



PCR related activities in OFFLU

The joint OIE-FAO
global network of expertise
on animal influenzas

Contributions from:

Ian Brown and Scott Reid, AHVLA, UK

Janice Pedersen, NVSL, USA

Oliver Lung, NCAD, Canada

John Pasick, NCFAD, Canada

Keith Hamilton, OIE/OFFLU

Overview



Constraints of influenza virus PCR in the veterinary field



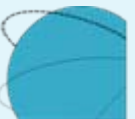
New developments



Porcine influenza



Novel PCR-linked detection methods



Proficiency testing

Comparison Study

- **Goal:** To compare the limit of detection for virus isolation and real-time RT-PCR (rRT-PCR) for avian influenza and Newcastle disease using tracheal/oropharyngeal and cloacal swabs from poultry and cloacal swabs from ducks.
- At the current time cloacal swabs from poultry and ducks are not acceptable sample matrixes for rRT-PCR.
- Tracheal/oropharyngeal swabs were used to establish equivalence margins since they are an acceptable sample matrix for rRT-PCR.
- If the limit of detection for virus isolation and rRT-PCR are comparable for cloacal swabs from poultry and ducks, cloacal swabs would be approved for use in the AI and ND rRT-PCR assays.

Comparison of LOD for VI and PCR Testing

Specimen	VI LOD	PCR LOD
CK TR Swab - AI	1.9 log ₁₀ EID ₅₀	1.0 log ₁₀ EID ₅₀
CK CL Swab - AI	1.7 log ₁₀ EID ₅₀	1.8 log ₁₀ EID ₅₀
CK TR Swab - ND	0.8-2.0 log ₁₀ EID ₅₀	1.5 log ₁₀ EID ₅₀
CK CL Swab - ND	3.2 log ₁₀ EID ₅₀	0.8-2.0 log ₁₀ EID ₅₀
Duck CL Swab - AI	1.5 log ₁₀ EID ₅₀	1.1-2.2 log ₁₀ EID ₅₀

Positive Controls

- **Commercial Controls**

- Qiagen: One-step viral RT-PCR with *in vitro* transcribed (RNA/DNA) IC
- Assuragen: Armored Enterovirus
 - Has similar thermal cycling conditions to M-gene rRT-PCR
 - Co-extracted on a per sample basis
 - Multiplex assay to detect presence of PCR inhibitors

- **Positive extraction controls**

- “Tuff RNA” contains M gene target engineered into cowpea mosaic virus:
 - Potential use as positive & extraction control
 - H5 & H7 “Tuff RNAs” planned for evaluation in EU FluLabNet Project.
 - Not yet available in armored form

AI rRT-PCR geographical differences

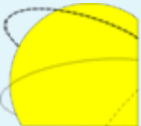
- **Generic AI detection:**
 - M gene rRT PCR, Spackman *et al* (2002)
- **AI RRT PCRs for detection of NAI (H5 & H7):**
 - H5: EU: Slomka *et al* (2007a), Monne *et al* (2008)
NA: Spackman *et al* (2007), does not detect current H5N2 Mexico!
 - H7: EU: Monne *et al* (2008), Slomka *et al* (2009)
NA: Spackman *et al* (2007)
- **N1 rRT PCR**
 - Payungporn *et al* (2006)
- **Conventional RT PCRs for molecular pathotyping (sequencing) of NAI**
 - EU: Slomka *et al* (2007b), NA: in-house assay
- **Lab protocols publicly available on AHVLA website:**

http://www.defra.gov.uk/vla/science/sci_ai_reflab_prot.htm

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RAPID COMMUNICATION

First Reported Incursion of Highly Pathogenic Notifiable Avian Influenza A H5N1 Viruses from Clade 2.3.2 into European Poultry

