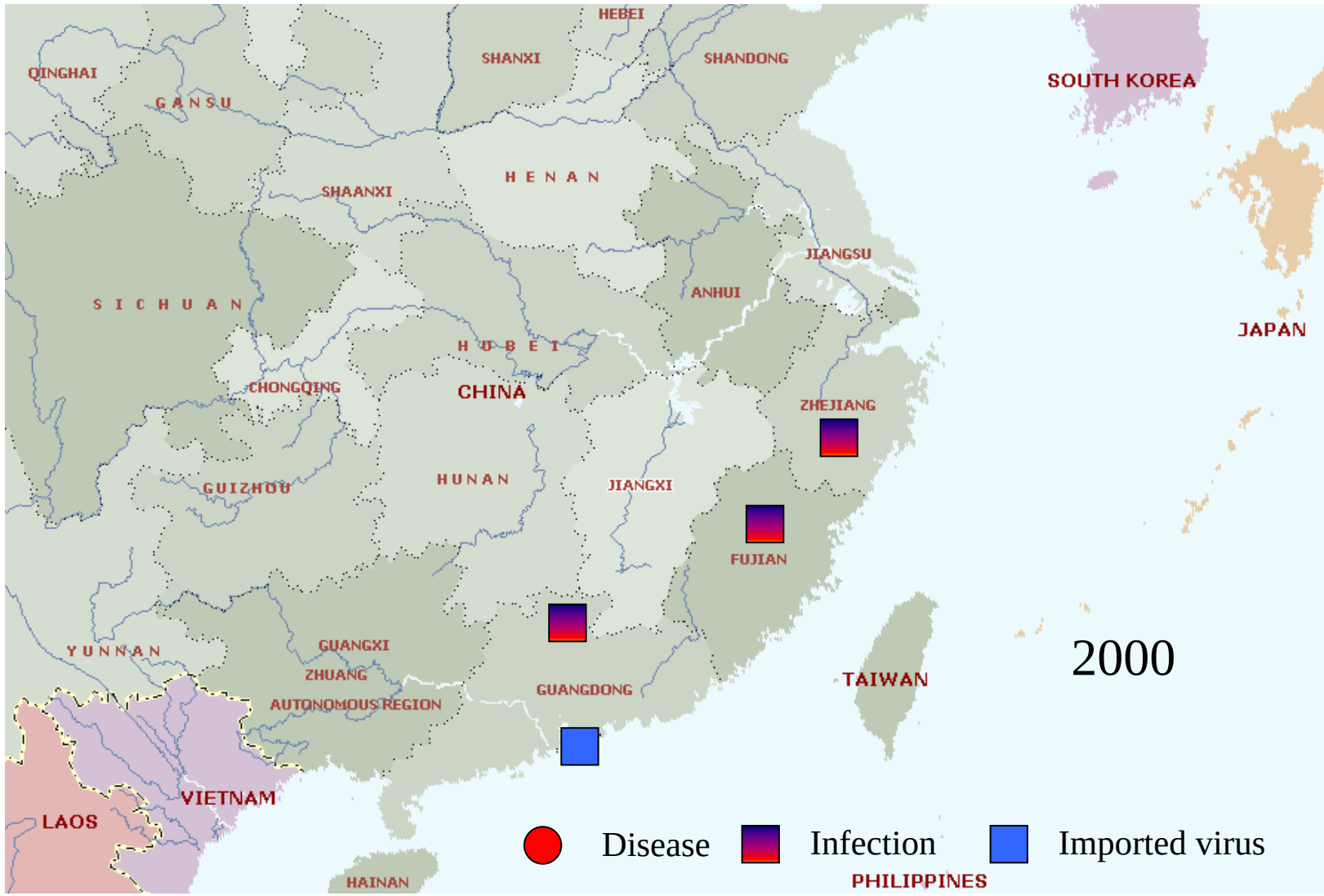
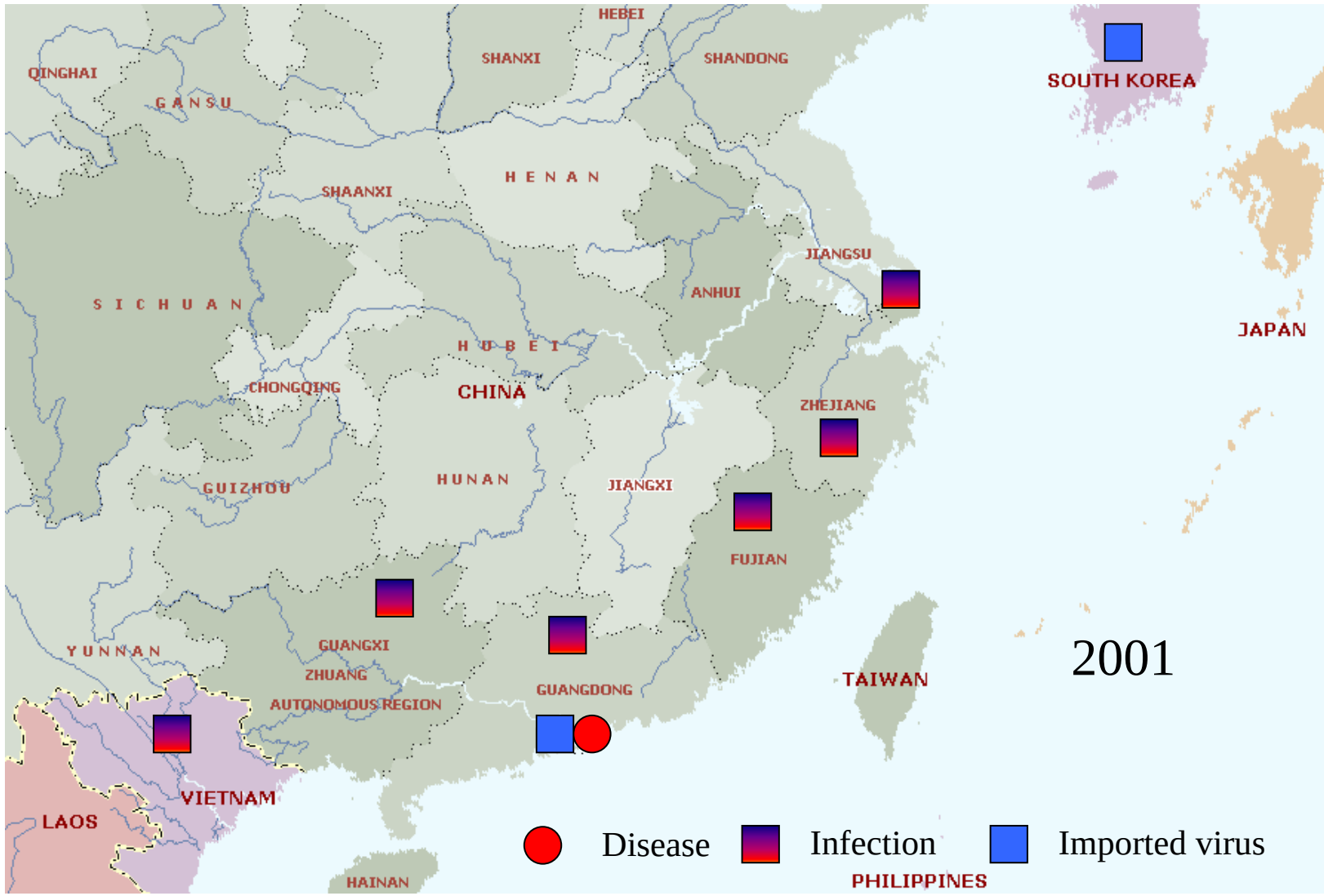
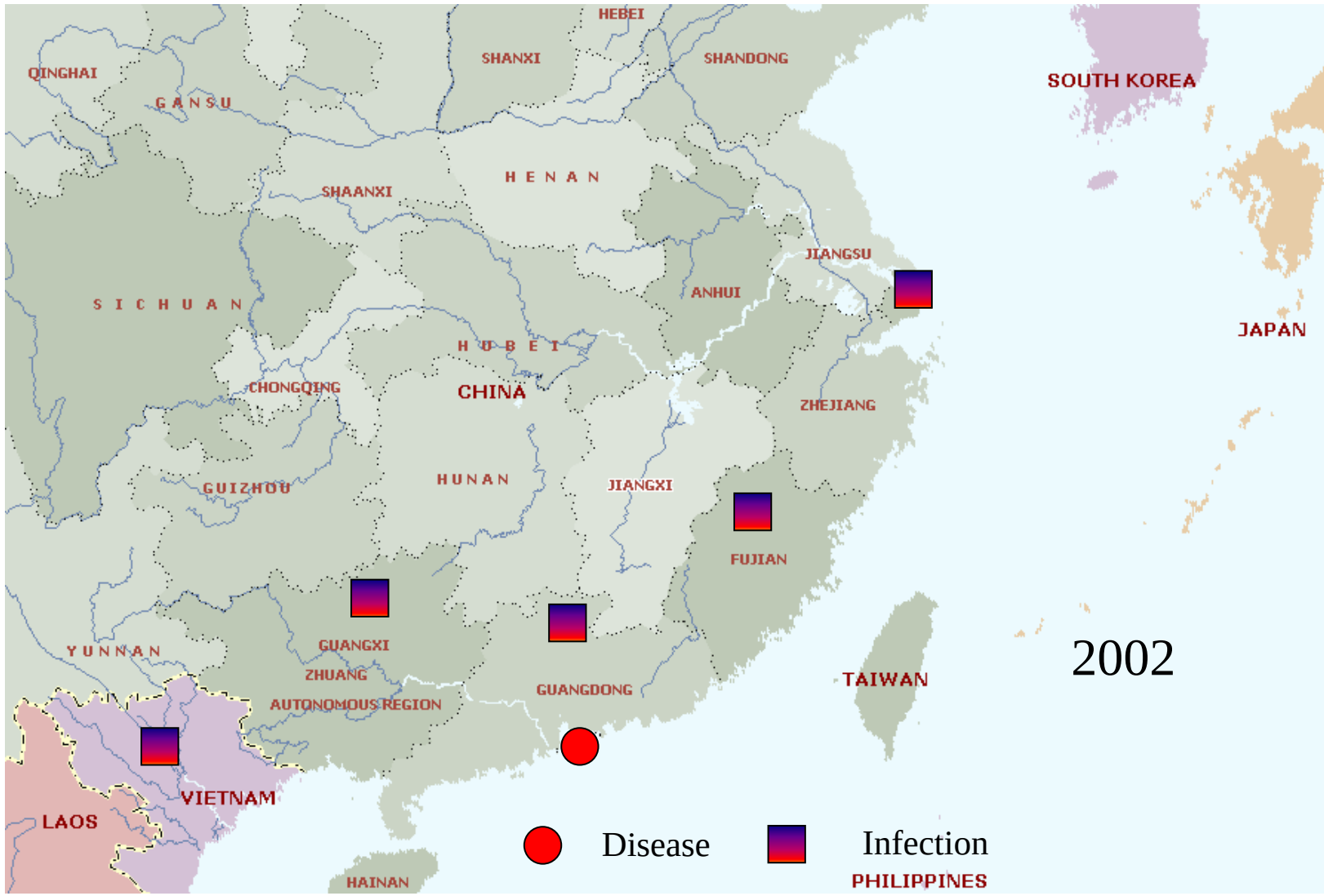


