Characterization and Pathotyping of AI V and APMV-1

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Identification of AIV

Type A Influenza can be identified by:
- AGID
- rRT-PCR – Matrix assay
- Antigen Detection assays

Influenza virus can be subtyped by:
- HI - 16 hemagglutinin subtypes
- NI – 9 neuraminidase subtypes
- Conventional RT-PCR or rRT-PCR N1 assay
- rRT-PCR – H5 and H7 assays
Avian Influenza
Factors Influencing Pathogenicity

- Polygenic
- Associated with H5 and H7 subtypes
- HA glycoprotein plays a dominate role
- Presence of multiple basic amino acids at the cleavage site of the HA glycoprotein
- Pathogenic strains evolve from nonpathogenic lineages
Definition of Highly Pathogenic Notifiable AIV (HPNAI)

1. Any AIV that is lethal for 6-8 of eight 4-8 wk. old susceptible chickens within 10 days - Intravenous lethality test

2. Any AIV that has a IVPI in 4-8 wk. old chickens of \(\geq 1.2\)

3. Any H5 or H7 subtype virus that does not meet the criteria in #1 & 2, but has an amino acid motif at the cleavage site of the hemagglutinin gene that is compatible with other HPAI viruses.
Low Pathogenic Notifiable AI (LPNAI)

- All influenza A viruses of H5 and H7 subtype that are not HPNAI viruses
Notifiable AI V (NAI)

- **HPNAI** - AI V or viral RNA detected in poultry or a poultry product
- **LPNAI** - AI V or viral RNA of the H5 or H7 subtypes detected in poultry or a poultry product
- **Antibodies to H5 or H7** that are not a consequence of vaccination, nor indicative of a non-specific reaction
Highly Pathogenic Avian Influenza

1. Any influenza that kills 6, 7, or 8 of 8 chickens

2. Any influenza virus that is not an H5 or H7 that kills 1 to 5 chickens and grows in cell culture in the absence of trypsin

3. Any H5 or H7 subtype that does not meet the criteria in item, but has an amino acid sequence at the cleavage site of the hemagglutinin that is compatible with other HPAI viruses

Nucleotide and amino acid sequencing of the HA gene

Phylogenetic analysis
Intravenous Pathogenicity Index (IVPI)
Intravenous Pathogenicity Index (IVPI)

- SPF or AIV antibody free commercial chickens

- AIV isolate free of bacterial contamination is diluted 1:10 in sterile diluent (TBTB, PBS)

- Ten 4-8 wk. old chickens are inoculated intravenously with 0.2 ml of the diluted isolate

- Number of normal, sick, morbid and dead chickens are recorded daily for 10 days
## Intravenous Pathogenicity Index (IVPI)

<table>
<thead>
<tr>
<th>State of Chickens</th>
<th>Day</th>
<th>Total</th>
<th>Weight</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sick</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Moribund</td>
<td>0</td>
<td>8</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Dead</td>
<td>0</td>
<td>82</td>
<td>3</td>
<td>246</td>
</tr>
</tbody>
</table>

### Total Recordings

Total Recordings: 100

\[
IVPI = \frac{262}{100} = 2.62
\]
Characterization of HPAI by IVPI

- Any isolate with an IVPI > 1.2 is pathotyped as HPAI

- Any isolate with an IVPI < 1.2 is pathotyped as LPAI
Identification and Pathotyping of Newcastle Disease Virus
Identification of APMV-1

- HI with APMV-1 monospecific antiserum
  - Cross neutralization with other PMV

- rRT-PCR
  - Matrix assay identifies all APMV-1
  - vNDV assay identifies virulent NDV & PPMV-1

- HI with monoclonal antisera
  - Identification of Pigeon paramyxovirus
Definition of Newcastle disease (ND) as defined by OIE

An infection in birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets the following criteria

- ICPI in day-old chicks of 0.7 or greater

- Multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 (NH2 terminus of F1)
Pathotyping Assays for ND