S. M. Reid¹, W. M. Shell¹, G. Barboi², I. Onita², M. Turcitu², R. Cioranu², A. Marinova-Petkova³, G. Goujgoulova⁴, R. J. Webby⁵, R. G. Webster⁵, C. Russell¹, M. J. Slomka¹, A. Hanna¹, J. Banks¹, B. Alton¹, L. Barrass¹, R. M. Irvine¹ and I. H. Brown¹

¹ OIE, FAO and EU Reference Laboratory for Avian Influenza and Newcastle Disease, Veterinary Laboratories Agency-Weybridge, Addlestone, Surrey, UK

² Institute for Diagnosis and Animal Health, National Reference Laboratory for Avian Influenza and Newcastle Disease, Bucharest, Romania

³ Regional Diagnostic Laboratory on Avian Influenza and Newcastle Disease in Birds, National Diagnostic and Research Veterinary Medical Institute, Varna, Bulgaria

⁴ National Diagnostic Laboratory on Avian Influenza and Newcastle Disease in Birds, National Diagnostic and Research Veterinary Medical Institute, Sofia, Bulgaria

⁵ Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, TN, USA

Papers

Initial incursion of pandemic (H1N1) 2009 influenza A virus into European pigs

M. D. Welsh, P. M. Baird, M. P. Guelbenzu-Gonzalo, A. Hanna, S. M. Reid, S. Essen, C. Russell, S. Thomas, L. Barrass, F. McNeilly, J. McKillen, D. Todd, V. Harkin, S. McDowell, B. Choudhury, R. M. Irvine, J. Borobia, J. Grant, I. H. Brown

The initial incursion of pandemic (H1N1) 2009 influenza A virus (pH1N1) into a European pig population is reported. Diagnosis of swine influenza caused by pandemic virus was made during September 2009 following routine submission of samples for differential diagnosis of causative agents of respiratory disease, including influenza A virus. All four pigs (aged six weeks) submitted for investigation from a pig herd of approximately 5000 animals in Northern Ireland, experiencing acute-onset respiratory signs in finishing and growing pigs, were positive by immunofluorescence for influenza A. Follow-up analysis of lung tissue homogenates by real-time RT-PCR confirmed the presence of pH1N1. The virus was subsequently detected on two other premises in Northern Ireland; on one premises, detection followed the pre-export health certification testing of samples from pigs presumed to be subclinically infected as no clinical signs were apparent. None of the premises was linked to another epidemiologically. Sequencing of the haemagglutinin and neuraminidase genes revealed high nucleotide identity (>99.4 per cent) with other pH1N1s isolated from human beings. Genotypic analyses revealed all gene segments to be most closely related to those of contemporary pH1N1 viruses in human beings. It is concluded that all three outbreaks occurred independently, potentially as a result of transmission of the virus from human beings to pigs.

Reassortant Pandemic (H1N1) 2009 Virus in Pigs, United Kingdom

Wendy A. Howard, Steve C. Essen, Benjamin W. Strugnell, Christine Russell, Laura Barrass, Scott M. Reid, and Ian H. Brown

Surveillance for influenza virus in pigs in the United Kingdom during spring 2010 detected a novel reassortant influenza virus. This virus had genes encoding internal proteins from pandemic (H1N1) 2009 virus and haemagglutinin and neuraminidase genes from swine influenza virus (H1N2). Our results demonstrate processes contributing to influenza virus heterogeneity.

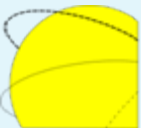
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PCR-Based Swine Surveillance