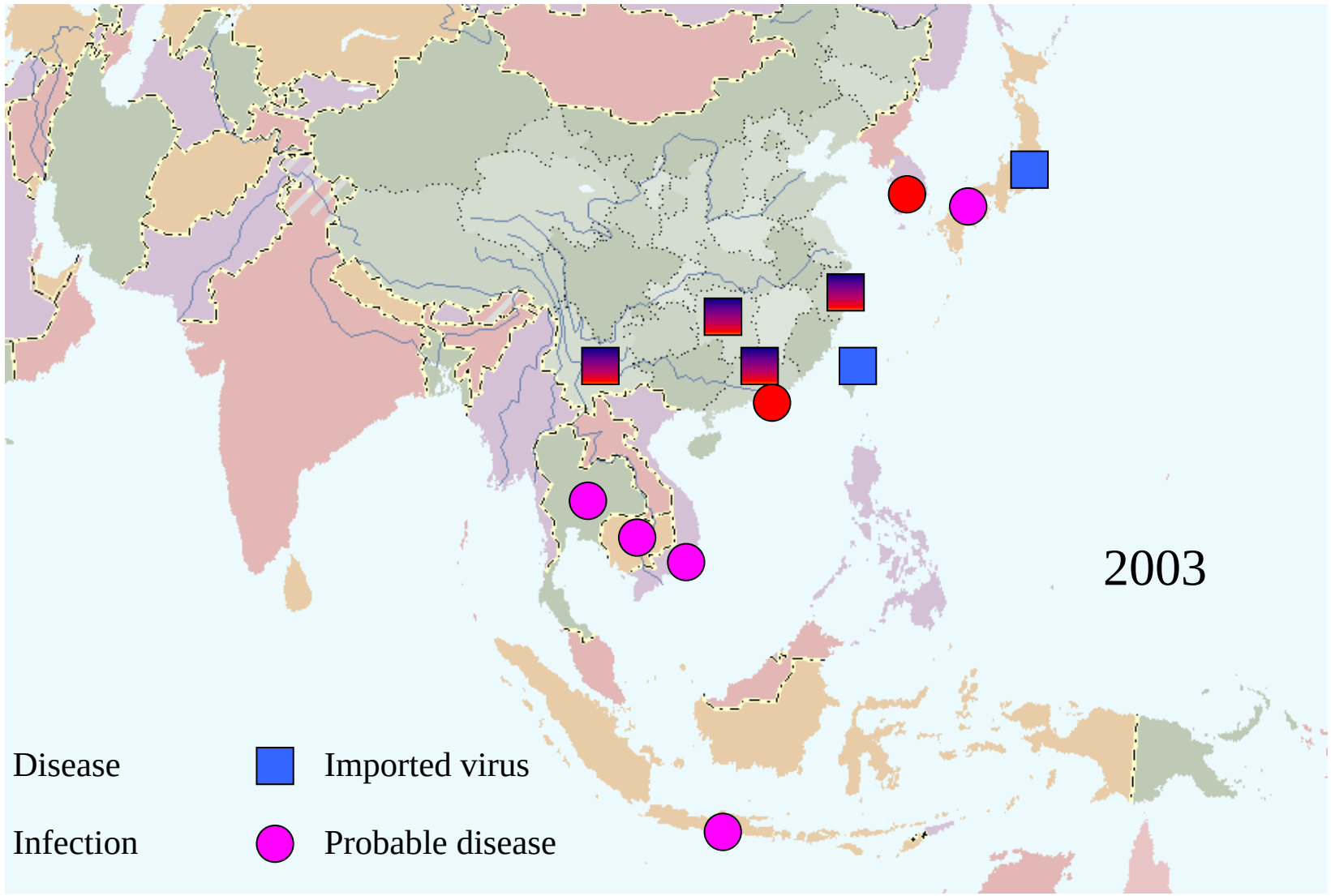
H5N1 HPAI in Asia



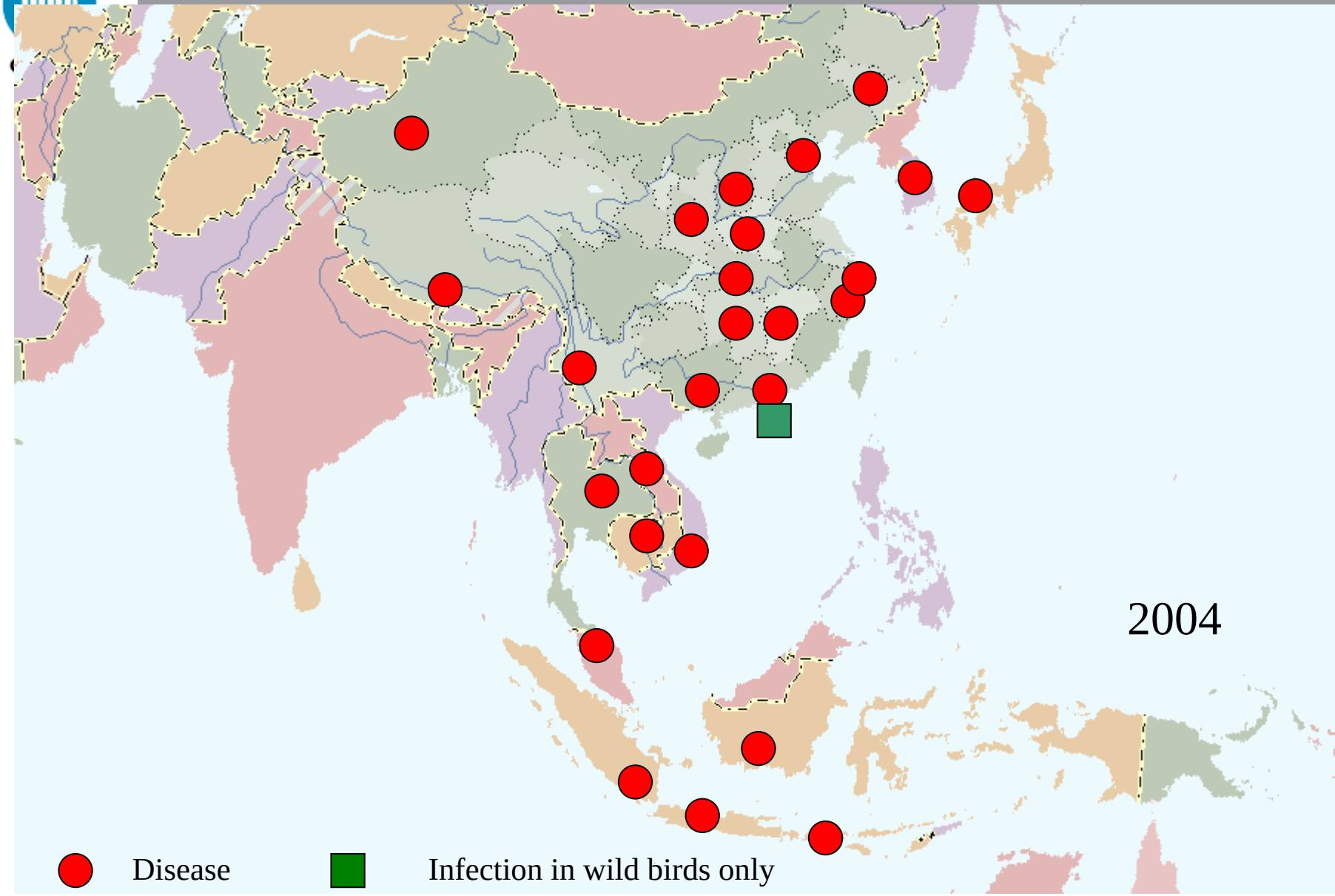
H5N1 HPAI in Asia



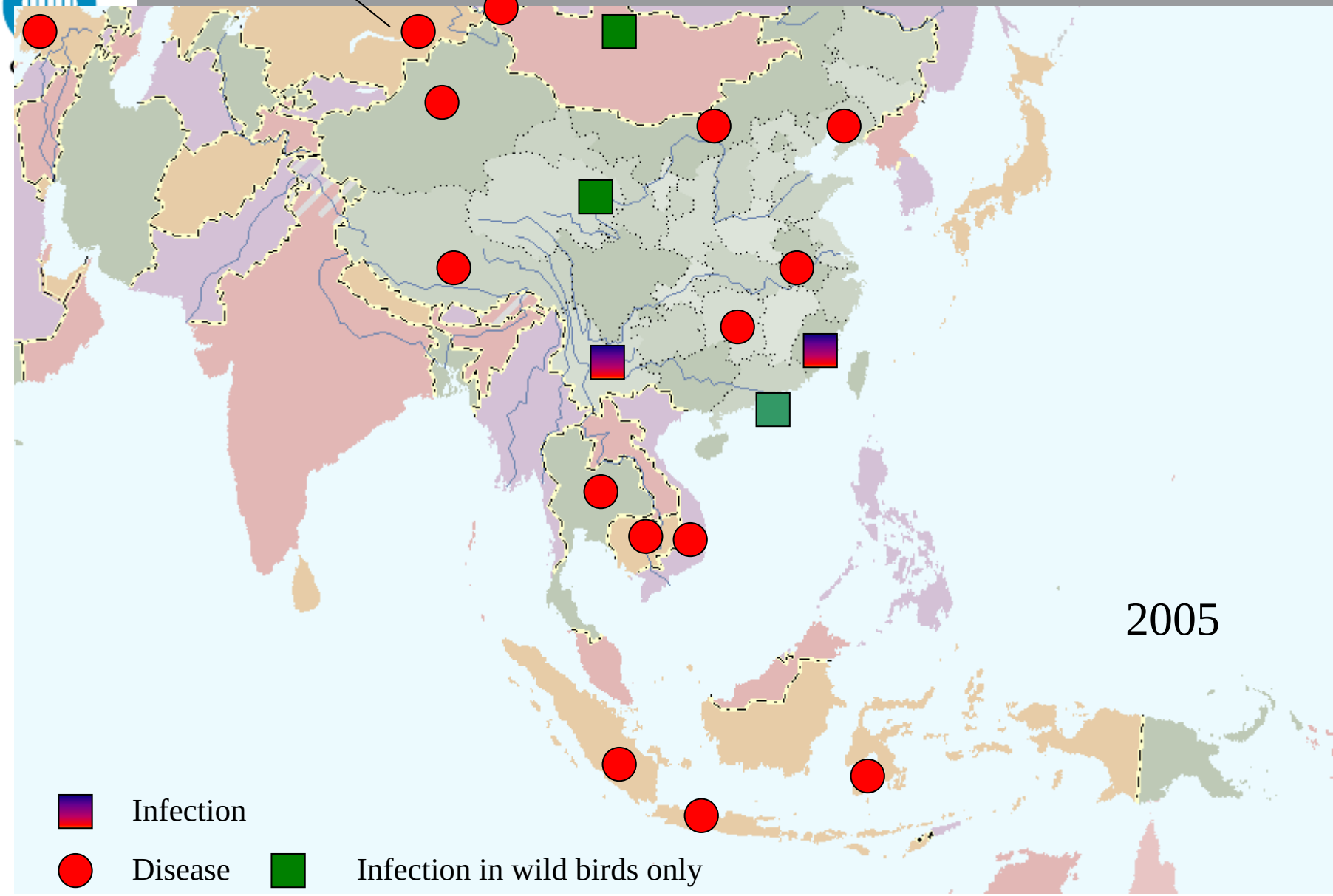
H5N1 HPAI in Asia

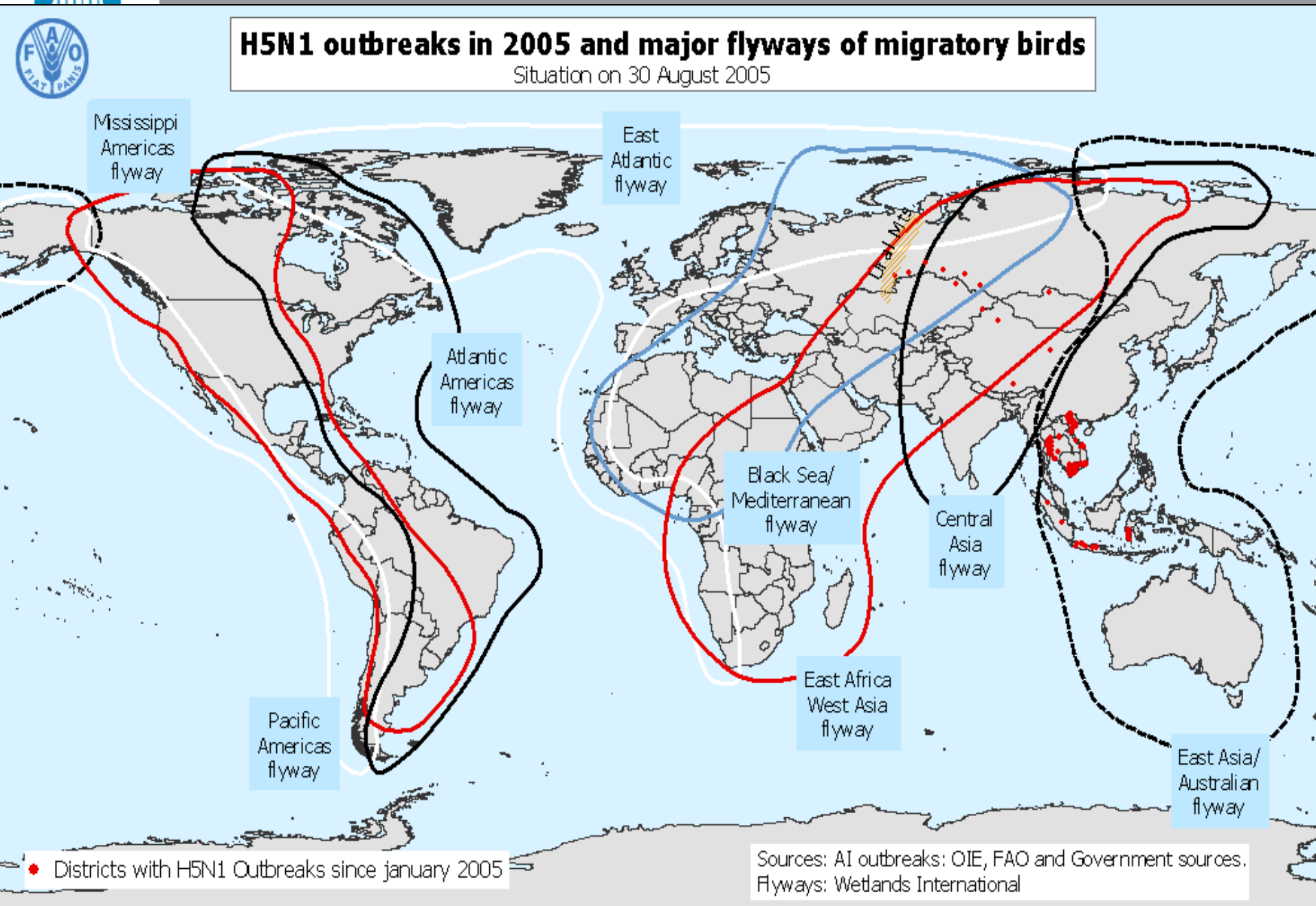


H5N1 HPAI in Asia



H5N1 HPAI in Asia




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

