Rapid and Accuracy Diagnosis of Highly Pathogenic Avian Influenza (H5N8) Virus used for the Control of the Outbreak in the Republic of Korea

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Animal and Plant Quarantine Agency
Republic of Korea
Organization of QIA

QIA (Animal and Plant Quarantine Agency)

General Service Division

Planning & Coordination Division

Emergency Preparedness and Response Center

Dep. Animal Disease Control & Quarantine

- Animal Disease Control
- Animal Quarantine
- Veterinary Epidemiology
- Animal Disease Diagnostic
- Import Risk Assessment
- Animal Protection & Welfare
- Veterinary Pharmaceutical
- Veterinary Drugs & Biologics

Dep. Plant Quarantine

- Plant Quarantine
- Export Management
- Risk Management
- Plant Pest Control

Dep. Animal & Plant Health Research

- Research Planning
- Bacterial Disease
- Foot and Mouth Disease
- Viral Disease
- Avian Disease
- Foreign Animal Disease
- Plant Quarantine Tech.

- 3 Department, 22 Division,
- 6 Regional Offices

Incheon Regional Office
Yeongnam Regional Office
Jungbu Regional Office
Seoul Regional Office
Honam Regional Office
Jeju Regional Office
Control Measures for HPAI

- **Stamping-out policy (culling)**
  - Prompt depopulation: infected farm, farms within 500m radius
  - Pre-emptive culling: within 3 km radius, dangerous contact

- **Zoning and movement restriction**
  - 500 m radius: contaminated zone
  - 500m – 3 km: protection zone
  - 3 km – 10km: surveillance zone

- **Vaccination prohibited**

- **Movement restriction were lifted 30 days after the last culling**
H5 HPAI Epidemics in Korea

- 03/04 H5N1
  - 17 farms
    - 8 Dk farms
    - 9 Ck farms

- 06/07 H5N1
  - 7 farms
    - 2 Dk farms
    - 4 Ck farms
    - 1 Qa farm

- 2008 H5N1
  - 33 farms
    - 6 Dk farms
    - 21 Ck farms
    - 6 Dk/Ck mixed

- 10/11 H5N1
  - 53 farms
    - 33 Dk farms
    - 18 Ck farms
    - 1 Qa, 1 Ph

- 2014 H5N8
  - 31 farms
    - 19 Dk farms
    - 11 Ck farms
    - 1 Gs
Outbreak of H5N8 HPAI in Korea

Poultry
- Date: 2014. 1. 16
- Region: Gochang in Jeonbuk(JB) province
- Farm: breeder duck farm
  - 20-53 week-old breeder ducks
  - 16,000 birds
- Reporting: farmer → regional veterinary office → QIA
- Clinical signs:
  - Decrease of egg production (above 50%)
  - Increase of death (1-2 bird → 7 birds)
  - Depression

Wild birds
- Date: 2014. 1. 17
- Region: Donglim reservoir near the index case farm
- Reporting: QIA team for epidemiological investigation
- hundreds of dead Baikal Teal in Donglim reservoir

Data: Epidemiological Division of QIA, 2014
## Isolation of H5N8 Viruses in Poultry & Wild Birds

- **H5N8 outbreaks:** 31 confirmed cases/37 suspect cases in poultry
- **H5N8 virus:** 278 viruses

<table>
<thead>
<tr>
<th></th>
<th>No. of cases</th>
<th>No. of virus isolation</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry</td>
<td>927</td>
<td>231</td>
<td>696</td>
</tr>
<tr>
<td>Wild bird</td>
<td>595</td>
<td>38</td>
<td>557</td>
</tr>
<tr>
<td>Environment</td>
<td>192</td>
<td>9</td>
<td>183</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1,714</strong></td>
<td><strong>278</strong></td>
<td><strong>1,436</strong></td>
</tr>
</tbody>
</table>

*170,153 samples  2014. 10. 12.*

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### Weeks number, 2014

- **Poultry**
- **Wild Birds**

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![Map of South Korea with regions colored to indicate H5N8 outbreaks](image)
Affected species in poultry and wild birds

