Criteria for Seropositivity: Standardization for Serologic Confirmation of Avian Influenza A (H5N1) Virus Infection

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Serologic Assays for Detection of H5N1 Virus Antibodies

- Hemagglutination-Inhibition (HI)
  - Horse or turkey/chicken RBC

- Neutralization assays
  - Microneutralization (MN) or Virus Neutralization (VN)
  - Pseudotype viral particle neutralization (PN)

- ELISA using rHA

- Single radial hemolysis (SRH) Assay

- Western blot
Criteria for Seropositive Results for Serologic Tests used in 1997 H5N1 Investigations

- Microneutralization assay
  - Titer of $\geq 1:80^*$ in 2 independent assay
  - Seroconversion ($\geq 4$-fold rise) between acute/convalescent paired sera

- Confirmatory assay to enhance specificity
  - Western Blot (CDC) with H5 rHA
  - SRH (Hong Kong Dept. of Health Lab)

*Using MN starting dilution = 1:20 convention*
Kinetics of Antibody Response in H5N1 Virus-infected Humans Determined by Microneutralization (MN) Assay

MN titer = 80
Age and Sensitivity, Specificity of H5 Serologic Tests

- **Children aged <15 yrs MN+WB tests**
  - Sensitivity: 88% (n=8); Specificity: 100% (n=24)

- **Adults aged 18-59 yrs MN+WB tests**
  - Sensitivity: 80% (n=8); Specificity: 96% (n=85)

- Frequency of Seropositives for H5 MN antibody in non-exposed baseline samples increased with age

- Person meeting clinical definition of H5N1 case AND:
  - Serological confirmation with appropriately timed paired sera:
    - >4-fold rise in H5N1 neutralization antibody titer
    - Convalescent neutralizing antibody titer \( \geq 1:80 \) (1:20 starting dilution)
  - Serological criteria for single serum collected \( \geq 14 \) days after symptom onset
    - H5N1 neutralization antibody titer \( \geq 1:80 \)
    - Positive result using a different assay
      - Horse RBC HI titer of \( \geq 1:160 \) or greater or positive H5-specific western blot result

*http://www.who.int/cdr/disease/avian_influenza/guidelines/case_definition2006*
<table>
<thead>
<tr>
<th>Population</th>
<th>Country/Year</th>
<th>Criteria</th>
<th>Confirm Assay?</th>
<th>Reported seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villagers</td>
<td>China, 2004</td>
<td>cRBC HI ≥ 20</td>
<td>MN</td>
<td>3%</td>
</tr>
<tr>
<td>Villagers</td>
<td>Turkey, 2006</td>
<td>ELISA + cRBC HI ≥ 20</td>
<td>MN ≥ 10</td>
<td>0%</td>
</tr>
<tr>
<td>Villagers</td>
<td>Thailand, 2008</td>
<td>MN ≥ 10</td>
<td>No</td>
<td>5.6%</td>
</tr>
<tr>
<td>Villagers</td>
<td>Cambodia, 2006</td>
<td>MN ≥ 80</td>
<td>WB</td>
<td>1%</td>
</tr>
<tr>
<td>Villagers</td>
<td>Cambodia, 2007</td>
<td>PN ≥ 20 (to screen)</td>
<td>MN ≥ 80</td>
<td>2.6%</td>
</tr>
<tr>
<td>Poultry workers</td>
<td>China, 2006</td>
<td>tRBC HI = 320</td>
<td>MN = 640</td>
<td>0.9%</td>
</tr>
<tr>
<td>Poultry workers</td>
<td>China, 2007-08</td>
<td>HI- no criteria</td>
<td>MN - no criteria</td>
<td>0.8%</td>
</tr>
<tr>
<td>Poultry workers</td>
<td>China, 2010</td>
<td>hRBC HI ≥ 160</td>
<td>No</td>
<td>2.6%</td>
</tr>
</tbody>
</table>
## Decline in Serum Antibody Response to H5N1 Virus over Time in Asymptomatic Persons*

*(Vong et al., JID 2009)*

<table>
<thead>
<tr>
<th>Subject</th>
<th>MN titer at estimated time post exposure</th>
<th>Fold drop in titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-2 months</td>
<td>10-11 months</td>
</tr>
<tr>
<td>A</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>320</td>
<td>40</td>
</tr>
<tr>
<td>C</td>
<td>1280</td>
<td>320</td>
</tr>
<tr>
<td>D</td>
<td>640</td>
<td>20</td>
</tr>
<tr>
<td>E</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td>F</td>
<td>640</td>
<td>80</td>
</tr>
<tr>
<td>G</td>
<td>160</td>
<td>20</td>
</tr>
</tbody>
</table>

* Using serological data from 11 severely ill H5N1 (cl 1.1) patients, a fractional polynomial regression model predicted rising titers of ≥80 2 weeks post onset, peak titer achievement at 5-6 weeks and a titer >80 beyond 2 years (Buchy et al., 2010)
Factors to Consider for Development of H5N1 Virus Antibody Seropositivity Criteria - I

- **Timing of sera collection in relationship to H5N1 virus exposure or illness onset**
  - >14 days post symptom onset and >> 14 days post exposure
  - Before waning of antibody response (6-9 months?)

- **Use of relevant clade/virus antigen in assays**

- **Sensitivity of assay**
  - Use criteria with high sensitivity to detect antibody in RT-PCR confirmed cases (mild to severe)
    - Cutoff of ≥1:10 ≥1:20 may be too low

- **Specificity of assay**
  - Use criteria that results in low/no detection of positives in age-matched unexposed persons
    - Adsorption of cross-reactive antibodies to seasonal influenza viruses (infection or vaccination)
Factors to Consider for Development of H5N1 Virus Antibody Seropositivity Criteria - II

- Do we need different criteria for different situations?
  - Persons with symptomatic illness and serological confirmation of H5N1 virus infection versus
    - Patients with more severe disease generally had higher antibody titers than those with clinically mild illness, regardless of age
  - Seroprevalence studies to identify asymptomatic or clinically mild illness

- Is a confirmatory assay still needed or recommended?
- If so:
  - hRBC HI \( \geq 160 \) may be too stringent?
  - hRBC HI may be insensitive for some H5N1 clades
  - Western blot lacks specificity

- Should the H5N1 seropositive criteria be applied to other avian influenza A virus subtypes (H7, H9)?