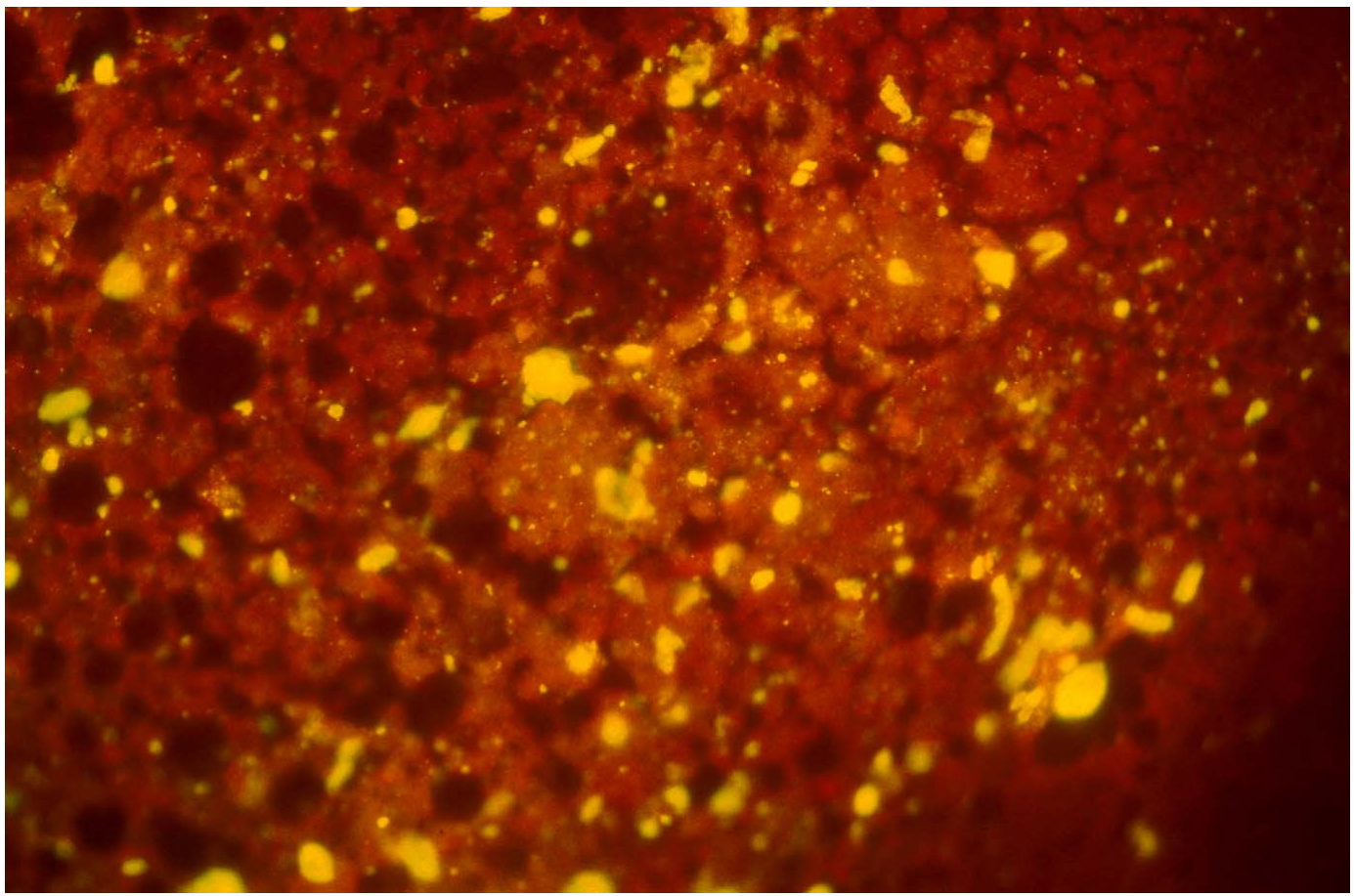
- H and N typing
- IVPI
- For a rapid test, the pancreatic impression smear

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

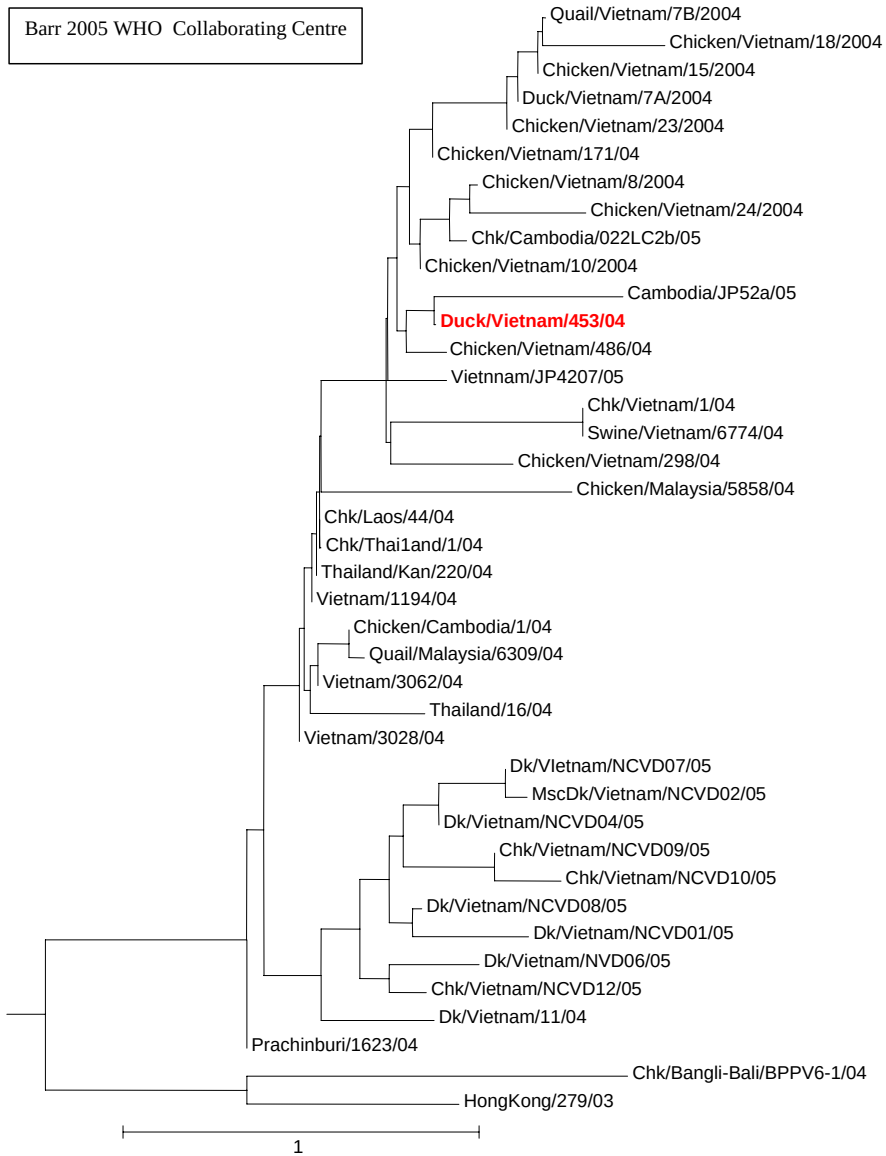
3 mechanisms for genetic evolution:

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