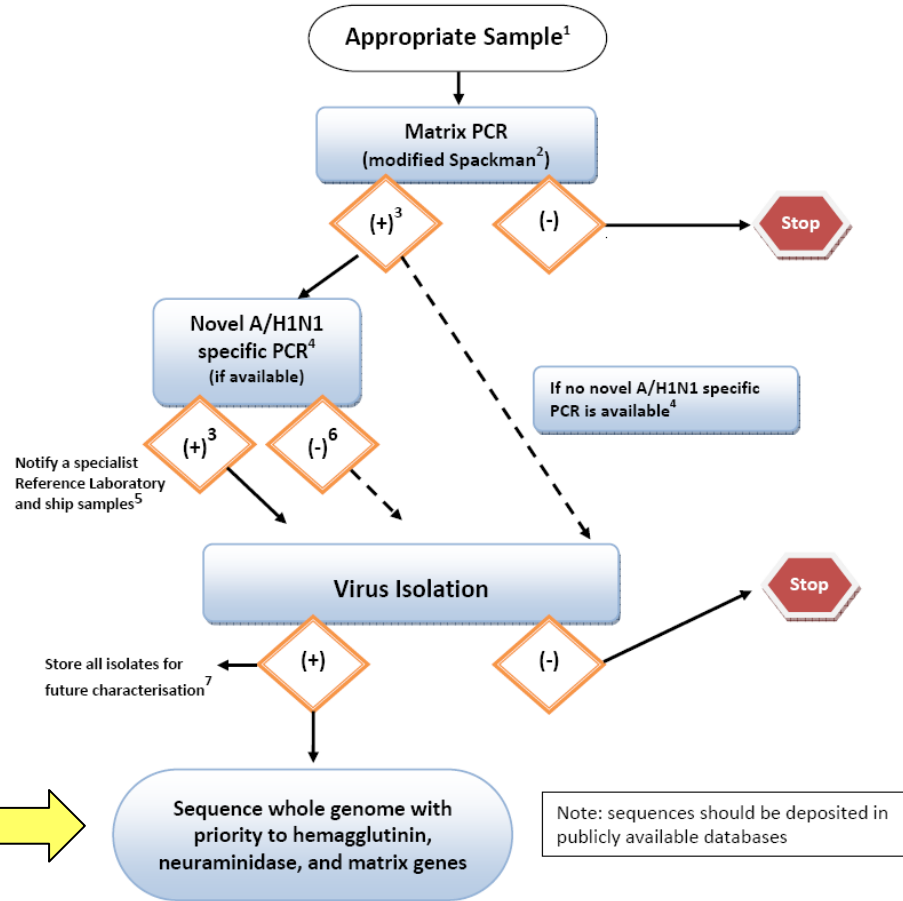
- **In Europe an H1 rRT-PCR assay has been validated to differentiate pH1N1 from established Eurasian SIVs**
 - Slomka *et al* (2010), *Influenza and other Respiratory Viruses* 4:277-293
- **In North America, detection of pH1N1 in domestic animals initially employed modified M gene and 2009 SEPRL N1 novel gene real-time RT-PCR assays**
- **Reassortants between pH1N1 and contemporary swine viruses have now been identified**

Collaboration with NVSL and NAHLN –

lab testing algorithm for pandemic H1N1

OFFLU interim guidance on detecting novel A/H1N1 in pigs laboratory testing algorithm for molecular tests

Originally designed for use in the USA by National Veterinary Services Laboratory (NVSL) and the National Animal Health Laboratory Network (NAHLN), USA. Kindly reproduced and modified for OFFLU with their permission.



