Combating RRD: NIFA-SCRI Project’s Diagnostic Objectives

Antibody-based Diagnostics

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Key to this effort to detect the virus and to control the disease is the development of efficient diagnostic tools to enable rapid, easy-to-use, accurate and affordable detection of the virus. Specifically –

Reagents will be designed and developed
- RRV-specific primers/probes (for nucleic acid-based assays)
- Monoclonal and/or polyclonal antibodies (for serological antibody-based assays)

Techniques & Assays will be developed and refined for lab and field use
- ELISA and immuno-dipstick tests
- IC-RT-PCR? Tissue-print?
- End-point RT-PCR, Taq-man qRT-PCR, SYBR Green qRT-PCR, RT-exoRPA
- RT-LAMP (Reverse transcription-Loop mediated isothermal amplification)
- Self-quenched primer (SqP) technologies (for laboratory detection systems)
- Lateral flow devices (LFD; both Ab-based & NA-based detection assays)
Progress Toward Development of a Serological Assay for the Detection of Rose Rosette Virus

Rose rosette virus (Emaravirus)

Antibody-based Diagnostics

Development and Characterization of RRV virus-specific PcAb & McAb antibodies


- A cloned and bacterially-expressed RRV 316 aa nucleocapsid (NP) was purified and used as a source of RRV NP for immunization of rabbits and mice and as a positive control protein in immunoassays.
Rabbit - Polyclonal antibodies

- Protein A-purified rabbit polyclonal antibodies react to RRV NP in Western-bLOTS and in both antigen coated plate (ACP-) and triple antibody sandwich (TAS-) ELISAs. [Titer > 1:512,000 in ACP-ELISA].
Development and Characterization of RRV-specific PcAb & McAb antibodies

Mouse - Monoclonal antibodies

- Monoclonal antibodies from 10 mouse hybridoma cell lines reacted with NP in ACP-ELISA and western-blot; 5 were selected for further study: Three that exhibited strong reactivity in both ACP- and TAS-ELISA and two that exhibited strong reactivity in ACP- and weak to zero reaction in TAS-ELISA.

Table 1. Immunoreactivities of selected monoclonal antibodies (McAb) to bacterially-expressed purified *Rose rosette virus* nucleoprotein (RRV-NP) in an antigen-coated plate ELISA (ACP-ELISA) and triple-antibody ELISA (TAS-ELISA).

<table>
<thead>
<tr>
<th>McAb Cell Line</th>
<th>ACP-ELISA</th>
<th>TAS-ELISA</th>
<th>ug mL⁻¹</th>
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<tbody>
<tr>
<td>NC</td>
<td>0.057³</td>
<td>0.002</td>
<td>ND</td>
</tr>
<tr>
<td>PTY-1</td>
<td>0.007</td>
<td>0.000</td>
<td>10.000</td>
</tr>
<tr>
<td>1E6A10</td>
<td>0.502</td>
<td>0.026</td>
<td>0.994</td>
</tr>
<tr>
<td>2B11C8</td>
<td>0.195</td>
<td>0.004</td>
<td>1.075</td>
</tr>
<tr>
<td>2D5D10</td>
<td>2.115</td>
<td>0.581</td>
<td>2.932</td>
</tr>
<tr>
<td>3A9F5</td>
<td>2.820</td>
<td>0.055</td>
<td>4.631</td>
</tr>
<tr>
<td>3D5H6</td>
<td>1.405</td>
<td>0.139</td>
<td>0.778</td>
</tr>
<tr>
<td>5A2B10</td>
<td>0.236</td>
<td>0.004</td>
<td>0.948</td>
</tr>
<tr>
<td>8D2F4</td>
<td>2.391 **</td>
<td>2.255</td>
<td>5.124</td>
</tr>
<tr>
<td>8E9E6</td>
<td>2.609 **</td>
<td>1.933</td>
<td>3.743</td>
</tr>
<tr>
<td>9A7E4</td>
<td>1.624 **</td>
<td>0.051</td>
<td>14.255</td>
</tr>
<tr>
<td>10D8E10</td>
<td>1.983</td>
<td>2.049</td>
<td>4.107</td>
</tr>
</tbody>
</table>

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Development and Characterization of RRV-specific PcAb & McAb antibodies

Mouse - Monoclonal antibodies

- Protein A-purified McAb 8D2F4 also reacts to bacterially-expressed RRV NP in Western-blots.

![Western-blot analysis](image)

- **Ags:** Lane 1 = RRV NP, 100 ng
  Lane 2 = RRV NP, 50 ng
  Lane 3 = Buffer
- **McAb:** 8D2F4, 1 μg/ml
- **NP:** 36.4 kDa
Development and Characterization of RRV-specific PcAb & McAb antibodies

**Rabbit - Polyclonal antibodies**
- Protein A-purified rabbit polyclonal antibodies react to RRV NP in Western-blot and in both antigen coated plate (ACP-) and triple antibody sandwich (TAS-) ELISAs.
- Alkaline phosphatase-conjugated PcAb did **not** work in DAS-ELISA as detecting Ab (with McAb 8D2F4 as trapping antibody).

