Avian influenza (AI) is considered an exotic disease in commercial poultry in the U.S. There have been few outbreaks of avian influenza in commercial poultry in the U.S. but they are always controlled very rapidly. It is important to mention that the H5N1 strain of avian influenza currently spreading through Asia has not yet been detected in the U.S. The primary line of defense against avian influenza is biosecurity. The poultry industry has done a tremendous job trying to keep avian influenza away from their facilities and it's working really well. However, biosecurity fails occasionally, and cases of avian influenza appear in commercial poultry. When this occurs, early detection is very important to initiate efficient control and eradication programs.

The natural hosts or reservoirs of avian influenza viruses are wild aquatic birds. All different types of avian influenza have been identified in wild aquatic birds and from these they have spread to other animal species. The ones of significant importance for the poultry industry are subtypes H5 and H7. These are viruses that can mutate from low pathogenicity to high pathogenicity. The highly pathogenic AI viruses are the ones that have the most impact in the poultry industry. They are also important now because those have been transferred to humans and, as you know, the mortality rate associated with highly pathogenic H5N1 virus in Asia is over 50-percent. There are also reports of H5N1 AI affecting cats; so H5N1 is increasing the spectrum of hosts.

In 2004 the National Program Improvement Plan developed the U.S. H5/H7 LPAI Monitoring Program for surveillance of H5 and H7 in commercial poultry. Since avian influenza is exotic in commercial poultry in the U.S. and most of the surveillance is done through diagnostics, I'm going to talk about what kind of diagnostic tests are available at this time for avian influenza.

In the last few years RT-PCR has been approved for use by OIE and also by the National Diagnostic Lab. This current test detects the highly conserved matrix gene and therefore can identify the presence of any influenza virus subtype. RT-PCR tests have also developed for H5 and H7 genes since viruses of these subtypes can mutate from low to high pathogenicity and these subtypes can be transmitted to humans. RT-PCR usually has a 3-h turnaround time with RNA isolation being the bottleneck.

The U.S. H5/H7 LPAI Monitoring Program is based mostly on the detections of antibodies against avian influenza. The gold standard is the agar gel immunodiffusion test that looks for antibodies to the M and NP proteins; these are both type specific antigens and they detect any type A influenza virus. The test is very specific but sometimes takes up to 72 h to get the result. There are two commercial ELISA tests that can detect antibodies against avian influenza but these have a significant number of false positives. Therefore, there is an increased need for improved diagnostic tests that can provide faster results with increased sensitivity specificity. In order to improve and develop new diagnostic test we need to produce large quantities of viral antigens and monoclonal antibodies.

We have used a baculovirus expression system for the expression of the avian influenza NP and M1 proteins. The viral proteins were purified and their authenticity showed by ELISA and Western blot with influenza virus antibodies produced in chickens.

We are currently working on the development of a competitive ELISA test that will allow the detection of influenza virus antibodies in any animal species. We are also using the baculovirus expressed protein to study the immune response of chickens to different viral proteins. This study will allow us to determine which viral protein is a better antigen in the development of diagnostic tests for the detection of influenza virus specific antibodies.

In conclusion, faster, more sensitive and specific diagnostic tests are required for the early detection of avian influenza in commercial poultry in order to control the spread of the virus.
Improved diagnostic tests for avian influenza (AI) surveillance

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Texas A&M University 1, FAZD Center 2, Synbiotics Corp.3, University of California Davis 4, Texas Veterinary Medical Diagnostic Laboratory 5
Influenza virus characteristics

- Orthomyxoviridae
- Pleomorphic, single stranded RNA virus
- Three antigenic types
  - A, B, and C
- Eight gene segments
- Vary in pathogenicity
Influenza virion

Hatta and Kawaoka, 2002 Trends in Microbiology (10) 340-344
Envelope proteins of AI virus

- **Hemagglutinin** (16 subtypes)
  - Attachment to cell receptors
  - Hemagglutinating activity of RBCs
  - Neutralizing antibodies
  - Major determinant of pathogenicity

- **Neuraminidase** (9 subtypes)
  - Responsible for virus release
  - Antibodies restrict spread and protect
Other antigenic proteins

- **NP**
  - Type specific antigen
  - Non neutralizing antibodies
- **M1**
  - Type specific antigen
  - Non neutralizing antibodies
- **NS1**
  - Antibodies are produced only during active viral replication
  - Non neutralizing antibodies
Ecology of influenza viruses

- Wild aquatic birds are the natural reservoirs of all influenza A viruses

Adapted from http://www.medicaledcology.org/diseases/influenza
Avian influenza diagnosis

- Early detection of avian influenza infection is very important to initiate efficient control and eradication programs.
- Several tests are available for avian influenza diagnosis.
Avian influenza diagnosis

- Nucleic acid detection
  - One-Step RT-PCR: fluorogenic Taqman probes
    - Matrix gene
    - H5 & H7 genes
  - 3 hour turn around time
  - Bottleneck
    - RNA isolation
Avian influenza diagnosis

- Antigen detection: Directigen
  - It has been used in the field
  - Drawbacks:
    - Expensive
    - Low sensitivity
Virus isolation and/or identification is essential

- 9-10 day embryonating chicken eggs (via allantoic cavity) (3-7 days)
- 3 days HA test
- If positive HI and NI test
Avian influenza diagnosis

