7th Annual CEIRS meeting
July 8 – July 10, 2013
Memphis, TN

Annual CEIRS Surveillance meeting
July 11, 2013
Memphis, TN

Memphis Marriott Downtown &
Cotton Row, Cook Convention Center
Memphis, TN

Sponsored by:
NIH/NIAID CEIRS Program

Hosted by:
St. Jude Children’s Research Hospital
Center of Excellence for Influenza Research and Surveillance
(SJCEIRS)
Dear Colleague,

On behalf of the Influenza Program at the National Institutes of Health/National Institute of Allergy and Infectious Diseases/Division of Microbiology and Infectious Diseases (NIH/NIAID/DMID) and the St. Jude Center of Excellence for Influenza Research and Surveillance (SJCEIRS), we welcome you to Memphis and the 7th Annual Meeting of the Centers of Excellence for Influenza Research and Surveillance (CEIRS) and the 3rd CEIRS Surveillance Meeting.

Memphis has served as “bookends” during this inaugural CEIRS award: 7 years ago, our 1st annual meeting was held on the St. Jude campus, and as a testament to the success of continued interest in the CEIRS program, our last annual meeting had to be moved to the Cook Convention Center to accommodate the growing number of registered participants. As in previous years, the meeting will focus on the latest scientific progress made at each of the CEIRS Centers, along with special panel discussions. This year, panels will discuss influenza vaccines and the A(H7N9) viruses responsible for the recent outbreaks in China. The wealth of unpublished data to be presented yet again demonstrates the cutting-edge research being conducted throughout the CEIRS Network.

New this year, we are introducing a series of “Perspectives” presentations at the beginning of each session. These presentations will be made by individuals who have been nominated by CEIRS investigators in recognition of their seminal contributions to influenza research. Also, because this is the last annual meeting of the current CEIRS funding period, each principal investigator will provide a summary of the research highlights made by their Center. Finally, watch for the video that will be presented by each CEIRS Center. There will most likely be singing, dancing, and all sorts of things that scientists really should not do; nevertheless, they promise to be a highlight.

Once again, we welcome you to Memphis. Although Memphis in July is no place for those who prefer snow, we hope that you will get out and enjoy some Southern hospitality. Within a trolley ride (or two) of the Convention Center, there is an abundance of live music, cultural attractions such as Sun Studios and the National Civil Rights Museum, the mighty Mississippi River, and all of the lip-smacking, artery-clogging, belt-loosening Southern food you could ever want to eat.

In the words of Memphis’ own Elvis Presley, the undisputed “King of Rock and Roll”—

Thank ya, thank ya very much!

Richard Webby, PhD
PI/Director
SJCEIRS

Diane Post, PhD
Project Officer
Respiratory Diseases Branch, NIH/NIAID/DMID
# 7th Annual CEIRS Network Meeting

Memphis Marriott Downtown & Cook Convention Center

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<td>37</td>
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<tr>
<td>Participants List</td>
<td>80</td>
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</tbody>
</table>
MEMPHIS MARRIOTT DOWNTOWN 2ND FLOOR:

HERITAGE BALLROOM (HERITAGE I-IV): Reception & Registration

ENTRANCE TO:
COOK CONVENTION CENTER

CEIRS MEETING
REGISTRATION
& RECEPTION
COOK CONVENTION CENTER FLOOR PLAN:

CEIRS ANNUAL NETWORK MTG: July 8th – 10th
COTTON ROW: Meeting Room
MISSISSIPPI/SULTANA: All Posters
STEAMBOAT: Meals & Breaks
CHICKASAW: Data Management Lunch

CEIRS SURVEILLANCE MTG: July 11th
CHICKASAW/MISSISSIPPI: Meeting Room
SULTANA: Surveillance Posters
RIVER BLUFF: Meals & Breaks
7th Annual CEIRS Network Meeting
July 8 –10, 2013
Cotton Row, Cook Convention Center
Memphis, TN

Arrival
Sunday July 7, 2013
5:30 – 7:00 pm  Registration (Heritage Ballroom entryway, Memphis Marriott Downtown)
7:00 – 9:00 pm  Welcome Reception (Heritage Ballroom, Memphis Marriott Downtown)

Monday July 8, 2013
7:00-5:00  Registration (Mezzanine Hallway, Cook Convention Center -CCC)
Poster Set-up (Sultana/Mississippi, CCC)
7:15-8:00  Breakfast (Steamboat, CCC)
8:00-8:05  Welcome – Richard Webby, SJCEIRS Director
8:05 - 8:40  CEIRS: Past, present and future –Irene Glowinski & Diane Post
8:40 – 9:10  SJCEIRS  Accomplishments Summary – Richard Webby

Session #1 – Surveillance
Session Chairs: Mike Osterholm and Rob Webster
9:10 - 9:40  Perspectives on Surveillance - Robert Webster (SJCEIRS)
9:40 – 9:55  Zoonotic transmission and molecular investigation of H3N2 influenza A viruses at agricultural fairs – Andrew Bowman (MCEIRS)
9:55 – 10:10  Active surveillance for variant influenza in swine, the environment, and employees at live animal markets – Montse Torremorell (MCEIRS)
10:10 - 10:30  Break (Steamboat, CCC)
10:30 – 10:45  Unraveling AIV dynamics in “boom and bust” Australian bird populations – Bethany Hoye (CRIP)
10:45 – 11:00  Filling the gaps to build gull influenza epidemiological models; experimental and field data – Josanne H. Verhagen (CRIP)
11:00 – 11:15  The emergence of the 2013 H7N9 and related H7N7 viruses in the Yangtze River Delta Region - Huachen (Maria) Zhu (SJCEIRS)

No US government appropriated funds were used to pay for food or beverages for this meeting.
11:15 -11:30 Evolution of HPAI H5N1 in South Asia – Vijaykrishna Dhanasekaran (SJCEIRS)

Panel Discussion #1 – H7N9: Lessons Learned

11:30 – 12:30 H7N9: Lessons Learned
Moderator: Daniel Perez
Panelists: Carol Cardona, Ron Fouchier, Yoshihiro Kawaoka, Mike Osterholm, Malik Peiris, John Treanor, Dave Topham, Ralph Tripp, Guan Yi

12:30 – 1:45 Lunch (Steamboat, CCC)
NEC Luncheon (Suite #1439, Memphis Marriott Downtown)
Data Manager Luncheon (Chickasaw, CCC- pick your lunch up from the Steamboat room)

1:45 – 2:15 IPIRC Accomplishments Summary – Walt Orenstein

Session #2 – Immunology
Session Chairs: Dave Topham and Peter Doherty

2:15 – 2:45 Perspectives on Influenza Immunology – Peter Doherty
2:45– 3:15 Live imaging of effector CD8 T cells in the influenza-infected airway – Dave Topham (NYICE)
3:15 – 3:45 Break (Steamboat, CCC)
3:45 – 4:00 Evaluation of CD4 T cell responses following heterosubtypic influenza infection or vaccination – Jennifer Nayak (NYICE)
4:00 – 4:20 Regulation of antibody responses to influenza by CD4 T cells: Implications for pandemic preparedness – Andrea Sant (NYICE)
4:20 – 4:45 High Resolution analysis of influenza vaccine responses in elderly and pregnant subjects – Tim Mosmann (NYICE)
4:45 – 5:00 Analysis of influenza virus specific plasma cells in the human bone marrow – Carl Davis (IPIRC)
5:00 – 5:15 Heads or Stalks: Dissecting the Antibody and Memory B cell Responses to Influenza HA - Ali Ellebedy (IPIRC)
## Tuesday July 9, 2013

<table>
<thead>
<tr>
<th>Time</th>
<th>Activity</th>
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<tbody>
<tr>
<td>7:00-5:00</td>
<td><strong>Registration</strong> (Mezzanine Hallway, CCC)</td>
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<td></td>
<td><strong>Poster Set-up cont.</strong> (Sultana/Mississippi, CCC)</td>
</tr>
<tr>
<td>7:15-7:55</td>
<td><strong>Breakfast</strong> (Steamboat, CCC)</td>
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<tr>
<td>7:55 - 8:00</td>
<td>Welcome and housekeeping</td>
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<tr>
<td>8:00 – 8:30</td>
<td>MCEIRS Accomplishments Summary – Mike Osterholm</td>
</tr>
</tbody>
</table>

### Session #2 – Immunology, continued

<table>
<thead>
<tr>
<th>Time</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:30 – 8:45</td>
<td>Increased expression of lung beta 6 integrin is associated with enhanced inflammation and lung damage during influenza infection - Victoria Meliopoulos (SJCEIRS)</td>
</tr>
<tr>
<td>8:45 – 9:00</td>
<td>The recruitment kinetics and functional heterogeneity of monocyte populations during influenza virus infection - Susu Duan (SJCEIRS)</td>
</tr>
<tr>
<td>9:00 – 9:15</td>
<td>Catching a moving target: Universal influenza virus vaccine constructs based on the conserved hemagglutinin stalk domain – Florian Krammer (CRIP)</td>
</tr>
<tr>
<td>9:15 – 9:30</td>
<td>Unanchored Lysine48-linked polyubiquitin chains positively regulate the type I IFN-mediated antiviral response – Ricardo Rajsbaum (CRIP)</td>
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</tbody>
</table>

### Panel Discussion #2 – Influenza Vaccines

<table>
<thead>
<tr>
<th>Time</th>
<th>Activity</th>
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</thead>
<tbody>
<tr>
<td>9:30 – 10:30</td>
<td>Influenza Vaccines</td>
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<tr>
<td></td>
<td>Moderator: TBD</td>
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<tr>
<td></td>
<td>Panelists: Florian Krammer, Walt Orenstein, Mike Osterholm, Peter Palese, Bali Pulendran, Paul Thomas, John Treanor, Rob Webster</td>
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<tr>
<td>10:30 – 11:00</td>
<td>Break (Steamboat, CCC)</td>
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</table>

### Session #3 – Transmission & Adaptation

**Session Chairs:** Stacey Schultz-Cherry and Barney Easterday

<table>
<thead>
<tr>
<th>Time</th>
<th>Activity</th>
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</thead>
<tbody>
<tr>
<td>11:00 – 11:30</td>
<td>Perspectives on Influenza Transmission – Barney Easterday</td>
</tr>
<tr>
<td>11:30 – 12:00</td>
<td>Use of hemagglutination inhibition for the detection of HPAI virus exposure in wild bird populations - Martin Gilbert (MCEIRS)</td>
</tr>
<tr>
<td>12:00 – 1:15</td>
<td>Lunch (Steamboat, CCC)</td>
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<tr>
<td></td>
<td><strong>Coordinator Luncheon</strong> (Suite #1439, Memphis Marriott Downtown)</td>
</tr>
<tr>
<td>1:15 – 1:45</td>
<td>The role of the matrix protein in facilitating transmission of influenza virus – John Steel (IPIRC)</td>
</tr>
<tr>
<td>1:45 – 2:00</td>
<td>Avian A(H7N9) virus; receptor binding, fusion, ferret transmission – Mathilde Richard (CRIP)</td>
</tr>
</tbody>
</table>
2:00 – 2:15  Transfection-based inoculation to understand H9 influenza virus transmission in the ferret model – Matthew Angel (CRIP)


2:45 – 3:00  Adaptation of an avian-like H2N3 virus in pigs - Qinfang Liu (SJCEIRS)

3:00 – 3:15  NYICE Accomplishments Summary – John Treanor

3:15 – 3:45  Break (Steamboat, CCC)

Session #4 – Molecular Virology
Session Chairs: Richard Compans and Peter Palese

3:45 – 4:15  Perspectives on Influenza Molecular Virology – Peter Palese

4:15 – 4:45  Structural determinants of HA stability: The ups and downs of fusion pH - David Steinhauer (IPIRC)

4:45 – 5:15  Functional analysis of PA-X protein in host shutoff– Toru Takimoto (NYICE)

5:15 – 6:15  Poster Session (Sultana/Mississippi, CCC)
Wednesday July 10, 2013

8:00-3:30  **Registration** (Mezzanine Hallway, CCC)  
**Poster Break-down** (Sultana/Mississippi, CCC)

8:30-9:00  **Breakfast** (Steamboat, CCC)

9:00-9:30  CRIP Accomplishments Presentation – Adolfo Garcia-Sastre

### Session #4 Molecular Virology, continued

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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</thead>
<tbody>
<tr>
<td>9:30 – 9:45</td>
<td>Influenza A virus NS1 and PI3K: strain and isotype specificity of a complex virus-host interaction – Juan Ayllon (CRIP)</td>
</tr>
<tr>
<td>9:45 – 10:00</td>
<td>H7N9 influenza viruses bind preferentially α2,3-linked sialic acids and induce activation of primary human dendritic cells.– Irene Ramos (CRIP)</td>
</tr>
<tr>
<td>10:00 – 10:15</td>
<td>Neuraminidase inhibitor oseltamivir protects mice against lethal challenge with A/Anhui/1/2013 (H7N9) influenza virus - Tatiana Baranovich (SJCEIRS)</td>
</tr>
<tr>
<td>10:30 – 10:45</td>
<td><strong>Break</strong> (Steamboat, CCC)</td>
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</table>

### Session #5 Pathogenesis

**Session Chairs:** Yoshihiro Kawaoka and Ron Fouchier

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
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</thead>
<tbody>
<tr>
<td>10:45 – 11:15</td>
<td>Perspectives on Influenza Pathogenesis – Ron Fouchier</td>
</tr>
<tr>
<td>11:15 – 11:45</td>
<td>Requirements for efficient influenza virus reassortment – Anice Lowen (IPIRC)</td>
</tr>
<tr>
<td>11:45 – 12:00</td>
<td>Molecular risk mitigation of gain-of-function studies with influenza viruses – Randy Albrecht (CRIP)</td>
</tr>
<tr>
<td>12:00 – 12:15</td>
<td>Susceptibility of swine to A/Anhui/1/2013 H7N9 virus (Anhui/1) - Kelly Lager (SJCEIRS)</td>
</tr>
<tr>
<td>12:15 – 1:15</td>
<td><strong>Lunch:</strong> boxed lunches available in the Steamboat, CCC from 11:30am-1:30pm</td>
</tr>
<tr>
<td>1:15 – 1:30</td>
<td>Characterization of Novel Influenza A(H7N9) Viruses – Yoshi Kawaoka (CRIP)</td>
</tr>
<tr>
<td>1:30 – 1:45</td>
<td>Characterization of the R292K mutation that confers resistance to the neuraminidase inhibitors in a novel H7N9 human isolate- Hui-Ling Yen (SJCEIRS)</td>
</tr>
<tr>
<td>1:45 – 2:15</td>
<td>Pathogenesis and transmission of novel influenza A(H7N9) virus in poultry – David Suarez (MCEIRS)</td>
</tr>
</tbody>
</table>

No US government appropriated funds were used to pay for food or beverages for this meeting.
2:15 – 2:45 Phenotyping swine-origin H3N2 influenza A viruses from Ohio agricultural fairs in relevant animal species – Carol Cardona (MCEIRS)

2:45 – 3:15 Contract Close Out Discussion – Diane Post

3:15 – 3:30 Meeting Summary and H7N9 Summary – Richard Webby
CEIRS Surveillance Meeting
July 11, 2013
Mississippi/Chickasaw, Cook Convention Center
Memphis, TN

Meeting Goals

- Enhance the scope and application of CEIRS surveillance-related research
- Move forward projects and discussion topics developed in previous surveillance meetings
- Restate and reaffirm research objectives and productivity through long-term collaborative projects between CEIRS investigators
- Provide update on specific cross-collaborative programs established at previous surveillance meetings with a goal to determine deliverables and timelines
- Determine next steps and closeout in cross-collaborative programs and projects

Arrival

7:30 – 8:30  Registration (Mezzanine Hallway, CCC)
Surveillance Poster Session & breakdown (Sultana, CCC)

7:30 – 8:25  Breakfast (River Bluff /Sultana, CCC)

8:25 – 8:30  Welcome and housekeeping

Session 1: CEIRS Cross-Collaborative Projects
Session Chair: Ralph Tripp

Long-term CEIRS collaborative efforts – Updates and Wrap-up

8:30 – 8:40  Epidemiology of AI in Gulls- Ron Fouchier (CRIP)

8:40 – 8:50  Blue-wing Teal – Dave Stallknecht (MCEIRS)

8:50 – 9:00  Risk analysis of H2N2 viruses from the avian reservoir – Scott Krauss and Jeremy Jones (SJCEIRS)

Cross-center projects – Updates and Wrap-up

9:00 – 9:10  Evaluation of global phylogeography of dabbling ducks – Gavin Smith (SJCEIRS)

9:10 – 9:20  Global geospatial map of influenza prevalence by species – Richard Scheuermann (IRD)

9:20 – 9:30  Surveillance in Delaware Bay– Dave Stallknecht (MCEIRS) and Scott Krauss (SJCEIRS)

9:30 – 9:40  Swine-Human interface and Characterization of 2009 pH1N1 virus – Yi Guan (SJCEIRS)

No US government appropriated funds were used to pay for food or beverages for this meeting.
9:40 – 10:10 Collaborative project close-out discussion

**10:10 – 10:25 Break** (River Bluff /Sultana, CCC)

### Session 2: Data and Surveillance Tools
**Session Chair: Maria Giovanni**

<table>
<thead>
<tr>
<th>Time</th>
<th>Session Details</th>
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<tbody>
<tr>
<td>10:40 – 10:55</td>
<td>Serologic tools to evaluate immunity—Justin Brown (MCEIRS)</td>
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<tr>
<td>10:55 – 11:10</td>
<td>Mapping transmission of influenza virus during an infection peak in wild ducks – Nichola Hill (CRIP)</td>
</tr>
<tr>
<td>11:10 – 11:40</td>
<td>Surveillance data and tools: Closeout and future directions – Maria Giovanni &amp; Alison Yao</td>
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</tbody>
</table>

### Session 3: Human and Human/Animal Interface Surveillance
**Session Chair: Malik Peiris**

<table>
<thead>
<tr>
<th>Time</th>
<th>Session Details</th>
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<tbody>
<tr>
<td>11:40 – 11:55</td>
<td>Human-animal interface studies: what have we learned and what we need to learn – Jeff Bender (MCEIRS)</td>
</tr>
<tr>
<td>11:55 – 12:10</td>
<td>Household transmission of Influenza in Nicaragua - Aubree Gordon (SJCEIRS)</td>
</tr>
<tr>
<td><strong>12:10-1:15</strong></td>
<td><strong>Lunch:</strong> boxed lunches available in the River Bluff /Sultana, CCC from 11:30am-1:30pm</td>
</tr>
<tr>
<td>1:15 – 1:30</td>
<td>Integrated surveillance of H7N9 in Human Cases and Avian Species in Taiwan - Chwan Chuen King (IPIRC)</td>
</tr>
<tr>
<td>1:30 – 1:45</td>
<td>Ecology of Influenza viruses in Egypt: Surveillance, Characterization and Vaccine Evaluation - Mohamed A. Ali (SJCEIRS)</td>
</tr>
</tbody>
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### Session 4: Animal Surveillance
**Session Chair: Carol Cardona**

<table>
<thead>
<tr>
<th>Time</th>
<th>Session Details</th>
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<tbody>
<tr>
<td>1:45 – 2:00</td>
<td>Epidemiological Studies of Avian, Equine, and Canine Avian Influenza Infection in Man - Greg Gray (SJCEIRS)</td>
</tr>
<tr>
<td>2:00 – 2:15</td>
<td>Prevalence of pH1N1 antibodies in seals and sea lions in California 2009-2012– Ignacio Mena (CRIP)</td>
</tr>
<tr>
<td>2:15 – 2:30</td>
<td>What is the risk of avian influenza viruses jumping into horses in Mongolia? – Pablo Murcia (MCEIRS)</td>
</tr>
<tr>
<td>2:30 – 2:45</td>
<td>Wild bird and swine influenza surveillance - update from Argentina – Ariel Pereda (CRIP)</td>
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<table>
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<tr>
<th>Time</th>
<th>Session</th>
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<tbody>
<tr>
<td>2:45 – 3:00</td>
<td>Avian influenza surveillance in wild birds in Guatemala, 2010-2012 – Ana Silvia Gonzalez-Reiche (CRIP)</td>
</tr>
<tr>
<td>3:00 – 3:15</td>
<td>Surveillance and characterization of avian influenza viruses circulating in the central region of Chile - Pedro Jimenez (SJCEIRS)</td>
</tr>
<tr>
<td>3:15 – 3:45</td>
<td>Animal Surveillance Closeout and Future Directions</td>
</tr>
<tr>
<td>3:45 -</td>
<td>Departures</td>
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</tbody>
</table>
# 7th Annual CEIRS Network Meeting

**Poster Session**

Tuesday, July 9th – 5:15-6:15PM  
Sultana/Mississippi, Cook Convention Center

<table>
<thead>
<tr>
<th>POSTER #</th>
<th>NAME</th>
<th>CENTER</th>
<th>TITLE</th>
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<tbody>
<tr>
<td><strong>Immunology</strong></td>
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<tr>
<td>01</td>
<td>Oshansky, C.</td>
<td>SJCEIRS</td>
<td>An innate immune profile predicts clinical outcomes in natural influenza infection</td>
</tr>
<tr>
<td>02</td>
<td>Keating, R.</td>
<td>SJCEIRS</td>
<td>mTOR regulates protection against lethal H5N1 influenza infection by modulating the antibody response</td>
</tr>
<tr>
<td>03</td>
<td>Lambert, K.</td>
<td>NYICE</td>
<td>Influenza tracheitis: a murine model system for studying T cell migration and function by intravitral multiphoton microscopy</td>
</tr>
<tr>
<td>04</td>
<td>Koutsonanous, D.</td>
<td>IPIRC</td>
<td>Effect of Age on Influenza Skin Vaccination</td>
</tr>
<tr>
<td>05</td>
<td>Miller, M.</td>
<td>CRIP</td>
<td>Neutralizing antibodies against previously-encountered influenza virus strains increase over time: A longitudinal analysis</td>
</tr>
<tr>
<td>06</td>
<td>Sharma, S.</td>
<td>SJCEIRS</td>
<td>mCMV alters the airway inflammatory milieu regulating protective and pathogenic heterologous immunity to influenza A virus infection</td>
</tr>
<tr>
<td>07</td>
<td>Sangster, M.</td>
<td>NYICE</td>
<td>The B cell response and hemagglutinin stalk-reactive antibody production in different age cohorts following 2009 H1N1 influenza vaccination</td>
</tr>
<tr>
<td>08</td>
<td>Knowlden, Z.</td>
<td>NYICE</td>
<td>Characterization of Follicular Helper T cell Responses to Influenza – Antigen Specificity and Influence upon B cell Responses</td>
</tr>
<tr>
<td>09</td>
<td>Leyva-Grado, VH.</td>
<td>CRIP</td>
<td>Local administration of a monoclonal antibody for therapeutic use in influenza infection in mice Clinical, immunological and virological characterization of hospitalized influenza A patients between 2011-2012</td>
</tr>
<tr>
<td>10</td>
<td>Barrera, A.</td>
<td>CRIP</td>
<td></td>
</tr>
<tr>
<td><strong>Immunology / Vaccine</strong></td>
<td></td>
<td></td>
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<tr>
<td>11</td>
<td>Goff, P.</td>
<td>CRIP</td>
<td>Induction of cross-reactive antibodies to novel H7N9 influenza virus by recombinant Newcastle disease virus expressing a North American lineage H7 subtype hemagglutinin</td>
</tr>
<tr>
<td>12</td>
<td>Eggink, D.</td>
<td>CRIP</td>
<td>Guiding the immune response against influenza hemagglutinin towards the conserved stalk domain by altering glycosylation</td>
</tr>
<tr>
<td>13</td>
<td>Margine, I.</td>
<td>CRIP</td>
<td>Induction of broadly neutralizing influenza virus group 2 HA antibodies through natural infection and novel vaccination strategies</td>
</tr>
<tr>
<td><strong>Transmission &amp; Adaptation</strong></td>
<td></td>
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<tr>
<td>14</td>
<td>Liu, Q.</td>
<td>SJCEIRS</td>
<td>H7N9 infection of pigs: Pathogenicity and transmissibility of the wild-type virus and avian-sigature mutants</td>
</tr>
<tr>
<td>No.</td>
<td>Author(s)</td>
<td>Institution</td>
<td>Title</td>
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<tr>
<td>15</td>
<td>Fabrizio, T.</td>
<td>SJCEIRS</td>
<td>Influenza virus transmission among pigs is not solely mediated by hemagglutinin and neuraminidase</td>
</tr>
<tr>
<td>16</td>
<td>Dlugolenski, D.</td>
<td>IPIRC</td>
<td>Infectivity, reassembly, and adaptation of IAV in Pteropus alecto (bat) epithelial cells</td>
</tr>
<tr>
<td>17</td>
<td>Zhu, H.</td>
<td>SJCEIRS</td>
<td>Infectivity, Transmission and Pathology of Human-isolated H7N9 Influenza Virus in Ferrets and Pigs</td>
</tr>
<tr>
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<td>Martínez-Romero, C.</td>
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Zoonotic transmission and molecular investigation of H3N2 influenza A viruses at agricultural fairs

Andrew S. Bowman, Sarah W. Nelson, Jacqueline M. Nolting, Richard D. Slemons
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Transmission of influenza A virus across the swine-human interface at agricultural fairs occurred at an unprecedented frequency in 2012. Across the United States, 309 human cases of infection with influenza A (H3N2) variant virus (H3N2v) resulted in 16 hospitalizations and one death; 107 of those H3N2v cases were reported in Ohio. Nucleotide sequences of 14 swine-origin H3N2 IAV isolates containing the M gene from the A(H1N1)pdm09 virus, termed H3N2pM, recovered from pigs at seven agricultural exhibitions in Ohio during 2012 were compared to seven H3N2v human isolates linked to each of those respective fairs. Nucleotide identity of the H3N2 isolates recovered from both humans and swine at all seven exhibitions was greater than 99%, indicating that the same swine-origin H3N2pM IAV infected exhibition swine at numerous exhibitions and was transmitted to humans in at least seven separate events occurring at geographically disparate locations across Ohio over a period of several weeks. Phylogenetic analyses showed this H3N2pM virus was similar to contemporary H3N2pM isolates recovered from North American swine indicating intra-species virus and/or gene flow occurs between exhibition and commercial swine. The molecular evidence provided here demonstrates that zoonotic transmission was widespread and confirms the epidemiological linkages between human H3N2v cases and swine exposure at agricultural exhibitions. The results of the current study demonstrate the need to define the ecology and evolution of IAVs in exhibition swine and highlight the need to develop veterinary and public health interventions to reduce inter- and intra-species transmission of influenza A viruses at swine exhibitions.

