Lyme Borreliosis, Syphilis & ANA

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March 14, 2020
Disclosure

- Employee and stock holder of Bio-Rad Laboratories
Outline

At the completion of the session, the participant will be able to:

• Describe the evolution of diagnostic tests utilized in the areas of Lyme disease, Syphilis, and Autoimmune (ANA) diagnostic testing

• Evaluate alternative testing algorithms in these three areas of diagnostic testing based on the clinical utility of the respective diagnostic tests

• Analyze the immunological reactivity between antigen and antibody and how their “ideal” pairing contribute to the performance of an assay (sensitivity, specificity, PPV, & NPV)
Lyme Topics

• Lyme borreliosis
• Epidemiology
• Lyme disease in California
• Diagnosing Lyme disease
• Current state of testing
• Overview of multiplex testing
Lyme Objectives

• Have a basic understanding of Lyme disease, its causative agents and clinical stages
• Describe the epidemiology of Lyme disease
• Recall what test methods/algorithms are used to diagnose Lyme disease
Lyme borreliosis
Lyme Disease (Lyme borreliosis)

The New York Times

We’ve Reached Peak Tick Anxiety
How the tiny arachnid became the hottest topic of the summer.

THE WALL STREET JOURNAL.

U.S. Edition | December 12, 2018 | Print Edition | Video

U.S. | THE NUMBERS

Lyme Disease: An Even Bigger Threat Than You Think
A look at why cases of the tick-borne illness are undercounted

By Jo Craven Mcinty
Updated June 22, 2018 12:05 p.m. ET

Rachael Amber
Lyme Borreliosis (LB)

- Tick borne disease
- Spirochete bacteria from the genus *Borrelia*
  - *B. burgdorferi* s.s.
  - *B. afzelii*
  - *B. garinii*
  - *B. mayonii*
Quick History

• Willy Burgdorfer
  • Isolated the bacterium in 1982
  • *Borrelia burgdorferi* is named for Willy
  • It was discovered later that there is more than one LB-causing bacteria…
True or False: *Borrelia burgdorferi* is the only Lyme disease-causing bacteria

**FALSE**

Although the disease-causing bacteria was originally named *Borrelia burgdorferi*, later research showed there are multiple Lyme disease-causing species of *Borrelia*.
**Borrelia burgdorferi sensu lato**

- sensu lato – “in the lax sense”
- Borrelia burgdorferi s.l.
  - spirochete group comprised of over 15 genospecies
  - 4 are known to cause Lyme disease

<table>
<thead>
<tr>
<th>Bacteria Name</th>
<th>Associated Clinical Manifestation</th>
<th>Geographic Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. burgdorferi s.s.</td>
<td>Lyme arthritis, carditis</td>
<td>US and Western Europe</td>
</tr>
<tr>
<td>B. mayonii</td>
<td>Diffuse rash</td>
<td>US</td>
</tr>
<tr>
<td>B. afzelii</td>
<td>ACA</td>
<td>Europe</td>
</tr>
<tr>
<td>B. garinii</td>
<td>Neuroborreliosis</td>
<td>Europe</td>
</tr>
</tbody>
</table>
Not all *Borrelia* species cause Lyme disease – but are still pathogenic to humans

• Tick-borne relapsing fever
  – *Borrelia miyamotoi*
  – *Borrelia hermsii*
  – *Borrelia cuoci durae*
  – *Borrelia spp.*
Clinical Presentation of Untreated LB

**Stage 1**
*Days-weeks*

- Early localized infection
- Erythema migrans

**Stage 2**
*Weeks-months*

- Disseminated infection
- Systemic symptoms
- Lyme Neuroborreliosis
  - Acute neurological involvement
- Carditis
  - 1st to 3rd degree atrioventricular block

**Stage 3**
*Months-years*

- Localized infection, usually without systemic symptoms
- Acrodermatitis chronica atrophicans
- Oedema (purplish in color), atrophy of the skin
- Lyme arthritis
  - Arthritis in one or a few joints, usually the knee

**Disease timeline (if untreated)**

North America/Europe
Treatment

- Lyme disease exists
- Treatable with antibiotics (2-4 wks)
- Post-Lyme disease Syndrome
  - ~10% of patients
  - No known effective treatment