**Mouse - Monoclonal antibodies**
- Protein A-purified McAb 8D2F4 also reacts to bacterially-expressed RRV NP in ACP- and TAS-ELISA, and Western-blots.
- Alkaline phosphatase-conjugated McAb 8D2F4 did work well in DAS-ELISA as the detecting Ab (with PcAb as trapping antibody).
Antigenic and Structural Analysis

- Predicted antigenic regions (dark red arrows) in the 316 amino acid sequence of RRV NP.
- Predicted secondary structure motifs in the protein (symbols above the NP sequence).
- Twelve synthetic 25-aa peptides containing the various antigenic regions were produced.
Progress Toward Development of a Serological Assay for the Detection of Rose Rosette Virus - 8

Development and Characterization of RRV-specific PcAb & McAb antibodies

**PcAb and McAb reactivity to RRV-NP peptides**
- Peptides 1-12: 25-aa NP peptides; Peptide 13: RRV-NP.
- Peptide 14-15: Flexiviridae Carlavirus coat protein peptides (Flex-CP 1 & 2); Peptide 16: Buffer blank.

✓ Note strong reactions with antigenic regions #3 and #11 (#13 = NP)

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Development and Characterization of RRV-specific PcAb & McAb antibodies

Reactivity of 10 RRV-specific McAbs to RRV-NP and seven selected peptides

- Peptides 1, 3, 7, 9, 11, 13 (NP), and 16 (Flexi-CP 2)
- NC: negative control (no antibody)
- PTY-1: Potyvirus-specific McAb

Note strong differential reactions with antigenic regions #3 and #11 (#13 = NP) by McAbs 8D2 and 8E9

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Progress Toward Development of a Serological Assay for the Detection of Rose Rosette Virus - 10

Development and Characterization of RRV-specific PcAb & McAb antibodies

3D model folding of RRV-NP [using Phyre2]
“NP surface-located” peptide regions 3 and 11 are identified [arrows].

- Peptide region #3 reactivities = PcAb, 1E6, 3A9, 8E9, 9A7
- Peptide region #11 reactivities = PcAb, 8D2, 10D8
Progress Toward Development of a Serological Assay for the Detection of Rose Rosette Virus - 11

Development and Characterization of RRV-specific PcAb & McAb antibodies

- Antigenic region #3 bound by PcAb and/or McAb 8E9
- Antigenic region #11 bound by PcAb and/or McAb 8D2

Proposed TAS-ELISA -->
1. Rabbit PcAb as trapping antibody.
2. Antigen/extract.
3. Admix of McAb 8D2 and 8E9 as detecting antibodies.
4. Then, Goat anti-mouse AP-conjugate.

<table>
<thead>
<tr>
<th>Trapping</th>
<th>Antigen</th>
<th>Detecting</th>
<th>Conjugate</th>
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<tr>
<td>PcAb</td>
<td>3-NP-11</td>
<td>McAb 8D2</td>
<td>anti-M*</td>
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<tr>
<td>PcAb</td>
<td>11-NP-3</td>
<td>McAb 8E9</td>
<td>anti-M*</td>
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</table>

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Progress Toward Development of a Serological Assay for the Detection of Rose Rosette Virus - 12

Development and Characterization of RRV-specific PcAb & McAb antibodies

- Now – Increase McAbs 8E9 and 8D2 (to milligram amounts Ab)
- Now – Evaluate various extraction buffers (x16)
- Now – Test fresh and frozen healthy and infected samples
- Now – Compare side-by-side with PCR-based protocols
- Now – Immuno-Electron Microscopy
  - Plants: naturally infected & clone-inoculated Arabidopsis (Jeanmarie Verchot & David Pang)
  - Next – Mites (Ron Ochoa & Gary Bauchan)

- Next – Protocol and reagents will be sent to collaborators for their in-house validation
- Next – Test parameters for tissue print immunoassay
- Next – Test parameters for immune-capture [IC-RT-PCR]
- Next – Test/Develop Lateral Flow Assay [LFA; ImmunoStrips]
Progress Toward Development of a Reliable, Efficient, Cost-effective, User-Friendly Diagnostic Assay for the Detection of Rose Rosette Virus

Table. Comparative analysis of the different potential diagnostic methods

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity</th>
<th>Skill required</th>
<th>Equipment needed</th>
<th>High throughput</th>
<th>Time required</th>
<th>Cost</th>
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<tbody>
<tr>
<td><strong>Nucleic acid-based Assays</strong></td>
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<tr>
<td>RT-PCR</td>
<td>High</td>
<td>High</td>
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<td>RT-LAMP</td>
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<td>RT-exoRPA</td>
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<td>NGS</td>
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<td>EDNA</td>
<td>Low?</td>
<td>Med/Low</td>
<td>Yes, Laptop</td>
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<td><strong>Antibody-based Assays</strong></td>
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</table>
Combating Rose Rosette Disease

USDA National Institute of Food and Agriculture (NIFA) Specialty Crop Research Initiative project, “Combating Rose Rosette Disease: Short Term and Long Term Approaches” (2014-51181-22644/SCRI)