Active surveillance for variant influenza in swine, the environment, and employees at live animal markets

Montserrat Torremorell¹, Mary J. Choi², Jeff Bender¹, D. Her, M. Jhung, Tim Uyeki, K. Wong, Sara Vetter, David Boxrud, Jim Ertl, J. Nguyen, Kirk Smith², Richard Danila², Ruth Lynfield²
¹Department of Veterinary Population Medicine, University of Minnesota
²Minnesota Department of Health

Variant influenza (VI) in humans is caused by swine-origin influenza A viruses (IAVs). Because swine can support genetic material exchange between avian, swine, and human IAVs, emergence of variant viruses with pandemic potential is a serious concern. During 2012, a VI outbreak linked to agricultural fairs sickened >300 persons in the United States. In Minnesota, half of all VI cases from 2008–2011, and the first VI cases of the 2012 outbreak, occurred among live animal market patrons. We initiated surveillance at two markets to identify factors contributing to VI transmission. We collected weekly air samples during October 8–January 12 from swine enclosures, environmental samples from high hand-contact surfaces (swine enclosure railings, door knobs, and patron sinks), pooled swine saliva samples, and slaughtered pigs’ lungs. Employees submitted weekly nasal swabs. Real-time reverse-transcription polymerase chain reaction for IAV gene targets was performed on all samples. IAVs were detected in
30/57 (53%) swine enclosure air samples, 16/34 (47%) swine enclosure railing samples, 48/50 (96%) swine saliva samples, and 70/149 (47%) swine lungs. Ill swine were observed. Weekly coughing scores (percent of pigs coughing) ranged from 2% to 18%. IAVs were detected in samples from 11/17 (65%) asymptomatic employees. Variant hemagglutinin gene segments were identified in 4/17 (23%) asymptomatic employees. Live animal markets facilitate close human-swine contact and represent a venue where interspecies IAV transmission can occur. This investigation confirmed the widespread presence of IAV at live animal markets, underscoring the potential for zoonotic transmission in this setting. Public health officials, employees, and patrons of live animal markets should understand the risk for variant IAV infection and take steps to minimize transmission.

Unravelling AIV dynamics in “boom and bust” Australian bird populations

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Climatic conditions in Australia are aseasonal, characterised by periods of intense rainfall followed by periods of protracted drought. This has considerable impact on the population dynamics and ecology of many Australian birds, including the waterbirds that form a reservoir for avian influenza viruses (AIV). In line with this, we have found temporal correspondence between long-term rainfall cycles and AIV outbreaks in Australian poultry. To elucidate the effect of these multi-year climate patterns on AIV dynamics in wild birds, we are conducting a longitudinal study of AIV infections and seroprevalence in Australian waterfowl and shorebirds species, in both permanent wetlands in south-east Australia and ephemeral wetland areas in Australia’s interior. We also include a few non-waterbird species in this survey that also show boom-bust dynamics and may serve as intermittent hosts between poultry and waterfowl. We present an overview and first results of these surveillance activities. We present an overview of our surveillance activities, including the major candidates for AIV reservoirs in Australia, and the links between their ecology and AIV susceptibility. The longitudinal data tentatively suggests that rainfall rather than seasonal dynamics drive AIV dynamics in wild birds. There are also indications that an increased number of young (i.e. naive) birds, rather than bird densities per se, drive infection peaks.

Filling the gaps to build gull influenza epidemiological models; experimental and field data

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¹Erasmus MC, department of viroscience, Rotterdam, the Netherlands, ²SOVON Dutch Centre for Ornithology, Nijmegen, the Netherlands, ³IREC, Ciudad Real, Spain, ⁴NVI RIVM, Bilthoven, the Netherlands.

Epidemiology of avian influenza A viruses (AIV) in wild waterbirds has been studied mainly in ducks (family Anatidae): AIV prevalence peaks in fall at pre-migratory staging sites and declines in the following months to reach the lowest prevalence in spring. A wide variety of AIV subtypes (H1-H12) has been isolated from dabbling ducks. However, subtypes H13 and H16 are almost exclusively isolated from gulls (family Laridae), which are therefore thought to act as their maintenance reservoir and may form an easier model to study the epidemiology of AIV than ducks. Based on a long-term field study, we investigated spatial and temporal dynamics of AIV infections in 7927 free-living Black-headed Gulls (Chroicocephalus ridibundus) sampled in the Netherlands year-round from 2006 to 2010. We show that H13 and H16 viruses cause annual epidemics in first-year Black-headed Gulls on their breeding colony sites around the time of fledging. This finding suggests that population characteristics of Black-headed Gull colonies at their breeding sites in summer are conducive for H13 and H16 virus epidemics, much like those of dabbling duck aggregations at their pre-migratory staging sites in autumn are conducive for AIV epidemics of other subtypes. Based on experimental infection, we investigate the effect of homosubtypic and heterosubtypic immunity on the excretion of H13 and H16 viruses, within and between
breeding seasons in Black-headed Gulls. The results of this experimental infection will fill the most important gaps in knowledge essential to develop mathematical models for AIV epidemiology in gulls.

**The emergence of the 2013 H7N9 and related H7N7 viruses in the Yangtze River Delta Region**

Huachen Zhu, Tommy T.Y. Lam, Jia Wang, David Smith and Yi Guan
Joint Influenza Research Center (SUMC/HKU), Shantou University Medical College, Shantou, China; and Centre of Influenza Research, The University of Hong Kong, Hong Kong SAR, China

The novel H7N9 influenza A virus first detected in March 2013 has caused more than 130 cases of human infection in China, resulting in 39 deaths. This virus is a reassortant of H7, N9 and H9N2 avian influenza viruses and carries some amino acids linked to mammalian receptor binding, raising concerns of a new pandemic. However, neither the source populations of the H7N9 outbreak lineage nor the conditions for its genesis are fully understood. Through a combination of active surveillance, screening of virus archives, and evolutionary analyses, we found that H7 viruses have independently transferred from domestic ducks to chickens in China on at least two occasions. Subsequently they reassorted with enzootic H9N2 viruses to generate the H7N9 outbreak lineage, and a related but previously unrecognized H7N7 lineage. The H7N9 outbreak lineage has spread over a large geographic region and is prevalent in chickens at live poultry markets that appear to be the immediate source of human infections. Whether the H7N9 outbreak lineage will, or has, become enzootic in China needs further investigation. Like the H7N9 virus, the H7N7 virus was also mainly isolated from chickens at live poultry markets, and could efficiently infect ferrets, be shed via the nasal and rectal routes, and cause severe pneumonia. This indicates that H7 viruses pose a broader threat than the current H7N9 virus. Continued prevalence of this family of H7 viruses in poultry could lead to further sporadic human infections, with an ongoing risk that the virus might acquire efficient human-to-human transmissibility.

**Evolution of HPAI H5N1 in South Asia.**

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Multiple outbreaks of the highly pathogenic avian influenza (HPAI) H5N1 virus have occurred in poultry in several countries in South Asia, including Bangladesh, Bhutan, India, Nepal and Pakistan, over the last eight years. Epidemiological data from these outbreaks indicate that with the exception of West Bengal in India and neighbouring Bangladesh where H5N1 viruses may have attained endemicity, all other outbreaks have been sporadic. More recently however, H5N1 viruses have also been implicated in large crow die-offs in India and Bangladesh and a turkey farm outbreak in southern India. To understand the changing ecology and epidemiology of H5N1 in South Asia, newly generated full and partial genomes of 37 highly pathogenic avian influenza H5N1 viruses isolated from poultry in India and Bhutan and from crows in India during 2011–2012, were analysed with sequences of >150 H5N1 viruses from Bangladesh, Myanmar, Nepal and Pakistan and reference H5N1 sequences from other countries in Asia. Phylogenetic analyses shows that (1) Clade 2.2 viruses that were endemic since 2006, may have been replaced by Clade 2.3.2.1 viruses since 2011, (2) the introduced Clade 2.3.2.1 viruses have reassorted with LPAI H9N2 viruses circulating in poultry in South Asia, and (3) both the Clade 2.3.2.1 viruses and their reassortants caused the crow die-off in India. To understand the effects of invasion of Clade 2.3.2.1, we investigated the spatial and temporal dynamics of these samples using phylogeographic analyses. For these analyses we acquired additional meta-data, including dates of isolation and GPS co-ordinates for the majority of these outbreaks in Bangladesh, Bhutan, India, Nepal and Myanmar.
Session #2: Immunology

Live imaging of effector CD8 T cells in the influenza-infected airway

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Influenza virus productively infects the epithelial cells that line the respiratory tract and is restricted there by a requirement for a trypsin-like enzyme that activates the viral hemagglutinin. During a primary influenza infection, cytotoxic CD8+ T cells (CTL) are the main effectors mediating elimination of infected cells. The CTL directly engage infected targets through class I MHC-viral peptide complexes expressed on the surface of infected cells. Therefore, the CTL must have mechanisms that allow them to migrate from lymphoid organs and enter the epithelium and mediate effector function. Until now, the methods used to assess T cell localization and memory in the tissues has relied primarily on static imaging and tissue disruption to isolate the T cells for study, methods that do not reveal dynamic functions. Therefore, by developing a model of influenza tracheitis, our lab has devised the means to image influenza-specific T cells in the infected and recovered, intact airways of live animals. We have combined dynamic and static imaging techniques with the ability to quantitate changes to the tissue architecture and composition that together reveal critical molecular interactions of T cells with the tissue microenvironment. We have observed the formation of distinct gaps in the lamina densa of the respiratory epithelial basement membrane through which lymphocytes appear to be passing into the airway epithelium. CD8+ T cell velocity and displacement progressively decrease from 6-8 days after infection, while the angle of migration progressively increases during that period. At the time point (day 9) when virus is typically cleared, both displacement and meandering index (a measure of directionality) decrease further, while velocity significantly increases. These observations are consistent with a model in which T cell infiltration from the circulation is followed by interstitial migration in the tissue and accumulation at the mucosal epithelium. When virus is cleared, the T cells switch to a rapid surveillance mode characterized by active sampling of cells in their immediate vicinity in the tissue, but relatively limited distance traveled. This is consistent with confinement of the T cells in the epithelial surface. Additional data also suggests that the cells transition to an alpha-1 integrin-dependent tissue resident memory (TRM) state after viral clearance. Collectively we show for the first time direct visualization of CD8 T cells during active viral infection in live animals. This system will be used to investigate the molecular mechanisms that regulate T cell migration and engagement of antigen-bearing target cells, as well as the formation of Tissue resident memory required for secondary immunity.

Evaluation of CD4 T cell responses following heterosubtypic influenza infection or vaccination

Jennifer L. Nayak*, Shabnam Alam and Andrea J. Sant
University of Rochester Medical Center, David H. Smith Center of Vaccine Biology and Immunology, New York Influenza Center of Excellence, Rochester, NY 14642

Humans are repeatedly exposed to influenza viruses containing both novel epitopes that elicit naïve T cell responses and genetically conserved determinants that stimulate memory T cells; however the effect of this potential competition between memory and naïve T cells on the specificity of the anti-influenza immune response is poorly understood. We used a mouse model to evaluate CD4 T cell and antibody responses following heterosubtypic influenza challenge. Striking and selective decreases in CD4 T cell reactivity to nonconserved HA epitopes were present following both secondary influenza infection and secondary intranasal challenge with a cold-adapted influenza virus. Surprisingly, these shifts in CD4 T cell specificity were associated with dramatic decreases in HA-specific antibody. Selective depletion of CD8 T cells had only minor effects on the HA-specific suppression of CD4 T cells, however a decrease in viral load was seen following secondary infection, suggesting that an early and robust memory CD4 T cell response may limit viral replication and contribute to the specificity shifts observed. These results
indicate that simultaneous boosting of CD4 T cells specific for conserved internal proteins may result in less robust HA-specific CD4 T cell responses and concurrent lower titers of neutralizing antibodies upon challenge with vaccines containing highly divergent HA proteins. Pre-pandemic vaccination with highly purified avian HA proteins or HA-based peptide vaccines may promote the development of HA-specific memory CD4 T cells, leading to more rapid and robust neutralizing antibody responses on challenge with a pandemic strain regardless of whether or not serological cross-reactivity is present.

**Regulation of antibody responses to influenza by CD4 T cells: Implications for pandemic preparedness**

Shabnam Alam, Francisco Chaves, Scott Leddon, Jennifer Nayak, Katherine Richards, Jacqueline Tung, Zackary Knowlden and Andrea J. Sant*.
University of Rochester Medical Center, David H. Smith Center of Vaccine Biology and Immunology, New York Influenza Center of Excellence, Rochester, NY 14642

The ever-present threat of pandemic influenza, as well as the toll of seasonal influenza, has prompted intense research to understand how to predict and potentiate influenza-specific immunity. Because of genetic variation in influenza and the need for frequent vaccination, the human influenza-specific immune repertoire will consist of both memory CD4 T cells specific previously encountered peptides, most commonly from internal virion proteins, and naïve CD4 T cells specific for novel epitopes most prevalent in HA and NA. It is not clear how this complex repertoire of CD4 T cells influences the host’s ability to respond to novel influenza viruses and vaccines. We have found that by establishing CD4 T memory enriched in reactivity towards HA, it is possible to potentiate the germinal center response and development of HA-specific B cells and to promote a more rapid production of circulating neutralizing antibody to influenza. These results agree with our earlier studies in human vaccine recipients that demonstrated that expansion CD4 T cells specific for HA correlates best with neutralizing antibody production. Our recent experiments have revealed that memory CD4 T cells specific for highly conserved internal virion proteins antagonize responses to new epitopes contained in HA and NA and that this modification of the CD4 repertoire by pre-existing memory cells is associated with dramatic blunting of the protective anti-HA antibody response. Together with our findings that CD4 T cell specificity can be modified by complex regulatory pathways involving gamma interferon and indolamine oxidase, our data suggest that the specificity of the CD4 T cells present both before and after confrontation with influenza will be a key determinant of whether the elicited immune response is ultimately protective to the host. These findings suggest strategies for pandemic preparedness.

**High resolution analysis of influenza vaccine responses in elderly and pregnant subjects**

David H. Smith Center for Vaccine Biology and Immunology, Departments of Electrical and Computer Engineering, Obstetrics and Gynecology, and Medicine, University of Rochester Medical Center.

Complex, high-dimensional flow cytometry data has not been fully exploited because of the difficulty of analyzing 20+ dimensions manually. We developed a clustering algorithm, SWIFT, that can detect populations at less than one part per million, in more than 25 million cells/sample, with >30 dimensions. We have now used competitive cluster assignment between SWIFT cluster templates to rigorously compare samples and sharpen the detection of differences. Adult and elderly subjects were vaccinated with seasonal influenza vaccine. SWIFT identified PBMC populations that were significantly altered in the elderly, even after Bonferroni corrections for multiple outcomes. Some populations were predicted, e.g. recently-activated Ki-67+ T cells. Importantly, using an agnostic training/test set strategy, SWIFT also detected alterations between adult and elderly subjects that had not been predicted, demonstrating that automated methods can identify novel cell sub-populations in samples too complex for exhaustive manual analysis. A similar analysis of influenza-
vaccinated pregnant subjects revealed an unusual influenza-specific Ki-67+ CD4 T cell population secreting high levels of IL-10, IL-4 and IFN gamma, only in pregnant day 5 samples. Thus the T cell response to influenza vaccination is qualitatively altered during pregnancy. This may be related to the proposed fetoprotective model of a shift in T effector cell responses away from Th1 (or towards Th2) during gestation.

Thus high-resolution automated flow cytometry analysis, particularly using the new competitive method, expands the potential of flow cytometry as a “big data” source. At a resolution of 5-10,000 clusters, flow cytometry approaches the complexity of other high-throughput methods, and cytomics becomes a good complement to transcriptomics, proteomics and metabolomics.

Analysis of influenza virus specific plasma cells in the human bone marrow.

Carl Davis
Microbiology & Immunology, Emory University School of Medicine, Atlanta, GA

Long-lived bone marrow resident plasma cells are thought to be the primary source of serum antibodies, but it is unclear how their numbers are regulated in response to vaccination in humans. We examined influenza-specific plasma cells in the bone marrow of healthy donors before and after seasonal influenza vaccination. Prior to vaccination, bone marrow plasma cell numbers correlated with circulating antibody levels. Vaccination induced a robust increase in bone marrow plasma cell numbers which predicted serum titers several months later. In most donors, influenza specific plasma cell numbers declined to near baseline levels by one year after vaccination, indicating that the majority of vaccine-induced bone marrow plasma cells were short-lived. Clonotypic analysis demonstrated the persistence of certain vaccine-induced plasma cell lineages after one year, indicating that seasonal vaccination can induce both short and long lived bone marrow plasma cell responses. Understanding the factors that determine which plasma cells take up long term residence in the bone marrow will be important for efforts to design a universal influenza vaccine.

Heads or Stalks: Dissecting the Antibody and Memory B cell Responses to Influenza HA

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Broadly neutralizing antibodies are the best protective measure against influenza infection. Neutralizing mAbs directed against conserved epitopes within the influenza HA stalk region have been isolated following human infection with or immunization against influenza. Here, we addressed two important questions; what are the baseline levels of antibodies and memory B cells specific to conserved HA-stalk epitopes? And to what extent these levels get boosted following immunization in comparison to those targeting the conventional HA head epitopes? First, we show that the baseline frequency of stalk-reactive IgG-secreting memory B cells is 5-fold lower than that of those directed against the HA head. Additionally, following either the 2009 pH1N1 monovalent (2009/10) or the TIV (2011/12) immunization, HA stem-specific memory B cells were poorly boosted showing only a 2-fold increase in frequency from day 0 to day 30 post-immunization in comparison to a 5-10 fold increase of their HA head-specific counterparts. Similar to memory B cell responses, anti-HA stalk antibodies, measured by two different strategies, were less abundant and were poorly boosted by immunization in comparison to anti-HA head antibodies. These findings support a model in which the subdominant HA stem-specific memory B cells are outcompeted by the overwhelmingly dominant HA head-specific ones for the limited vaccine antigen. Therefore, rational vaccine design to target memory B cells that are specific to conserved HA epitopes would be the critical next step along the path to a universal influenza vaccine.
Increased expression of lung beta 6 integrin is associated with enhanced inflammation and lung damage during influenza infection

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Integrins are key modulators of cellular function, regulating the innate immune response. The tightly regulated β6 integrin (Itgb6) is unique in that it is only expressed in epithelial cells during development and injury. Several studies have demonstrated a critical role for Itgb6 in controlling acute lung injury and the development of fibrosis through the regulation of transforming growth factor-β (TGF-β). Given that during influenza infection, Itgb6 is upregulated in epithelial cells, and that TGF-β is required during influenza infection, we challenged Itgb6−/− KO mice and found that they were less susceptible to lethal infection. Mechanistically, the Itgb6−/− KO mice have significantly lower pro-inflammatory cytokines in the bronchoalveolar lavage and lower numbers of neutrophils in the lungs, but increased numbers of macrophages, accompanied by improved lung function as measured by edema, permeability, and lung damage/fibrosis. Surprisingly, the Itgb6−/− KO mice had significantly less spread of the virus to the lower lung, although there were no differences in overall titers. Preliminary studies suggest that matrix metalloproteinases (MMPs) secreted by epithelial cells may be responsible. Finally, we found that the Itgb6−/− KO mice were also more resistant to bacterial infections, both primary and secondary to influenza infection. Based on our results we hypothesize that the upregulation of Itgb6 during influenza infection, and potentially other respiratory pathogens, leads to the increased expression of pro-inflammatory cytokines and destructive MMPs leading to increased lung damage and inflammation. Studies are underway to determine if modulation of Itgb6 could be used as a therapy during severe influenza infection.

The recruitment kinetics and functional heterogeneity of monocyte populations during influenza virus infection

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Influenza infection in mice is characterized by waves of recruitment of innate immune cells into the lung. We have previously described TNF-α/iNOS-producing monocytes (TipDC) as promoting the anti-influenza adaptive immune response and contributing to disease pathogenesis. Here, we report the identification of a novel subset of monocytes, distinct from TipDCs and lung resident innate populations at morphology and phenotypic lineage marker, which is recruited into the lung during influenza infection in mice. These monocytes are recruited into the lung mainly at inflammation resolution phase, as opposed to TipDCs that are recruited during acute inflammation phase. The global transcriptional profile of these monocytes showed that they are developing alveolar macrophage phenotypes. Both CCR2 and CX3CR1signaling contributed to the recruitment of this monocyte subset into the lung, but to different extents. Functionally, both these monocytes and TipDC do not have the ability to stimulate CD4 T cell proliferation, and only the monocytes from the resolution phase possessed the ability to suppress CD4 and CD8 T cell proliferation. Interestingly, these cells also induced high levels of Th17-associated cytokines in co-cultures with CD4 T cells. Our results highlight the kinetic changes in monocyte population recruitment and their functional heterogeneity in anti-viral immunity to influenza infection. This heterogeneity may be harnessed for immune modulation strategies and as novel therapeutics for influenza-associated disease.
Catching a moving target: Universal influenza virus vaccine constructs based on the conserved hemagglutinin stalk domain

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Influenza virus infections remain a significant cause of morbidity and mortality worldwide. Current vaccines show good efficacy against antigenically matched viruses, but fail to protect against drifted and pandemic strains since they do not induce broadly neutralizing antibodies. Due to the rapid antigenic drift of influenza viruses, these vaccines have to be re-formulated, generated and administered through a cumbersome and expensive process every year. The membrane-proximal stalk domain of the viral hemagglutinin exhibits a high degree of both, sequence and structural conservation across influenza virus subtypes. Furthermore, antibodies directed against this region typically show broad neutralizing activity. We therefore hypothesize that a vaccine strategy that stimulates a robust immune response towards this region of the hemagglutinin could provide universal influenza virus protection. Such a vaccine would thus abolish the need for annual vaccine reformulation and further enhance our pandemic preparedness. We developed a universal influenza virus vaccine based on the conserved stalk domain of group 1 and group 2 hemagglutinins. By sequential vaccination of mice with these chimeric hemagglutinin constructs we were able to boost broadly neutralizing antibody titers against conserved epitopes in the hemagglutinin stalk domain. Mice vaccinated with our constructs were protected from morbidity and mortality induced by infection with a panel of heterologous and heterosubtypic influenza A viruses. Further, we used passive transfer and CD8⁺ T-cell depletion experiments to show that the observed protection is solely antibody mediated. It is of note that our vaccination regimen protected animals from a stringent H7N1 challenge and also induced high titers of antibodies that reacted strongly with the H7 HA protein derived from the H7N9 strain A/Shanghai/1/13. These findings are of high importance in the light of the emergence of the novel H7N9 strain in China. The present data suggest that this vaccine strategy could be successfully developed in humans to provide broad influenza virus protection and enhance our pandemic preparedness. A universal influenza virus vaccine, which - similar to the ones developed for polio and measles viruses - requires a single or only a few immunizations, would represent a major advance towards the control of influenza worldwide.