The bad news is...you have Lyme disease. The good news is, I don't believe in that disease so you're fine!
True or False: Post Lyme disease Syndrome had the same symptoms of Lyme disease

TRUE

PLDS is characterized by a continuation of symptoms despite the clearance of infection and the absence of the Lyme disease-causing bacteria.
Epidemiology
Clinical Background
Confirmed LB cases by month of disease onset – US, 2001-2006
Vector Distribution

Ixodes Distribution
- I. pacificus
- I. scapularis
- I. ricinus
- I. persulcatus
Reported Cases of Lyme Disease—United States, 2016

Each dot represents one case of Lyme disease and is placed randomly in the patient’s county of residence. The presence of a dot in a state does not necessarily mean that Lyme disease was acquired in that state. People travel between states, and the place of residence is sometimes different from the place where the patient became infected.
Endemic Areas

Tick Season in Maine

Ah! The Glory of Nature!
The epidemiology of Lyme disease is affected by:
A) The life-cycle of the tick
B) The vector hosts
C) The weather
D) All of the above

D) All of the above

The life-cycle of the tick and its ability to reproduce and move are all factors that contribute to the distribution of Lyme disease.
Lyme is Spreading

• Lyme vector is detected in 50% more counties in 2015 than 1996
• Incidence
  – US: 2004-2016, nearly double
  – Canada: 2009-2015, six-fold increase
Climate Change

- Longer summers
- Warmer average temperatures
- More hospitable climates for vectors
- Changing migratory pattern of vectors
  - Birds
  - Deer
  - Mice

Lyme disease in California
True or False: Lyme disease is *endemic* to California

FALSE

Lyme disease is not endemic to the state of California, but it is endemic in some counties in California
Public Service Announcement

- The black-legged tick is found in 56 of 58 counties in CA
- Endemic in:
  - Marin County
  - Santa Cruz County
  - Sonoma County
  - Mendocino County
  - Trinity County
  - Humboldt County
  - Mono County
  - Mariposa County
  - Nevada County
  - Amador County

Incidence of Lyme disease in 2011, source: CA Department of Public Health
Lyme Question #5

Nymph ticks are the size of a:
A) Small Pea
B) Sunflower seed
C) Marble
D) Poppy seed

D) Poppy seed
Public Service Announcement

Ticks can be the size of a poppy seed. Can you spot all 5 ticks in this photo? Learn how to prevent tick bites.
[bit.ly/2rjox6U]

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CDC @CDCgov · 7 May 2018

Sorry we ticked some of you off! Don’t let a tick bite ruin your summer. Protect yourself: [bit.ly/2K2XAF].

💬 46  🔴 80  ❤️ 521
Diagnosing Lyme disease
• Symptoms:
  – Erythema migrans (EM) – Bull’s-eye rash
  – Fever, fatigue, malaise
  – Symptom checklists

• Risk Factors:
  – Hiking
  – Location
  – Season
  – Memory of a tick bite
Testing: Culture

• Basic research tool
• Labor intensive
• Expensive
• 12 week incubation time before considered negative
• Only useful for untreated patients
Testing: Molecular (PCR and NGS)

- Basic research tool
- Need a high initial bacterial DNA concentration
  - EM scraping
  - Inflamed joint fluid drawing
- Difficult to reproduce
- Inhibitors in samples
- No molecular tests have been FDA cleared
Testing: NGS

• Push to use NGS
• Benefits:
  • Screen for all tick-borne disease at one time
  • Epidemiological tool
• Limitations:
  • It is a molecular method
  • Expensive
  • All tick-borne disease are treated the same way
Testing: Serology

- Quick, cheap, easy
- Protein-based
- 94 FDA cleared tests (1987-2018)
- <20 routinely used today
- Screening: highly sensitive
- Western blot: highly specific
Two-Tiered Testing for Lyme Disease

**First Test**
- Enzyme Immunoassay (EIA)
- Immunofluorescence Assay (IFA)

**Positive or Equivocal Result**
- Signs or symptoms ≤ 30 days
  - IgM and IgG Western Blot
- Signs or symptoms > 30 days
  - IgG Western Blot ONLY