- H5N8 viruses from poultry (231 cases)
  - Duck (174): 75%
  - Chicken (48): 21%
  - Others (9): 4%

- H5N8 viruses from wild birds (38 cases)
  - Dead bird (20)
  - Captured live bird (9): Mallard(3), spot-billed duck(2), common teal(4)
  - Feces (9)

2014.10.12
Avian Influenza Viruses in Eastern China during 2009-2011

- **H5N2**: A/duck/Eastern China/1111/2011
- **H5N8**: A/duck/Jiangsu/k1203/2010
- **H11N9**: A/environment/Jiangxi/28/2009

**Group A**
- Wild birds: 27 cases
- Poultry: 18 cases

**Group B**
- Wild birds: 1 case
- Poultry: 1 case

- Layer Chicken farm, Kumamoto prefecture in Japan (4.13)
- Duck in LBM, Zhejiang of China 2013

*EID online (5.6)*

HPAI H5N8 virus of Korea, 2014

- Total 47 viruses were analyzed in all 8 segments
Procedures for HPAI Suspected Cases

Farmer
- Recognize clinical signs
- Report to Provincial Veterinary Service

Provincial Veterinary Service
- History taking and sampling
- Screening test using antigen detection kit (Type A)
- Transportation samples to QIA

QIA
- Direct RNA detection by RT-PCR, real-time RT-PCR
- Virus isolation by egg inoculation (HA, RT-PCR & Sequencing)
- Assessment of pathogenicity
- ELISA, HI test
The Application of Antigen Detection Kit

- It was developed in 2004 during first H5N1 HPAI outbreak
- Detection Limit: 0.06-0.13 HA unit, $10^{3.9-4.2}$ EID$_{50}$/0.1ml
## The Application of Antigen Detection Kit

<table>
<thead>
<tr>
<th>Suspected cases</th>
<th>Species</th>
<th>Virus isolation</th>
<th>Type A</th>
<th>Suspected cases</th>
<th>Species</th>
<th>Virus isolation</th>
<th>Type A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>Duck</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>16th</td>
<td>Chicken</td>
<td>+</td>
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<tr>
<td>2nd</td>
<td>Duck</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>17th</td>
<td>Duck</td>
<td>+</td>
</tr>
<tr>
<td>3rd</td>
<td>Duck</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>18th</td>
<td>Chicken</td>
<td>-</td>
</tr>
<tr>
<td>4th</td>
<td>Duck</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>19th</td>
<td>Duck</td>
<td>+</td>
</tr>
<tr>
<td>5th</td>
<td>Chicken</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>20th</td>
<td>Chicken</td>
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<tr>
<td>6th</td>
<td>Duck</td>
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<td>ND</td>
<td>+</td>
<td>21th</td>
<td>Chicken</td>
<td>+</td>
</tr>
<tr>
<td>7th</td>
<td>Duck</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>22th</td>
<td>Chicken</td>
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<tr>
<td>8th</td>
<td>Duck</td>
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<td>ND</td>
<td>+</td>
<td>23th</td>
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<td>+</td>
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<tr>
<td>9th</td>
<td>Duck</td>
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<td>ND</td>
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<td>24th</td>
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<td>+</td>
</tr>
<tr>
<td>10th</td>
<td>Duck</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>25th</td>
<td>Chicken</td>
<td>+</td>
</tr>
<tr>
<td>11th</td>
<td>Duck</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>26th</td>
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</tr>
<tr>
<td>12th</td>
<td>Chicken</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td>27th</td>
<td>Duck</td>
<td>+</td>
</tr>
<tr>
<td>13th</td>
<td>Duck</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>28th</td>
<td>Chicken</td>
<td>+</td>
</tr>
<tr>
<td>14th</td>
<td>Chicken</td>
<td>-</td>
<td>ND</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15th</td>
<td>Chicken</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**ND:** Not done or no information from regional Veterinary Service