Unanchored Lysine48-linked polyubiquitin chains positively regulate the type I IFN-mediated antiviral response

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Type-I interferons (IFN-I) are essential antiviral cytokines produced upon microbial infection. IFN-I elicits this activity through the upregulation of hundreds of IFN-I stimulated genes (ISGs), many of which have known antiviral activity. The full breadth of ISG induction requires activation of a number of cellular factors including the IkB kinase epsilon (IKKe). However, the mechanism of IKKe activation upon viral infection or IFN receptor signaling remains elusive. Here we show that TRIM6, a member of the E3-ubiquitin ligase tripartite motif (TRIM) family of proteins, interacts with IKKe and promotes induction of
IKKε-dependent ISGs that are essential for establishment of an efficient antiviral response to influenza virus. Some studies have suggested a role for unanchored K63-linked polyubiquitin chains, which are not conjugated to any protein, in regulation of kinase activity. However, no role has yet been established for unanchored K48-linked polyubiquitin chains in kinase activation. We show that TRIM6 and the E2-ubiquitin conjugase UbE2K cooperate in the synthesis of unanchored K48-linked polyubiquitin chains, which activate IKKε for subsequent STAT1 phosphorylation. Mechanistically, these unanchored ubiquitin chains promote IKKε oligomerization and autophosphorylation, required for downstream signaling. Our work defines a previously unrecognized activating role of K48-linked unanchored polyubiquitin chains in kinase activation and identifies the UbE2K-TRIM6-ubiquitin axis as critical for IFN signaling and antiviral response.

Session #3: Transmission & Adaptation

Use of haemagglutination inhibition for the detection of HPAI virus exposure in wild bird populations

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Surveillance for highly pathogenic avian influenza viruses (HPAIV) in wild birds is logistically demanding due to the very low rates of virus detection. Serological approaches that require smaller sample sizes to identify exposed populations may be a more cost effective strategy. We hypothesized that antigenic differences between H5 subtype low pathogenic avian influenza viruses (LPAIV) and H5N1 HPAIVs may be used to differentiate populations where HPAIVs have been circulating, from those where they have not. To test this we performed hemagglutination inhibition (HI) assays to compare the HI titers of field-derived serum samples from wild birds in Mongolia (where HPAIV has been circulating) and northern Europe (where HPAIV has been rare or absent) to a panel of reference viruses including classic LPAIV H5, and five HPAIV H5N1 antigens of the Asian lineage A/Guandong/1/96. A clear bias in HI titers to HPAIV antigens was found in the Mongolian sample that was absent in the European sera. This suggests that a proportion of the Mongolian population has been surviving exposure to HPAIV, and that serological assays may enhance the targeting of traditional HPAIV surveillance toward populations where isolation of HPAIV is more likely.

The role of the matrix protein in facilitating transmission of influenza virus

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The 2009 pandemic virus (pH1N1) spread efficiently among humans, with an estimated 60 million cases of infection occurring between April 2009 and April 2010 in the US. The virus emerged following reassortment of strains from two distinct lineages (N. American TRIG lineage, which donated 6 segments to the pandemic strain, and Eurasian “avian-like” swine lineage (EAsw), which donated M and NA segments). Of note, viruses derived from either lineage are poorly transmissible between humans. Experiments were performed with reassortant viruses in which M, NA, M/NA, NA/HA or M/NA/HA segments from the pH1N1 strain A/NL/602/09 (NL602) were introduced into the background of A/Puerto Rico/8/34 (PR8). PR8/NL602 M+NA+HA virus recapitulated the replication, virion morphology, and contact transmission phenotype (in guinea pigs), of the rNL602wt strain. All other reassortant viruses showed reduced transmission, and altered virion morphology. Additionally, PR8/NL602 M virus exhibited significant contact transmission (12/16 animals) while a PR8-based virus containing the M segment from EAsw lineage exhibited lower replication and slightly reduced transmission between guinea pigs.
Additionally, the NA activity of virion preparations, and virion morphology, were distinct between these two strains. A reassortant TRIG:pH1N1 (5:3) virus, KAN07/NL602 M+HA+NA recapitulated the wt rNL602 transmission phenotype. In comparison, KAN07/EAsw M/NL602 HA+NA virus had lower peak replication and slightly delayed kinetics of transmission. Nonetheless KAN07/EAsw M and Kan07/EAsw M+NA viruses showed improved transmission relative to rKAN07 wt virus. Interestingly, viruses with NL602 M+HA+NA or EAsw M+NA showed lower infectious dose, and transmitted from lower inoculation dose in guinea pigs than A/swine/Kansas/77778/07.

**Avian A(H7N9) virus; receptor binding, fusion, ferret transmission**

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Recently, avian-origin A/H7N9 viruses were transmitted to humans, causing severe respiratory tract infections and deaths in China. A/H7N9 viruses harbour genetic traits that have been associated with human adaptation of avian viruses and increased transmission between mammals. However, no sustained human-to-human transmission of A/H7N9 viruses has been reported to date. We assessed the airborne transmissibility of an A/H7N9 human isolate, A/Anhui/1/13 (AN1), in the ferret model. The experimental set-up is designed to prevent direct contact between donor and recipient animals but to allow transmission via the airborne route. AN1 virus was transmitted to 3 out of 4 recipient ferrets. Virus recovered from a recipient ferret was used for a subsequent transmission experiment, in which 1 out of 4 recipient ferrets became infected. Next generation sequencing revealed a rapid selection of substitutions N123D and N149D in HA and M523I in PB1 after two subsequent transmission experiments. Residues N123 and N149 are adjacent to the receptor binding site but do not interact directly with 2.3 and 2.6 linked sialic acids. Binding to α2.3 linked sialic acids of AN1N123D, AN1N149D and AN1N123D,N149D viruses was increased slightly by 2 to 4-fold compared to AN1 virus. No change in HA mediated cell-to-cell fusion nor in HA stability thought to increase transmission were conferred by the substitutions N123D and N149D. Here, we showed that the airborne transmissibility of an A/H7N9 human isolate, A/Anhui/1/2013, in the ferret model appears to be limited, intermediate between that of typical human and avian viruses and that there is no evidence for natural selection of viruses with increased transmissibility. A/H7N9 virus outbreak highlights the need for increased understanding of determinants of efficient airborne transmission of avian influenza viruses between mammals.

**Transfection-based inoculation to understand H9 influenza virus transmission in the ferret model**

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Influenza A H9N2 viruses are a common poultry pathogen that regularly infects swine and humans. It has been shown previously that H9N2 viruses are capable of reassortment and can generate novel viruses with increased transmissibility and pathogenicity. Here we demonstrate the modeling power of a novel transfection based inoculation system to select a reassortant under in vivo selective pressure. Plasmids containing the genes from an H9N2 and a pandemic H1N1 (pH1N1) were transfected HEK 293T cells to generate the full panel of possible H9 reassortants. These cells were then used to inoculate ferrets and population dynamics were studied. An aerosol transmissible H9N1 was selected by our method, indicating a selective
pressure in ferrets for the novel combination of surface genes. These results show that a transfection
based inoculation system is a fast, efficient method to model reassortment and highlight the risk of a
reassortment between H9N2 and pH1N1. *author

Influence of HA acid stability on the interspecies adaptation of emerging influenza viruses

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The prefusion form of the HA surface glycoprotein is trapped in a metastable conformation and triggered
by low pH to undergo irreversible structural changes that cause membrane fusion in the endosome. Our
surveillance studies reveal that HA proteins from human seasonal influenza viruses are activated at pH
5.1-5.4 while those from highly pathogenic avian H5N1 influenza viruses are activated at pH 5.5-6.0. Using sequence analysis, x-ray crystallography, and biochemistry, we have identified numerous
mutations that alter the HA activation pH of H5N1 influenza viruses. Using reverse genetics, we have
generated recombinant H5N1 influenza viruses that vary in HA activation pH but not in other properties
such as HA protein expression, cleavage, or receptor binding. We have found that the optimal HA
activation pH for high H5N1 virus growth, virulence, and transmission in avian species (including
chickens and ducks) is ~5.6-6.0. An HA2-K58I mutation that decreases the H5N1 HA activation pH from
5.9 to 5.4 was found (a) to attenuate virus growth and eliminate virulence and transmission in ducks, and
(b) enhance virus growth in the upper respiratory tracts of mice and ferrets. Overall, HA acid stability is
identified as a novel molecular marker for influenza virus adaptation to mammals and constitutes an
important risk factor for pandemic potential. Ongoing experiments are elucidating the role of HA acid
stability in the interspecies adaptation of H1N1 and H7N9 viruses.

Adaptation of an avian-like H2N3 virus in pigs

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How avian influenza viruses (AIVs) adapt to a mammalian species is not fully understood yet. We
reported the first H2 subtype influenza mammalian isolate in the past 40 years which was isolated from
diseased pigs in 2006. This swine H2N3 virus was partially adapted to the mammalian host having the
mammalian-like signature leucine (L) at position 226 and the avian-like signature Glycine (G) at position
228 (226L/228G) within the HA receptor binding site (RBS). To explore how avian influenza viruses
adapt to pigs, an H2N3 mutant containing an avian-like HA RBS defined by 226Q and 228G was
generated and passaged in cultured cells and in pigs. The avian-like 226Q/228G HA was maintained for
8 serial passages in either MDCK or MDCK-SIAT1 cells, and for 12 passages in swine PK-15 cells. In
contrast, a mammalian-like 226L/228G adaptation was found in lung lavage from H2N3 mutant infected
pigs already 5 days post infection. These results showed that the HA Q226L substitution occurred in the
pig respiratory tract, but not in cultured cells. These data suggest that factors present in the live pigs (e.g.
target cells, immune system, receptors etc) enable adaptation of an avian-like H2N3 virus to mammalian-
like influenza receptors. Our results confirm that swine play an important role in adaptation of AIVs to
mammalian hosts.
Session #4: Molecular Virology

Structural determinants of HA stability: The ups and downs of fusion pH

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Recent studies have highlighted potential roles for HA stability in transmission, adaptation, and pathogenicity, and we have now characterized well over 100 mutant HAs that either stabilize or destabilize the glycoprotein. These include representatives of all avian subtypes, and numerous HAs derived from human isolates. The locations of mutations that alter the fusion pH map to several different regions of the HA structure, but their mechanisms of action can clearly be interpreted based on biochemical, antigenic, and high resolution structural data. Group-1 and Group-2 HAs show significant differences in domains that rearrange during fusion, and data derived from numerous assays for acid-induced structural rearrangements and membrane fusion phenotype indicate that Group-1 HAs display slower fusion kinetics than Group-2 representatives. The structures of acid-stable and acid-labile HA mutants provide insights regarding the phenotype, and further mutational analysis identifies residues that may be critical for initiating the acid-induced structural rearrangements. Overall, we attempt to relate the structural and functional data to biological properties, as well as resistance to antiviral compounds.

Functional analysis of PA-X protein in host shutoff

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Influenza infection causes host protein synthesis shutoff while allowing viral protein production. Inhibition of cellular protein synthesis is expected to aid in dampening the anti-viral response and immune response. Therefore, it is considered to be a major factor that determines viral pathogenicity and immunogenicity. Recently, Jaggar et al., reported that a novel viral protein (PA-X) is expressed from PA mRNA by frame-shifting, and shuts off host gene expression (Science 337:199-204). We found that avian virus PA-Xs are more active than those of human viruses in shutoff activity, and various regions in PA-X are responsible for the difference (Desmet et al., J. Virol 87:3108-18). To further analyze the functions of PA-X protein, we rescued A/California/04/09 containing mutations at the frame-shift motif (Cal-PAXFS). Host shutoff activity of Cal-PAXFS was reduced compared with wt Cal, showing a direct correlation between PA-X expression and host shutoff in infected cells. PA-X contains an endonuclease domain, and mutations at the active site completely eliminated shutoff activity, suggesting that mRNA degradation is responsible for host shutoff by PA-X. PA-X inhibited luciferase protein production expressed from cDNAs, but not from transfected capped mRNA, suggesting that PA-X targets transcription by RNA polymerase II, but not translation of mRNAs. Characterization of various truncation mutants indicated that unique PA-X C-terminal domain is required for the shutoff activity. Our results clearly indicate the role of PA-X in virus-induced host shutoff, which is likely to contribute to viral pathogenicity and escape from immune recognition of influenza viruses.

Influenza A virus NS1 and PI3K: strain and isotype specificity of a complex virus-host interaction

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The NS1 protein of influenza A viruses is a multifunctional virulence factor with a broad array of interactors within the infected cell. Amongst them, NS1 is known to bind and activate the host class IA
phosphoinositide-3-kinase (PI3K), a critical regulatory node in multiple cell signaling networks that influences cell physiology at various stages including cell growth, survival, trafficking and immune function. The biological purpose of the activation of PI3K by NS1 remains, however, unclear. Here, we show how this activation contributes to the virus replication and virulence in vivo and how this relevance is viral strain specific despite all the NS1 tested being equally able to activate PI3K. Furthermore, we have found that there is an additional layer of specificity within the host factor itself: class I PI3K are obligate heterodimeric enzymes composed of a regulatory, inhibitory subunit (mainly p85α or p85β) and a catalytic subunit, p110, with three isotypes designated α, β and δ. NS1 is known to specifically bind and repress the inhibition caused by p85β. Here, we show that NS1 differentially redistributes and activates heterotypic PI3K complexes depending on their catalytic subunit isotype. We postulate that different NS1 strains induce heterotypic PI3K complexes to signal from distinctive platforms and through different pathways, thus affecting overall viral fitness in varying degrees. Our findings suggest that activation of PI3K by influenza A virus NS1 has been diversely shaped through evolution in distinct viral strains to take advantage of the variability within PI3K signaling, providing a challenging example of a complex and multi-variant virus-host interaction.

H7N9 influenza viruses bind preferentially α2,3-linked sialic acids and induce activation of primary human dendritic cells.

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The recent human outbreak of H7N9 avian influenza A virus has caused worldwide concerns. Receptor binding specificity is critical for transmission and viral pathogenicity, and still not thoroughly studied for this emerging virus. Analysis of the first three available hemagglutinin (HA) sequences from the novel Chinese H7N9 viruses (A/Shanghai/1/13, A/Shanghai/2/13 and A/Anhui1/13) indicated the presence of mutations in the receptor binding site (RBS) that are associated with enhanced binding of H5 and H7 HAs to α2,6-linked sialic acids (SAα2,6). Interaction with SAα2,6 is usually associated with high levels of replication in the human upper respiratory tract which facilitates aerosol-based transmission. In this study we evaluated the receptor specificity of the HA of two human H7N9 isolates (A/Shanghai/1/13 and A/Anhui/1/13) through a solid phase binding assay and a flow cytometry based assay. Also, we compared it with those from several HAs from human and avian influenza viruses. We observed that the HA from the novel H7 isolates strongly interacted with α2,3-linked sialic acids (SAα2,3). Importantly, they also showed low levels of binding to α2,6-linked sialic acids, but significantly higher than other avian H7s. Finally, we evaluated the impact of this unique binding specificity on viral pathogenicity through characterizing the cytokine profile induced by an H7N9 bearing virus in human dendritic cells.

Neuraminidase inhibitor oseltamivir protects mice against lethal challenge with A/Anhui/1/2013 (H7N9) influenza virus

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High mortality rates of humans infected with A (H7N9) influenza viruses and limited information about the effectiveness of neuraminidase inhibitors (NAIs) are of public health concern. Here, we determined the susceptibility of 15 avian influenza viruses to NAIs in vitro, and developed a mouse model to address the antiviral activity of NAI oseltamivir. All 15 N9 avian influenza viruses tested were susceptible to NAIs with IC50 values ranging from 0.3 to 1.6 nM. Influenza A/Anhui/01/2013 (H7N9) virus caused lethal infection in BALB/c mice without prior adaptation with an MLD50 of ~102.5 PFU. In early post-exposure regimen,
administration of 80 mg/kg/day oseltamivir resulted in complete protection and significant reduction of virus replication in the lungs of mice. 24 hours delayed treatment with 20 and 80 mg/kg/day oseltamivir protected 80% and 90% of mice, respectively. When administrated 48 hours p.i. 60% mice survived in both groups. Analysis of arterial blood gases revealed hypoxemia and acute respiratory acidosis as early as 3 day p.i., with the resolution of hypoxemia observed in a dose-dependent manner. Sanger sequencing and cloning analysis revealed a lack of emergence of the oseltamivir-resistant variants. Thus, influenza A (H7N9) viruses are comparable to currently circulating seasonal viruses in susceptibility to NAI oseltamivir in vitro and in a mouse model. Early initiation of NAI treatment is crucial in achieving high levels of protection.

Fecal influenza: selection of novel variants?

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In aquatic birds, influenza A viruses mainly replicate in the intestinal tract without significantly affecting the health of the host, but in mammals they replicate in the respiratory tract and often cause disease. Occasionally, influenza viruses have been detected in stool samples of hospitalized patients and in rectal swabs of naturally or experimentally infected mammals. In this study, we compared the biological and molecular differences among four wild-type avian H1N1 influenza viruses and their corresponding fecal and lung isolates in DBA/2J and BALB/cJ mice. All isolates were more pathogenic than the original wild-type viruses, when inoculated into mice of both strains. The increased virulence was associated with the acquisition of genetic mutations. Most of the novel genotypes emerged as PB2 E627K or HA F128V, F454L, or H300P variants, and double-mutants frequently occurred in the same isolate. However, influenza strain- and host-specific differences were also observed in terms of selected variants. The avian H1N1 virus of shorebird origin appeared to be unique in its ability to rapidly adapt to BALB/cJ mice via the fecal route, compared to the adaptability of the H1N1 virus of mallard origin. Furthermore, bimodal distribution was observed in fecal shedding in mice infected with the fecal isolates, while normal distribution was observed after infection with the lung isolates or wild-type virus. Fecal isolates contained HA mutations that increased the activation pH of the HA protein. We conclude that influenza variants that emerge in fecal isolates in mammals might influence viral transmission, adaptation to mammals, and viral ecology or evolution.

Session #5: Pathogenesis

Requirements for efficient influenza virus reassortment

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Reassortment is central to the evolution of influenza viruses and frequently drives the emergence of novel epidemic and pandemic strains. Previous studies indicate that reassortment is restricted by segment mismatch, arising from functional incompatibilities among the components of two viruses. Additional factors that dictate reassortment efficiency remain poorly characterized. To examine these additional factors, we developed a system for studying reassortment in the absence of segment mismatch. Silent mutations were introduced into A/Panama/2007/99 virus such that high-resolution melt analysis could be used to differentiate all eight segments of the wild-type and the silently mutated variant virus. The use of phenotypically identical parent viruses ensured that all progeny were equally fit, allowing measurement of reassortment without selection bias. Using this system, we found that reassortment occurred with approximately 90% efficiency following high multiplicity infection, suggesting that the process is not appreciably limited by intracellular compartmentalization. That co-infection is the
major determinant of reassortment efficiency in the absence of segment mismatch was confirmed by an exponential relationship between the frequency of reassortant viruses and that of co-infected cells. The number of reassortant progeny shed from co-infected guinea pigs was likewise dependent on dose: with 103 and 106 PFU inocula, respectively, 30% and 59% of isolates collected at 2 dpi were reassortants. The introduction of a delay between infections allowed definition of time windows during which super-infection led to reassortment in culture (8 h) and in vivo (18 h). Overall, our results indicate that reassortment between two like influenza viruses is efficient but also highly dependent on dose and timing.

Molecular risk mitigation of gain-of-function studies with influenza viruses

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Gain-of-function studies have elucidated the minimal amino acid changes that are sufficient to permit aerosol transmission of H5N1 between mammals. To address concerns regarding the risks and benefits of this type of research, endogenous miRNAs were explored as a means to confer intrinsic molecular biocontainment to influenza viruses, and to provide an added level of risk mitigation to studies assessing pathogenicity and transmissibility of zoonotic influenza viruses. The H3 and H5 genomic sequences encoded by A/Wyoming/3/2003 (H3N2) and A/Vietnam/1203/2004 (H5N1), respectively, were genetically modified to insert scrambled non-coding RNA or four fully complementary miR192 target sites. Target sequences for miR192 were selected as this miRNA is expressed in lung tissues of mice and humans but is not expressed in lung tissue of ferrets which is the classic animal model used for influenza virus transmission studies. Whereas in vitro replication of wild-type and control influenza viruses were similar in human lung epithelial cells, the replication of miR192-targeted viruses was attenuated. Northern blot and western blot analyses revealed a correlation between the level of endogenous miRNA expression and the degree of knockdown of H3 or H5 expression. Whereas miR192-targeted H5N1 influenza virus did not induce morbidity or mortality following intranasal infection of mice, wild-type and control virus infection resulted in significant morbidity and all animals succumbed to infection. However, in ferrets which do not express miRNA192 in lungs wild-type, control, and miR192-targeted H3N2 influenza viruses replicated and transmitted with comparable efficiencies. Incorporation of species-specific microRNA target sites in the influenza virus HA results in unperturbed replication and transmissibility in desired species, while eliminating pathogenicity in others. This strategy introduces a new biosafety approach for the molecular biocontainment of pathogens that can be added to present risk-minimizing strategies.

Susceptibility of swine to A/Anhui/1/2013 H7N9 virus (Anhui/1)

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The emergence in early 2013 of the H7N9 influenza A virus in China demonstrates again the dynamic ecology of influenza viruses. New regulations for live bird markets in April were quickly followed by a decline in human infections indicating the source of infections may have been related to these markets.
This avian-human linkage represents one of two predominant pathways for zoonotic transmission of influenza A viruses, the other involves swine. There are no reported natural infections of swine with this recent H7N9 lineage. The study objective was to determine the susceptibility of swine to infection with the Anhui/1 virus, and the potential for transmission to direct and indirect contacts (5 pigs each). Fifteen 4-week-old pigs were intranasally inoculated with $1 \times 10^6$ EID$_{50}$ virus at 0 days-post-inoculation (dpi). Five challenge pigs were necropsied at 3 and 5 dpi, and all remaining pigs were necropsied at about 24 dpi. Nasal swabs were collected at selected times as well as bronchoalveolar lavage fluid at necropsy to test for virus by PCR and virus isolation. Serum was tested for influenza A antibody by ELISA. No clinical disease was recognized and only minor lung lesions were observed at 3 and 5 dpi. Although the experiment has not been completed, preliminary results indicate most challenged pigs did replicate virus, and all 5 pigs at 14 dpi were antibody positive indicating swine are susceptible to this lineage of virus, but this isolate is mildly pathogenic. Results are pending on the direct and indirect contact pigs.

Characterization of Novel Influenza A(H7N9) Viruses

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Since February/March 2013, more than 130 people have been infected with a previously unrecognized avian influenza A virus of the H7N9 subtype (A(H7N9)). These infections have caused concern due to the appreciable case fatality rate (>25%), potential instances of human-to-human transmission, and the lack of pre-existing immunity among humans to these viruses. We, therefore, characterized two early human A(H7N9) isolates, A/Anhui/1/2013 (Anhui/1) and A/Shanghai/1/2013 (Shanghai/1). These viruses replicated efficiently in the respiratory tract of mice, ferrets, and nonhuman primates. Importantly, Anhui/1 transmitted via respiratory droplets in one of three pairs of ferrets. Glycan arrays (carried out by Dr. Paulson, The Scripps Research Institute, La Jolla, CA) demonstrated that Anhui/1, Shanghai/1, and also A/Hangzhou/1/2013 (another human A(H7N9) virus) bind to human virus-type receptors, a property that may be critical for virus transmissibility in ferrets. These findings suggest that A(H7N9) viruses have pandemic potential.

Characterization of the R292K mutation that confers resistance to the neuraminidase inhibitors in a novel H7N9 human isolate

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We characterized the A/Shanghai/1/2013 virus isolated from the first confirmed human case of A/H7N9 disease in China. The A/Shanghai/1/2013 isolate contained a mixed population of R (65%; 15/23 clones)/K (35%; 8/23 clones) at the neuraminidase (NA) residue 292, as determined by clonal sequencing. A/Shanghai/1/2013 with mixed R/K at residue 292 exhibited a sensitive phenotype to zanamivir and oseltamivir carboxylate by the enzyme based NA inhibition assay and to zanamivir in vitro. The plaque purified A/Shanghai/1/2013 with dominant K292 (94%; 15/16 clones) showed decreased sensitivity to zanamivir by 30-fold and to oseltamivir carboxylate by >100-fold compared to its plaque purified wild-type counterpart possessing dominant R292 (93%, 14/15 clones). In MDCK cells, the plaque purified A/Shanghai/1/2013-NA$^K$ virus exhibited no reduction in viral titer under increasing concentrations of oseltamivir carboxylate (range 0-1000 μM), whereas the replication of the plaque purified A/Shanghai/1/2013-NA$^R$ and the A/Shanghai/2/2013 viruses were completely inhibited at 250 μM and 31.25 μM of oseltamivir carboxylate, respectively. Although the plaque purified A/Shanghai/1/2013-NA$^K$ virus exhibited lower NA enzyme activity and a higher $K_m$ for 2'-4-
methylumbelliferyl)-α-D-N-acetylneuraminic acid than that of the wild-type A/Shanghai/1/2013-NA^{R292}

virus, the A/Shanghai/1/2013-NA^{K292} virus formed large plaques and replicated efficiently in vitro. Our
results confirmed that the NA R292K mutation confers resistance to oseltamivir and zanamivir in the
novel human H7N9 viruses. Importantly, detection of the resistance phenotype may be masked in the
clinical samples containing a mixed population of R/K at NA residue 292 while applying the in vitro based
assay or the fluorescence based NA inhibition assay.