- **Intracerebral Pathogenicity Index (ICPI)**
  - Differentiates vNDV from avirulent APMV-1
  - ICPI for PPMV-1 are quite variable

- **Intracloacal Inoculation Pathogenicity Test**
  - Differentiates clinicopathological forms (VVNDV, NVNDV, mesogenic)

- **Sequence Analysis**
  - Nucleotide and amino acid sequencing of the F gene cleavage site
  - Phylogenetic Analysis
## Monoclonal Antibody typing (HI) of Newcastle Disease Virus (NDV) Isolates

<table>
<thead>
<tr>
<th>Pathotype</th>
<th>B79</th>
<th>10D11C</th>
<th>15C4</th>
<th>AVS-1</th>
<th>161</th>
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</thead>
<tbody>
<tr>
<td>VV-NDV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NV-NDV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M-NDV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-NDV</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pigeon-NDV</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Intracerebral Pathogenicity Index (ICPI)

- Determined by inoculating 0.05 ml of a 1:10 dilution of infective, bacterial-free AAF in sterile isotonic saline (w/o antibiotics) in the brains of 10 one-day-old SPF chicks.

- Chicks are observed daily for 8 days and the number of normal, sick and dead chicks are recorded.

- ICPI is calculated by dividing the weighted mean by the number of observations.
## Intracerebral Pathogenicity Index (ICPI)

<table>
<thead>
<tr>
<th>State of chicks after inoculation</th>
<th>Day</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Total</th>
<th>Score</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Normal</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Sick</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dead</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Total recordings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>80</td>
<td>3</td>
<td>57</td>
</tr>
</tbody>
</table>

\[
\text{ICPI} = \frac{\text{Weighted Mean}}{\text{Number of Observations}} = \frac{3 + 57}{80} = 0.75
\]
Intracloacal Inoculation Pathogenicity Test

- Determines virulence and tropism differentiating WNDV from NVNDV
- The cloaca of four 6-to-8-week-old SPF chickens are swabed with a 1:10 dilution of infective allantoic fluid
- Birds are observed for 10 days
- All dead birds are necropsied and the lesions are scored
Scoring of Lesions for the Intracloacal Inoculation Pathogenicity Test

- **+4** = edema of head and neck, hemorrhage in trachea, hemorrhage and necrosis/ulceration throughout the gastrointestinal tracts
- **+3** - **+1** = lesions in respiratory and intestinal tracts but in decreasing severity
- Viruses are viscerotropic (VVNDV) if one bird shows **+4** lesions or at least two birds show **+2** or **+3** lesions
- NVNDV – virus causes death in birds which display neurological signs
Pathotyping AI V and APMV-1 by Nucleotide Sequencing
Nucleotide Sequencing & Analysis of AI HA Gene

- Sequence analysis is conducted on all H5 and H7 viruses
- Single tube one step RT-PCR for H5
- Conventional 2 step RT-PCR for H5 & H7
  - Specific H5 and H7 PCR primers are selected for amplification of cDNA
  - Eurasian isolate
  - North American isolate
Nucleotide Sequencing & Analysis of AI NA Gene

- Conventional RT-PCR for the detection of N1
  - WHO assay
  - 6.5 hrs. needed for results

- rRT-PCR for the detection of N1
  - WVDL assay
  - 4 hrs needed for results
Nucleotide Sequencing & Analysis of F Gene for ND

- Sequence analysis is conducted on the F gene

- PCR is conducted to amplify a portion of the F gene including the cleavage site
  - 254 bp amplicon for analysis of cleavage site
  - 1000 bp amplicon for phylogenetic analysis (includes portions of the matrix and fusion genes)
<table>
<thead>
<tr>
<th>Position</th>
<th>113</th>
<th>114</th>
<th>115</th>
<th>116</th>
<th>117</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avirulent</td>
<td>R/K</td>
<td>Q</td>
<td>G</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Virulent</td>
<td>R/K</td>
<td>Q</td>
<td>R/K</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>
Automated Sequencing

- Sanger sequencing with fluorescently labeled DNA fragments is the most frequently used technique.
- Tagged ddNTP’s are detected during electrophoresis with a four-color dye system.
- Sequence is automatically recorded to chromatograph which is interpreted by computer software into DNA sequence (ABI 3730 DNA analyzer).
Sequencing PCR Amplicons

Chromatograph from automated DNA sequencer of NDV F nucleotide sequence
Chromatograph Analysis

- The height of each of the 4 colored lines are indicative of fluorescence that corresponds to each of the 4 labeled dideoxynucleotides.