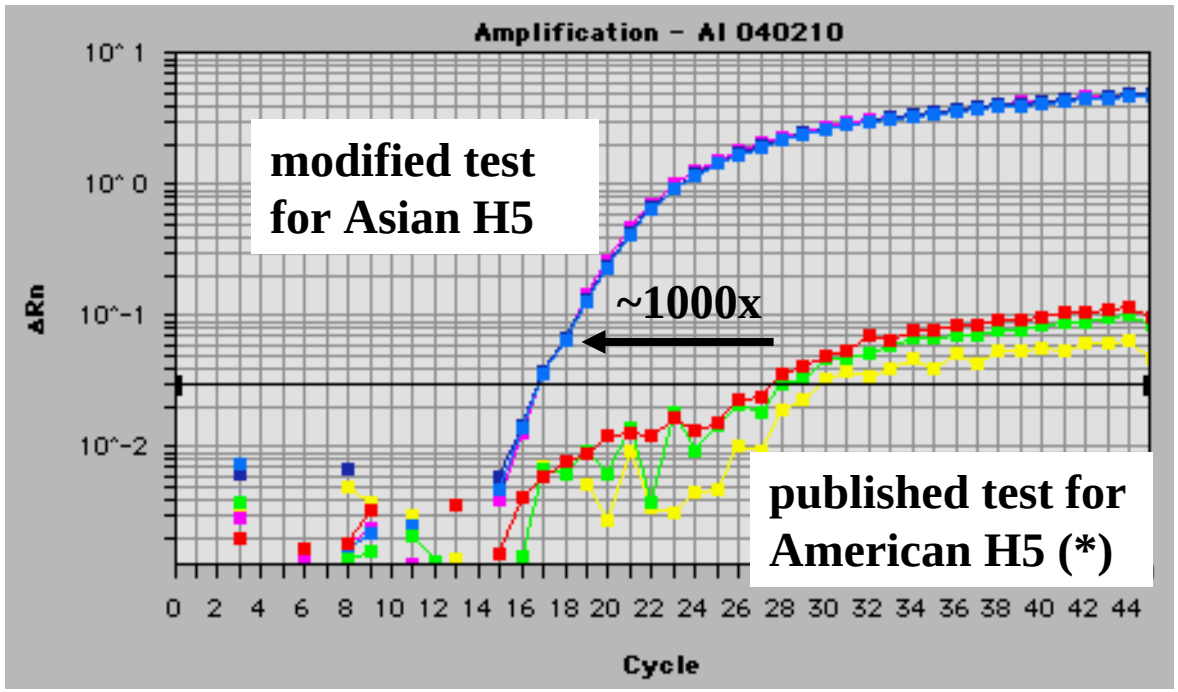
Increasing the sensitivity of both the H5 and the FLU A tests for the detection of the outbreak strain

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)

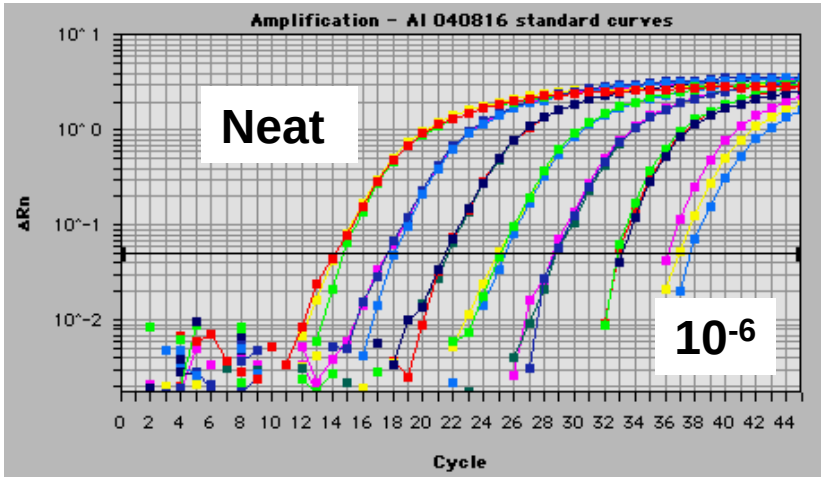


A/chicken/Viet Nam/8/2004 (H5N1)