Pathogenesis and transmission of novel influenza A (H7N9) virus in poultry

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The recent outbreak of H7N9 influenza in China has resulted in many human cases with a high fatality
rate. Poultry is suspected as the source of infection based on sequence analysis and virus isolation from
live-bird markets, but it's unclear which species of birds are most likely to be infected and shedding
sufficient levels of virus to infect humans. In our recent experimental trials, intranasal inoculation of
chickens, turkeys, Japanese quail, pigeons, Pekin ducks, Mallard ducks, Muscovy ducks, and Embden
geese with $10^6$ EID₅₀ of the A/Anhui/1/2013 virus resulted in infection but no clinical signs. Virus
shedding in quail, chickens, and Muscovy ducks was much higher and prolonged than in the other
species tested. Quail effectively transmitted the virus to direct contacts but pigeons and Pekin ducks did
not. In all poultry species tested, virus was detected at much higher titers from oropharyngeal swabs than
cloacal swabs. The HA gene from samples collected from infected chickens and quail were sequenced to
examine changes in the virus after passage in these species. Three amino acid differences were
observed when compared to A/Anhui/1/2013: N123D, N149D, and L217Q. Different combinations were
present indicating most likely that the inoculum contained virus subpopulations that were selected after
passage in birds. In conclusion, these experimental studies corroborate that poultry species are an
important reservoir of the H7N9 virus. The high level of viral replication in the upper respiratory tract is
characteristic of poultry-adapted influenza viruses, and consequentially testing of bird species should
preferentially be conducted with oropharyngeal swabs for highest sensitivity.

Phenotyping swine-origin H3N2 influenza A viruses from Ohio agricultural fairs in relevant animal
species

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Viral phenotyping is critical to understand the threat presented by any influenza isolate to public health.
We have developed an approach to phenotyping that characterizes the capability of an influenza A viral
isolate to emerge in a series of hosts that have the potential to play a variety of roles in the emergence of
a human pathogen. As an example of this approach, results from the phenotyping of H3N2 swine
influenza virus isolates from Ohio agricultural fairs from 2009 to 2012 will be presented. The phenotyping
results of viruses that appeared in swine prior to the occurrence of human cases and those that were the
sources of human infections will be presented. The outcomes of both in vivo and in vitro phenotyping
methods in relevant animal host species for early and later viruses will be discussed.
Risk analysis of H2N2 viruses from the avian reservoir

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The H2N2 pandemic of 1957-58 caused significant morbidity, mortality and economic burden worldwide. This subtype disappeared from human circulation after only a decade resulting in a present day population that is largely naïve to the H2 antigen. Historical evidence demonstrates the 1957 pandemic viruses arose from avian virus ancestors, and H2N2 infections persist in wild and domestic birds. The potential for reemergence in humans, particularly from the avian reservoir, must be addressed through surveillance, characterization, and antiviral testing. The goal of these studies was to understand the potential risk of avian H2N2 isolates to mammals by evaluating a panel of 22 viruses for the following criteria: 1) pathogenicity, virulence and transmission \textit{ex vivo} and \textit{in vivo}, 2) antigenicity and receptor binding phenotypes, 3) and susceptibility to current antiviral drugs. All viruses remained antigenically similar to avian H2 viruses isolated over the past 50 years and to human pandemic viruses; however, they were dissimilar to human viruses from the end of the H2N2 circulation period. All viruses showed a preference for the \(\alpha_2\)-3–linked sialylglycopolymers over those with \(\alpha_2\)-6–linkages. Despite antigenic and receptor binding patterns typical of avian viruses, most isolates were pathogenic in primary human cells and mice without prior adaptation. Replication in swine tracheal tissues was low or absent. Several isolates were able to replicate in ferrets (5/9 tested) and transmit to cage mates (3/9), but no aerosol transmission was observed. M2 gene sequence analysis and neuraminidase inhibition assays suggest that all viruses are susceptible to both adamantanes and neuraminidase inhibitors. The pathogenicity of these avian H2N2 viruses in multiple mammalian models elevates their risk potential. However, all viruses remain highly susceptible to FDA-approved antiviral drugs, suggesting they may be used as a control strategy should H2 viruses emerge once again in humans.

Surveillance in Delaware Bay

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Surveillance for avian influenza virus (AIV) in migrating shorebirds has been performed annually by St. Jude Children’s Research Hospital (SJCRH) at Delaware Bay since 1985. In 2000 a concurrent AIV surveillance project to study the ecology of influenza viruses at Delaware Bay was undertaken by the University of Georgia (UGA). A formal collaboration between SJCRH and UGA uniting these studies was established in 2008 under the auspices of the NIAID Centers of Excellence for Influenza Research and Surveillance. Collectively, results from these studies have established that Delaware Bay becomes a seasonal hotspot for AIV when influenza virus amplification is fueled by infection of ruddy turnstones.
Sample collection strategies, virus detection and isolation methods, and virus identification assays were cross-validated by the two studies. AIV subtypes were found to be diverse and variable from year-to-year. Pursuit of a full understanding of the natural history of AIV and the ecology of Delaware Bay will continue collaboratively into the foreseeable future.

Session #2: Data and Surveillance Tools

Animal Surveillance Data in the Influenza Research Database (IRD)

The Influenza Research Database Team\textsuperscript{1,2,3,4,5,6,7}

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The Influenza Research Database (IRD, www.fludb.org) is a freely available, one-stop database and analysis resource to support influenza research developed through the NIAID Bioinformatics Resource Center program. Through a close collaboration with the Centers of Excellence of Influenza Research and Surveillance (CEIRS) network, the IRD has developed a comprehensive infrastructure to support standardized avian and non-human surveillance data. Of the 229,827 avian and 22,047 non-human mammalian specimens tested through the CEIRS program whose data is supported in IRD, 15,502 (6.7\%) and 1097 (5.0\%) were found to contain detectable influenza virus by culture and/or PCR based assays, respectively. Of the flu positive specimens, 2369 avian (15\%) and 224 non-human mammalian (20\%) complete genome sequences have been determined and are available for comparative genomics sequence analysis in IRD. Because the surveillance records are described using structured and standardized metadata, reliable comparison of flu prevalence are now possible. For example, in the case of avian hosts, Anseriformes (ducks, geese, swans) and Charadriiformes (waders, gulls) appear to be important wild bird reservoirs. But the question arises as to whether they are able to support the spread of all influenza subtypes equally. A preliminary comparison of all HA and NA subtype combinations revealed that major differences exist in the subtype distribution of influenza A virus found in these avian orders, with the predominant H3N8 subtype found in Anseriformes, virtually absent in Charadriiformes. (Anseriformes - total 2520 records: H3N8 - 550 records > H4N6 - 438 records > H10N7 - 165 records > H6N1 - 126 records; Charadriiformes - total 360 records: H10N7 - 49 records > H4N6 - 31 records > H12N5 - 26 records > H16N3 - 22 records > H13N8 - 16 records > H3N8 - 3 records). While additional analysis will be required to control for sampling bias and confounding factors that may contribute to these numbers, it is likely that significant skewing in the distribution of influenza A subtypes in different wild bird species is being observed, suggesting that host range restrictions exist between avian species.

Mapping transmission of influenza virus during an infection peak in wild ducks

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Wild birds are the major reservoir for avian influenza virus (AIV) however their precise role in the spread and evolution of the virus remains unclear. Studies of AIV evolution that rely on analysis of publicly available sequences have advanced our broad understanding of viral dynamics in nature. However mechanisms that drive virus gene flow in wild birds, remain elusive without consideration of host ecological ‘metadata’. The influence of host ecology, physiology and behaviour on transmission represents a large knowledge gap in part because virology and ecology remain two disparate fields that rarely overlap during the investigation of AIV in wild birds. We sampled wild ducks at a major breeding ground in North America – Minto Flats, Interior Alaska. Our goal was to sample ducks during the peak of
infection to characterize infection patterns at high temporal resolution. We sampled over 15,000 ducks between 2009-2010, collecting swabs, bloods and morphological measurements achieving the densest sampling of a wild bird population to date. Using phylogenetic analyses, an index for interspecies transmission between wild ducks was generated for each subtype. In addition, the rates of genetic shift (reassortment) or genetic drift (point mutations) were assessed to determine the primary evolutionary dynamic shaping the genetic structure of subtypes. This metric is being developed as a potential predictor of subtypes likely to switch hosts and infect other wild bird species or poultry.

**Session #3: Human and Human/Animal Interface Surveillance**

**Human-animal interface studies: what have we learned and what we need to learn**

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Over the past decade, there have been several spillover events documenting the importance of understanding the human-animal interface for influenza virus transmission. Specific challenges include H5N1 and the recent detection of H3N2v in the United States and H7N9 in China. These events have highlighted the need for appropriate response, coordination, and updated policies. Efforts to respond to such incidents can result in economic consequences, consumer education challenges, and stakeholder coordination issues. Often, new partnerships and coordination are needed to address our established misperceptions about viral, reservoir, and human behavior. These ongoing challenges will require us to take a fresh look at our prevention, education, and engagement strategies.

**Household Transmission of Influenza in Nicaragua**

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The Nicaraguan Household Transmission of Influenza Study is a case-based ascertainment study of influenza transmission in Managua, Nicaragua. Index influenza cases who are positive for influenza by rapid test within 48 hours of illness onset and are the only person in their household who has had influenza-like symptoms in the previous two weeks are identified and enrolled. The index case’s household is then visited by study personnel that day, and all household members are invited to participate in the study. At enrollment, study staff record detailed information about clinical history, socioeconomic status, and individual and household risk factors and collect a respiratory sample. Study staff then visit the household every other day to collect clinical information and a respiratory sample for a total of five visits. A blood sample is collected at enrollment and 30-45 days later to assess influenza infection and nutritional status. To date, we have enrolled 77 participants from 12 households. Study acceptance among household contacts has been consistently high, and 91% of the potential participants approached gave consent for participation in the study. Participants ranged in age from 0 to 65 years old, with a mean age of 21.2 years. A total of 341 respiratory samples have been collected during household visits, with an average of 4.4 respiratory samples per participant. Of the 12 index cases, 11 tested positive for influenza by RT-PCR. Of the 63 household contacts of influenza-positive individuals, 10 were RT-PCR-positive for influenza virus during the 10-day follow-up period, yielding a secondary attack rate of 15.9%. This study will provide important information about the transmission of influenza in a tropical urban area.
Integrated Surveillance of H7N9 in Human Cases and Avian Species in Taiwan

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The emerging epidemics of the highly pathogenic avian influenza (HPAI) H5N1 virus since 2005 and the pandemic influenza H1N1 virus in 2009 plus recent outbreaks of H7N9 in China all have enlighten us to emphasize the crucial importance of integrated surveillance and timely genetic analyses of the influenza viruses isolated from human and animal hosts. Unfortunately, the first case of H7N9 outside China occurred in Taiwan. To have better integrated surveillance and data analyses, the viral sequences of the two avian H7N9 strains, and the selected 35 H7 and 12 N9 viruses isolated from wild birds in different years from February of 1998 to March of 2013 in Taiwan were compared with those viral sequences obtained from NCBI of the human H7N9 throat and sputum isolates. The results showed that the avian H7N9, H7 and N9 viruses isolated from wild birds flying into Taiwan were quite different from the Taiwan’s human H7N9 isolate. In addition, The HA sequences of these wild bird isolates revealed temporal variations with various groupings of phylogenetic trees by different years. All the NA segments of wild-bird derived N9 and H7N9 viruses did not have the five amino acid deletions as those human H7N9 viruses implying that the NA gene of human H7N9 viruses probably went through further molecular adaptation from waterfowls to chickens or other unidentified hosts. In conclusion, continuous surveillance and monitoring molecular changes and adaptation of avian influenza viruses in different host species is very important not only to understand the origin of viral genes but also to prevent large-scale epidemics of severe human influenza cases and possible emergence of novel influenza viruses leading to future pandemics.

Ecology of Influenza viruses in Egypt: Surveillance, Characterization and Vaccine Evaluation

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In mid-February 2006, Egypt was attacked by AI H5N1 virus causing many outbreaks in poultry and humans. In 2008, Egypt was declared endemic for AI H5N1. By the year 2009, Egypt recorded the highest number of H5N1 human cases in the World. By 2011, H9N2 virus emerged and the two viruses (H5N1 & H9N2) currently co-circulating in Egypt. The purpose of our collaboration with SJCRH and NIAID was to study the ecology of influenza viruses in Egypt. The goals of this project were: (i) Establish a laboratory capable of studying influenza viruses whether of animal or human origin; (ii) Study the ecology of avian influenza viruses in Egyptian poultry; (iii) Characterize Egyptian influenza viruses genetically and antigenically; (iv) Study the efficacy of commercial poultry vaccines under laboratory and field settings; (v) Train our NRC staff at SJCRH on laboratory procedures. All goals were achieved and as a result of our work the Egyptian Organization for Veterinary Services now rely on our lab for virologic data on influenza viruses. Furthermore, the selection of poultry vaccine strains and challenge viruses are based on our data and the reverse-genetic viruses produced in our lab. One of the important achievements of the NRC/SJCRH cooperation is that we received a new large laboratory space and were awarded a fund from the Egyptian Ministry of Scientific Research to become the Center of Scientific Excellence.
Excellence for Influenza Viruses which is envisioned to become a state of the art influenza laboratory capable of conducting research at BSL2 to BSL3+.

Epidemiological Studies of Avian, Equine, and Canine Avian Influenza Infection in Man

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During the last 8 years, our research team has conducted a series of epidemiological studies of zoonotic influenza A virus transmission among workers with intense exposure to animals. The cross-sectional and prospective studies have involved collaborations with research partners in Cambodia, China, Mongolia, Nigeria, Romania, Thailand, and the United States. Such seroepidemiological research is challenging because evidence for these infections is often rare, the laboratory assays are difficult and imprecise, and the most definitive studies require intensive resources. In particular, serologic detections of these infections in man may be confounded by cross-reacting antibody, waning antibody from the infection of interest, inaccurate matching of the enzootic virus and the laboratory strain, laboratory error, and weakly powered statistical comparisons. In our body of work we have found considerable evidence that swine workers and less commonly poultry, equine, and canine-exposed workers have experienced zoonotic influenza A virus infections. Modeling data suggest that agriculture workers may contribute to generation of novel viruses and serve as a bridging population and accelerate influenza virus spread between animals and large populations of humans. In this presentation, the speaker will summarize research finding thus far for avian, equine, and canine influenza viruses.

Prevalence of pH1N1 antibodies in seals and sea lions in California 2009-2012

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Marine mammals are in contact with important reservoirs of influenza virus, including aquatic birds and humans. After its emergence in humans in 2009, pandemic H1N1 (pH1N1) influenza virus was isolated from 2 Northern elephant seals (NES, Mirounga angustirostris) on the coast of central California in the spring of 2010 (Goldstein et al. 2013, PLOS One, 8:e62259).

To better understand the extent of pH1N1 infection we have used haemagglutination inhibition assay to analyze serum samples from the three most common marine mammals in California: NES (222 samples), California sea lions (CSL, Zalophus californianus, 183 samples) and harbor seals (HS, Phoca vitulina, 140 samples). Samples from 2009 through 2012 were obtained from free-ranging individuals as well as stranded animals hospitalized at The Marine Mammal Center (Sausalito, CA). Our results show that pH1N1 spread very rapidly in NES populations, with seroprevalence rising from nearly 0% before the spring of 2010 to almost 50% in 2011. Pups from seropositive mothers had pH1N1 specific antibodies, with titers often higher than those of their mothers, but a few 16 month old juveniles sampled in 2012 were seronegative, suggesting that the epizootic had a limited duration and the virus might not be circulating in NES anymore.

Contrary to NES, CSL and HS had low prevalence (less than 10%) of pH1N1 specific antibodies. This could be explained by differences in susceptibility to influenza infection, by specific adaptive mutations acquired in the NES virus strains and/or by more efficient intra-species transmission related to animal behavior.
What is the risk of avian influenza viruses jumping into horses in Mongolia?

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H3N8 avian influenza viruses have jumped to horses at least twice and horses provide a long-term reservoir for these viruses. A major outbreak of H3N8 virus in horses occurred around 1963 and resulted in the currently circulating virus, while a separate epidemic of equine influenza in Asia in 1989 was caused by an avian-like virus (A/equine/Jilin/1989) and resulted in the death of tens of thousands of horses (80% attack rate and 20% mortality). This virus also circulated the following year (A/equine/Heilongjiang/1990) with lower morbidity and no mortality. Since 1990 this virus has not been isolated, although there is seroepidemiological evidence of continued circulation in the early 1990s. Mongolia is a unique ecosystem to study avian-to-equine cross-species transmission because it exhibits a large horse population (approximately 3 million), very little domestic poultry, and high incidence of H3N8 viruses in wild birds. We are currently determining the likelihood of H3N8 AIVs to emerge in horses in Mongolia. We have performed phylogenetic analysis of H3N8 AIVs and EIVs in Mongolia as well as field seroepidemiology in Mongolian horses. We have also performed experimental infections of horse tracheal explants with H3N8 AIVs. Our results suggest that horses have been exposed to H3N8 AIVs, and that these viruses can infect the respiratory tract of the horse, albeit with low efficiency. Sequence analyses of EIVs isolated from horses show no reassortment between EIVs and AIVs.

Wild bird and swine influenza surveillance - update from Argentina

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Influenza A viruses (IAVs) are important pathogens responsible for economic losses to the swine industry and represent a constant threat to public health. Little is known about the ecology and evolution of wild bird influenza viruses in Central and South America (including Mexico and the Caribbean). Regarding this, in Argentina since 2006, we started a systematical collection of samples from wild waterfowl captured at the Parana River delta and at the Argentinean Atlantic coast; and since 2008 this program was enhanced with the collaboration with University of Maryland, College Park (UMCP) through CRIP-CEIRS. These long-term efforts represent the first in South America and have provided important insights into the ecology and evolution of IAVs in an otherwise poorly studied part of the world. More than 50 AIV positive samples have been detected from over 7,500 cloacal swab samples. Isolated viruses belong to a cluster of phylogenetically related AIVs in South America. Virus subtypes identified so far include H13N9 (from kelp gull), H6N8, H6N2, H7N9, H7N7, H10N7, H4N8, H1N1, and H5N3 (from various Anas spp). Our results are the first to describe the presence of AI viruses in wild birds in Argentina and, most importantly, that the gene constellation of this virus may evolve independently from influenza viruses in other latitudes. Exchange of genetic material between viruses from the South American cluster and those from North American or Eurasian lineages appears very limited, although sample size does not allow for a definite conclusion. For these reasons it is remarkable to continue the sampling in these areas and to incorporate specific species to be sampled and geographic tracking by GPS in coordination with sampling activities in Central and North America to try to assess the ecology of AIVs in the American continent.

Also we started in 2008 a study focused in evaluate the presence of Swine Influenza Viruses (SIVs) in Argentina. Previously to this effort, reports about swine influenza virus (SIV) were scarce in South America, and neither virus isolation nor detection of SIV by RT-PCR has been reported. Interestingly,
Argentinean SIVs that were isolated (a wholly human H3N2 virus, H1N1 pandemic virus and reassortant viruses between the pandemic virus and human-like H1 cluster δ SIVs) are distinguishable from similar subtypes in North America and likely represent independent human-swine transmission events. We do not know at this stage, whether reassortment among SIVs in Argentina is a common occurrence and/or reflect the exponential growth of the swine industry. It is also puzzling to find that a virus that was detected in 2008 remained undetected for 3 years until it shows up again in the form of a reassortant. These observations highlight the complexities of swine production systems and the role they play as a reservoir for SIV. In conclusion, the SIVs isolated in Argentina are distinguishable from similar SIVs in North America and represent independent transmission events. Therefore it becomes essential to continue sampling pig farms in Argentina through longitudinal studies to study the SIV dynamics and the influence of several activities on the perpetuation of SIV inside those farms. Also it is of great importance to study the Animal-Human interphase and the relevance of commercial pigs as reservoirs with potential to transmit viruses that are not circulating in humans and/or to population with no or low immunity to these subtypes.

Avian influenza surveillance in wild birds in Guatemala, 2010-2012

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Historically, surveillance efforts for avian influenza virus in Latin America have been localized in places where outbreaks in poultry have occurred. As a result, virus nucleotide sequence data for avian influenza circulating in wild birds is still limited in comparison to other regions of the world. Overall AIV reports are sparse across the region and the extent of the co-circulation of two distinct genetic lineages from North and South America is still puzzling. As a continuation of previously established surveillance, cloacal swab samples were collected from hunted-harvested waterfowl and coastal birds during the north wintering migration of 2010-11 and 2011-12 in two different locations across the pacific coast of Guatemala. A total of 649 birds were sampled from 19 species and 10 different orders, in their majority Anseriformes. Influenza A virus was detected in 170 (26.2%) samples by rRT-PCR. Thirty-five virus isolates were obtained from the duck species Anas discors (32 isolates) and A. clypeata (3 isolates). Twelve different HA and NA subtype combinations were found, including H5 and H7, and five other HA (H1, H3, H4, H12, H14) and one NA (N5) subtypes not previously reported for the region. 454 sequencing was used to obtain full-genome virus sequences, providing a better level of resolution in the case of co-infections. To our knowledge Guatemala remains as the only country in Central America from which avian influenza viruses circulating in wild waterfowl are being described.

Surveillance and Characterization of Avian Influenza Viruses circulating in the central region of Chile

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Waterfowl and shorebirds are the main reservoirs of avian influenza viruses (AIV) in nature, particularly for low pathogenicity (LPAI) strains. Bird migration is considered one of the main routes of viral spread throughout large geographical areas. Chile forms part of the Pacific Migratory flyway, a bird migration route that stretches from the furthermost tip of Alaska to the South American Patagonia. However, influenza surveillance along this flyway has been scarce. During winter 2012 and summer 2013, 2757
environmental fecal samples were collected in distinct geographical locations within the central region of Chile; 597 samples belonging to wild birds and 2160 to domestic poultry. 40 samples tested positive by real-time RT-PCR for the influenza M gene with a prevalence of 3.35% in wild birds and 0.92% in poultry. Viral isolation was successful on one of the wild bird samples (Anas georgica spinicauda) and genetic analysis revealed a non-pandemic H1N1 virus. Interestingly, the hemagglutinin (HA) gene is closely related to one other South American influenza isolate, while its neuraminidase (NA) gene relates to both South and North American wild bird AIV isolates. This is the first isolate of AIV from a wild bird in Chile, hence the importance of its finding. Pathogenicity studies in BALB/c mice showed no weight loss or disease, although viral particles could be detected in lung homogenates until day six post infection. Altogether, these findings highlight the importance of influenza surveillance in order to fully understand the spread and impact of AIV on wildlife, poultry production and human health in Chile and other South American countries.
Immunology

1. An innate immune profile predicts clinical outcomes in natural influenza infection

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Influenza infection can result in mild or serious illness in some individuals, and studies suggest that increased inflammatory mediators have a role in disease severity. To investigate the role of inflammation in clinical disease, we performed a prospective, longitudinal study, sampling symptoms, blood, nasal lavages, and viral loads from participants during natural influenza infection. Surprisingly, clinical disease defined by symptoms or hospitalization did not correlate with viral dynamics, but consistent with other clinical reports, age was associated with hospitalization. There was no association between age and viral dynamics, though a strong inverse correlation between age and cytokine levels in the nasal lavage was maintained even when adjusted for prior immunity, indicating that children mount more aggressive inflammatory responses independently of pathogen dynamics. Correcting for this age-association, analysis of cytokine levels in nasal lavages revealed that MCP3 and IFNα were predictors of respiratory symptoms, while plasma MCP3 and IL-10 predicted symptom severity and hospitalization. In all cases, associations were not dependent on viral load. The association between IL-10 and symptom severity was confirmed in an independent cohort. In summary, children suffer significantly higher local inflammation, and a small subset of cytokines, including MCP3 and IL-10, serve as diagnostic markers for clinical outcome of influenza infection in all age groups.