- **Field:** Samples were oropharyngeal and cloacal swab at the farm
- **Lab:** Samples were tissues of dead birds

Total: 23/28, 8/9, 23/28
Laboratory Test for AIV Diagnosis

**Direct RNA Detection**
- Standard RT-PCR & Sequencing analysis
- Real time RT-PCR

**Virus Isolation & Identification**
- Egg Inoculation, HA
- RT-PCR & Sequencing Analysis
- Pathogenicity test in Animal

**Serological Test**
- AGID
- C-ELISA
- HI test
Direct RNA Detection by RT-PCR

- Materials & Methods
  - AIV multi RT-PCR kit (in-House; Intron Co.): premix with specific Primer sets
  - Traditional RT-PCR: H typing and N typing

![RT-PCR Electrophoresis Images](Image)

**H5**
- M, NP gene
- Negative

**N8**
- M, NP and H5 + N8 positive

**Product of H5 cleavage site**

**Pathotyping (sequencing)**

**AIV-**

**H5 or H7 AIV**
Comparison of RT-PCR & real time RT-PCR

<table>
<thead>
<tr>
<th>H5N8 Virus*</th>
<th>Real time RT-PCR**</th>
<th>Traditional RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Virus dilution)</td>
<td>M gene (Ct)</td>
<td>H5</td>
</tr>
<tr>
<td>H5N8 (10^-1)</td>
<td>17.55</td>
<td>20.04</td>
</tr>
<tr>
<td>H5N8 (10^-2)</td>
<td>24.18</td>
<td>27.04</td>
</tr>
<tr>
<td>H5N8 (10^-3)</td>
<td>28.51</td>
<td>32.06</td>
</tr>
<tr>
<td>H5N8 (10^-4)</td>
<td>32.17</td>
<td>N/A</td>
</tr>
<tr>
<td>H5N8 (10^-5)</td>
<td>34.79</td>
<td>N/A</td>
</tr>
<tr>
<td>H5N8 (10^-6)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>H5N8 (10^-7)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* H5N8 virus: A/duck/Korea/Buan2/2014 (H5N8)
** real time RT-PCR: QIA use the same primer and probe set with OIE reference lab(VLA)
## Setting of real time RT-PCR for pathotyping

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence of primers and probes</th>
<th>Nucleotide position</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rH5_960F</td>
<td>GGGGAATGCCCCAAAATATGT</td>
<td>960-979</td>
<td>Hofmann et al.,</td>
</tr>
<tr>
<td>rH5_1195R</td>
<td>TTTTGTCATTGAGTTGACCTTATTTG</td>
<td>1195-1224</td>
<td>QIA</td>
</tr>
<tr>
<td>rH5_Conserve_HEX_1061</td>
<td>HEX-TTGGAGCTATAGCAGTTTTTATAGAGG-BHQ</td>
<td>1061-1083</td>
<td>Hofmann et al.,</td>
</tr>
<tr>
<td><strong>Probe</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rH5_CS_FAM_1029_A*</td>
<td>FAM-AGAGARAGAAGAGGAAGAGGACT-TAMRA</td>
<td>1029-1055</td>
<td>QIA</td>
</tr>
<tr>
<td>rH5_CS_FAM_1029_B**</td>
<td>FAM-AGAGAAAGAAGAAAAAGAGACTTGT-TAMRA</td>
<td>1029-1055</td>
<td>QIA</td>
</tr>
<tr>
<td>rH5_CS_FAM_1029_C***</td>
<td>FAM-AGAGAGAAGAAGAAAGAGAGACTA-TAMRA</td>
<td>1029-1055</td>
<td>Hofmann et al.</td>
</tr>
</tbody>
</table>

*Detection for cleavage site of 2008/2010/2014 (clade 2,3,2,1, clade 2.3.4.6 <Buan2>) H5 HPAI virus of Korea