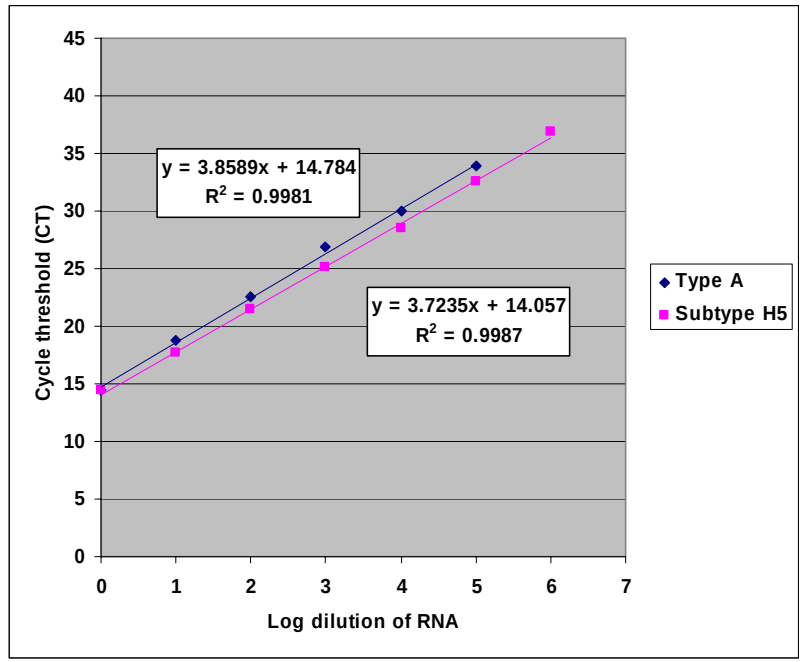
Conclusion:

~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)



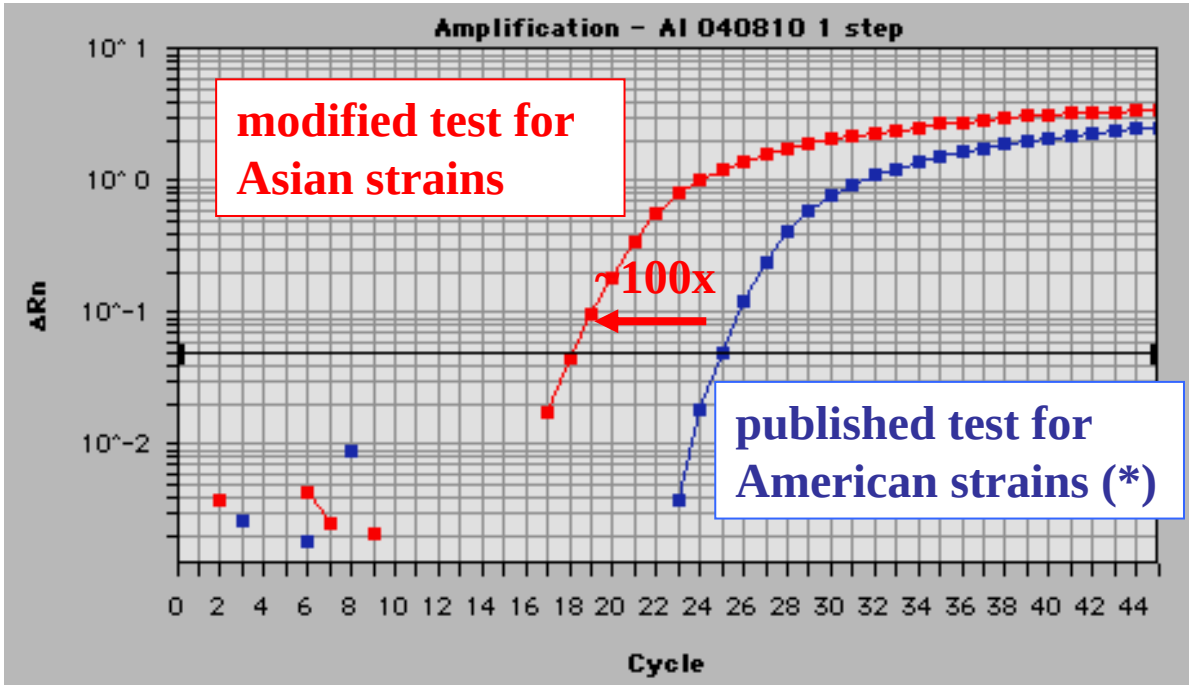
Conclusion:

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)

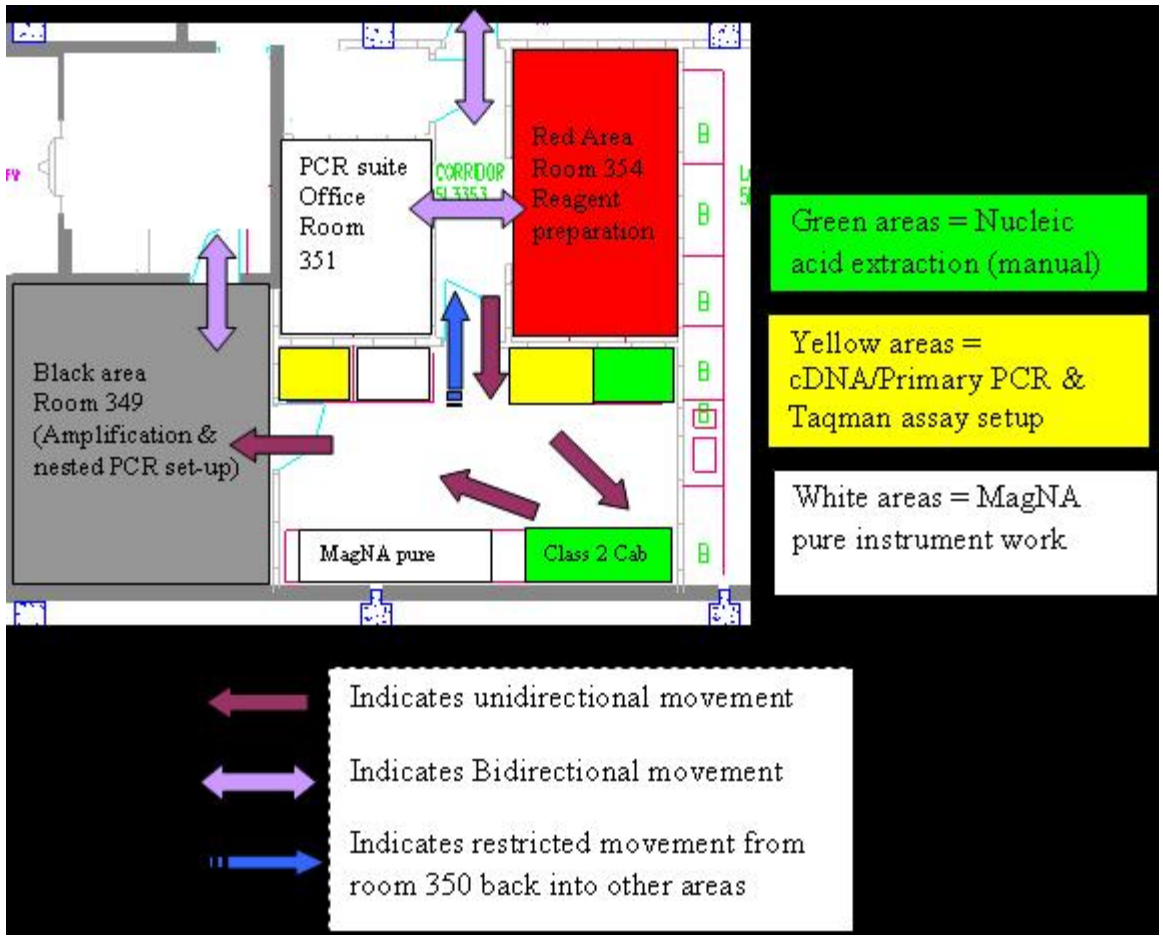
Conclusion:

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

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

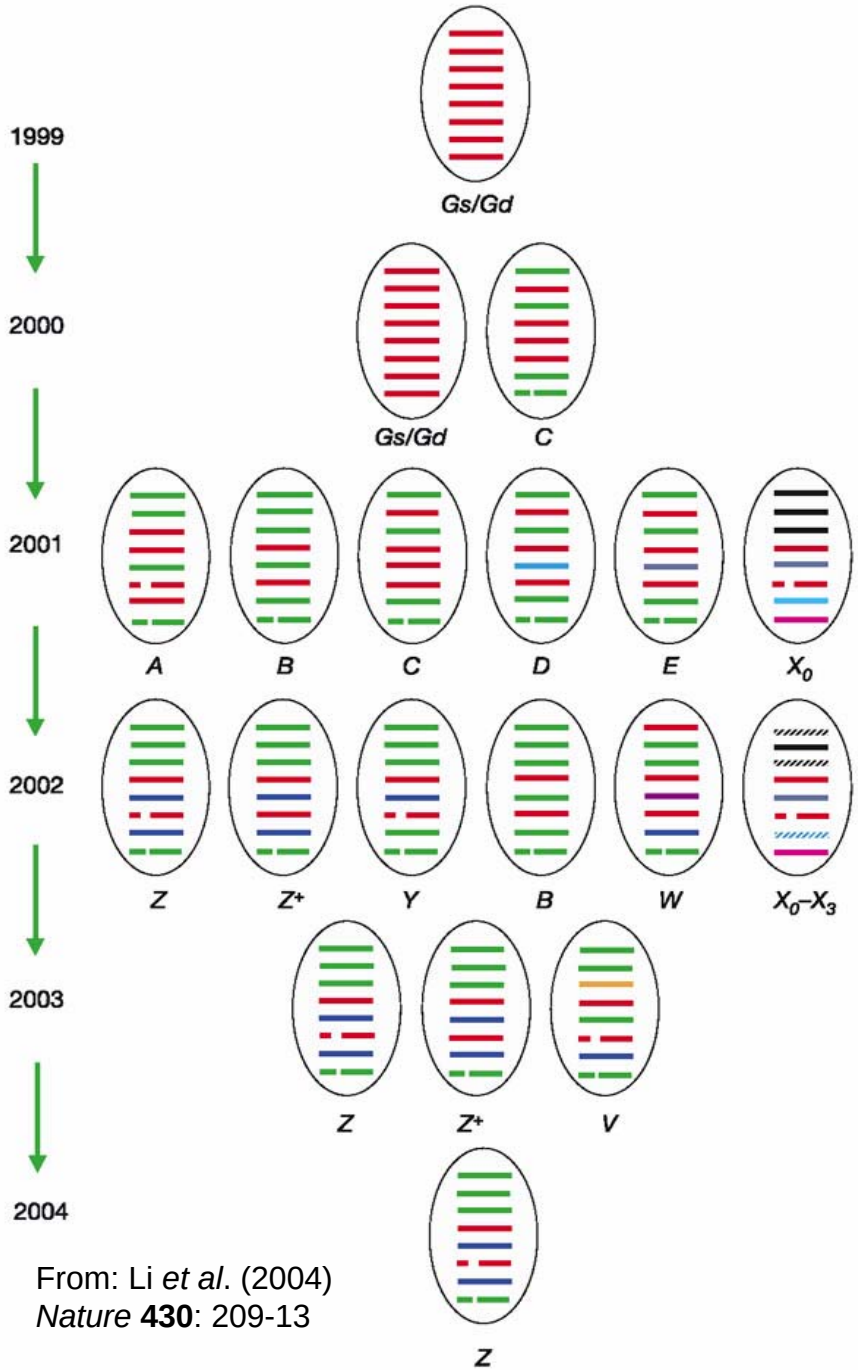
3 mechanisms for genetic evolution:

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)

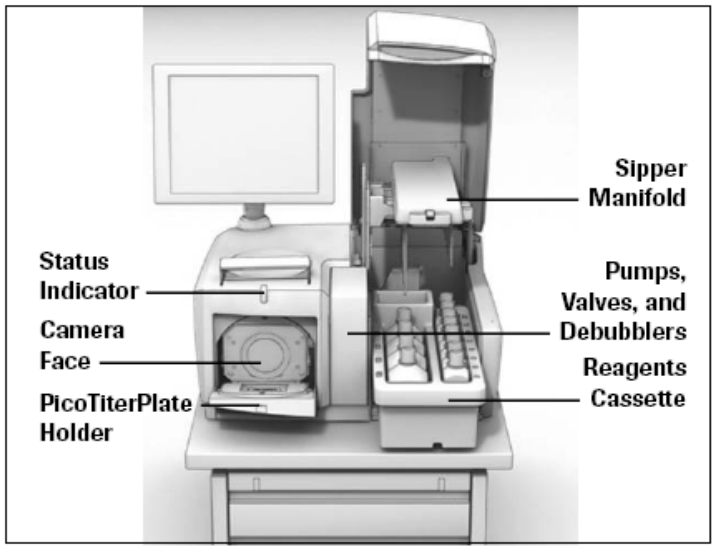
Diagnostic challenges

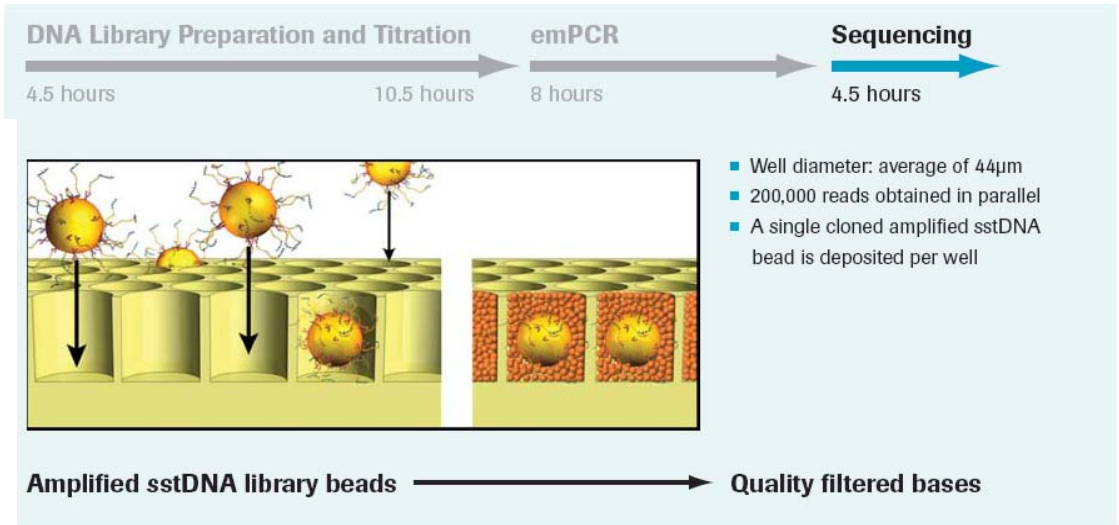
- Primer/probe design
- **Whole genome sequencing**