2. mTOR regulates protection against lethal H5N1 influenza infection by modulating the antibody response

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Highly pathogenic H5N1 avian influenza viruses pose a continuing global threat. Current vaccines will not protect against novel pandemic viruses. Creating “universal” vaccines has been unsuccessful because the immunological mechanisms promoting heterosubtypic immunity are incompletely defined. We show that rapamycin, an immunosuppressive drug that inhibits mTOR, promotes cross-strain protection against a lethal H5N1 infection when administered during H3N2 virus immunization. Rapamycin reduced germinal center formation and inhibited B cell class-switching, yielding a unique repertoire of antibodies that mediated heterosubtypic protection. Our data establish a requirement for mTORC1 in B cell class-switching and demonstrate that rapamycin skews the antibody response away from high affinity variant epitopes, targeting more conserved elements of hemagglutinin. These findings have intriguing implications for influenza vaccine design.
3. Influenza tracheitis: a murine model system for studying T cell migration and function by intravital multiphoton microscopy

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Influenza virus productively infects the epithelial cells that line the respiratory tract and is restricted to this site by a requirement for a trypsin-like enzyme that activates the viral hemagglutinin. During a primary influenza infection, cytotoxic CD8+ T cells (CTL) must traffic to the mucosal epithelium to mediate elimination of infected cells. After the virus is cleared, long-term, virus specific “tissue resident memory” (TRM) CD4 and CD8 T cells become established in the respiratory mucosa. In spite of the importance of T cell migration into the airways, the dynamic processes that regulate these interactions are only understood at a very gross level. Light and electron microscopy of extracellular matrix (ECM) show substantial remodeling of the tissue during infection, corresponding to ECM specific integrin expression by the T cells. To quantify T cell interactions with ECM, we developed a model of influenza tracheitis that we use to study real-time behavior of CD8 CTL in the infected airways. Using intravital multiphoton microscopy, we show the transition of effector CTL, between 8 and 9 days, to a surveillance mode of motility as TRM is formed. These observations demonstrate the ability to visualize the formation of memory T cells in the mucosal tissue. Future experiments aim to determine the molecular mechanisms that regulate T cell migration and interaction with (ECM) during viral clearance and formation of tissue-resident memory.

4. Effect of Age on Influenza Skin Vaccination

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Skin has gained attention recently as a vaccine target organ due to its immunological properties, which include an increased number of professional antigen presenting cells (APCs). Since the young and aged represent the populations at high risk for influenza infection, we compared the immune responses in these two groups upon skin delivery of influenza vaccine. As representatives for the aged group, Balb/c mice that were kept in sterile conditions for 15 months received 5 ug of A/Ca/07/09 influenza subunit vaccine through the skin using MN patches or IM. For the young population, two week old pups received 5 ug of A/Ca/07/09 through MN or IM routes. As controls, three month old adult controls received the same amount of the vaccine through MN or IM routes. Young mice that received influenza vaccine using MN demonstrated an improved antibody response (IgG1 and IgG2a) when compared to the young IM group, accompanied by higher numbers of ASCs in the bone marrow and improved protection after lethal challenge. In the aged group, either IM or MN immunization led to reduced immune responses when compared to the adult controls and suppression of IgG2a accompanied by reduced number of influenza-specific antibody secreting cells (ASCs) in the bone marrow. Our results indicate that influenza vaccine delivery through the skin could be beneficial for high risk populations from influenza infection.
5. Neutralizing antibodies against previously-encountered influenza virus strains increase over time: A longitudinal analysis

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Antigenic diversity shapes immunity in distinct and unexpected ways. This is particularly true of the humoral response generated against influenza A viruses. While it is known that immunological memory developed against previously-encountered influenza A virus strains impacts the outcome of subsequent infections, exactly how sequential exposures to antigenically variant viruses shape the humoral immune response in humans remains poorly understood. To address this important question, a longitudinal analysis of antibody titers against various pandemic and seasonal strains of influenza virus spanning a 20-year period (1987-2008) was performed using samples from 40 individuals (d.o.b. 1917-1952) obtained from the Framingham Heart Study. Longitudinal increases in neutralizing antibody titers were observed against previously-encountered pandemic H2N2, H3N2 and H1N1 influenza A virus strains. Antibody titers against seasonal strains encountered later in life also increased longitudinally at a rate similar to that against their pandemic predecessors. Titers of cross-reactive antibodies specific to the hemagglutinin stalk domain were also investigated, since they are known to be influenced by exposure to antigenically diverse influenza A viruses. These titers rose modestly over time, even in the absence of major antigenic shifts. No sustained increase in neutralizing antibody titers against an antigenically more stable virus (human cytomegalovirus) was observed. The results herein describe a role for antigenic variation in shaping the humoral immune compartment, and provide a rational basis for the hierarchical nature of antibody titers against influenza A viruses in humans.

6. mCMV alters the airway inflammatory milieu regulating protective and pathogenic heterologous immunity to influenza A virus infection

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Prior immunity to apparently unrelated pathogens can affect the course of subsequent acute infections. The mechanisms of heterologous regulation in most circumstances remain poorly defined. In mice co-infected with murine cytomegalovirus (CMV) and Influenza A virus (IAV), we show that prior infection with CMV results in significantly reduced weight loss upon acute challenge with IAV as well as improved recall responses. Tetramer and ex-vivo ICCS assay showed an early recruitment of CMV specific CD44hi CD62Llo IFNγ⁺ TNFα⁺ CD8 T cells in the bronchoalveolar lavage (BAL) of both acutely and latently infected animals following x31 challenge that was absent in mice infected with CMV-alone. This protection was dependent on IFNγ production by these mature anti-CMV CD8 T cells, the protective phenotype was completely ablated in co-infected IFNγ⁻/⁻ animals or in animals co-infected simultaneously. However, no cross-reactive T cells were detectible. Furthermore, comparative kinetics of CMV specific CD8 T cell responses showed that IAV infection resulted in the attrition of the CMV specific CD8 T cell response in both acutely and latently infected animals. Finally, analysis of the IAV-specific T cell receptor repertoire by multiplex nested PCR suggested that prior CMV infection altered the recruited IAV-specific T cell repertoire. These data demonstrate that CMV co-infection can determine the outcome of subsequent IAV infection by altering the airway microenvironment.
7. The B cell response and hemagglutinin stalk-reactive antibody production in different age cohorts following 2009 H1N1 influenza vaccination

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The 2009 pandemic H1N1 (pH1N1) influenza virus carried a swine-origin hemagglutinin (HA) that was closely related to the HAs of pre-1947 H1N1 viruses, but highly divergent from the HAs of recently circulating H1N1 strains. Consequently, prior exposure to pH1N1-like viruses was mostly limited to individuals over the age of about 60 years. We related age and associated differences in immune history to the B cell response to an inactivated monovalent pH1N1 vaccine given intramuscularly to subjects in three age cohorts: 18-32 years, 60-69 years, and ≥70 years. Day 0 pH1N1-specific hemagglutination inhibition (HAI) and microneutralization (MN) titers were generally higher in the older cohorts, consistent with greater pre-vaccination exposure to pH1N1-like viruses. Most subjects in each cohort responded well to vaccination, with early formation of circulating virus-specific Ab-secreting cells and ≥4-fold increases in HAI and MN titers. However, the response was strongest in the 18-32-year cohort. Circulating levels of HA stalk-reactive Abs were increased after vaccination, especially in the 18-32-year cohort, raising the possibility of elevated levels of cross-reactive neutralizing Abs. In the young cohort, an increase in MN activity against the seasonal influenza virus A/Brisbane/59/07 after vaccination was generally associated with an increase in anti-Brisbane/59/07 HAI titer, suggesting an effect mediated primarily by HA head-reactive rather than stalk-reactive Abs. Our findings support recent proposals that immunization with a relatively novel HA favors the induction of Abs against conserved epitopes. They also emphasize the need to clarify how the level of circulating stalk-reactive Abs relates to resistance to influenza.

8. Characterization of Follicular Helper T cell Responses to Influenza – Antigen Specificity and Influence upon B cell Responses

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Seasonal influenza and the risk of widespread influenza pandemics are an ongoing major world health concern. In the United States alone, influenza infection results in more than 200,000 hospitalizations and up to 41,000 deaths each year. The current vaccine strategies focus on eliciting neutralizing antibodies against hemagglutinin and neuraminidase, the major surface glycoproteins involved with viral entry and exit from a host cell. Because of the highly mutable nature of influenza, the vaccine must be reformulated each year – highlighting the necessity for an alternate approach that provides broad and long-lasting protection. We have approached this problem by asking if CD4 T cell memory, elicited by peptide priming, is sufficient to accelerate T cell and B cell responses to influenza infection. After immunization with immunogenic peptides derived from A/New Caledonia/20/99 followed by infection with the same virus, we observed an increased frequency of virus specific CD4 T cells 7 days post infection. Additionally, there was an enhanced B cell response both kinetically and in magnitude. We have also observed a strong correlation between the frequencies of follicular T helper cells and germinal center B cells in the peptide primed/infected cohorts that are not seen in the control primed/infected group. Our results suggest that T cell help for a primary B cell response to infection is limiting, and that peptide priming is sufficient to generate a functional memory CD4 pool.
9. Local administration of a monoclonal antibody for therapeutic use in influenza infection in mice

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Infection with influenza viruses continues to be a medical and economic burden for humanity. Just in the United States alone, approximately 30,000 people die every year due to influenza-related illnesses. Vaccination is still the best method to prevent virus infection, despite the inherent uncertainties in predicting the strains to be included in the vaccine formulation each year. However, vaccination coverage is far from universal, leaving more than 50% of the population still susceptible to infection. Influenza neuraminidase inhibitors are the only effective therapeutics currently licensed for human use in the US. One of the main drawbacks of this class of drugs is their potential to induce drug-resistance, a phenomenon recently seen with the rapid increase in oseltamivir resistance arising in H1N1 influenza viruses in 2007-2009. This highlights the critical need to discover new therapeutics that can be used for the treatment of influenza virus-induced disease. We have generated and characterized a pan-H1 anti-stalk monoclonal antibody (MAb), 6F12, which has a broadly neutralizing spectrum against several viruses of the H1 subtype. Here we tested this monoclonal antibody in a delay of treatment regimen using nasal administration to determine its potential therapeutic use. Groups of 6-8 weeks old BALB/c mice were infected with an influenza A H1N1pdm virus and then received 15 mg/kg of 6F12 via intranasal (IN) or aerosol (Aero) routes on days 3, 4, 5, or 6 post-infection (pi). One hundred percent survival was observed in mice treated with 6F12 at the onset of clinical signs (day 3 pi) both after IN and Aero administration. When the treatment was given on day 4 pi, 100% of the mice in the IN group and 60% in the Aero group survived the infection. Sixty percent survival was observed in mice from the IN group receiving treatment on day 5 pi, while 80% survival was observed for the mice that received 6F12 by the aerosol route on day 5 pi. When treatment was given on day 6 pi, 20% (IN) and 0% (Aero) survival was observed. Results demonstrate that MAb 6F12 delivered via the respiratory tract can be used as a therapeutic agent since treatment was effective even when delayed 4 or 5 days after infection. In addition, the amount of antibody administered via the respiratory tract is half that needed to achieve similar protection when given by the intraperitoneal route. This report highlights the potential for the use of anti HA stalk monoclonal antibodies as a therapy for during influenza-associated disease.

10. Clinical, immunological and virological characterization of hospitalized influenza A patients between 2011-2012

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Influenza A virus, affects mainly the upper respiratory tract and occasionally can cause viral pneumonia in some individuals. Disease outcome is modulated by both viral and host factors. The efficient transmissibility and pathogenesis shown by the 2009 H1N1 pandemic strain (H1N1pdm2009) during its emergence, emphasized the value of monitoring the clinical evolution of infected individuals to establish efficient mitigation and clinical treatment strategies, and to elucidate susceptibility factors in the general population. To date no in-depth longitudinal studies have been conducted with influenza virus patients, integrating epidemiological data, pathogenesis, clinical findings (e.g. presence of comorbidities or
susceptibility factors), analysis of immune responses of the host, and a complete characterization of the antigenic properties, virulence and genome of the virus. In this study, for the years 2011 and 2012, serial of nasopharyngeal swabs and blood samples were collected from a total of 78 patients hospitalized with influenza A. Clinical data was obtained from each patient utilizing the CEIRS Network Metadata fields (including symptoms, epidemiological history, morbidities, treatments, etc). Preliminary analysis shows that the majority of patients considered to be severe, had risk factors by either pre-existing comorbidities or because they belonged to high-risk age groups (individuals < 2 or >65 years). We also identified that a large proportion (44.3 %) of severely ill individuals had some level of immunosuppression. Subtyping by qRT-PCR or inhibition of hemagglutination (HI) test, established that 87.5% of the 2011 samples corresponded to H1N1pdm09 and 64.7% of the samples of the 2012 corresponded to H3N2. We identified 5 individuals who showed a decrease in seroconversion titers (day 21) against the homologous virus strain, in comparison to the level of antibodies in pre-infection sera (day 1). In some cases this may be related to immune suppression or alternatively it might be due to antigenic drift of the virus during infection. To investigate genomic variation of the virus (quasispecies) during the acute phase of infection, we are currently sequencing 86 samples collected from 23 individuals during the first 7 days of infection. To complement our data we are also investigating the status of the innate immune response of these patients by ELISA and qRT-PCR in serum and cells of the nasopharyngeal cavity, respectively.

Immunology / Vaccine

11. Induction of cross-reactive antibodies to novel H7N9 influenza virus by recombinant Newcastle disease virus expressing a North American lineage H7 subtype hemagglutinin

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Severe human disease caused by the emerging H7N9 influenza virus in China warrants rapid response. Here, we present a recombinant Newcastle disease virus expressing a North American lineage H7 influenza virus hemagglutinin. Sera from immunized mice are cross-reactive to a broad range of H7 subtype viruses and inhibit hemagglutination by the novel H7 hemagglutinin. Immunized mice were protected against a heterologous H7 subtype challenge, and genetic analysis suggests that cross-protective antibodies recognize conserved antigenic sites.

12. Guiding the immune response against influenza hemagglutinin towards the conserved stalk domain by altering glycosylation

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Recently, several influenza hemagglutinin (HA) targeting antibodies have been described that show broad reactivity within HA subtypes or even neutralize HAs of different subtypes. It was shown that these broadly neutralizing antibodies target the stalk domain of HA. This domain is responsible for membrane fusion and is more conserved than the receptor binding head domain of HA. In general, the immune response against influenza vaccines focuses on the head domain and neutralization is mostly caused by inhibition of receptor binding. However, the high variability in the head domain makes this immune response strain specific for the vaccine used and antibodies show little to no cross-reactivity to drifted variants or strains from different subtypes. Fewer antibodies directed against the stalk domain are elicited which could be more broad protective. We have explored a method in which we try to focus the immune response towards the stalk domain and lower the immune response to the head domain. We do
so by introducing N-linked glycosylation sites in the head domain, covering the immuno dominant antigentic sites with glycans. At the same time we remove glycans of the stalk domain, thereby opening up this domain to make it better accessible to the immune system. The A/Puerto Rico/8/34 (PR8) HA contains six potential N-glycosylation sites of which five are thought to be glycosylated. Four of these are situated in the stalk domain, while one is present in the head domain of HA. We are able to show that one can introduce up to eight additional glycosylation sites into the PR8 head domain, resulting in the loss of binding of PR8 antiserum while keeping the ability to bind stalk directed monoclonal antibodies. In parallel, two glycosylation sites in the stalk domain were removed, which did not affect protein expression and binding of several stalk directed antibodies. This suggests that the stalk domain is still properly expressed and folded. These constructs are currently tested in their potential to elicit stalk directed neutralizing antibodies and provide broad protection in mice.

13. Induction of broadly neutralizing influenza virus group 2 HA antibodies through natural infection and novel vaccination strategies

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Broadly neutralizing antibodies directed against the conserved stalk domain of the viral hemagglutinin have attracted increasing attention in the recent years. However, only three antibodies directed against the stalk regions of group 2 influenza hemagglutinins have been isolated so far, providing very little information about the epitopes found in these domains. Also, little is known about the general level of induction of these antibodies by influenza virus vaccination or infection. We performed a quantitative and qualitative characterization of the anti-stalk humoral response in the mouse model as well as in humans, by employing chimeric hemagglutinin constructs previously developed in our group. Influenza virus infection induced significant titers of stalk-reactive antibodies in both humans and mice, while immunization with inactivated influenza virus vaccines failed to do so. Furthermore, the hemagglutinin stalk-directed antibodies induced in mice and humans had broad reactivity and neutralizing activity in vitro and in vivo. We also demonstrate that a novel vaccination strategy based on the stalk domain of the H3 hemagglutinin is able to induce broadly neutralizing anti-stalk antibodies in mice that are highly cross-reactive to various H3, H7 (including the novel Chinese H7N9), H10, H14 and H15 hemagglutinins. These observations are relevant for the development of universal influenza virus vaccines and are encouraging, as they suggest that broadly neutralizing stalk-reactive antibodies against group 2 HAs can be induced in humans.
14. H7N9 infection of pigs: Pathogenicity and transmissibility of the wild-type virus and avian-signature mutants

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More than one hundred of human cases infected with a novel avian H7N9 in China have raised concerns that this strain has pandemic potential. To date, the animal reservoir, routes of transmission and the scope of the spread of this virus among people and animals remains unclear. Herein, we used a pig infection model to evaluate the pathogenicity and transmissibility of 4 different H7N9 viruses: the human H7N9 isolate A/Anhui/1/2013 (WT), recombinant WT virus (RG-WT, which is synthetically created from the consensus sequence and contains mammalian-signatures HA/226L and PB2/627K) and two mutant viruses encoding avian-signature substitutions in the HA (HA L226Q) or the PB2 (PB2 K627E) proteins. All viruses replicated in the lungs of intratracheally infected pigs, but nasal shedding was limited. The WT and the avian-signature HA-226Q viruses did not transmit to sentinels. In contrast, the RG-WT and the avian-signature PB2-627E viruses transmitted to contact animals. These data demonstrate that the mammalian-signature HA 226L, but not the mammalian-signature PB2 627K of the zoonotic H7N9 viruses plays a critical role in replication, pathogenesis and transmissibility in pigs, suggesting that the circulating H7N9 viruses may have potential for human-to-human transmission.

15. Influenza virus transmission among pigs is not solely mediated by hemagglutinin and neuraminidase

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Prior to 1998 there was limited antigenic and genetic diversity in the classical H1N1 swine influenza viruses (SIV) circulating in the US pig population. This changed in 1998 when reassortant H3N2 SIV were detected in the context of the triple reassortant viruses. At least three independent introductions of human virus H3 HA’s were observed in the ensuing year, all reassortants with the triple reassortant virus. As it has been shown that wholly human H3N2 viruses do not replicate well in swine, we hypothesized that reassortment of the HA and NA with a swine adapted backbone was critical to the emergence of the H3N2 viruses in swine. To determine if an innate incompatibility between the classical H1N1 and human H3N2 viruses was responsible for the lack of prior detection of H3N2 reassortants in swine we created a panel of reassortant viruses and tested their ability to transmit in pigs. As expected, the wholly human H3N2 virus did not transmit to any contact animals although it replicated in infected animals. When, however, reassortant H3N2 viruses on both the triple reassortant and classical swine backbones were assayed, both were able to transmit to all contact animals. Addition of a human-like PB1 to the classical swine reassortant abolished transmission. This data indicates that a lack of human H3N2 virus transmission among pigs is likely due to post entry steps rather than viral attachment and that gene incompatibility was not the reason for the lack of emergence of H3N2 viruses in US swine prior to 1998.
16. Infectivity, reassortment, and adaptation of IAV in *Pteroptus alecto* (bat) epithelial cells

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Waterfowl are primary hosts for influenza A viruses (IAV), however there is sporadic infection of swine and other species that pose a risk for zoonotic spread. Yellow-shouldered bats were shown to be hosts of an IAV constituting a novel reservoir. This finding led to the investigation of whether current strains of influenza virus can replicate in bat epithelial cells, and whether bats can be a reservoir for the generation of novel influenza viruses through reassortment. We show that *Pteroptus alecto* kidney cells (PaKi) are susceptible to infection by a human H1N1 (A/WSN/33) isolate and a high pathogenic avian H5N1 (A/Vietnam/1203/04) isolate. We show that co-infection of PaKi cells with a currently circulating human influenza virus isolate and a swine isolate results in generation of novel reassortants. In addition, we determine whether IAV will undergo selective adaptation during serial passaging in PaKi cells.

17. Infectivity, Transmission and Pathology of Human-isolated H7N9 Influenza Virus in Ferrets and Pigs

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The emergence of the H7N9 influenza virus in humans in Eastern China has raised concerns that a new influenza pandemic could occur. Here, we used a ferret model to evaluate the infectivity and transmissibility of A/Shanghai/2/2013 (SH2), a human H7N9 virus isolate. The study proves that the avian influenza A (H7N9) virus can infect ferrets and cause relatively mild clinical signs. This virus can transmit via direct contact and air-borne exposure, albeit the latter less efficiently. As high titers of virus start to be shed before symptomatic infections of animals and the infected animals do not necessarily develop fever and other related clinical signs, the possibility for asymptomatic infections could not be excluded in humans. Pigs can also be productively infected by the H7N9 virus, leading to mild visible clinical signs and pneumonia. However, sustained transmission is not observed among pigs via close contact or airborne exposure. Under appropriate conditions, this virus may further evolve to acquire human-to-human transmissibility and form the basis of a future pandemic threat. Public health measures should thereby be well maintained and surveillance of this virus in humans and animals is needed to prevent the epidemic from spreading further.

18. Substitutions T200A and E227A in the hemagglutinin of pandemic 2009 influenza A virus and their role in replication, lethality and transmission

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We describe that swine influenza virus-like substitutions T200A and E227A in the hemagglutinin of the 2009 pandemic influenza A H1N1 virus alter its replication, pathogenesis and transmission. Viral replication is increased in mammalian cells. Infected mice with the mutated virus show increases in disease as measured by weight loss and in lethality. This is confirmed by a stronger inflammation response and increased histopathological lesions. Transmission in ferrets is slightly decrease in the presence of both substitutions, suggesting that 200T and 227E HA aminoacids are adaptive changes in
the HA of swine-origin influenza viruses associated with increased transmission and decreased pathogenesis.

19. Studies on the effect of adaptation of the influenza virus A (H1N1) pdm09 to structural changes in the tissues of the lung, brain, liver, kidney and heart in experimental mice

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In spring 2009, a previously unknown variant of the influenza virus A (H1N1) emerged in North America and Mexico. This new variant of the influenza virus was highly contagious and can spread rapidly, causing the first influenza pandemic in the 21st century. Currently, there is concern that if the virus A (H1N1) pdm09 retains the ability to efficiently spread from person to person and thus strengthen its virulence by adapting to humans, a new adapted version could have a very significant effect on public health. The aim of our research is to study the adaptation of influenza A (H1N1) pdm09 with pandemic potential in an experimental model in mice. We carried out virological, molecular biological, morphological and immunohistochemical analyses of changes in the lung, brain, liver, kidney and heart of mice (BALB/c and C57BL/6) under the influence of an adapted version of the strain A/Tomsk/13/2010pdm09. As a result of the strain adaptation in the laboratory FSRC VB "Vektor", a variant with 100% lethality in mice was obtained (7th passage «lung to lung» in BALB/c mice and 13th passage «lung to lung» in C57BL/6 mice). It was shown that the adaptation of the strain A/Tomsk/13/2010pdm09-MA causes significant pathomorphological changes in the study of internal organs of infected mice. In lung tissue, we observed the development of pneumonia, hemorrhage, and cellular infiltration. In the brain of infected animals we observed perivascular edema, hemorrhage, and vascular congestion. In the liver, kidneys and heart we also observed dystrophic changes. Thus, it was shown that adaptation in mice increases the virulence of the pathogenic properties of the influenza A (H1N1) pdm09 virus.

20. Respiratory and Direct Contact Transmission of Highly Pathogenic H7N1 Influenza in Ferrets

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Highly pathogenic avian H7 influenza viruses are recognized as potential pandemic viruses. H7 infections in humans typically cause mild conjunctivitis; however, the H7N9 outbreak in March 2013 caused severe respiratory disease. To date, no H7 viruses have acquired the ability to transmit by respiratory droplets in mammals. Thus, we sought to determine if a highly pathogenic influenza A H7N1 (A/H7N1) virus could be adapted to transmit in ferrets. After 10 serial passages, A/H7N1 developed the ability to transmit via direct contact and respiratory droplets. Four amino acid mutations (PB2 T81I, NP V284M, M1 R95K and Q211K) in the internal genes and a minimal change in the HA gene (K/R313R) were consistently associated with transmission. These findings indicate that an avian H7N1 can acquire the ability to transmit by respiratory droplet in a mammalian host and support continued H7 pandemic vaccine development.
21. The 2009 pandemic H1N1 influenza M, NA, and HA segments together allow for efficient contact transmission of recombinant PR8 viruses

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The recent 2009 influenza pandemic H1N1 (pH1N1) virus caused approximately 60 million US cases within its first year of circulation. However, the genetic factors responsible for the high pH1N1 transmissibility remain incompletely defined.