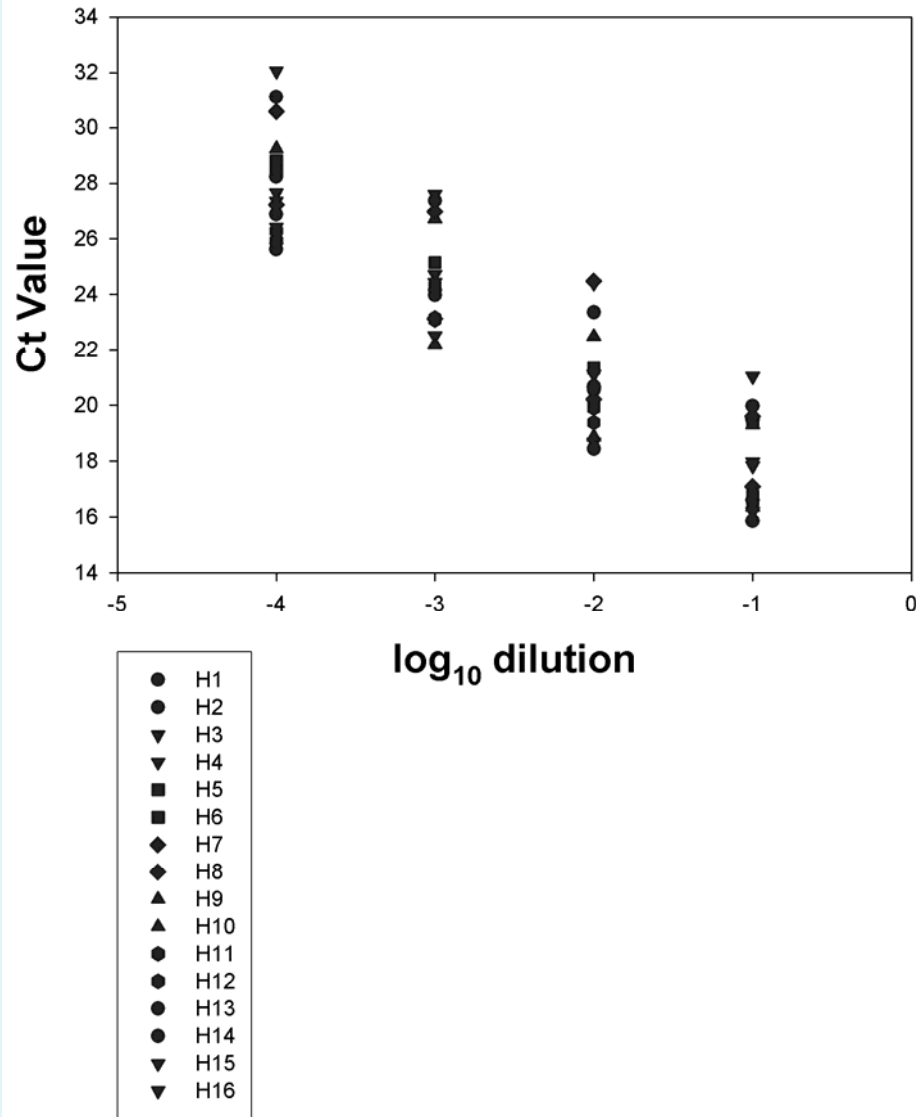
1. For sample guidance http://www.oie.int/eng/normes/mmanual/2008/pdf/2.08.08_SWINE_INFLUENZA.pdf
 2. Adapted Matrix PCR protocols will increase test sensitivity for novel A/H1N1 in pigs; for guidance please contact an OFFLU listed Reference Laboratory <http://offlu.net/docs/Swine%20Influenza.doc#e1d8>
 3. If the test results for any sample cross the threshold, they are considered positive.
 4. Currently for North America there is a novel A/H1N1 specific N1 PCR, for areas where Eurasian strains of H1N1 are circulating this N1 differentiation PCR will not differentiate novel A/H1N1 from those Eurasian strains and other specific tests will be required as they are developed. Otherwise virus isolation and sequencing will be necessary for further characterisation. **If in doubt please contact your Reference Laboratory.**
 5. Contact one of the OFFLU listed Reference Laboratories and provide the following information a) Test results, b) Laboratory internal referral number and c) Courier tracking number.
- Sample shipment:** Samples should be refrigerated and shipped on ice packs and shipped in accordance with OIE guidelines for sample shipment. http://www.oie.int/eng/normes/mmanual/2008/pdf/1.1.01_COLLECTION.pdf
<http://www.fao.org/ag/againfo/programmes/en/empres/AH1N1/docs/SampleShipmentGuidanceSwine.pdf>
6. A subset of these samples may be tested for further characterisation depending on the type of surveillance strategy being implemented by your National Veterinary Services. If no PCR differentiation test is available virus isolation and sequencing will be required.
 7. **Isolate storage and shipment:** Isolates should be frozen at -70C or colder. You may consider sending these samples to a Reference Laboratory.