- Good sequence data
  - Well defined peaks
  - Peaks easily distinguished from background noise

- Poor sequence data
  - Poorly defined peaks
  - Low signal-to-noise resulting in ambiguities
Peaks are well defined and distinguishable from background noise.

Lower signal to noise results in occasional ambiguities.

Peaks are less well defined and ambiguities increased.

Unreliable sequencing.
Sequence Analysis

- Chromatograph is edited for quality of sequence and analyzed by commercial sequence analysis software.
- The nucleotide sequence is translated and the amino acid sequence at the cleavage site is identified.
- Multiple basic amino acids?
  - Fusion gene – NDV
  - Hemagglutinin gene - AIV
The amino acid sequence at the fusion gene cleavage site of avirulent APMV-1
SGGGKQGR / LI GAI

Basic amino acids
Arginine (R) and Lysine (K)
# Amino Acid Sequences at the Cleavage Site of the Fusion Protein

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pathotype</th>
<th>AA Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LaSota</td>
<td>L</td>
<td>SGGGGRQRGR/LIGAI</td>
</tr>
<tr>
<td>Roakin</td>
<td>M</td>
<td>SGGRQRKR/FIGAI</td>
</tr>
<tr>
<td>GB Texas</td>
<td>NV</td>
<td>SGGRQRKRFVGAI</td>
</tr>
<tr>
<td>CK/CA/03</td>
<td>VV</td>
<td>SGGRQRKRFVGAI</td>
</tr>
<tr>
<td>PG/NY/01</td>
<td>PPMV-1</td>
<td>SGVRRRKFRFIGAI</td>
</tr>
<tr>
<td>PG/NC/01</td>
<td>PPMV-1</td>
<td>SGERQRKRFFIGAI</td>
</tr>
</tbody>
</table>
The amino acid at the fusion gene cleavage site of PPMV-1
## Amino Acid Sequence at the Fusion Gene Cleavage Site

<table>
<thead>
<tr>
<th>Isolate</th>
<th>F2</th>
<th>F1/117</th>
<th>Pathotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX GB/48</td>
<td>Gly Gly</td>
<td>Arg Arg Gln Lys Arg</td>
<td>Phe Iso Gly</td>
</tr>
<tr>
<td>Pigeon/Italy/00</td>
<td>Gly Gly</td>
<td>Arg Arg Gln Lys Arg</td>
<td>Phe Iso Gly</td>
</tr>
<tr>
<td>Turkey/ND/92</td>
<td>Arg Gly</td>
<td>Arg Arg Gln Lys Arg</td>
<td>Phe Val Gly</td>
</tr>
<tr>
<td>Mexico/1/00</td>
<td>Gly Gly</td>
<td>Arg Arg Gln Lys Arg</td>
<td>Phe Val Gly</td>
</tr>
<tr>
<td>TX/248306/03</td>
<td>Gly Gly</td>
<td>Arg Arg Gln Lys Arg</td>
<td>Phe Val Gly</td>
</tr>
<tr>
<td>CA/211472/02</td>
<td>Gly Gly</td>
<td>Arg Arg Gln Lys Arg</td>
<td>Phe Val Gly</td>
</tr>
<tr>
<td>Dove/CA/9547/03</td>
<td>Gly Gly</td>
<td>Arg Arg Gln Arg Arg</td>
<td>Phe Val Gly</td>
</tr>
<tr>
<td>Chick/CA/634/03</td>
<td>Gly Gly</td>
<td>Arg Arg Gln Arg Arg</td>
<td>Phe Val Gly</td>
</tr>
</tbody>
</table>
Influenza A Virus Hemagglutinin