Utilizing plasmid-based reverse genetics, we generated wt recombinant, as well as reassortant viruses containing segments (M, NA, M/NA, HA/NA or M/NA/HA) from a human pH1N1 strain (A/Netherlands/602/09; NL602), in the A/Puerto Rico/8/34(H1N1) (PR8) background.

rNL602 virus spread efficiently among guinea pigs, whereas the rPR8 virus did not transmit between co-caged animals. Swapping of the HA, NA and M segments of NL602 into the PR8 background yielded a virus with similar transmissibility to the wild-type pandemic strain. As previously reported, introduction of the pandemic M segment alone resulted in an intermediate phenotype of approximately 75% transmission efficiency.

Since the M, NA and HA segments of influenza virus have each been reported to affect virion morphology, we evaluated the morphology of each reassortant virus using scanning and transmission electron microscopy.

A correlation between efficient transmission and filamentous morphology was observed: PR8 virions were largely spherical, whereas PR8/NL602 M+NA+HA virus exhibited a similarly filamentous morphology to that of rNL602 virus, and the virus carrying only the NL602 M segment had an intermediate morphological phenotype. Interestingly, increases in the virion length also correlated with increased neuraminidase activity in in vitro assays.

22. The M segments of a Eurasian avian-like swine, and a 2009 pH1N1 influenza strain confer differences in contact transmission, morphology and NA activity of recombinant PR8 viruses

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The first influenza pandemic of the 21st century is believed to have occurred as a result of an epizootic swine influenza outbreak around 2009. The specific molecular changes, if any, which allowed the precursor strain to attain high transmissibility in human populations are not understood.

Previous publications indicate that introduction of the M segment of pandemic influenza virus (pH1N1) improves transmissibility of A/PR/8/34 (PR8). To investigate whether the M segment of a Eurasian avian-like swine influenza strain (putative precursor lineage of pH1N1) could similarly affect transmissibility of PR8, we generated recombinant PR8 viruses harboring either a pandemic (A/Netherlands/602/09; NL602) or an avian-like Eurasian swine consensus (EAsw) M segment.

Faster replication kinetics, and higher peak titers were generated by PR8/NL602M than PR8/EAswM in vivo. Additionally, PR8/EAswM appeared to transmit with slightly reduced and delayed contact transmission kinetics between guinea pigs. Electron microscopy revealed that the PR8/NL602M possessed a sub-population of virions (>1um filaments) that was absent PR8/EAswM. The absence of long filaments was recapitulated in rEAsw(wt), but not in rNL602(wt), virion preparations. PR8/EAswM virion preparations possessed lower neuraminidase activity than virion preparations containing the NL602 M segment in in vitro assays.

Interestingly, the phenotypes of a PR8/NL602M-S30G-N207S-M248I(C19Y) mutant virus, which revert the matrix protein (and 1 amino acid of the M2 protein) to the EAsw lineage, recapitulate those of PR8/EAswM virus, however, PR8/EAswM-G30S-S207N-I248M(Y19C) mutant replicated poorly, and did not transmit between guinea pigs, suggesting an interplay between the M1 and M2 proteins is important to fitness.
23. Specific residues delineating the consensus 2009 pH1N1 and Eurasian swine influenza M1 proteins affect pH1N1 virus morphology, neuraminidase activity, replication, and transmission in the guinea pig model

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Although there have been previous human infections with swine lineage influenza viruses, the recent 2009 pandemic H1N1 (pH1N1) virus was the first to efficiently transmit within the population. However, the viral factors underlying replication and transmission of pH1N1 have yet to be fully elucidated.

We have previously shown that relative to the avian-like Eurasian swine lineage consensus (EAsw) M segment, a pH1N1 M segment (from A/NL/602/09; NL602) confers improved replication and transmission in guinea pigs, in an A/Puerto Rico/8/34 (PR8) background.

To assess the contribution of individual residues of the matrix protein that differ between EAsw and pH1N1-consensus M segments (S30G, N207S and M1-M248I(M2-C19Y)) to phenotypic differences, we generated recombinant (PR8) viruses with single amino acid changes in the M segment.

The PR8/NLM S30G virus replicated efficiently, while exhibiting a largely spherical morphology, decreased neuraminidase activity against the fluorogenic sialoside, MUNANA, and inefficient transmission between guinea pigs compared to a control strain (PR8/NL M).

The PR8/NLM N207S mutant virus showed a slight reduction in NA activity, but this was not reflected in changes in replication kinetics in vitro or in vivo, morphology, or transmission efficiency.

The PR8/NLM M248I(M2-C19Y)) mutant virus demonstrated a reduction in replication kinetics both in vitro and in vivo, as well as inefficient transmission, and changes in virion morphology suggestive of a loss of structural integrity, but unaltered neuraminidase activity. We are currently testing the effect of these mutations in vitro and in vivo, in the context of the rNL602 strain.

24. Residue 41 of the A/swine/Spain/53207/04 matrix protein modulates virion filament length and viral fitness

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Alanine 41 of the influenza A virus matrix protein is highly conserved in human and avian lineages. Nonetheless, the Eurasian avian-like swine lineage strain A/swine/Spain/53207/04 (SPN04) encodes a proline at this position, and several other EAsw isolates carry valine. Of note, the M1 A41V polymorphism is also associated with adaptation to mice and cell culture. To assess the impact of the naturally occurring matrix 41P polymorphism on viral fitness, we utilized a reverse genetics system for SPN04 to generate viruses encoding M1 41P and 41A respectively.

The parental virus grew with reduced kinetics in MDCK cells, and in guinea pigs exhibited attenuated growth and less efficient contact transmission. Moreover, the single amino acid mutation P41A altered the morphology of the virion from a predominantly long-filamentous to predominantly short-filamentous phenotype.

Six reassortant viruses between A/PR/8/34 (PR8) and SPN04 were also rescued: PR8/SPN04 M, PR8/SPN04 M+NA, PR8/SPN04 M+NA+HA, PR8/SPN04 M P41A, PR8/SPN04 M P41A +NA, and PR8/SPN04 M P41A +NA+HA. In each case, the virus which possessed 41P was attenuated in vitro (MDCK) and in vivo (guinea pig) relative to the isogenic virus possessing 41A. Interestingly, the morphology of the viruses possessing the 41P polymorphism varied depending upon overall gene constellation. The short-filamentous to long-filamentous change was most pronounced in the context of the SPN04 HA and NA, suggesting interactions between M1 residue 41 and HA and/or NA affect morphology. In summary, we have identified a naturally occurring mutation in the matrix protein that is attenuating and alters virion morphology in a context dependent manner.
25. Transmission of the human influenza viruses A/Panama/2007/99 and A/NL/602/09, but not the triple reassortant A/swine/Kansas/77778/07, occurs efficiently from low infectious dose in guinea pigs

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Swine influenza viruses of triple reassortant (TRIG), Eurasian avian-like (EAsw) or classical lineages do not transmit efficiently among humans. Neither the genetic and molecular factors responsible for these phenotypes, nor their mechanistic bases, are fully delineated. Here we present data indicating differences in infectious dose of specific strains representing TRIG, EAsw, and human pH1N1 and H3N2 lineages, in the guinea pig model. Utilizing plasmid-based reverse genetics, we generated recombinant A/Panama/2007/99 (H3N2); A/NL/602/09 (H1N1) [NL602]; A/swine/Kansas/2007 (H1N1) [KAN07]; and A/swine/Spain/53207/04 M1P41A (H1N1) [SPN04] viruses. Specific reassortant viruses containing segments from two or more backgrounds were also generated. All wild-type recombinant viruses grew efficiently in HTBE cells, indicating no gross replication deficiencies associated with the human and swine influenza strains. However, only human strains could transmit efficiently from an inoculum of 100 PFU in a guinea pig contact transmission model. Interestingly, the EAsw lineage strain appeared intermediate in infectivity between the TRIG and the human influenza virus phenotypes. A reassortant TRIG:pH1N1 (5:3) virus, KAN07/NL602 M+HA+NA, recapitulated the transmission phenotype of wild-type rNL602. In contrast, KAN07/EAsw M/NL602 HA+NA virus, that differs only in the M segment, had lower peak replication and slightly delayed kinetics of transmission. Nonetheless, KAN07/EAsw M and Kan07/EAsw M+NA viruses showed improved transmission relative to rKAN07wt virus. We suggest that infectivity from low dose contributes to the ability of specific influenza lineages to spread within their target populations, and intend to exploit the guinea pig model and other assays to investigate whether morphology, NA activity, or stability contribute to the observed differences in transmission.

26. Spherical influenza viruses have a fitness advantage in embryonated eggs, while filament producing strains are selected in vivo

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It has long been observed that influenza virus can assume two predominant morphologies: filamentous and spherical. The filamentous morphology occurs mainly in primary or low-passage isolates while the spherical morphology is seen in strains that have been grown extensively in laboratory substrates such as eggs. The fact that the filamentous morphology is maintained in nature but not in the laboratory raises the question of whether the filamentous morphology confers a selective advantage in the host that is not necessary for growth in laboratory substrates. To address this question, we assessed the effect of serial passage in vitro and in vivo on virion morphology and growth. Two filamentous strains, A/Netherlands/602/2009 (H1N1) and A/Georgia/M5081/2012 (H1N1), were passaged in eggs, the common laboratory substrate believed to select for spherical virions. Conversely, the spherical laboratory strain A/Puerto Rico/8/1934 (H1N1) was passaged 12 times in guinea pigs. After passaging, we found that, in eggs, a conversion to a spherical morphology was not required for increased growth. We did, however, identify two point mutations in the M1 matrix protein of egg passage 10 isolates that confer both a spherical morphology and increased growth in eggs, suggesting that spherical influenza viruses can have a fitness advantage in eggs. Conversely, adaptation to guinea pigs was associated with the emergence of filamentous virions and point mutations were identified within the guinea pig passage 12 virus pool which, when introduced individually into the PR8 background, lead to robust filament production. These data suggest a functional role for filaments in vivo.
27. Automated production and analysis of evergreen phylogenetic trees for avian Influenza

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We recently published a report on the potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host (Russell et al., Science, 2012). We showed phylogenetic trees from avian and human samples color coded by whether viruses had particular substitutions, and by the number of nucleotide mutations from the Imai et al. 2012 and Herfst et al. 2012 viruses. We have now created an automatic pipeline where the nucleotide sequences for all gene segments are downloaded from the IVR sequence database, aligned and phylogenetic trees generated and coloured for all substitutions of interest. We have extended this work to include all substitutions that are potentially functionally-equivalent in hemagglutinin (HA) receptor binding. In addition, we have generated phylogenetic trees color coded by all substitutions of concern in the other seven gene segments, as detailed by the US CDC inventory of H5N1 Genetic Changes (http://www.cdc.gov/flu/pdf/avianflu/h5n1-inventory.pdf).

In addition to the generation of phylogenetic trees, other analysis of the data, such as tracking genetic changes over time, is also routinely performed. The resulting analysis is freely available from http://antigenic-cartography.org/surveillance/evergreen/H5 and will be automatically updated monthly with the latest sequence data, and as more substitutions of interest in any gene segment are identified. This automated pipeline can also be applied to other subtypes of the influenza virus as well as other host species.

Molecular Virology

28. Mutations in PA cause a loss of the temperature sensitive phenotype conveyed by the PB2 gene of the Live Attenuated Influenza Vaccine

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Influenza viruses infect millions of persons each year and are directly responsible for between 3,000 and 49,000 deaths annually in the United States. This morbidity and mortality has led to vaccination efforts against influenza A and B. Due to the low effectiveness of the triple inactivated virus (TIV), a live attenuated influenza virus (LAIV) was successfully developed to increase efficacy. The current LAIV has recently been recommended as the primary vaccination strategy in persons ages 2-49. Therefore, the mechanism underlying the stability of this attenuation is of great importance and not fully understood. This virus has been attenuated through cold adaptation and subsequent work has determined the attenuating segments. These are contained in the polymerase (PB1, PB2, PA) and nucleoprotein segments. It has been shown through viral recombination that while the introduction of the attenuating PB2 segment into a wild type background could convey temperature sensitivity, rescue mutations could restore the ability to grow at elevated temperatures. These revertant viruses are of great interest as they provide an insight into the mechanism of attenuation by providing genetically similar viruses with vastly differing phenotypes and are medically significant as they provide an insight into vaccine safety.

We were gifted with these viral isolates from the lab of John Treanor and have isolated plaque purified viruses possessing impaired growth at 39°C as well as genetically similar phenotypic revertant viruses. Characterization utilizing a minigenome assay has confirmed the residues responsible for this phenotypic reversion to be within the PA segment.
29. Challenges in temporal phylogenetics and selection analysis of H7N9 and other rapidly emerging zoonotic viruses

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Phylogenetic analysis is a powerful tool for investigating the emergence pathway of zoonotic influenza viruses and in identifying the source population(s) and times of interspecies transmission and reassortment events. However, rapidly evolving RNA virus such as influenza exhibit a significant excess of low frequency non-synonymous mutations on external branches of a phylogeny when compared to internal branches. These are most likely deleterious or slightly deleterious non-synonymous mutations that are not fixed in the virus population and would normally escape detection. Furthermore, virus sequences from previous zoonotic influenza outbreaks have shown an increased ratio of non-synonymous to synonymous (d_N/d_S) mutations that could be due to the increased detection of mildly deleterious mutations resulting from intensive epidemic surveillance or these mutations could be adaptations to the new host species. Irrespective of the cause of the increased d_N/d_S in outbreak sequences, its presence can confound date estimates in temporal phylogenetics. Using pandemic H1N1 and avian H7N9 sequences as examples, we compared dN/dS values, both averaged and of external and internal branches of the phylogeny, of outbreak versus non-outbreak data sets to approximate the degree of excess non-synonymous mutations in the outbreak sequences and show that this leads to older estimates of interspecies transmission and reassortment events.

30. Fitness of influenza B viruses with neuraminidase inhibitor-resistant mutations in vitro

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The neuraminidase (NA) inhibitors (NAIs) are the only FDA-approved class of antivirals for treatment of influenza B infection. To date the level of NAI resistance among influenza B viruses is low but the risk of emergence of uncompromised and transmissible variants must be examined. We investigated the role of single NA mutations on fitness of influenza B viruses in vitro. Using reverse genetics we introduced catalytic (R371K) or framework (E119A, I222T, D198E, D198Y, H274Y, N294S) NA mutations into B/Yamanashi/166/1998 virus. A fluorometric-based assay demonstrated different impact of these mutations on oseltamivir susceptibility: low reduction (IC_{50} <15-fold as compared to WT) was caused by I222V and H274Y mutations; medium reduction (15 < IC_{50} < 50-fold) was conferred by D198E and N294S mutations and high reduction (IC_{50} >50-fold) by E119A, D198Y, and R371K mutations. The NA mutations did not significantly alter K_m and V_max values when compared to WT, except for R371K exhibiting lower affinity. All viruses had replicative capacities comparable to WT in NHBE cells, except restricted replication of D198Y and R371K viruses. Only the R371K mutation reduced both surface NA protein expression in 293T cells and NA activity as compared to WT. Three other mutations (E119A, D198E and D198Y) reduced NA activity without loss of protein expression. Therefore, NAI-resistance associated mutations can affect fitness of B/Yamanashi/166/1998 influenza virus by distinct mechanisms either by altering NA expression or activity. Importantly, of three NA mutant viruses conferring high NAI resistance, fitness of D198Y and R371K viruses was severely impaired, but E119A virus was genetically stable with fitness comparable to WT. Thus the emergence of influenza B virus with E119A mutation would be a public health concern and should be closely monitored.
31. Determination of neuraminidase kinetic constants using whole influenza virus preparations and correction for spectroscopic interference by a fluorogenic substrate

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The influenza neuraminidase (NA) enzyme cleaves terminal sialic acid residues from cellular receptors, a process required for the release of newly synthesized virions. A balance of NA activity with sialic acid binding affinity of hemagglutinin (HA) is important for optimal virus replication. NA sequence evolution through genetic shift and drift contributes to the continuous modulation of influenza virus fitness and pathogenicity. A simple and reliable method for the determination of kinetic parameters of NA activity could add significant value to global influenza surveillance and provide parameters for the projection of fitness and pathogenicity of emerging virus variants. The use of fluorogenic substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) and cell- or egg-grown whole influenza virus preparations have been attractive components of NA enzyme activity investigations. We describe important criteria to be addressed when determining Km and Vmax kinetic parameters using this method: (1) determination of the dynamic range of MUNANA and 4-methylumbelliferone product (4-MU) fluorescence for the instrument used; (2) adjustment of reaction conditions to approximate initial rate conditions, i.e. ≤ 15% of substrate converted during the reaction, with signal-to-noise ratio ≥ 10; (3) correction for optical interference and inner filter effect caused by increasing concentrations of MUNANA substrate. The results indicate a significant interference of MUNANA with 4-MU fluorescence determination. The criteria proposed enable an improved rapid estimation of NA kinetic parameters and facilitate comparison of data between laboratories.

32. Characterizing non-structural protein 1 (NS1) from bat influenza virus H17N10

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Bats have emerged as a potential reservoir species for influenza virus after isolation of genomic material from a novel influenza virus, H17N10, from bat tissue. H17N10 has yet to be shown to replicate in bats or to be grown in a laboratory setting; however, recent published data as well as unpublished data from our lab indicates that at least several bat cell lines from different species are able to support influenza infection. Given the well-characterized role of influenza non-structural protein 1 (NS1) in promoting viral replication by antagonizing the host immune response, we have chosen to characterize the H17N10 NS1 protein. Sequence analysis shows that the H17N10 NS1 shares around a 50-60% degree of homology to various influenza A strain NS1 proteins. Several important features of NS1 are conserved in the H17N10 NS1, including residues involved in binding to dsRNA and PI3 kinase. Preliminary results indicate that H17N10 NS1 protein is a weaker but functionally equivalent interferon (IFN) antagonist in human cells. For example, while IFN β promoter reporter assays show that H17N10 NS1 is less efficient than H1N1 PR8 NS1 in blocking IFN production, these NS1 proteins show a similar ability to block IRF3 nuclear translocation upon cellular stimulation. Interestingly, H17N10 NS1 localizes in a manner distinct from other characterized NS1 proteins in mammalian cells, with a higher degree of membrane localization and perinuclear foci formation. Studies in bat cell lines are currently being done to better understand these findings.
33. Generation of Baculovirus Transducer for Influenza A Viruses Rescue

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We demonstrate a new strategy to generate Flu vaccine candidates, which combined Bac-to-bac baculovirus system with Bacuodirect Gateway cloning method. First, we amplified all the eight segments cassettes of PR8, Ty04att, WF10att, and VN1203 virus flanking with CMV-hpol1 ambisense expression elements. We subcloned all the internal segments into one shuttle plasmid, and also subcloned HA and NA segment cassettes into an entry vector. Using LR recombination of Gateway cloning technique, we got an all-in-one plasmid carrying all the eight segments expression cassettes. Compared to the classical reverse genetics system, the all-in-one plasmids showed a much higher efficiency in virus rescue in Vero cells. After transposition into DH10Bac competent cells, we generated a set of recombinant baculoviruses carrying the full genome of flu virus. These baculoviruses could transduce efficiently into mammalian cells and produce flu viruses or reassortants. Thus, our approach suggested a complete departure from other conventional or non conventional influenza vaccine strategies. Intranasal and/or oral delivery of a recombinant baculovirus carrying a reverse genetics competent LAIV will be carried out to result in the generation of live influenza viruses in the host and the stimulation of neutralizing immune responses against influenza.

34. The effects of deleterious intermediates on within-host pathogen evolution

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Models on the evolution of virus populations, which include processes of host-adaptation, frequently involve the study of fitness effects. These may be ultimately neutral or beneficial substitutions, but similarly certain intermediate mutants may be deleterious (such as a loss of binding affinity, before gaining affinity for a different cell type). Through studying the number of routes through which a given virion can obtain a required set of mutations, using a deterministic probability model of virus evolution over time, a more intuitive understanding and description of the accumulation of mutations is provided. Results will be given for a range of fitness costs, variations on the number of intermediate mutants being deleterious, and for the situation in which the fully adapted mutant has a fitness advantage over the starting (wild type) virion. We also demonstrate and develop intuition on the effect of any requirement of a certain order of mutations. In summary, the results show that deleterious costs in intermediate mutants can have a great effect on the viral population outcome, if many mutants are deleterious. However, the effect of deleterious mutations in general is much smaller than may be initially expected from the name "deleterious", because viruses can avoid any large fitness cost by acquiring multiple mutations at once: in this way, the deleterious cost is never incurred.

35. Investigating the effect of natural variation on an unusual H9 wild isolate strain’s viral fitness

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Wild birds are the natural reservoirs of avian influenza viruses (AIVs), which occasionally cross the species barrier to infect domestic poultry and mammals. H9N2 is one important subtype that is currently on the watch list of the WHO as potentially pandemic because of its history of trans-species transmission to humans and pigs. A careful examination of wild H9N2 viruses identified an interesting reassortant isolate from a wild Alaskan duck. Preliminary genetic analysis of this virus revealed acquisition of segments from H5N2 and H7N3 viruses. Acquisition of a PB2 segment from an H5N2 virus (A/snow goose/Montana/466771-4/2006) conferred 10 unique amino acid changes to this important component of
the viral polymerase complex that has been shown to be involved in mammalian adaptation. Using a reporter assay to quantitatively measure polymerase function, we show that the newly acquired H5N2 PB2 segment slightly increased viral polymerase activity by about two fold. We also show that a basic amino acid (R or K) at position 591 significantly increased H9N2 polymerase activity by 3-5 fold. Q591K mutation has been recorded in naturally circulating H9N2 viruses in China and Saudi Arabia. Homology modeling indicates that replacing Q (neutral) with a positively charged R or K might increase the binding affinity to other interacting partners. A double mutant of Q591K and E627K or D253N didn’t offer any synergistic effect to the H9N2 polymerase function. However, A double mutant of Q591R and E627K or D253N increased polymerase activity by at least two fold.

36. Interactions among the Influenza A virus RNP Components and the Retinoic Acid-Inducible Gene I: Impact on Interferon Production and Viral Polymerase Activity

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The influenza A virus genome possesses eight negative-strand RNA segments in the form of viral ribonucleoprotein particles (vRNPs) in association with the three viral RNA polymerase subunits (PB2, PB1, and PA) and the nucleoprotein (NP). Through interactions with multiple host factors, the vRNP components play vital roles in replication, host adaptation, interspecies transmission, and pathogenicity. In order to gain insight into the potential roles of vRNP components in the modulation of the host’s innate immune response, we investigated the interactions of each RNP subunit with Retinoic Acid-Inducible Gene I protein (RIG-I) from mammalian and avian species. Studies using Bimolecular Fluorescence Complementation (BiFC), co-immunoprecipitation (Co-IP), and co-localization using confocal microscopy, provided direct evidence for the binding of PB2, PB1, PA, and NP with RIG-I from various hosts (human, swine, mouse, and duck). Expression of the viral NS1 protein, which interacts with RIG-I, did not interfere with, but rather enhanced, through stimulation of protein expression, the association of RNP components with RIG-I. The association of PB2, PB1, and PA, but not NP, with RIG-I led to reduced activation of the IFNβ promoter and Interferon Stimulated Response Elements (ISRE) elicited by RIG-I in transient reporter assays. In addition, RIG-I from different species suppressed polymerase activity of influenza A viruses to various degrees in both IFN-competent and IFN-deficient cell systems. These observations argue for opposing forces between RNP activity and the RIG-I signaling pathway, aimed at cancelling each other.

37. Influenza A virus NS1 protein blocks and prevents different complexes in the RIG-I like receptor pathway

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The innate immune system plays a critical role in the induction of an antiviral state in viral infected cells. The secretion of several cytokines is activated with the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Among PRRs, retinoic acid inducible gene (RIGI) like receptors (RLR) plays an important role in the identification of RNA viruses. RIGI, upon contact with viral RNA motifs, interact with the IFN-β promoter stimulator 1 (IPS-1) which triggers downstream signaling resulting in the production of cytokines such as type I and type II IFNs. Some viruses have developed strategies to inhibit the induction of IFN system and survive in the host cell, by escaping from the PRRs detection system or by inhibiting the function of PRRs interacting directly or indirectly with some of the proteins that participate in the regulation of the RLR pathway. In order to study in more depth the molecular mechanisms involved in the interaction virus-host cell, we have developed a
Bimolecular Fluorescence Complementation (BiFC) assay. The non-structural protein 1 (NS1) from Influenza A virus (IAV) interferes in the complex formation RIGI-MAVS and TRIM25 dimerization specifically, but does not have any effect on the interaction RIGI-TRIM25. These interactions take place in different compartments in the host cells. The use of BiFC technique has provided us a new and powerful tool to analyze known and novel protein interactions of the RLR pathway among themselves and with viral proteins in living cells. We have been able to isolate and track directly protein interactions, analyzing the localization in the host cell and the effect of these complexes in the type I IFN production.