Note: If you have any queries please contact one of the listed OFFLU Reference Laboratories or the OFFLU Secretariat (offlu@oie.int)

Spackman M gene RRT-PCR Modifications

Primer	Nucleotide Sequence
Spackman M +25	5'-AGA TGA GTC TTC TAA CCG AGG TCG-3'
CFIA Degenerate M +25	5'-AGA TGA GTC YTC TAA CCG AGG TCG-3'
Spackman M -124	5'-TGC AAA AAC ATC TTC AAG TCT CTG-3'
SEPRL modified Spackman M -124	5'-TGC AAA GAC ACT TTC CAG TCT CTG-3'
CFIA Degenerate M -124	5'-TGC AAA RAC AYY TTC MAG TCT CTG-3'

Evaluation of Degenerate Matrix Primers on Avian Influenza Virus Subtypes H1-H16



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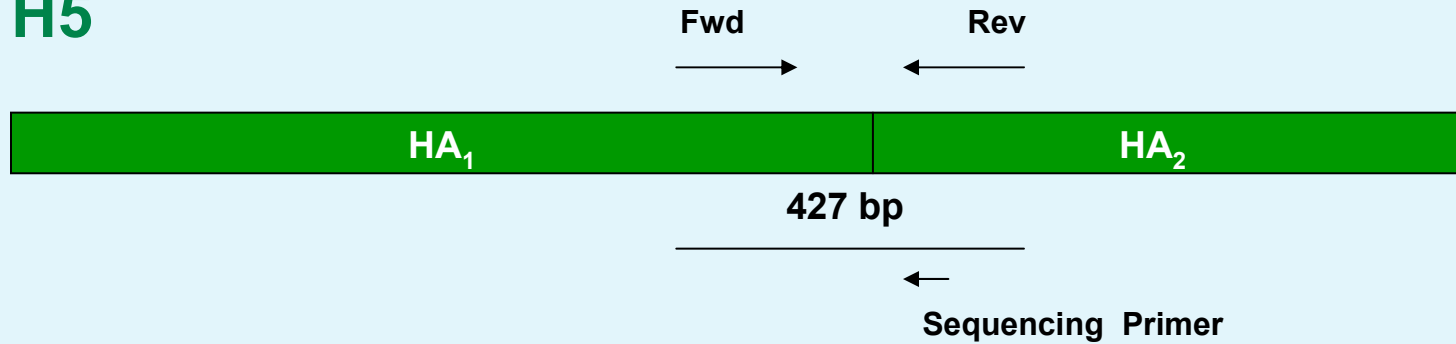
Novel PCR-linked detection methods