**Negative Result**
- Consider alternative diagnosis
- OR
  - If patient with signs/symptoms consistent with Lyme disease for ≤ 30 days, consider obtaining a convalescent serum
CDC Testing Algorithm

Modified Two-Tiered Testing (MTTT) for Lyme Disease

First Test

- ELISA 1
  - Enzyme Immunoassay (EIA)
  - Positive OR Equivocal
  - Negative
  - MTTT Negative

Second Test

- ELISA 2
  - Enzyme Immunoassay (EIA)
  - Positive OR Equivocal
  - Negative
  - MTTT Negative
  - MTTT Positive
Testing For Lyme disease
History: Lyme testing’s “bad rap”

• The first Lyme disease test was cleared by the FDA in 1987
  • IFA test for antibodies to *Borrelia burgdorferi* – Zeus Scientific
  • 18 other tests introduced by 2000
• Used whole cell lysates
  • High cross-reactivity
  • Low specificity
  • High sensitivity
History: Western Blot

- Introduction of the Western blot algorithm shortly after
- Western blots:
  - 100% specificity
  - Low sensitivity
    - 62% Early (0-3 mons)
    - 81% Convalescent (3-12 mons)
    - 100% Late (>12 months)

Current State: Antigen testing

• 2007 first assay released using Lyme-specific antigens (VlsE)
• Most commonly used assays have Lyme-specific antigens
• Common antigens:
  • FlaB – flagellar protein
  • OspC – outer surface protein
  • VlsE – outer membrane protein
  • p58 – membrane protein
  • DbpA – membrane protein
• This increased specificity significantly
Current State: Problems

- Western blotting technology has not changed
  - Extremely low sensitivity
  - Early and Convalescent Lyme disease cases (0-12 mon)
- Movements to remove WBing from the algorithm are underway
Using a whole cell lysate in a diagnostic test:
A) Increases specificity
B) Increases sensitivity
C) Decreases specificity
D) Increases specificity

C) Decreases specificity

Whole cell lysates are very non-specific and have good sensitivity, but high cross-reactivity leading to decreased specificity
Multiplex Lyme Total Overview
Beads coated with *B. burgdorferi* antigens
Bead 1: FVlsE (fusion of flagellar and modified VlsE peptide)
Bead 2: OspCB (recombinant outer surface protein)
Bead 3: p58 (recombinant membrane protein)
Lyme Total Multiplex Testing

Analyte beads are manufactured and calibrated individually

- **p58**
- **FVlsE** (Fusion peptide FlaB & modified VLsE)
- **OspCB**

**Internal QC beads**
- ISB
- SVB

Beads Combined into a Single “Bead Reagent” for Multiplex Analysis
Lyme Question #6

Which was the first recombinant protein that was added to an assay:
A) FlaB
B) p58
C) VlsE
D) DpbA

C) VlsE

In 2007 the first assay that did not contain a whole cell lysate was released and contained VlsE
Intended Use Statement

The BioPlex 2200 Lyme Total kit is a multiplex flow immunoassay intended for the qualitative detection of total (IgM/IgG) antibodies to *Borrelia burgdorferi* in human serum or plasma (EDTA, heparin). This assay should be used to test patients with history and/or symptoms of infection with *B. burgdorferi*. The BioPlex 2200 Lyme Total assay is intended for use with the Bio-Rad BioPlex 2200 System. All reactive and equivocal specimens should be tested with a second tier test such as Western blot. Positive second tier results are supportive evidence of infection with *B. burgdorferi*. Diagnosis of Lyme borreliosis should be made based on the presence of *B. burgdorferi* antibodies, history, symptoms, and other laboratory data. Non-reactive first tier or negative second tier results should not be used to exclude borreliosis.
**Review**

**Sensitivity** (also called the **true positive rate**)
- measures the proportion of actual positives that are correctly identified as such
- e.g., the percentage of Lyme disease patients who are correctly identified as having LB

**Specificity** (also called the **true negative rate**)
- measures the proportion of that are correctly identified as such
- e.g., the percentage of LB negative patients who are correctly identified as not having Lyme disease
Lyme Question #7

Statistically, which is likely to be the most sensitive algorithm?
A) 2 different serological assays
B) 1 serological assay
C) 1 serological assay + 1 WB
D) 2 different WBs