38. PI3K/FAK signaling: linking receptor-mediated PI3K activation and influenza virus-endoosomal trafficking

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Virus entry is mediated by host-cell signaling cascades induced by viral attachment to extracellular receptors. Phosphatidyl-inositol 3-kinase (PI3K) is activated through multiple pathways including receptor tyrosine kinases. PI3K activation by influenza A virus (IAV) attachment is required for efficient IAV entry and infection. Integrin-mediated PI3K signaling is dependent on focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase component of focal adhesion complexes associated with the actin cytoskeleton. FAK activation leads to Y397 phosphorylation, which serves as a binding site for Src kinases and PI3K that in turn mediate maximal FAK activation through Y576/577 phosphorylation. Active FAK phosphorylates paxillin-Y118 to affect actin reorganization. FAK-regulated virus entry was reported for Kaposi's sarcoma herpes-virus (KSHV) and herpes simplex virus 1 (HSV-1) but not for influenza virus. Therefore, we investigated the role of FAK during influenza virus infection. We observed that viral attachment to sialic acid was required for efficient PI3K-dependent FAK-Y397 phosphorylation; this correlated with Y118 phosphorylation of paxilin, a FAK effector protein. FAK inhibitor treatment resulted in dose-dependent viral titer reductions; also confirmed in NHBE cells. Additionally, overexpression of a kinase-dead FAK mutant in A549 cells produced lower titers compared to cells overexpressing wild-type FAK (FAK-WT). Virus infectivity and NP expression were reduced in FAK inhibitor treated cells 24 hpi. We observed minimal colocalization of virus with early endosomes in the presence of FAK inhibitor that also resulted in virion accumulation at the periphery of cells. Neither FAK-pY397 inhibition nor FAK-KD overexpression affected cortical actin or microtubules but did affect the actin meshwork and stress fibers. Our data highlights a novel regulatory role of FAK in influenza virus entry linking receptor-mediated PI3K signaling to IAV-induced actin reorganization and endosomal sorting.

Pathogenesis

39. Influenza virus attachment patterns in the upper and lower respiratory tracts of guinea pigs

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Viral attachment is an important determinant of the cell and tissue tropism of influenza viruses in animals. The guinea pig model is increasingly used to study the growth and transmission of influenza viruses in a mammalian host. However, the tissues to which different influenza viruses bind in guinea pigs are not fully understood. To gain a better understanding of the influenza virus binding patterns in guinea pig tissues, we examined the attachment of a human strain [A/Netherlands/213/03 (H3N2)] and an avian-like virus [carrying the H3 HA of A/dk/Ukraine/63] on nasal turbinate and lung tissues using a histochemistry-based technique, called virus-histochemistry. The human virus attached to the apical side of ciliated epithelial cells in the respiratory epithelium and to the apical side of the olfactory mucosa in the nasal cavity. Attachment of the human strain was not seen in the epithelium of the lower respiratory tract, specifically the bronchioles, alveoli or alveolar macrophages; however this virus did bind endothelial cells
in these areas. The avian-like virus exhibited binding to the apical side of ciliated epithelial cells in the respiratory epithelium and to the apical side of olfactory mucosa in the nasal cavity, although to a lesser degree than the human virus. The avian-like virus also bound epithelium in the lower respiratory tract, specifically to the apical side of bronchiolar epithelium, alveolar epithelial cells and alveolar macrophages. Taken together, this study shows that there are marked differences in the attachment pattern between human and avian influenza viruses in the guinea pig respiratory tract, which could be important for transmission efficiency.

40. Experimental co-infection of poultry with avian influenza and Newcastle Disease viruses

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Avian influenza virus (AIV) and Newcastle disease virus (NDV) are two of the most important viruses affecting poultry worldwide. Co-infections with AIV and NDV in poultry are problematic for clinical management and accurate diagnosis. We conducted three experiments in which we infected chickens, turkeys and Pekin ducks with lentogenic, mesogenic or velogenic strains of NDV and with low pathogenic (LP) or highly pathogenic (HP) AIV by giving the viruses simultaneously or sequentially. No clinical signs were observed in chickens co-infected with a lentogenic NDV strain and a LPAIV, or in chickens infected with the two viruses given separately. However, the pattern of virus shedding was different, with co-infected birds initially shedding lower amounts of virus than birds infected with the single viruses. All turkeys inoculated with the same LPAIV, co-infected or not, presented similar mild clinical signs. Like chickens, co-infected turkeys had altered patterns of virus shedding, which was especially evident in the group that received the LPAIV followed by NDV. We also found that previous infection of chickens with more virulent NDV viruses interfered with replication of a HPAIV, in some cases even preventing infection. Likewise, previous infection of ducks with a velogenic NDV interfered with infection with a LPAIV, and vice versa. In conclusion, previous or simultaneous infection of NDV and AIV can affect replication dynamics and disease caused by these viruses in poultry. This virus interference will depend on the virulence of the two viruses co-infecting the birds and the timing of the infections.

41. Characterization of an H5N1 highly pathogenic avian influenza virus strain isolated from a domestic duck in Vietnam in 2012

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The continued spread of highly pathogenic avian influenza virus (HPAIV) subtype H5N1 among poultry in Vietnam has posed a potential threat to animals and public health. To estimate the possible role of infected ducks as a source of viral replication as well as virus spread, an experimental infection with HPAIV subtype H5N1 was carried out in domestic ducks. Ducks were infected with 10^{7.2} TCID_{50} of A/duck/Vietnam/QB1207/2012 (H5N1), which was isolated from moribund domestic duck. In the infected ducks, clinical signs of disease including neurological signs were observed. Ducks started to die at 3 days-post-infection (dpi), and the mortality reached 67%. Viruses were recovered from oropharyngeal and conjunctival swabs until 7 dpi, and from cloacal swabs until 4 dpi. In the ducks that died or were sacrificed on 3, 5, or 6 dpi, viruses were recovered from the lung, brain, heart, pancreas and intestine, among which the highest virus titers were in the lung, brain or heart. Results of virus titration were confirmed by real-time RT-PCR for viral gene detection. Genetic and phylogenetic analysis of the HA gene revealed that the isolate belongs to clade 2.3.2.1 similarly to the other H5N1 viruses isolated in Vietnam in late 2012. The present study demonstrated that recent HPAI H5N1 virus of clade 2.3.2.1 retains the neuron-oriented feature, and further developed properties of amplification and shedding virus
from the oropharynx and conjunctiva rather than from cloaca, which is posing a higher risk of virus spread through cross-contact and/or environmental transmission.

42. Lung cytokine gene expression is correlated with increased severity of disease in mice infected with H4N8 virus isolated from shorebirds

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We reported that an H4N8 subtype virus, which was isolated in our AIV surveillance study in shorebirds in Japan, caused severe respiratory disease in mice. The H4N8 isolate was genetically highly related to an Australian H4N8 strain isolated from a shorebird, except for significant differences in the PB1 and some differences in the NS gene segments. Both strains proliferated in mouse lungs to a similar level, but the Australian strain did not cause disease in mice. In order to elucidate the role of host immune responses in the disease severity in these mice, we investigated the lung cytokine gene expression profiles of mice infected with these two strains of H4N8 viruses isolated from shorebirds, and reference H4 viruses from ducks. In mice with H4N8 virus induced pneumonia, overall expression of TNF-α, IL-6, and IL-12 genes was markedly higher than in mice infected with other H4 viruses tested, although gene expression of type I interferon was not increased until day 4 post viral infection. In contrast, in the mice infected with the Australian H4N8 strain, gene expression of type I interferon peaked on day 1 post viral infection. Overall, the cytokine response corresponds with the severity of disease caused by shorebird H4N8 virus. The results obtained in this study provide valuable information to understand the immunopathology induced by a low pathogenic AIV, which may be useful in preparation for outbreaks of novel influenza A virus.

43. Pathogenicity and fitness of recent reassortant avian H5N1 viruses in birds, NHBE cells and ferrets

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Highly pathogenic avian influenza (HPAI) viruses of subtype H5N1 were identified in healthy domestic ducks during systematic surveillance in Lao People’s Democratic Republic during 2010 including natural reassortants. The genotypes of identified viruses did not contain the major known pathogenicity markers such as PB2627E or 701N, or HA222L. The three reassortants (clade 2.3.2.1 x clade 2.3.4(NP); 2.3.4.2x2.3.2.1(PB2); 2.3.2.1xH9N2(PB1)) were inoculated into adult mallards (ocular/nasal/oral; 4 log EID50). Symptoms were none to transient conjunctivitis and shedding had differing preference for trachea and cloaca. One reassortant was assessed for transmission and found to transmit to 2/3 direct contact mallards and to 3/3 direct contacts chickens. The three reassortants and a clade 2.3.4.1 duck isolate (A/duck/Lao/1023/2010) grew to low titers in differentiated normal human bronchial epithelial (NHBE) cells compared to control A/Brisbane/59/07 (seasonal H1N1). Three-month old outbred male ferrets were inoculated intranasally with 6 log TCID50 and showed different levels of pathogenicity (non-pathogenic to lethal/clade 2.3.4.1 virus) and virus shedding (none to 6 log TCID50/mL/clade 2.3.4.1 virus.) The clade 2.3.4.1 virus showed some ability for direct contact transmission. Most surviving inoculated ferrets seroconverted to low titers (5/6; detectable with horse red blood cells only). In conclusion, despite the absence of pathogenicity markers, two of the viruses examined showed potential to cause disease in humans as assessed in the ferret model, and one virus showed potential for mammalian transmission. These results reiterate the call for systematic pathogenicity assessment of novel H5N1 viruses even in the absence of known pathogenicity markers.
44. Isolation of a H4N2 virus from quails with a dibasic-amino acid insertion in the hemagglutinin cleavage site

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Highly pathogenic avian influenza strains (HPAI) are of significant agricultural and public health concerns due to its high mortality rate in infected birds and humans. The increased pathogenicity is associated with the presence of a multibasic cleavage site (MBCS) in the hemagglutinin (HA) protein which leads to increased tissue tropism and multiorgan infection. Thus far, the multibasic cleavage site motif and high pathogenicity phenotype have only been reported for the H5 and H7 subtypes, leading to the general thought that acquisition of MBCS is restricted to these two subtypes. In this study, we report the isolation of an H4N2 virus, from an outbreak at a quail farm, with two basic amino acids –RR- inserted 2 position upstream of the HA cleavage site forming a MBCS-like motif. Phylogenetic analyses of the HA and neuraminidase (NA) genes revealed that this virus was most closely related to an H4N2 virus isolated from domestic duck in 2006 but had undergone reassortment and acquired internal genes from contemporary avian strains circulating in North American wild birds. The NA gene also had the 17-amino acid deletion in the stalk region, suggesting that it had adapted to poultry species. Phenotypic characterization showed that the quail virus is of low pathogenicity in chickens, but had a higher binding affinity for the mammalian-type receptor compared to the duck isolate. Findings from this study demonstrate that the acquisition of the MBCS-like motif can occur in other subtypes in nature but does not render them highly pathogenic in avian species.

45. Depletion of Alveolar Macrophages during Influenza Infection Facilitates Bacterial Super-infections

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Viruses such as influenza suppress host immune function by a variety of methods. This may result in significant morbidity through several pathways, including facilitation of secondary bacterial pneumonia from pathogens such as Streptococcus pneumoniae. PKH26-PCL dye was administered intranasally to label resident alveolar macrophages (AMs) in a well-established murine model prior to influenza infection to determine turnover kinetics during the course of infection. More than 90% of resident AMs were lost in the first week after influenza, while the remaining cells had a necrotic phenotype. To establish the impact of this innate immune defect, influenza-infected mice were challenged with S. pneumoniae. Early AM-mediated bacterial clearance was significantly impaired in influenza-infected mice - about 50% of the initial bacterial inoculum could be harvested from the alveolar airspace 3 hours later. In mock-infected mice, by contrast, more than 95% of inocula up-to-50-fold higher was efficiently cleared. Co-infection during the AM depletion phase caused significant body weight loss and mortality. Two weeks after influenza, the AM population was fully replenished with successful re-establishment of early innate host protection. Local GM-CSF treatment partially restored the impaired early bacterial clearance with efficient protection against secondary pneumococcal pneumonia. We conclude that resident AM depletion occurs during influenza infection. Among other potential effects, this establishes a niche for secondary pneumococcal infection by altering early cellular innate immunity in the lungs resulting in pneumococcal
outgrowth and lethal pneumonia. This novel mechanism will inform development of novel therapeutic approaches to restore lung innate immunity against bacterial super-infections.

46. Exploration and Comparison of Host Factor Responses to Viral Infection in the Influenza Research Database (IRD)

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Host cell responses to viral infection can be monitored by a variety of difference high throughput experimental methodologies in order to understand the biological systems involved. Gene expression studies can be used to identify host factors required for viral replication. RNAi screens can be used to identify host factors required for viral replication. The Influenza Research Database (IRD, www.fludb.org), a free online database resource supported by the U.S. National Institute of Allergy and Infectious Diseases (NIAID) Bioinformatics Resource Centers (BRC) program, has recently implemented a new Host Factor component that contains results from experiments investigating host responses to viral infections. At present, results from 33 host factor experiments generated by NIAID-sponsored Systems Biology for Infectious Diseases Research programs - Systems Virology (www.systemsvirology.org) and Systems Influenza (www.systemsinfluenza.org) - are available. These datasets currently include both transcriptomic and proteomic experiments; future datasets being added include lipidomics, metabolomics, and RNAi screens of both influenza A and Dengue virus infections. Within the Host Factor component, one can investigate the design and results of individual experiments, search for comparable experiments via a faceted navigation menu drawn from the structured experiment metadata, or directly search for results for individual host factors. After locating experiments of interest, one can explore the trends within an experiment by viewing the patterns of responses, or compare results from different experiments by choosing from several methods of combining data, including “subtraction” and “intersection” operators. Finally, one can view the pathway membership of selected host factor biosets in the Reactome resource, a free online tool for visualizing biological pathways. As the Host Factor component of IRD expands both in its functionality and scope, it will become a one-stop shop for the exploration of a comprehensive collection of viral host factor studies.

Surveillance

47. Juveniles and migrants as drivers for seasonal epizootics of avian influenza virus

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The prevalence of low pathogenic avian influenza viruses (LPAIV) has shown to exhibit marked seasonal variation in wild birds. However, mechanisms driving this seasonal variation have yet to be tested. We investigated the validity of three previously suggested drivers for the seasonal dynamics in LPAIV infections in wild birds: (1) immunologically-naïve juveniles, (2) susceptible migrants, and (3) host density. We sampled a key LPAIV host species, the mallard Anas platyrhynchos, on a small-spatial scale throughout a complete annual cycle. Using a mallard feather isoscape, based on stable hydrogen isotope ratio measurements of mallard feathers of natal origin, we could discriminate between residents, local (short-distance) and distant (long-distance) migrants. We found a minor peak in LPAIV prevalence
in summer and a dominant peak in autumn, during which half of the sampled population was infected. The summer peak of LPAIV prevalence coincided with the entrance of naïve chicks in the population, who were more likely to be infected and less likely to have AIV antibodies than adult birds. The arrival of migratory birds appeared to drive the autumn peak in LPAIV infection, with migrants more likely to be infected than residents. Remarkably, there was no difference in AIV antibodies between migrants and residents. Host density increased throughout autumn, it peaked in winter, however showing no direct correspondence with either of the LPAIV infection peaks. This study exemplifies the importance of understanding host demography and migratory behaviour when examining seasonal drivers of infection in wildlife populations.

48. Surveillance for Avian Influenza Viruses in Western Siberia (Russia), 2008-2011

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The territory of Western Siberia (Russia) is situated in the center of the Eurasian continent. It is located under three major bird migration routes, combining bird populations from Asia, Africa and Europe. Western Siberia’s geographic location and climatic conditions favors virus evolution and promotes maintenance of avian influenza viruses (AIV)s in water and soil for extended periods of time. Seasonal bird migrations support the distribution of different virus variants, including highly pathogenic AIVs of H5 and H7 subtypes, over long distances. AIVs from wild birds can be transmitted to poultry, humans and other mammals, which causes significant economic loses and menace to human health. During surveillance for AIV in Western Siberian (Novosibirsk, Omsk and Altai Regions) conducted during 2008-2011, 4678 samples from wild birds of 11 orders were collected. The most abundant bird species sampled were Anseriformes, Charadriiformes and Passeriformes. In total, 105 AIVs were isolated and 21 different viral subtypes were detected. No isolated influenza virus possessed a hemagglutinin multibasic motif and thus all were categorized as low pathogenic. The viruses were isolated from Anseriformes (n=98), Charadriiformes (n=8), Gruiformes (n=2) and Ciconiiformes (n=1). The most common virus subtypes were H3N8 (n=38) and H4N6 (n=16). Moreover, rare virus subtypes (H8N8, H15N4, H16N3, LPAI H5N1) were isolated during these surveillance studies which were not previously identified Western Siberia or elsewhere (according to databases). Phylogenetic analyses of viral genes showed a close relationship of Russian viruses with strains from different European, African, and Asian countries as well as from Australia. This surveillance study demonstrates the importance of the Western Siberia territory in distribution and evolution of AIVs due to the ecological intermingling of bird populations of Russia and different countries of Asia, Europe, Africa as well as Australia.

49. Seasonal dynamics of avian influenza outbreaks in Central Africa

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Understanding how seasonal climate cycles affect the timing of disease outbreaks can help predict and potentially prevent future epidemics. Early warning systems already exist for Rift Valley fever, Chikungunya virus, and malaria, which use changes in rainfall or temperature to predict abundance of arthropod vectors and outbreaks. However, how climatic factors influence influenza epizootics in animal hosts in the tropics is largely unknown. To investigate this, we analyzed a time series of influenza infections in wild and domestic birds in Cameroon, which experienced an outbreak of highly pathogenic H5N1 avian influenza in 2006 in ducks. Results indicate that influenza infections in tropical songbirds exhibit significant seasonality synchronized with Cameroon’s two annual rainy seasons (autocorrelation
Chi-squared test = 24.52, \( p = 0.0004 \), Akaike weight for precipitation = 0.87). A mechanism to explain this is that the rainy season serves as cue for birds to congregate and breed, increasing the rate of contact between infected and susceptible individuals. Consistent with this hypothesis, we found that passerines that are communal breeders have higher prevalence than non-communal breeders. It is expected that climate change effects could increase precipitation by as much as 44% by 2050 in Cameroon, which has the potential to cause a major increase in influenza cases in wild birds.

50. Identifying areas with a high risk of human infection with the avian influenza A(H7N9) virus in East Asia

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In February 2013, a new influenza A virus of subtype H7N9 spilled over from birds to humans in eastern China. Within two months H7N9 had been reported in eight provinces, Beijing, Shanghai, and Taiwan, infecting 131 people and causing 35 fatalities, a 27% mortality rate. By comparison, the Spanish influenza pandemic of 1918 that killed 50 million and only had a 1% fatality rate. Due to its significant mortality, it is crucial to detect H7N9 in birds to limit transmission to humans. However, H7N9 may be asymptomatic in poultry, which complicates detection. Thus, there is an urgent need for better methods to predict the location of H7N9 outbreaks. To address this, we developed spatial models to identify sites with high ecological suitability for H7N9 in East Asia. The novel H7N9 virus arose through reassortment (exchange of genes) among influenza strains that circulated in birds including H9N2 and an ancestral strain of H7N9. We predicted ecological suitability for H9N2 and ancestral H7N9 using chicken, duck, and human population density and the percent land and water per site. We then calculated the probability that both H9N2 and ancestral H7N9 would occur at a site, which could lead to reassortment in birds. Results indicate that sites that are ecologically suitable for reassortment between ancestral H7N9 and H9N2 in birds overlap significantly with human cases of novel H7N9. Our model identifies Chinese provinces with a high risk of future H7N9 emergence such as Chongqing, Guangdong, Hebei, and Liaoning, which can be prioritized for surveillance.

51. Characterization of Asian reassortant H13N8 influenza virus isolated from gull

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Central Asia is an important region for control and research of influenza virus spreading by birds during migration, because it is crossed by main flyways connecting China, Southeast Asia, and Russia and it has its own specific avifauna. Mongolia is separated from Alaska and Far East where reassortments of American and Eurasian genes in AIVs isolated from gulls have been documented and we investigated reassortment events in this region. We isolated influenza A(H13N8) virus A/herring gull/Mongolia/454/08 (GenBank: JF775470-JF775477) from herring gull \((Larus argentatus)\) that was caught at Uvs Nuur Lake (Mongolia). All genes of A/herring gull/Mongolia/454/2008(H13N8) virus except PA and NS were of
classical avian-like Eurasian lineages. The PA gene is clustered with classical avian-like LP AIV strains of different subtypes isolated in Africa, Asia, and Europe, and also to HPAI H5N1 viruses isolated from ducks in 2008-2010 in Russia, Mongolia and China. The NS gene (A allele) of this Mongolian H13N8 strain belongs to gull-like viruses and is related to H13 and H16 viruses from Mongolia, Ukraine and Alaska. The remaining four internal segments belong to a gull-like clade and are related to H13 and H16 viruses from Eurasia and North America. We analyzed the viral pathogenicity in chickens and mice and investigated the predicted functional amino acid and glycosylation changes in this virus. Our data highlights the relevance of the present study identifying rare influenza virus in Asia, its importance in understanding AIV evolutionary ecology in gulls, and the needs for surveillance of novel reassortant virus that may have pathogenic potential.

52. Prevalence and characterization of influenza viruses in diverse species in Los Llanos, Colombia

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While much is known about the prevalence of influenza viruses in North America and Eurasia, their prevalence in birds and mammals in South America is largely unknown. To fill this knowledge gap and provide a baseline for future ecology and epidemiology studies, we conducted 2 years of influenza surveillance in the Llanos region of Colombia. qRT-PCR identified influenza viruses in wild birds, domestic poultry, swine and horses. Prevalence ranged from 2.6% to 13.4% across species. Swine showed the highest prevalence and were infected primarily with 2009 pandemic H1N1 viruses genetically related to those in humans. In addition, we isolated H5N2 viruses from two resident species of whistling ducks (genus Dendrocygna) that differed completely from previous South American isolates, instead genetically resembling North American wild bird viruses. Both strains caused low pathogenicity in chickens and mammals. The prevalence and subtype diversity of influenza viruses isolated from diverse species within a small area of Colombia highlights the need for enhanced surveillance throughout South America, including monitoring of the potential transmissibility of low-pathogenic H5N2 viruses from wild birds to domestic poultry and the emergence of reassortant viruses in domestic swine.

53. The evolutionary dynamics of influenza A virus migration and competition in North American wild birds

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The geographical separation of potential avian host species has structured the avian influenza A virus (AIV) gene pool into independently evolving Eurasian and American populations. Phylogenetic evidence has shown viral migration between these populations. The co-circulation of antigenically similar viruses creates suitable conditions to study viral competition in natural populations. In the mid 1990’s H6 subtype influenza viruses with hemagglutinin (HA) gene derived from Eurasian ancestors were detected in North American wild birds. Since 2003 the endemic H6-HA lineage has not been detected. The introduced lineage has displaced the endemic population. However, bird migratory habits and the discontinuous distribution of susceptible hosts may produce long periods of co-circulation of competing virus strains before lineage extinction occurs. Recent studies of AIV in North American wild birds have shown that persistence of AIV was independent of bird migratory flyways suggesting that an introduced virus could rapidly spread across flyways by causing outbreaks in major congregation sites. For this study we sequenced the full genome of 300 H6 subtype and other subtype viruses collected from wild bird systematic surveillance to investigate the evolutionary dynamics associated with competition between introduced and endemic viral lineages. We thereby estimate a wave front of viral invasion and the decreasing habitat of the displaced lineage. The circulation and spread of highly pathogenic H5N1 viruses highlights the need to incorporate viral migration in bird populations in pandemic preparedness planning. This study can inform prediction of viral movement and highlight potential population bottlenecks for disease control efforts.