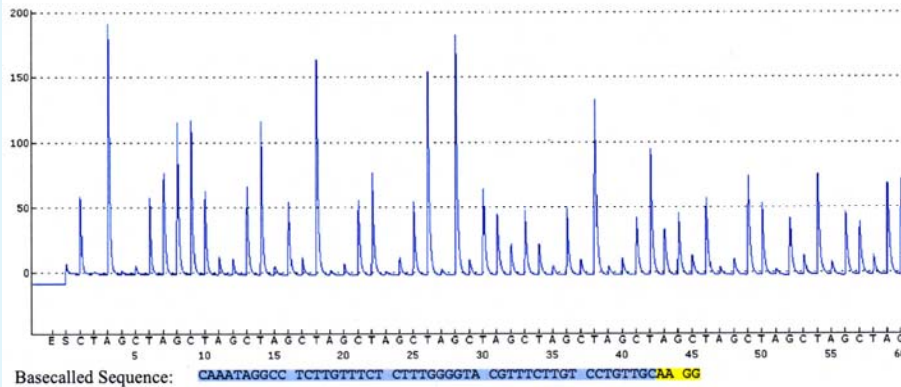


Proficiency testing

PCR-Pyrosequencing Coupled Assays

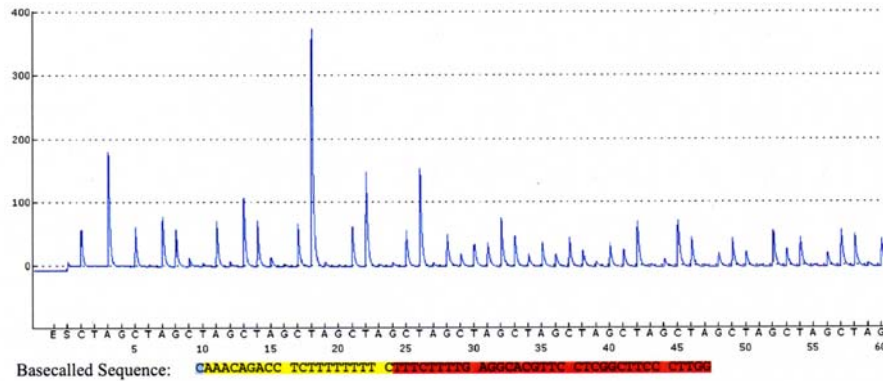
H5





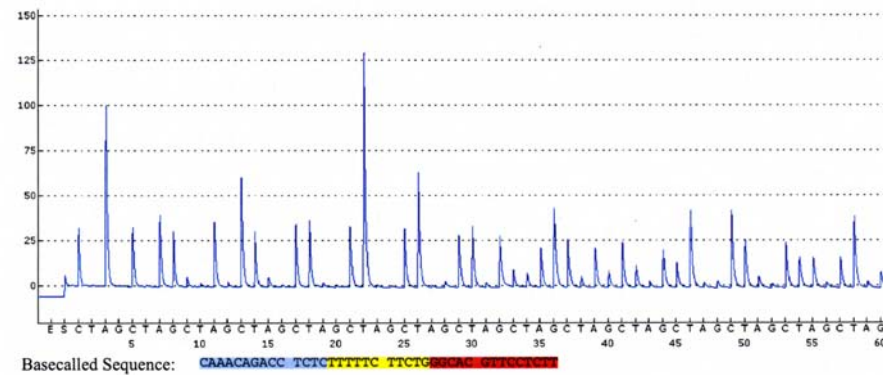
**A/Tk/BC/FAV-8/05 (H5N2)
LPAI**

RTRNVPQRETR/GLF



**A/Tk/ON/7732/66 (H5N9)
HPAI**

PQRKKKR/GLF

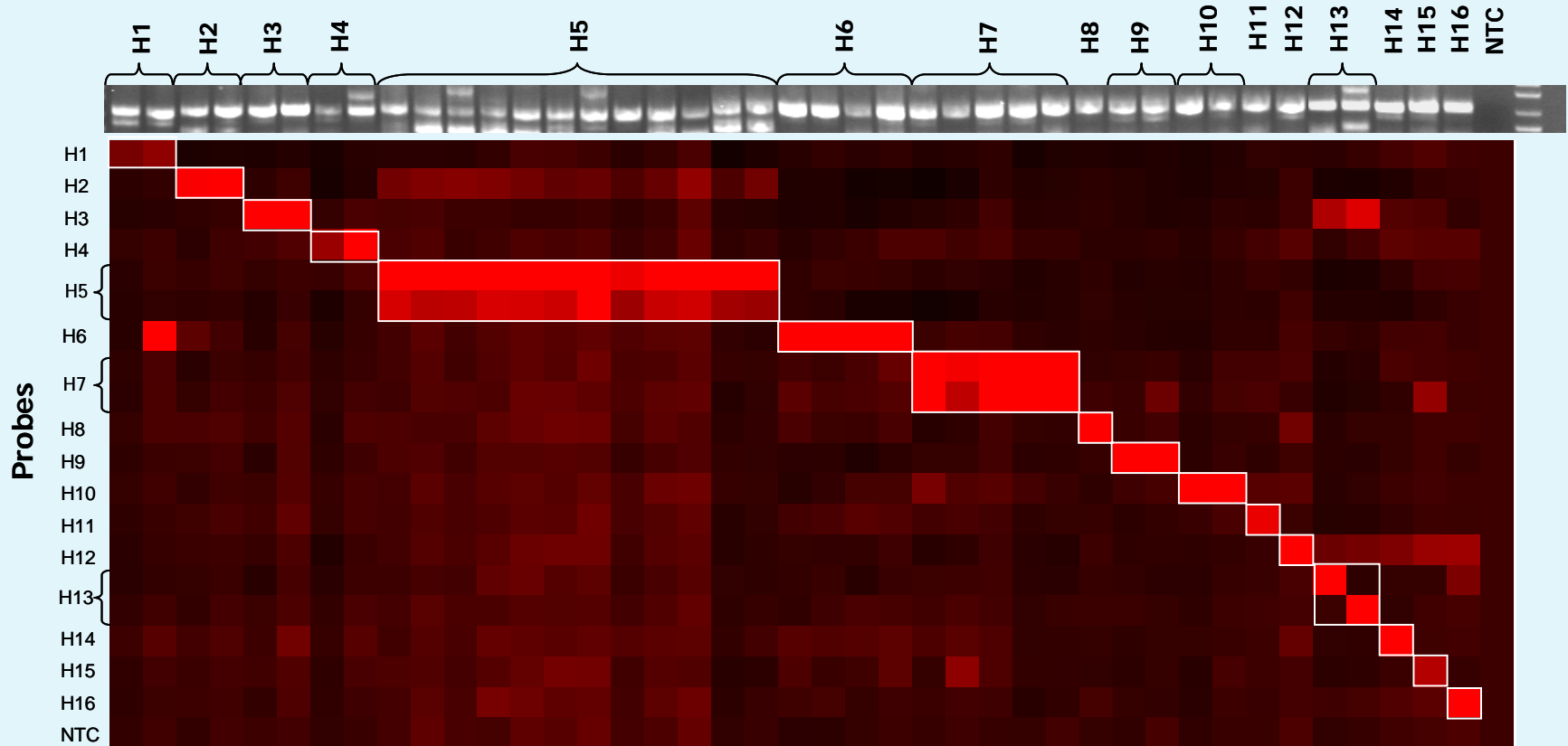


**A/Ck/PA/1370/83 (H5N2)
HPAI**

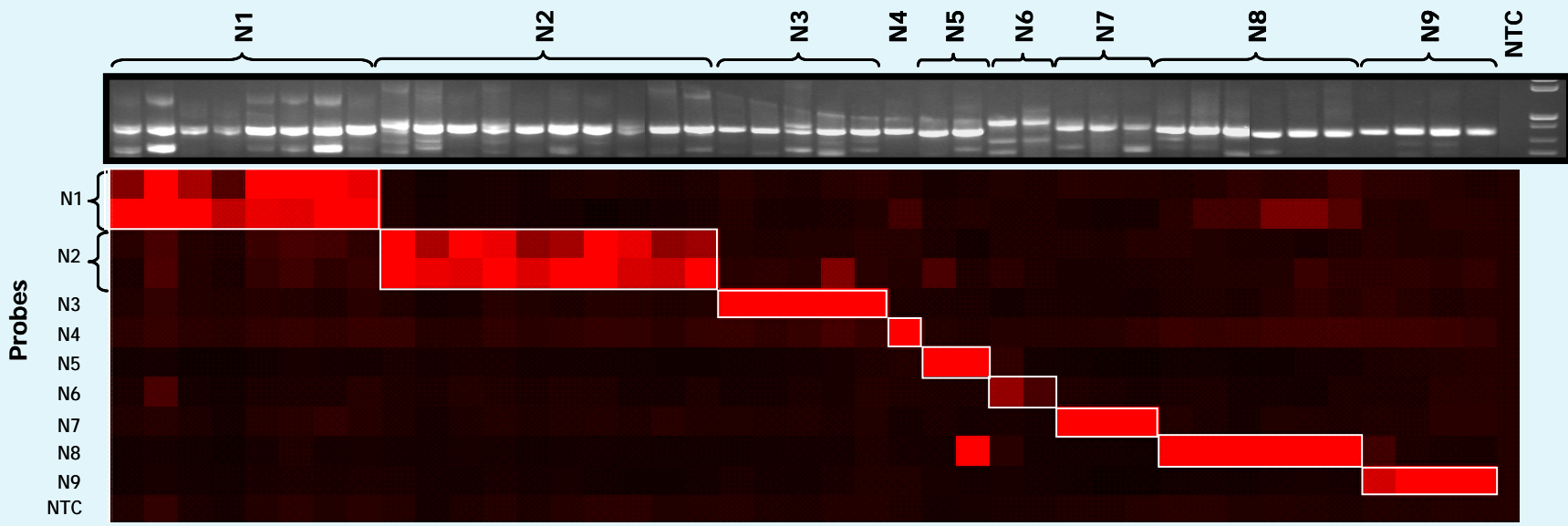
PQKKKR/GLF

Microarray for Detection of AIV

- **Electronic microarray to detect and subtype all 16 HAs and 9 NAs**
 - North American bias
 - Not validated against HPAI H5N1 (in progress)
- **Simultaneous detection of multiple HA/NA subtypes in cases of mixed infections**



Analyzed data depicted as a ratio of each sample to a negative RT-PCR sample (P:N ratio), samples considered positive if P:N ratio ≥ 2.0



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Proficiency testing

2011 EU AI PCR Proficiency Panel

