Challenges during Development and Validation of Functional Antibody Assays to measure Vaccine Immunogenicity

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Overview

- Different types of functional NAb assays to measure vaccine immunogenicity
- Factors that affect performance of vaccine NAb assays
- Considerations during optimization and validation of NAb assays.
  - *Clostridium difficile* neutralizing antitoxin assay
  - *Streptococcus pneumoniae* multiplex opsonophagocytic activity (MOPA) assay
- Ligand binding assays as a surrogate for Nab activity
Neutralizing Antibodies—Desired for Vaccines

- Clinical assays are one of the bases for licensure for all vaccine products
  - May serve as a surrogate for efficacy / substitute for a primary endpoint
  - Correlate of Protection: a laboratory parameter that has been shown from adequate and well-controlled studies to be associated with protection from clinical disease

- Examples of correlates of protection for vaccines include:
  - Toxin neutralization- Diphteria (0.01 IU/mL)
  - Viral neutralization- Polio (1:8 titer)
  - Serum bactericidal activity- A,C, Y,W-135 meningococcus (hSBA ≥ 1:4, rSBA ≥ 1:8)
  - gpELISA- Varicella (5 Ab units)
  - EIA- S. pneumoniae (0.35 μg/mL)
  - EIA- Rubella (10 IU/mL)
Functional Neutralizing Antibody Activity can be measured

Directly
- Virus or Toxin Nab assays
- Ligand binding assays

Indirectly
- Serum Bactericidal Activity (SBA)
- Opsonophagocytosis (OPA)

Singleplex

Multiplex
- MOPA, Luminex, ECL (Meso Scale Discovery)
Increased degree of complexity

from M. Huber & A. Trkola (2007) Journal of Internal Medicine, 262(1)
## Examples of Functional Nab Assays and Mechanisms

<table>
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<th>Neutralization</th>
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<td><strong>C. difficile</strong></td>
<td>Neutralizing antitoxin assay</td>
<td><em>N. meningitidis</em> A, C, W135 and Y Serum Bactericidal Activity (SBA) assay</td>
<td><em>S. pneumoniae</em> multiplex opsonophagocytic activity (mOPA) assay</td>
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<td><strong>Human Cytomegalovirus (HCMV)</strong> Neutralization Assay</td>
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<td><em>S. aureus</em> opsonophagocytic activity (OPA) assay</td>
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<td></td>
<td>(Tang et al; Vaccine 2011)</td>
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<td>(Cook et al; Human Vaccine 2009)</td>
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<td><strong>HPV high throughput pseudovirion-based neutralization assay (HT-PBNA)</strong></td>
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<td>(Sehr et al; PLoS One 2013)</td>
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Key factors that affect NAb assay performance

- Neutralization time
- Infection time
- Cell line selection
- Cell input
- Virus or Toxin source
- Virus input dilution
- Toxin concentration
- Neutralization time
- Infection time
- Cell line selection
- Cell input
- Virus input dilution
- Virus or Toxin source
- Toxin concentration
- Complement
- Target bacteria
- Effector cells
**Clostridium difficile** neutralizing antitoxin assay

- **Clostridium difficile** is a leading cause of nosocomial diarrhea worldwide. Pathogenic strains of *C. difficile* produce two exotoxins commonly referred to as TcdA and TcdB.

- Antitoxin antibodies, especially neutralizing antibodies, have been shown to be associated with a lower incidence of *C. difficile* infection recurrence.

- The *C. difficile* NAb assay measures the ability of antibodies to neutralize the function of TcdA and TcdB *in vitro*.
  - The assay measures the changes in cytoplasmic content of F-actin as a function of treatment of target cells with TcdA and TcdB.
  - The change in F-actin content is reflected by an overall change of the cell surface, which is the primary signal reported by this assay.

(Xie et al; Clin Vacc Immunol 2013)
C. difficile optimization

- Goal was to identify the conditions that allow for:
  - Enhanced sensitivity for TcdA and TcdB
  - Automated assay readout that correlates with cytotoxicity.

- Biological components that required optimization included:
  - Cell line selection: Differential sensitivity of cell lines to TcdA and TcdB.
  - Cell seeding density (no. of cells/well)
  - Toxin source and concentration: source, purity and potency of toxin preparations are critical for enhancing assay sensitivity and robustness.

- The assay conditions were evaluated in terms of:
  - the signal-to-noise ratio,
  - the toxicity of the toxin (TC50),
  - the level and variability of the NAb titers (ED50) for human and monkey samples,
  - and the linearity of the NAb titers across the range of monkey spikes tested.