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

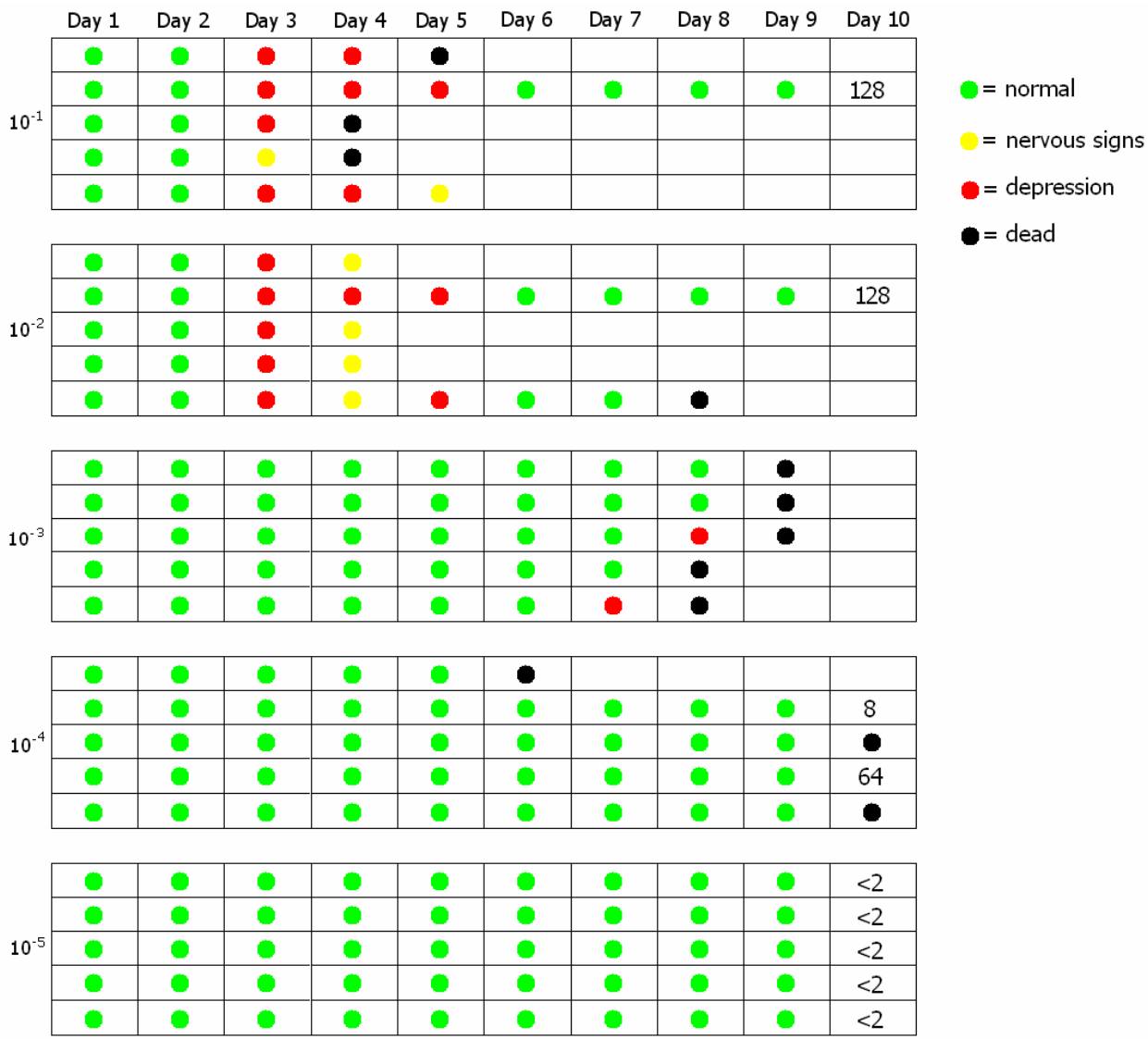
10^{-1} to 10^{-5} infected AF

I/N; I/Oral; I/Ocular

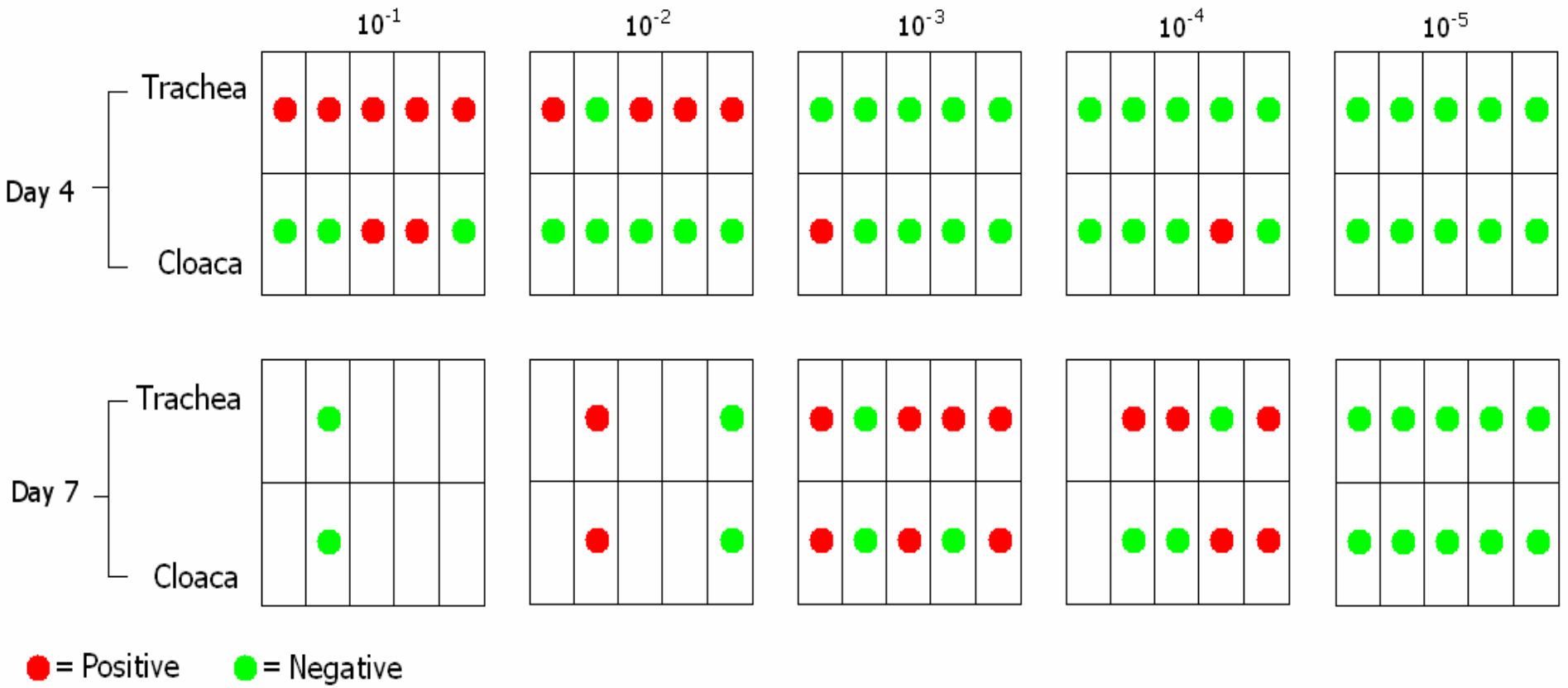
Observed for 10 days

- 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

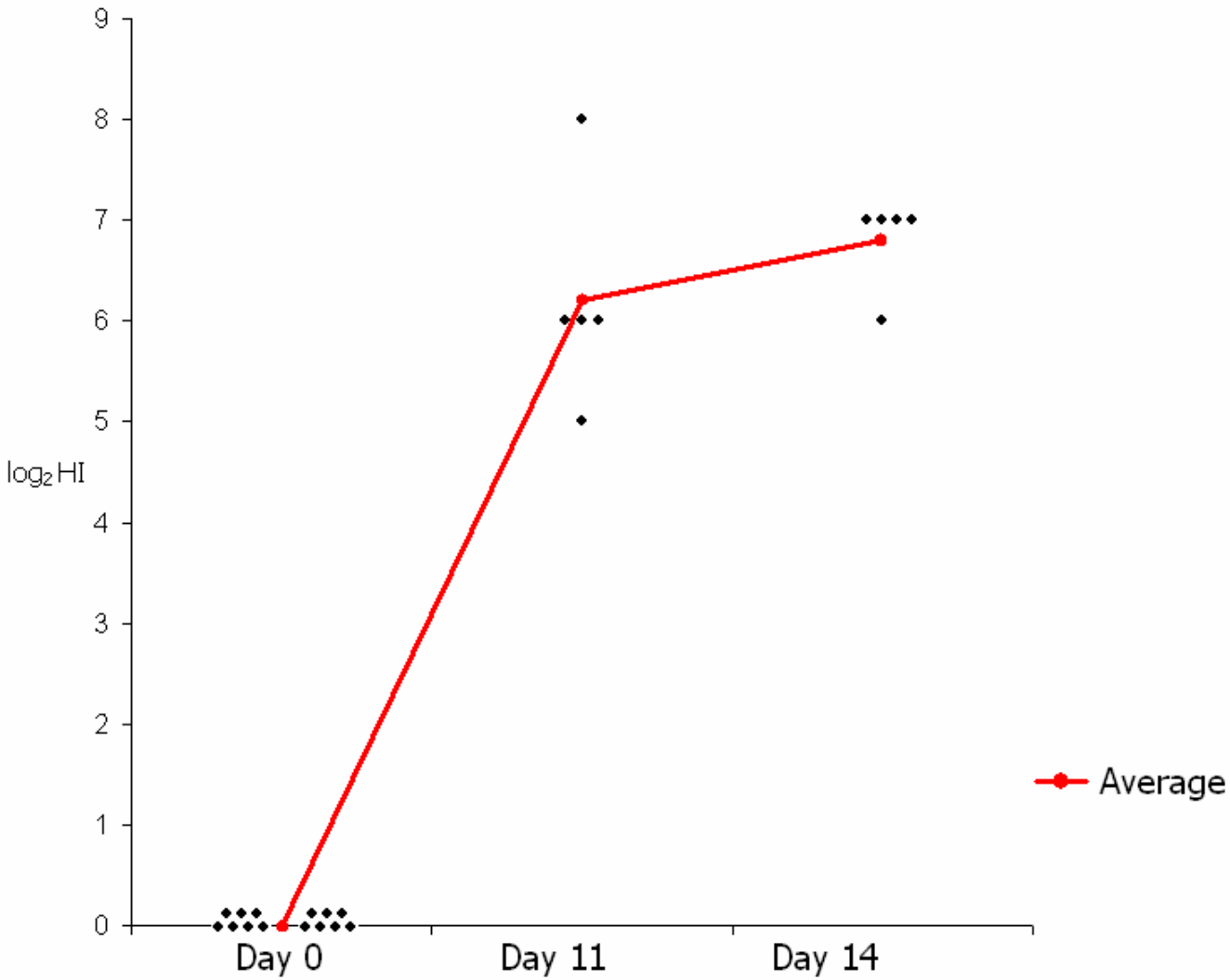
Poulvac i-AI H5N3 n=15

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

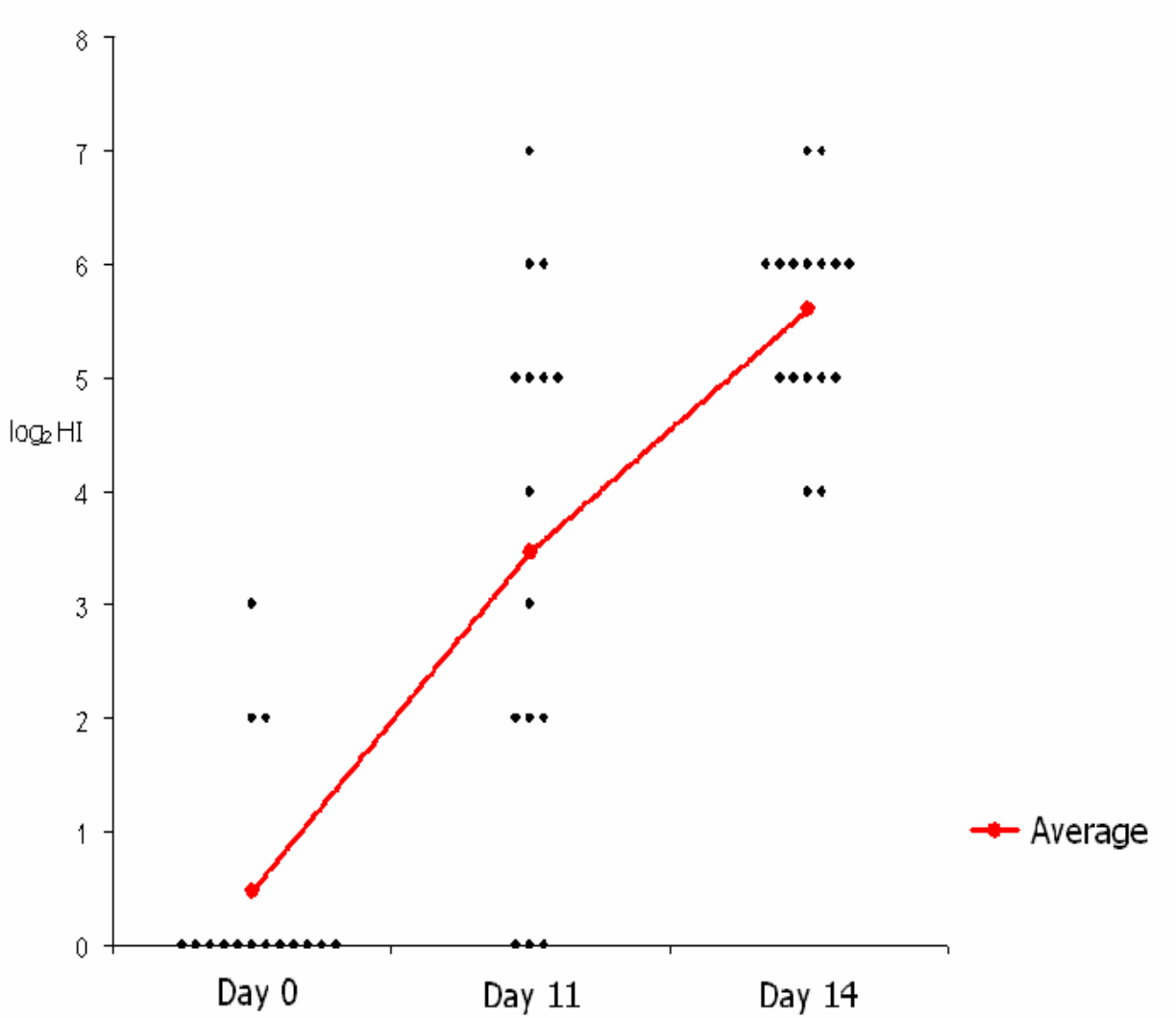