- **A/England/195/09 (H1N1p)**
- NDV B1 vaccine strain
- **A/Goose/Czech Rep/1848/09 (H7N9) LPAI**
- **A/Swan/England/08 (H5N1)**
- NDV AV2388/08
- NDV AV1294/07
- **A/Duck/Romania/107-144/09 (H5N3) LPAI**
- **A/Chicken/Spain/6279/09 (H7N7) HPAI**
- NDV AV470/09
- NDV AV776/10
- **A/Buzzard/Bulgaria/15325/10 (H5N1) HPAI**
- **A/Teal/N. Ireland/07 (H9N1)**
- NDV AV234/09
- **A/Turkey/England/09 (H6N1)**
- **A/Duck/Finland/Li9201/10 (H7N3) LPAI**

2011 EU AI PCR Proficiency Panel

- **Generic AI – Positive / Negative**
- **H5, H7 & pandemic H1N1**
- **Pathogenicity of H5 and H7 viruses**
- **N1 detection for HPAI H5N1**

2011 EU AI PCR Proficiency Panel

- 45 laboratories participated
- 10/45 scored perfect results
- 43/45 with perfect generic PCR result correct
- 6/45 with a single mistake or an omission – LPAI versus HPAI, H1N1v
- 3/45 with false positive results
- Issues detecting Romanian (H5N3) virus 29/45 (unable to type/pathotype)

2011 OFFLU proficiency panel testing

- **Goal:** “to standardize diagnostic testing for AIV through participation in a world-wide proficiency testing system for international reference laboratories”
- **Reference labs must participate 1/year**
- **Blind panel 10 specimens**
 - H5 & H7 from NA, H5, H7 & H10 from EU, APMV-1, non-H5/H7 AIV
 - Clinical and low titre specimens
- **Details:**
 - Identify type A influenza, Subtype H5/H7; pathotype
 - Results compiled as +/- and Ct values, low/high pathogenicity
 - Labs may use current PCR platform
- **Secondary goal - to determine if NA primers/probes can detect EU AI and *vice versa***



OIE/FAO Network of Expertise on Animal Influenza

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On behalf of all contributors:

Thank you for your attention!

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offlu@oie.int