- Statistical DOE approach facilitated assay optimization

(Xie et al; Clin Vacc Immunol 2013)
**Statistical Design of Experiment (DOE)**

DOE - a statistical tool that allows for the evaluation of all of the key assay parameters to determine the optimal conditions for the assay, as well as determine if there are any interactions of these parameters on the response of the assay.

- need to know how these factors *collectively* affect a measured response

- can use the extreme combinations of each factor to estimate the main effects of each factor, interactions of each factor with each other and overall assay performance

Figure 1: Full Factorial DOE design. Red circles indicate points to be tested

Figure 2: Partial Factorial DOE design. Red circles indicate points to be tested
**Streptococcus pneumoniae Opsonophagocytic Assay (OPA)**

- *S. pneumoniae* is a major cause of infant mortality, especially in the developing world
  - Bacteremia and meningitis
  - Acute otitis media (AOM)

- The primary mechanism of host defense against pneumococcal disease is antibody and complement mediated opsonophagocytosis.

- OPA is an antibody mediated killing assay with the help of complement and phagocytic effector cells to kill *S. pneumoniae* serotypes.
  - Labile assay components include bacteria, complement, and effector cells.

- OPA has been widely accepted as the reference method for measuring the protective capacity of antibodies to pneumococci.
Opsonophagocytic Assay OPA

- **Opsonization**: Specific antibody (IgG, IgM) binds to the bacteria and activates complement. Complement protein C3b coats the bacteria.

- **Phagocytosis**: Effector cells bind the opsonized bacteria through cellular antibody Fc receptors and C3b receptors and internalize the opsonized bacteria into phagocytic vesicles (phagosomes)

- **Killing**: Phagosomes fuse with lysosomes and ingested bacteria are killed by lysosomal enzymes and toxic oxygen metabolites
Multiplex OPAs (MOPAs)

Bacteria + sera + C’

Mix bacteria with effector cells and incubate 37ºC

Each serotype is resistant for a different antibiotic (4 per reaction)

Drip onto agar plate containing antibiotic

DMF + 5 days

Differentiated to express CR1

Undifferentiated Effector cells

(Burton and Nahm, Clin Vacc Immunol 2006)
MOPA optimization

- Growth state of the target bacteria
- Antibiotics
  - Each *S. pneumoniae* serotype has been genetically altered to be resistant to one of four antibiotics. Strains are grouped into assay groups based on their antibiotic resistance.
  - Require optimal antibiotic concentrations that result in a more consistent bacterial growth profile
- Baby rabbit complement source
  - Screen and select lots of complement that have low non-specific killing activity specific to each serotype
- HL-60 cell line
  - Mammalian promyelocytic cell line
    - Differentiated into granulocytes for use as effector cells
    - Phagocytizes the *S. pneumoniae* serotypes in a complement-dependent manner
  - Various serotypes may respond differently depending on age of HL60 cell line
Validation of Functional Assays - Performance parameters

- Specificity
- Precision - Intra and Inter-assay precision
- Linearity
- Ruggedness to factors that can vary during routine operation
- Sensitivity, Limit of Detection (LOD)/Quantifiable range and Lower Limit of Quantitation (LLOQ)
- Relative accuracy/dilutability and matrix interference
- Serum stability

Validation of Functional Assays - Challenges

- Reliance on biologically active (labile) components and lack of standardization of reagents
- For LLOQ assessments, high-titered serum samples should not be diluted
- Demonstrate that OPA activity is Ab-mediated, and not non-specific
- Control identification
- Identify samples with titers spanning the entire range.
  - Assays are only considered validated for the working range that has been demonstrated using incurred samples and matrix relevant samples.
HPV Competitive Luminex Assay (cLIA) for Detection of HPV Type-Specific Neutralizing Antibodies

VLP-Bead with bound mAb-PE

HPV Type-specific mAb

Luminex Beads

Phycoerythrin fluorescent tag

Analyte (VLP)

Green Laser 532 nm

Red Laser 635 nm

578 nm

658 nm

712 nm
Competitive Binding of HPV Specific Serum Abs and HPV Specific mAbs for Neutralizing Epitopes

Summary

- The optimization and standardization of functional assays to obtain favorable performance characteristics are particularly challenging because of the use of multiple labile components in the assay, including viable bacteria, freshly isolated or cultured phagocytic (effector) cells, and complement.

- Optimization of biological components is critical to minimize assay variability and generate reliable data.

- The measurement of neutralizing antibodies and/or functional activity will provide key information on antibody threshold levels required for protection from these diseases and may allow for the establishment of an immune correlate of protection if they are associated with efficacy.
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