**Detection for cleavage site of 2014 (clade 2.3.4.6, Gochang1>) H5 HPAI virus of Korea

***Detection for cleavage site of 2003/2006 H5 (clade 2.5, clade 2.2) HPAI virus of Korea
# Efficacy test of real time RT-PCR for pathotyping H5N8 Virus*

<table>
<thead>
<tr>
<th>H5N8 Virus* (Virus dilution)</th>
<th>Real time RT-PCR**</th>
<th>Real time RT-PCR for pathotyping</th>
<th>Traditional RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M gene (Ct)</td>
<td>H5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H5-cleavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>H5</td>
</tr>
<tr>
<td>H5N8 (10^{-1})</td>
<td>17.55</td>
<td>20.04</td>
<td>20.40</td>
</tr>
<tr>
<td>H5N8 (10^{-2})</td>
<td>24.18</td>
<td>27.04</td>
<td>28.94</td>
</tr>
<tr>
<td>H5N8 (10^{-3})</td>
<td>28.51</td>
<td>32.06</td>
<td>32.65</td>
</tr>
<tr>
<td>H5N8 (10^{-4})</td>
<td>32.17</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>H5N8 (10^{-5})</td>
<td>34.79</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>H5N8 (10^{-6})</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>H5N8 (10^{-7})</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* H5N8 virus: A/duck/Korea/Buan2/2014 (H5N8)
** real time RT-PCR: QIA use the same primer and probe set with OIE reference lab(VLA)
Virus Isolation by Egg Inoculation

• **Virus Isolation**
  • **Advantage**
    – Gold Standard
    – Virus available for further characterization
    – Sensitive for avian viruses
  • **Disadvantage**
    – SPF eggs expensive
    – Can be time consuming
    – Safety issues with handling infectious material
Antigenic Subtyping

• **Advantages**
  - Used to identify isolates
  - No non-specific inhibitors

• **Disadvantages**
  - Time consuming
  - Complex
  - Requires experience to interpret
  - Requires well-characterized reagents
Antibody Detection Methods

- **Agar gel immunodiffusion (AGID)**
  - Group-specific test for influenza A virus

- **Enzyme-linked immunoassay (ELISA)**
  - Alternative method to the AGID
  - Competitive ELISA was developed and applied

- **Haemagglutination inhibition test (HI)**
  - H type specific test
  - Antibodies are subtyped as H5 and H7
Antibody detection in poultry & wild birds

- Results
  - Chickens are all negative for H5 Ab
  - Some of ducks and goose are positive for H5 Ab
  - H5 antibody positive rate is unusually high in wild bird of this year

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of tested farm</th>
<th>No. of tested birds</th>
<th>No. of Ab positive farms</th>
<th>No. of H5 Ab positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>294</td>
<td>14,929</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ducks</td>
<td>403</td>
<td>21,519</td>
<td>22</td>
<td>674</td>
</tr>
<tr>
<td>Goose</td>
<td>8</td>
<td>59</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Others (mixed)</td>
<td>22</td>
<td>927</td>
<td>3</td>
<td>55</td>
</tr>
<tr>
<td>Wild birds</td>
<td>-</td>
<td>927</td>
<td>-</td>
<td>107</td>
</tr>
</tbody>
</table>
**Diagnostic Procedures**

**Clinical signs and Gross Lesions**
- Sample: Tissue of dead bird, OP·CL swab, serum
- Clinical signs: egg production ↓, depression, death
- Gross lesions: hemorrhage in ovary, necrosis in pancreas

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**Virus isolation**
- Virus isolation by egg inoculation
  - Egg inoculation
  - HA test
- RT-PCR
- Sero-typing

**Gene detection**
- Direct RT-PCR & real time RT-PCR
- Sequence analysis
Acknowledgements

Emergency Preparedness and Response Center
Department of Animal Disease Control & Quarantine
  - Animal Disease Control Division
  - Veterinary Epidemiology Division
Department of Animal & Plant Health Research
  - Avian Disease Division

Regional Office for Animal Disease Control

Veterinary School