All birds challenged with H5N1 at 6wo

Clinical observations – vaccine study

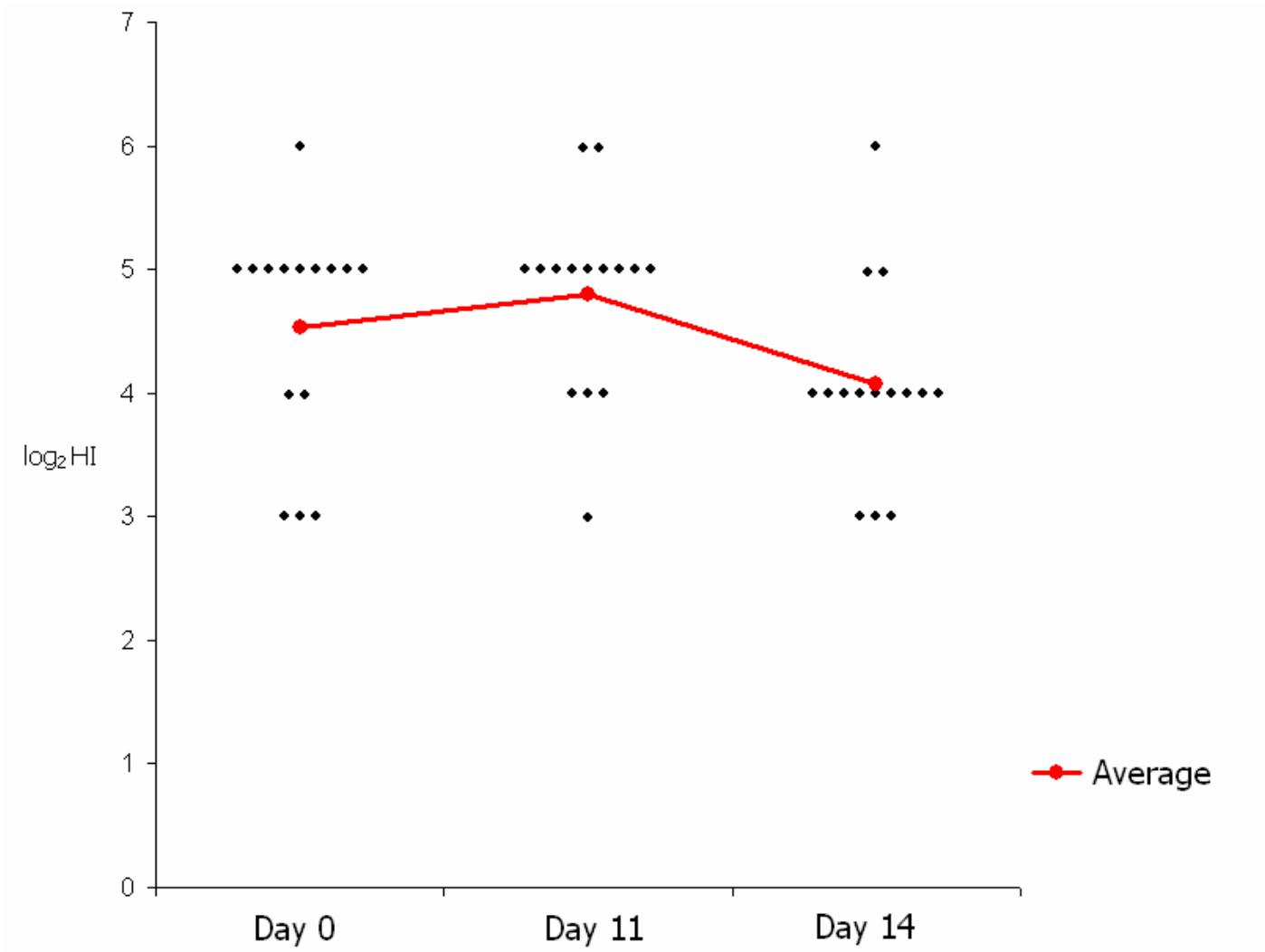
Serology - controls



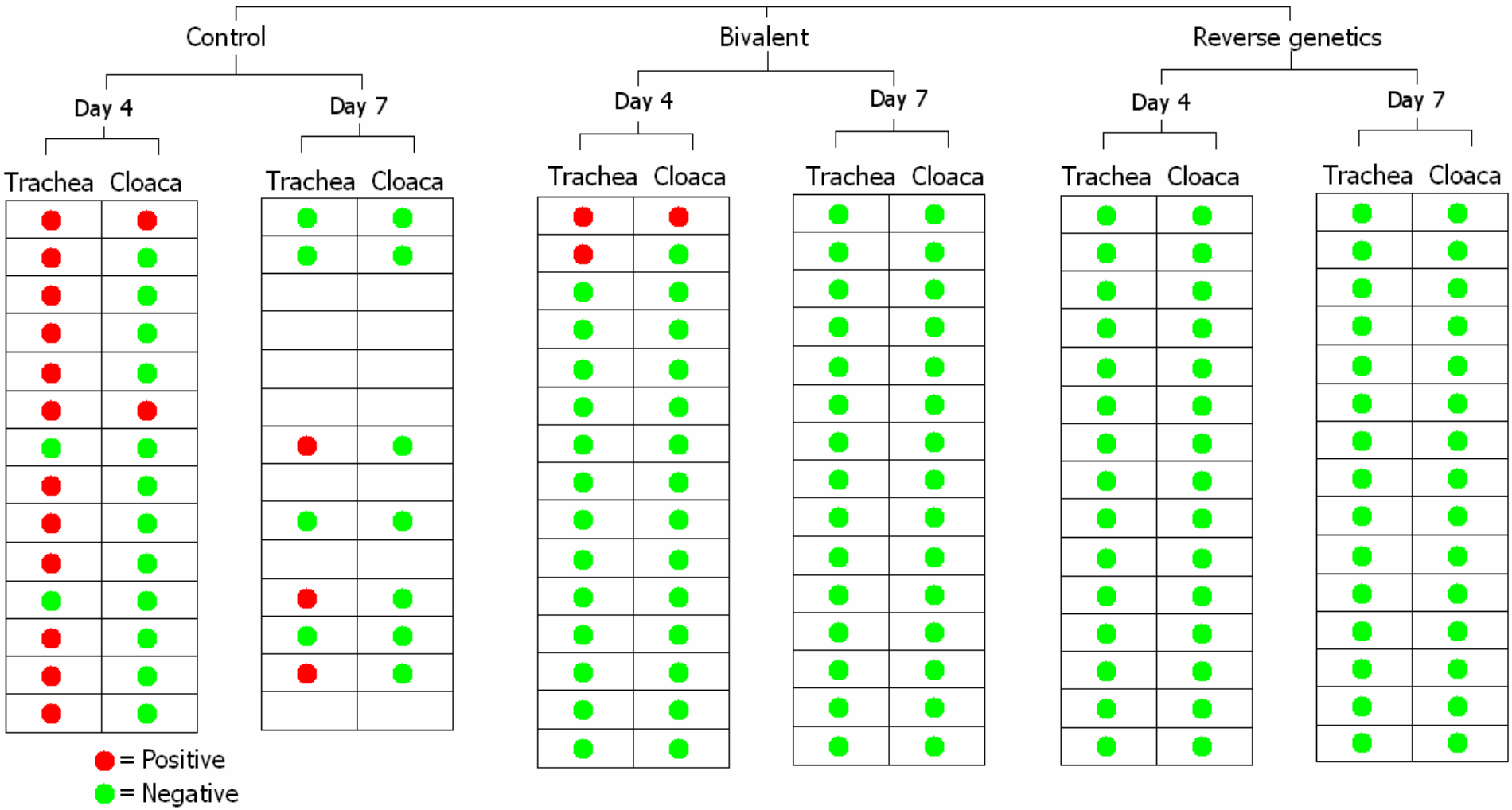
Serology – bivalent vaccine



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.

CSIRO Livestock Industries

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Thank You

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