Theoretical minimum sequence for high pathogenicity

HA1 / HA2

-4 -3 -2 -1 +1

BASIC – ANY – BASIC – ARG / GLY

N-terminus of HA2

C-terminus of HA1

Basic amino acids

Arginine (R) and Lysine (K)
## Amino Acid Sequences at the Cleavage Site of the Hemagglutinin for Selected H7 Viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virulent</th>
<th>Amino Acid Sequence</th>
<th>HA1/Ha2</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPV/34</td>
<td>+</td>
<td>Pro Glu Pro Ser Lys Lys Arg Glu Lys / Gly</td>
<td></td>
</tr>
<tr>
<td>Avirulent H7</td>
<td>-</td>
<td>Pro Glu Asn Pro – – – Lys Thr Arg / Gly</td>
<td></td>
</tr>
<tr>
<td>A/P.R./CA/94</td>
<td>-</td>
<td>Pro Glu Ile Pro – – Lys Arg Arg Arg / Gly</td>
<td></td>
</tr>
<tr>
<td>A/Ck/NY/95</td>
<td>-</td>
<td>Pro Glu Asn Pro – – – Lys Thr Arg / Gly</td>
<td></td>
</tr>
<tr>
<td>A/Ck/NJ/95</td>
<td>-</td>
<td>Pro Glu Asn Pro – – – Lys Pro Arg / Gly</td>
<td></td>
</tr>
<tr>
<td>A/Emu/TX/95</td>
<td>-</td>
<td>Pro Glu Asn Pro – – – Lys Thr Arg / Gly</td>
<td></td>
</tr>
<tr>
<td>A/Tk/UT/95</td>
<td>-</td>
<td>Pro Glu Asn Pro – – – Lys Thr Arg / Gly</td>
<td></td>
</tr>
</tbody>
</table>

Minimum sequence for HPAI = B Any B Arg / Gly
Pathogenic Strains Evolve from Nonpathogenic Lineages

**LBM H7N2 LPAI: Cause for Concern**

<table>
<thead>
<tr>
<th>Year Range</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1994 - 1995</td>
<td>PENPKTR/GLF</td>
</tr>
<tr>
<td>1995 - 1998</td>
<td>PENPKPR/GLF</td>
</tr>
<tr>
<td>1999 - 2001</td>
<td>PEPKPPR/GLF</td>
</tr>
<tr>
<td>2002</td>
<td>PEPKKR/GLF</td>
</tr>
<tr>
<td>2003 (33)</td>
<td>PEPKPPR/GLF</td>
</tr>
<tr>
<td>2004 (12)</td>
<td>PEKKKR/GLF</td>
</tr>
<tr>
<td>2004 (178)</td>
<td>PEKPPR/GLF</td>
</tr>
<tr>
<td>2004 (3)</td>
<td>PERPKR/GLF</td>
</tr>
</tbody>
</table>

Minimum for HPAI = B X B R / G
### Connecting Peptide Sequence of Selected H5 Viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virulence</th>
<th>Amino Acid Sequence</th>
<th>HA1/ HA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK/Penn/83</td>
<td>Virulent</td>
<td>Pro Gln Lys Lys Lys</td>
<td>Arg/Gly</td>
</tr>
<tr>
<td>Ck/Scott/59</td>
<td>Virulent</td>
<td>Pro Gln Arg Lys Lys</td>
<td>Arg/Gly</td>
</tr>
<tr>
<td>Dk/Penn/84</td>
<td>Avirulent</td>
<td>Pro Gln Arg Glu Thr</td>
<td>Arg/Gly</td>
</tr>
<tr>
<td>Ck/Penn/93</td>
<td>Avirulent</td>
<td>Pro Gln Arg Lys Thr</td>
<td>Arg/Gly</td>
</tr>
</tbody>
</table>

**HA1 / HA2**

-4 -3 -2 -1 +1

BASIC – ANY – BASIC – ARG / GLY

**Amino Acid Sequence**

- **ACA**: G A
- **Arg**: Lys
Phylogenetic Analysis

Phylogenetic analysis is the study of evolutionary relationships. Objective: to determine which data (sequences) are most closely related – how this family of viruses have evolved.