54. The recent establishment of North American lineage H10 influenza viruses in Australian wild birds

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Influenza A H10N7 virus with a hemagglutinin gene of North American origin was detected in Australian chickens and poultry abattoir workers in New South Wales in 2010 and in chickens in Queensland on a mixed chicken and domestic duck farm in 2012. We investigated their genomic origins by sequencing full and partial genomes of H10 viruses isolated from wild aquatic birds and poultry in Australia and analysed them with all available avian influenza virus sequences from Oceania and representative viruses from North America and Eurasia. Our analysis showed that the H10N7 viruses isolated from poultry were similar to those circulating since 2009 in Australian aquatic birds; and their initial transmission into Australia occurred during 2007–2008. The H10 viruses that appear to have developed endemicity in Australian wild aquatic birds, were derived from several viruses circulating in waterfowl along various flyways. Their hemagglutinin gene was derived from aquatic birds in the western states of the USA, whereas the neuraminidase was closely related to viruses previously detected in waterfowl in Japan. The remaining genes were derived from Eurasian avian influenza lineages.

55. Evolutionary dynamics of Australian avian influenza viruses

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We conducted large-scale phylogenetic analyses of all available avian influenza virus sequence data from Australia to understand the population structure of avian influenza viruses. Our aims were to understand the effects of repeated introduction of Eurasian lineage viruses, the apparent recent introduction of the North American viruses on the population structure of avian influenza in Australia and to identify research gaps in avian influenza surveillance in the region. Our analysis of virological data spanning 40 years in Australia indicate that the long-term evolutionary dynamics of avian influenza
viruses in Australia may be determined by climatic changes. The introduction and long-term persistence of avian influenza virus lineages were observed during periods with increased rainfall, whereas bottlenecks and extinction was observed during phases of widespread decreases in rainfall. These results extend our understanding of factors affecting the dynamics of avian influenza and provide important considerations for surveillance and disease control strategies.

56. Multiple subtypes of influenza A virus isolated from free-grazing ducks in 2012-2013, Thailand

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Surveillance for influenza A viruses (IAVs) in free-grazing ducks of Thailand was conducted from September 2012 to March 2013. In a cross-sectional survey, six provinces in central and lower northern Thailand with high densities of ducks and a history of previous HPAI virus outbreaks located were surveyed. Twenty-seven free-grazing duck flocks were sampled, including 290 oropharyngeal swabs, 290 cloacal swabs, and 280 serum samples collected from 1-17 months old ducks. Virologic testing showed that 14 of 580 (2.4%) swab samples were positive for IAV by real-time RT-PCR (M gene). To date, 12 IAVs were isolated and subtyped as H4N6 (n=5), H11N6 (n=5) and H11N9 (n=2). The 12 IAVs were isolated from 1-5 month old ducks in 4 flocks raised in 2 provinces (Ang Thong and Phitsanulok). Two of 4 flocks were found with multiple subtypes of IAVs circulating in the same flock. Serology testing showed that 26 of 27 flocks (96.3%) were positive for influenza A antibodies using blocking-ELISA (FlockChek® AI multiS-Screen diagnostic kit, IDEXX). In a longitudinal survey, 2 free-grazing duck flocks (flock A, 6 months old, and flock B, 1 month old) in Kanchanaburi were subjected to longitudinal sample collection for 3 months. 360 samples (120 each of oropharyngeal swabs, cloacal swabs and serum samples) were collected. One swab sample (2.5%, 1/40) collected from a 2-month old duck of flock B was positive for AIV, which was isolated and identified as H11N9. Serology results showed that serum samples of flock A and B were positive for influenza A antibodies, 85% (51/60) and 28.3% (17/60) respectively. In summary, this study showed that multiple subtypes of IAV are circulating in free-grazing ducks in the central and lower northern parts of Thailand. More than one subtype (H4N6, H11N6 and H11N9) can be found in the same free-grazing duck flock. This study is also the first to report influenza A H11N6 and H11N9 subtypes in poultry in Thailand.

57. The co-circulation of swine influenza subtypes in Thai swine farms, 2012-2013

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Swine influenza virus (SIV) surveillance was conducted from June 2012 to February 2013 on 15 swine farms in 7 provinces with high-density pig production. In total, 865 samples (434 nasal swabs and 431 serum samples) were collected from both healthy and sick pigs of different ages: 1-4 weeks (suckling pigs) 44.12%; 5-12 week (nursery pigs) 39.18%; 13-20 weeks (fattening pigs) 9.11%; 21-24 weeks (finishing pigs) 4.56%; and sows 2.73%. Virological results showed that 11.75% (51/434 samples) were positive for influenza A virus on real-time RT-PCR (M gene). The positive samples then underwent virus isolation using egg inoculation and/or cell culture. 24 swine influenza viruses (SIVs) were isolated from 5 swine farms: farm A (n=1), farm B (n=3), farm C (n = 9), farm D (n =9), and farm E (n=2). Preliminary analysis of HA and NA genes indicated that the 24 viruses are H1N1 (n=8), H3N2 (n=14) and H1N2 (n=2). Two different subtypes of SIV were isolated from farm C in two different visits, in December 2012 (H1N1; n=2 and H1N2; n=2) and in February 2013 (H1N1; n=5). Detailed subtype and lineage identification of the SIVs is in process. Serological analysis using NP-ELISA showed that 114 of 431
serum samples (26.45%) had influenza A antibodies: HI positive for influenza A antibodies were swH3N2 (2.78%), swH1N1 (10.90%), and pH1N1 (10.67%). Interestingly, in 2012-2013 SIV surveillance, the predominant subtype was H3N2 while the result from 2012-2012 was H1N1 (swH1N1, pH1N1 and rH1N1). In conclusion, we found SIV subtypes H1N1, H3N2 and H1N2 circulated in Thai swine farms. The co-circulation of different subtypes of SIVs in Thai swine farms was evident throughout our 3-year surveillance effort, a situation that may increase the potential of influenza virus reassortment in Thai swine populations.

58. North Atlantic migratory bird flyways provide linkages for inter-hemispheric movement of avian influenza viruses

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Intercontinental movement of avian influenza viruses by migratory birds in the North Atlantic region has not been systematically investigated. Since 2008, we have sampled wild waterfowl, gulls, shorebirds, and sea birds in Greenland, eastern Canada, and Iceland. As part of these efforts we isolated 29 AI viruses from Iceland (2010, 2011), sequenced their genomes, and phylogenetically compared those sequences with Eurasian and North American influenza lineages. Of the 3 unique virus subtypes isolated from Icelandic waterfowl (H6N8, H6N5, H3N6), all gene segments were from Eurasian lineages. In contrast, viruses isolated from gulls (H11N2, H16N3, H5N2, H4N8, H2N5) were much more diverse with viruses containing genomes entirely from North America, entirely from Eurasia, and several were hybrids of genetic elements from both continental lineages. These results show that migratory birds, especially gulls, in Iceland represent a mechanism of genetic mixing where viruses from North America and Eurasia can reassort creating new virus genetic combinations. We also show that the North Atlantic is a likely route of influenza virus transport between North America and Eurasia and that further investigation and surveillance of AI viruses in the North Atlantic is necessary.

59. Are we detecting what we think we are? CEIRS QA/QC testing results

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Quality assurance and control (QA/QC) testing was implemented during year 6 of NIAID’s CEIRS program to evaluate molecular detection of influenza virus and serological detection of influenza antibodies in CEIRS laboratories that conduct animal surveillance. As of July 2013, three testing cycles were completed. During each cycle, three panels of reagents were offered: one for testing avian samples by molecular methods, one for testing mammalian samples, and one for serology. Over the course of the three testing cycles, 37 labs from 4 of the 5 CEIRS centers requested material, 31 labs completed and returned results, and 14 labs completed more than one cycle of testing. Although most labs were able to successfully detect type A influenza virus or antibodies to influenza, the most common errors were false negatives with all three panels (79%-95% of all errors depending on the panel). Two types of common problems were noted: (1) identification was highly inaccurate in the avian panel; subtypes were identified correctly for only 47% of the samples when attempted and false negatives were the most common subtype identification error, although misidentification also occurred frequently; and (2) data integrity was inconsistent; missing or incorrectly recorded data were observed with individual results from numerous labs. Overall, a positive outcome from the CEIRS QA/QC testing is that four labs reported that they modified their procedures based on the results of the QA/QC testing and two of those labs have completed testing after the update and improved their results.
60. Detection and quantification of influenza A virus in swine environmental samples

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Assessing the sources of environmental contamination of influenza A virus (IAV) is important in order to determine risk of exposure to people. In this study we evaluated the sources and level of environmental contamination of swine-origin IAV in commercial swine farms and live animal markets in Minnesota. In commercial swine farms air samples were collected from inside the barn, at the air exhaust point and downwind from the infected population (up to 2.1 km). In the live animal markets, samples were collected from the air of swine enclosures, air from pork processing areas, swine pen railings, doors leading to swine pens and the patrons sinks. Samples were tested by RT-PCR, viral genetic material quantified, and virus isolation attempted in MDCK cells. Swine origin IAV was readily detected and isolated in air samples from enclosures of both commercial swine barns and live animal markets. IAV was also isolated from the exhausted pig air and PCR detected downwind from the infection source. IAV was also detected in hand contact surfaces from live animal markets. Levels of virus load in the air of swine commercial enclosures averaged 3.20E+05 IAV RNA copies/m³ of air. Overall our data provides evidence of environmental contamination of swine origin IAV in commercial farms and live animal markets and a better understanding of the routes for potential exposure to people.

61. Preliminary examination of factors contributing to the presence of influenza A virus in swine at agricultural fairs

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Influenza A virus infections occur in exhibition swine populations at agricultural fairs. Zoonotic viral transmission at fairs resulted in more than 300 cases of variant IAV during 2012. We examined several potential fair-level risk factors contributing to the occurrence of influenza A virus infection in pigs at agricultural fairs in Ohio. Results show that the adjusted odds of having influenza A virus infected pigs at a fair were 1.27 (95% CI: 1.04-1.66) higher for every 20 pig increase in the size of the swine show. Also, four of the five fairs hosting breeding shows in addition to their junior market swine shows had pigs test positive for influenza A virus, illustrating that breeding swine may play a significant role in carrying virus with them to and from these fairs. While the study was limited to one year in Ohio, the findings will be helpful to veterinary and public health officials exploring mitigation strategies to decrease the intra- and inter-species transmission of influenza A virus at fairs.

62. Evidence for the circulation and inter-hemispheric movement of rare influenza A virus genetic diversity

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The recovery of three unique H14 isolates from sea ducks in Wisconsin in 2010 raised concern about gaps in surveillance for influenza A virus (IAV) antigenic and genetic diversity among wild birds. The recent genotyping of an additional three H14 IAVs and one H10 IAV recovered during the same 2010 season provides further support for this undetected diversity in the North American wild bird reservoir. Full-length genomic sequencing of five of the six 2010 H14 isolates demonstrated that the hemagglutinin (HA) gene from the 1982 and 2010 isolates showed 89.6% nucleotide and 95.6% amino acid identity and phylogenetic analysis of these viruses placed them strongly within the H14 subtype lineage. The HA sequence of the sixth 2010 H14 isolate, while congruently divergent from the 1982 isolates, showed an additional 14 amino acid changes from the other five 2010 H14 North American isolates, providing strong
support for a separate H14 subtype lineage circulating undetected in North American waterfowl. The nonstructural (NS) segments from several of these 2010 isolates were placed in a Eurasian NS clade isolated infrequently over the last several decades and include the NS segment from a previously reported 1982 H14 isolate. The NS segment from a H10N6 virus was also placed in this rare NS clade but showed nearly 5% (43/865) nucleotide divergence from the 2010 H14 NS segments, indicating persistence of and circulation in an undetected reservoir. Further, this H10N6 had extreme branch length values for the matrix, polymerase PB1 and PB2 segments, as well as a unique Eurasian HA segment. Three of the H14 IAVs and the H10 showed inter-continental movement in several segments. These findings highlight surveillance gaps for IAV genetic and antigenic diversity in North American wild birds.

63. Influenza A viruses from spring migrating waterfowl in the United States

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Reported influenza A virus surveillance in migratory waterfowl in the United States is primarily conducted in autumn, during southern migration. Therefore, little is known about the presence and ecology of influenza A viruses in waterfowl as they fly north in the spring. In spring of 2006, 2007, and 2013, cloacal swabs were collected from a total of 498 northern migrating birds in Ohio and Michigan, resulting in the recovery of 18 (3.6%) influenza A virus isolates. Antigenically these isolates represent 11 distinct HA-NA combinations. The genomes of these isolates are most similar to other North American waterfowl-origin influenza A virus isolates published in public databases. Although limited in size and scope, this effort demonstrates the presence of diverse influenza A viruses in the spring migrating waterfowl population in the Lake Erie Basin. Additionally, comparisons are made to both preceding and subsequent autumn isolates.

64. Risk analysis of a novel avian H3N8 virus isolated from harbor seals

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The recent isolation of avian H3N8 influenza viruses that naturally acquired several of the changes associated with increased transmissibility of H5N1 viruses from New England harbor seals has generated a great deal of scientific and public interest. To elucidate the potential human health threat from these viruses, we evaluated the virulence and transmissibility of a panel of avian and mammalian H3N8 viruses in vitro and in vivo including a panel of avian H3N8 viruses phylogenetically related to the seal virus. Our studies demonstrate that although the seal and genetically similar wild-bird viruses replicate faster in human lung cells as compared to other mammalian (canine and equine) and avian H3N8 viruses, only the harbor seal virus caused severe disease in mice. More importantly, the seal and genetically similar wild-bird viruses readily transmit via direct contact in ferrets and the harbor seal virus transmitted by aerosol droplet to 2 out of 3 contacts. Therefore, it is imperative to understand how this particular clade of viruses may have evolved over time to become readily transmissible in ferrets. Our results suggest that the avian H3N8 viruses warrant closer examination and enhanced surveillance to monitor potential risks to human health as they exhibit a number of pre-pandemic characteristics. Further study will observe how current vaccination protocols can provide protection against the seal and genetically similar viruses in human populations.
65. The emergence of H9N2 virus in Egypt

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Avian influenza virus (AIV) subtype H9N2 has been widespread in the Middle East since the 1990s. For ambiguous reasons, H9N2 was not detected in Egypt until the summer of 2011. Circulation of H9N2 viruses in Egyptian poultry in the presence of the endemic highly pathogenic AI H5N1 subtype adds a huge risk factor to the Egyptian poultry industry. This work describes the prevalence of H9N2 viruses in poultry through active surveillance in various Egyptian governorates during the last 2 years. The genomic signatures and protein sequences of several H9N2 isolates were analyzed. Phylogenetic data showed that Egyptian isolated H9N2 viruses were closely related to viruses of the G1-like lineage isolated from neighboring countries. These viruses showed impressive replication rate in embryonated chicken eggs and mammalian cells. To investigate the causative segment for increasing replication rate of H9N2 viruses, we constructed a recombinant influenza virus strain of A/PR8/34 (PR8) expressing individual genes from an avian H9N2 influenza strain A/chicken/Egypt/S4456B/2011(H9N2). Using several in vitro models of virus replication, we observed an increasing in replication rate for a reassortant virus expressing the neuraminidase (NA) gene of A/chicken/Egypt/S4456B/2011(H9N2) relative to that of either parental virus or reassortant PR8 expressing other genes. This finding suggests that replication and antigen content of PR8-derived H5N1 influenza vaccine viruses can be improved by incorporation of NA segment of H9N2 to improve in ovo growth of vaccine strains and offers a promising strategy to differentiate infected from vaccinated animals (DIVA).

66. Genetic evolution of H5N1 viruses in Egypt

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Highly pathogenic avian influenza H5N1 viruses in Egypt have been rapidly evolving genetically and antigenically. The first outbreak in early 2006 was caused by clade 2.2 viruses. By 2008 viruses were reclassified as clade 2.2.1 and in 2010 clade 2.2.1.1 viruses emerged. In this study different isolates of H5N1 from 2006-2012 were sequenced to explore their genetic diversity. Three clusters were defined based on hemaglutinin sequences, the majority of viruses isolated in 2010-2012 fell into two of these clusters. A potential glycosylation site was introduced at position 72-74 due to a P74S substitution. Viruses lost a potential glycosylation site at position 154-6 due to T156A substitution. Some important mutations were observed at critical positions of antigenic sites (K140G, S141P and K140R). Other substitution were observed at the receptor binding pocket of HA-1. Human like host specific markers were also observed. Amino acid changes associated with sensitivities to various antiviral drugs were found in Egyptian H5N1 isolates. Given the continuous evolution of H5 viruses in Egypt, surveillance and whole genome sequencing should be routinely performed.

67. Epidemiology and control of human infections with avian influenza A(H7N9) virus in China: empirical assessment

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The novel influenza A(H7N9) virus recently emerged, while influenza A(H5N1) virus has infected humans since 2003 in mainland China. Both infections are thought to be predominantly zoonotic. An integrated database was constructed with information on demographic, epidemiological, and clinical variables of laboratory-confirmed A(H7N9) and A(H5N1) cases that were reported to the Chinese Center for Disease
Control and Prevention up to May 28, 2013. The database was used to describe disease occurrence by age, sex and geography and to estimate key epidemiologic parameters. Among 130 and 43 patients with confirmed A(H7N9) and A(H5N1) respectively, the median ages were 62y and 26y. Among A(H7N9) and A(H5N1) cases, 75% and 71% reported recent exposure to poultry. The mean incubation periods of A(H7N9) and A(H5N1) were 3.1 and 3.3 days, respectively. The hospitalization fatality risk was 35% (95% CI: 25%, 44%) for A(H7N9) and 70% (95% CI: 56%, 83%) for A(H5N1). Depending on assumptions about the coverage of the sentinel ILI network and health-care seeking behavior, we estimated that the symptomatic case fatality risk was between 160 and 2,800 per 100,000 symptomatic cases. Closure of live poultry markets in Shanghai, Nanjing and Hangzhou reduced the risk of human infection by 92%. If A(H7N9) follows a similar pattern to A(H5N1), it is possible that the A(H7N9) epidemic may reappear in the fall. This potential lull should be an opportunity for discussion of definitive preventive public health measures, as well as capacity building in the region given the possibility that A(H7N9) may spread outside China’s borders.

68. The genesis and source of the H7N9 influenza viruses causing human infections in China

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A novel H7N9 influenza A virus first detected in March 2013 has since caused more than 130 cases of human infection in China, resulting in 32 deaths. Preliminary analyses suggest the virus is a reassortant of H7, N9 and H9N2 avian influenza viruses and carries some amino acids linked to mammalian receptor binding, raising concerns of a new pandemic. However, neither the source populations of the H7N9 outbreak lineage nor the conditions for its genesis are fully understood. Through a combination of active surveillance, screening of virus archives, and evolutionary analyses, we find that H7 viruses have independently transferred from domestic duck to chicken populations in China on at least two occasions. We show that they subsequently reassorted with enzootic H9N2 viruses to generate the H7N9 outbreak lineage, and a related previously unrecognised H7N7 lineage. The H7N9 outbreak lineage has spread over a large geographic region and is prevalent in chickens at live poultry markets, mainly in chickens that are thought to be the immediate source of human infections. Whether the H7N9 outbreak lineage will, or has, become enzootic in China and neighbouring regions needs further investigation. The recognition of a related H7N7 influenza virus in chickens, with the ability to experimentally infect mammals, suggests that threats beyond the H7N9 virus exist. The continuing prevalence of H7 viruses in poultry could lead to further sporadic human infections, with a continued risk of the virus acquiring efficient human-to-human transmissibility.

69. Antigenic evolution of avian influenza A/H5N1 clade 2.1 viruses

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Clade 2.1 viruses are responsible for A/H5N1 influenza virus remaining to be endemic in Indonesia. Since the first isolation of A/H5N1 viruses in Indonesia in 2003 clade 2.1 viruses have evolved into multiple different subclades. To map the genetic and antigenic diversity of clade 2.1 viruses, a Maximum Likelihood tree was built using hemagglutinin sequences from the WHO H5N1 tree (January 2011) and
101 additional sequences from recent isolates. We then selected contemporary viruses to represent the most prominent branches within the subclades. We characterized their antigenic phenotypes by hemagglutination inhibition (HI) assays using ferret antisera raised against the representative viruses and analyzed the results using antigenic cartography methods. Mutants were constructed by site-directed mutagenesis to map the molecular basis for antigenic differences from the A/Indonesia/5/05 virus. The representative viruses differentiated into six distinct antigenic phenotypes. Surprisingly, antigenic distances between viruses from the same subclade were often found to be larger than those between viruses belonging to different subclades. Seven different amino acid changes in six positions close the receptor binding site were sufficient to explain all antigenic differences from the A/Indonesia/5/05 virus. HI assays using antisera from immunized chickens show that mutant viruses can escape antibody recognition similar to what we found in HI assays using ferret antisera. These results may help to more rapidly identify emerging antigenic variants and selection of vaccine strain candidates, but stress the importance of continued and stringent surveillance.

Data & Resource Management

70. Integrating Surveillance and Genotypic Data to Understand Influenza Emergence

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The ongoing risk of emerging avian influenza viruses (AIV) has been exemplified by pathogenic H5N1 (1997-present) and H7N9 (2013) outbreaks in humans. There remains an intense need for expanded surveillance, and for research into viral genotypes and host factors governing species specificity, transmission and pathogenicity. In its first six years, the Center for Research on Influenza Pathogenesis (CRIP) has collected over 140,000 samples from global surveillance for influenza viruses in wild and domesticated animals and human patients. Of 100,870 avian samples screened, 11,981 (11.9%) were positive for IAV when analyzed by immunoassay, RT-PCR, and/or virus isolation. Validated surveillance records were formatted according to CEIRS Minimum Data Field standards and reported in the Influenza Research Database (IRD). In addition, 1535 complete viral genomes were sequenced by CRIP, the majority in collaboration with the Influenza Genome Sequencing Project (J. Craig Venter Institute). The objective of integrating influenza virus data is to make new kinds of analyses possible, for example, ecological modeling of viruses and hosts to improve hypothesis-driven surveillance, and to provide rich data sets for studies of virus evolution, antigenicity, and pathogenesis. We are developing a phylogenetic-ecological risk model using H5N1 and H7N9 AIV genotypes, host characteristics, and open access outbreak reports and ecological data. With the deployment of new high throughput sequencing technologies, the need to adopt universal data standards for next-generation sequencing of influenza
viruses is a critical step towards developing algorithms that predict how viral genotypes might contribute to pathogenic phenotypes, and potentially to the emergence of novel AIV strains in humans.

71. Influenza Research Database (IRD): an Integrated Public Bioinformatics Resource for Influenza Virus Research

The Influenza Research Database Team

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The Influenza Research Database (IRD, www.fludb.org) is a freely available, one-stop database and analysis resource to support influenza research developed through the NIAID Bioinformatics Resource Center program. IRD provides access to influenza virus sequences, IRD-computed consensus sequences, enriched sequence annotations, proteins domains and motifs, curated influenza phenotypic characteristic data, experimentally-determined and computationally-predicted immune epitopes, Sequence Feature Variant Types, 3D protein structures, human clinical metadata, animal surveillance and serology data generated through the CEIRS program, host factor data, and other data types through an intuitive web-based search interface. IRD data or custom user data can be analyzed and visualized through web-based tools including: BLAST, short peptide search, point mutation identification, multiple sequence alignment, phylogenetic tree construction and visualization, Metadata-driven Comparative Analysis Tool for Sequences, sequence variation determination, Sequence Feature Variant Type analysis, 3D protein structure visualization, PCR primer design, nucleotide sequence annotation and GenBank submission, pandemic H1N1 classification, highly pathogenic avian influenza H5N1 clade classification, and sequence format conversion. Personal Workbench spaces are also provided for IRD users to save and share data working sets, searches, and analysis results for future use. The suite of data, analysis and visualization tools can be used to facilitate the research and development of diagnostics, prophylactics/vaccines, and therapeutics to influenza virus. Visit our exhibit booth during the poster session to learn more about what IRD has to offer.

72. BEI Resources – Supporting Infectious Disease Research

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BEI Resources provides unique, quality-assured reagents to the scientific community for use in basic research and product development involving biodefense and emerging infectious diseases. These include microorganisms (up to Biosafety Level-4) on the National Institute of Allergy and Infectious Diseases (NIAID) and Centers for Disease Control and Prevention (CDC) lists of Category A, B and C priority pathogens. In addition to live microorganisms, related products such as polyclonal antisera, monoclonal antibodies, isolated nucleic acid preparations, overlapping peptide arrays, purified proteins, and assay kits are also available. Many of these materials have direct or indirect applications in influenza research. These reagents are available free of charge to all registered investigators, regardless of funding source or affiliation. Acquisition of new reagents for the repository is one of the critically necessary and challenging tasks for BEI Resources. Therefore, investigators are encouraged to deposit relevant items, so as to provide access to materials, relief from the burden of distribution, protection of intellectual property rights, and secure storage. In addition, BEI Resources has the capability of contracting for the preparation of specific reagents. If there is a resource needed to advance a specific research area, contact an NIAID program officer or use the "suggest a reagent" option on the BEI Resources homepage, (www.beiresources.org). BEI Resources is funded by the National Institute of Allergy and Infectious Diseases and managed by ATCC.
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