- Serology (as part of surveillance programs)
  - Antibodies to type A influenza:
    - AGID (NP,M)
      Specific but low sensitivity
    - Commercial ELISA
      False positives
  - Subtyping: HI, NI, IFA

IFA
Avian influenza diagnosis

• There is need for improved diagnostic tests:
  – Increased sensitivity (AGID)
  – Increased specificity (ELISA)
  – Increased range (real time RT-PCR for additional subtypes)

• Pen site tests

• There is need for pan-species serological tests for detection of antibodies to influenza virus in all bird species

• Current problems: lack of readily available reagents
Development of diagnostic tests

- Expression of avian influenza proteins in baculovirus expression system
- Development of monoclonal antibodies
Development of diagnostic tests

• Diagnostic tests:
  
  – *Antibody detection*:
    • ELISA
    • Luminex
  
  – *Antigen detection*:
    • Luminex,
    • Strip test
Baculovirus expression of AI proteins

- Expression of NP, M1 and NS1 proteins using baculovirus expression system

Sf-9 cells

Sf-9 cells/NP (AIV)

Mouse α-NP
Purified NP protein

SDS-PAGE purified NP
Purified M1 protein

SDS-PAGE purified M1

<table>
<thead>
<tr>
<th>Fractions</th>
</tr>
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<tbody>
<tr>
<td>1  2  3  4  5  6  7  8</td>
</tr>
<tr>
<td>9</td>
</tr>
</tbody>
</table>
Purified NS1 protein

SDS-PAGE purified NS1
Immunoreactivity of purified NP, M1 and NS1 proteins

<table>
<thead>
<tr>
<th></th>
<th>NS1</th>
<th>M1</th>
<th>NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie Blue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chicken α-Al</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Coomassie Blue

MAb α-NP
Antibody detection tests

- **ELISA**
  - Indirect ELISA
    - NP
    - NS1
    - M1
  - Competitive ELISA
    - NP
    - M1
- **Luminex**
  - NP
  - NS1
  - M1
  - HA (16)
  - NA (9)
Indirect ELISA

bound NP

AI positive

bound NP

AI negative

chicken serum

anti-chicken HRP
Evaluation of NP response to convalescent serum

OD (405 nm)

OD (+ve control)

μg/ml NP

0 0.02 0.04 0.06 0.08 0.1

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
Avian influenza antibody titers

NP Indirect ELISA

<table>
<thead>
<tr>
<th>Flock Antisera</th>
<th>Mean Titers</th>
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<tbody>
<tr>
<td>High</td>
<td>2500 ± 100</td>
</tr>
<tr>
<td>Medium</td>
<td>800 ± 50</td>
</tr>
<tr>
<td>Low</td>
<td>400 ± 25</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
</tbody>
</table>
NP protein indirect ELISA

<table>
<thead>
<tr>
<th></th>
<th>Chicken Sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Avian Pneumovirus</td>
</tr>
<tr>
<td>2</td>
<td>Chicken infectious anemia virus</td>
</tr>
<tr>
<td>3</td>
<td>Infectious bronchitis virus</td>
</tr>
<tr>
<td>4</td>
<td>Infectious bursal disease virus</td>
</tr>
<tr>
<td>5</td>
<td>Infectious laryngotracheitis virus</td>
</tr>
<tr>
<td>6</td>
<td>Newcastle disease virus</td>
</tr>
<tr>
<td>7</td>
<td>Avian reovirus</td>
</tr>
<tr>
<td>8</td>
<td>Avian pmetaneumovirus</td>
</tr>
<tr>
<td>9</td>
<td>Avian adenovirus</td>
</tr>
<tr>
<td>10</td>
<td>AIV negative</td>
</tr>
<tr>
<td>11</td>
<td>AIV weak positive</td>
</tr>
<tr>
<td>12</td>
<td>AIV strong positive</td>
</tr>
</tbody>
</table>
Competitive ELISA

- Faster than traditional ELISA
- Can be used for any species

![Diagram of Competitive ELISA]

- Bound NP
- AI positive
- Chicken serum
- HRP-NP MAb

- Bound NP
- AI negative
NP competitive ELISA
Competitive ELISA based on NP

Antibody Dilution

OD

Mab-C1
Mab-B2

Competitive ELISA
Antibody response to avian influenza proteins
Luminex for AI antibodies detection

Positive sample

Negative sample
Antigen detection tests

- Strip test
  - NP
  - M1
- Luminex
  - NP
  - NS1
  - M1
  - HA (16)
  - NA (9)
FLU DETECT™ strip test

- Antigen capture assay
- Based on the detection of NP type specific antigen
- Uses NP MAb
• FLU DETECT™ strip test assay shows 80 – 100% correlation with virus isolation and Real Time RT-PCR methods during the first 3 to 5 days post-infection

• FLU DETECT™ strip test is more sensitive than other similar commercially available assays

• The sensitivity of FLU DETECT™ is $10^3$ to $10^4$ EID$_{50}$

• FLU DETECT™ test is efficacious for the surveillance of AI infection in chicken, ducks and quails
Ongoing research

- Expression of AI HA proteins:
  - H5
  - H7
  - H6

- Development of monoclonal antibodies to NP, M1, HA and NA

- Development of a Luminex system for detection and subtyping of AI antibodies and viruses
Acknowledgments

- National Center for Foreign Animal and Zoonotic Disease Defense (FAZD)
- USDA/AI CAP
Thank You