- **Sequence phylogenetics** – 2 sequences are the same at 95% of the nucleotide positions.
- **Protein phylogenetics** – 2 proteins are the same at 95% of the amino acid positions.
Phylogenetic Analysis Cont.

- 2 sequences that are highly related (high % homology) will be located as neighboring branches – joined to a common branch.

- Analysis can be conducted with sequence (taxa) from a conserved gene (matrix) or non-conserved (HA/F).

- An important tool for epidemiology:
  - From what virus did the virus of interest most likely evolve?
  - Is the virus of interest a new introduction of the disease, or an expansion of a previously introduced virus?
Phylogenetic Analysis Of California 2002/03 v-NDV with South and Central American NDVs
Phylogenetic Analysis of Ck/DE/04 H7N2 and other U.S. H7 Viruses

Hemagglutinin Gene Phylogeny

Phylogenetic tree courtesy D. Suarez, SEPRL
California 2002/03
V-NDV Fusion
Gene Phylogeny
H5 tree for Parrot/CA/04

Mexican-lineage
H5N2 viruses

Eurasian H5

10 changes
Matrix Gene Phylogeny

10 changes

Pigeon-origin NDV Isolates

Chicken/U.S./BeaudetteC/52
Chicken/U.S./Roakin/48
Chicken/U.S.(TX)/GB/48
Chicken/U.S./B1./48
Turkey/U.S./VGGA/89
Chicken/U.S./LaSota/46
Dove/Italy/2736/00
Duck/Japan/D26/78
Chicken/Australia/QV4/66
Chicken/NorthernIreland/Ulster/64
Chicken/Australia/AV/32
Chicken/Italy/Milano/45
Chicken/Mexico/37821/96
Chicken/Honduras/H15/00
Pigeon/U.S.(CA)/5658/75
Psittacine/U.S(FL)/Largo/71
Anhinga/U.S.(FL)/44083/93
Turkey/U.S.(ND)/430084/92
Chicken/Argentina/TL/71
Cockatoo/Indonesia?/14698/90
Parakeet/Tanzania?/28710/93
Chicken/3286/Italy/00
Pigeon/U.S.(GA)/21042/98
Pigeon/U.S.(TX)/17498/98
Pigeon/Italy/1166/00
Pigeon/U.S.(NY)/84
Pigeon/U.S.(MD)/3981/84
Pigeon/Argentina/Capital/97
Pigeon/Argentina/Tigre/99
Chicken/Korea/12a/89
Chicken/U.S./CA1083(Fontana)/71
Alignment and BLAST Search Data

- Process of comparing a new sequence with all other known sequences and determining homology (similarity)
- Powerful tool to quickly determine relatedness
- BLAST search should be conducted using a comprehensive and up-to-date repository
  - National Center for Biotechnology Information (NCBI)
  - GeneBank
- Alignment is conducted using selected genomic sequence
  - Hemagglutinin – variable gene related to pathogenicity
  - Matrix – more conserved gene, not used for pathogenicity analysis
Alignment and BLAST Search Analysis

- Comparing a new sequence with all other known sequences
- Can differentiate genotypes
  - LBM H7N2 viruses have a 24 nt deletion in the hemagglutinin gene
Nucleotide sequence alignment of AI H7 viruses
24 nt deletion differentiates the two H7 genotypes
Nucleotide sequence alignment of two avirulent APMV-1 isolates

Amino acid motif at cleavage site – SGGRQGR / GLFGAI
and SGGKQGR / GLFGAI
Thank You For Your Attention!

NVSL, Ames, Iowa
OIE, FAO Reference Laboratory:
Avian Influenza
Newcastle disease