B) 1 serological assay
When testing with only 1 serological assay you will not have any disagreement between methods and you there is no probability of loosing a true positive
Statistically, which is likely to be the most specific algorithm?
A) 2 serological different assays
B) 1 serological assay + 1 WB
C) 1 WB
D) both B and C

D) Both B and C

Western blots should always be 100% specific, so your specificity shouldn’t be affected if you use it alone or after a serological assay.
## CDC LSR Premarketing Panel Summary

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Reactive</th>
<th>Equivocal</th>
<th>Non-Reactive</th>
<th>% Agreement with Clinical Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BioPlex 2200 Lyme Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>39</td>
<td>33</td>
<td>0</td>
<td>6</td>
<td>84.6%</td>
</tr>
<tr>
<td>Convalescent</td>
<td>31</td>
<td>29</td>
<td>0</td>
<td>2</td>
<td>93.5%</td>
</tr>
<tr>
<td>Late</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>Look-alike Diseases</td>
<td>90</td>
<td>1</td>
<td>2</td>
<td>87</td>
<td>96.7%</td>
</tr>
<tr>
<td>Healthy Controls</td>
<td>100</td>
<td>1</td>
<td>2</td>
<td>97</td>
<td>97.0%</td>
</tr>
</tbody>
</table>

Analysis: *The CDC LSR Premarketing panel are the most highly characterized samples that were tested.*

*Each assay gets a slightly different panel with different compositions.*
## CDC Panel: Head-to-Head Comparison

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>BioPlex 2200 Lyme Total</th>
<th>Immunetics C6</th>
<th>Zeus Borrelia VlsE1/pepC10 IgG/IgM</th>
<th>VIDAS IgG+IgM</th>
<th>MarDx (Western Blot)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute</strong></td>
<td>39</td>
<td>84.6%</td>
<td>71.8%</td>
<td>82.1%</td>
<td>79.5%</td>
<td>61.5%</td>
</tr>
<tr>
<td><strong>Convalescent</strong></td>
<td>31</td>
<td>93.5%</td>
<td>90.3%</td>
<td>90.3%</td>
<td>83.9%</td>
<td>80.6%</td>
</tr>
<tr>
<td><strong>Late</strong></td>
<td>20</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>NA*</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Total Sensitivity</strong></td>
<td>90</td>
<td>91.1%</td>
<td>84.4%</td>
<td>88.9%</td>
<td>85.6%**</td>
<td>76.6%</td>
</tr>
<tr>
<td><strong>Total Specificity</strong></td>
<td>190</td>
<td>96.8%</td>
<td>97.9%</td>
<td>89.5%</td>
<td>84.7%</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Not enough sample for the late stage of disease  
**Assumed 100% sensitivity for the Total calculation

*All tested with the same CDC panel. Zeus, VIDAS, and BioPlex 2200 in-house, Immunetics C6 from the CDC.*
Lyme Testing Agreement: CDC Panel

**CDC Premarketing Panel**

Overall Clinical Sensitivity & Specificity

- bioMérieux VIDAS Lyme II (IgM + IgG) 84.7%, 85.6%
- ZEUS Borrelia VlsE1/pepC10 89.5%, 88.9%
- Immunetics Lyme C6 97.9%, 84.4%
- MarDx WB (IgM + IgG) 100%, 76.7%
- BioPlex 2200 Lyme Total 96.8%, 91.1%
Additional Key Points to Remember

- Most Lyme disease tests detect antibodies (Abs) in response to infection.
- Abs can take several weeks to develop, so patients may test negative if infected only recently.
- Abs normally persists in the blood for months to years after the infection is gone; the test can’t be used to determine cure.
- Infection with other diseases, including tick-borne, or some viral, bacterial, or AI, can result in false positive test results.
- Some tests give results for 2 types of Ab, IgM & IgG. Positive IgM results should be disregarded if the patient has been ill for > 30 days.
Syphilis Testing
Syphilis Objectives

Upon completion of this Syphilis module, the learner will be able to:

• Describe the common test methods used for Syphilis testing

• Describe the common test algorithms used for Syphilis screening and confirmation

• Describe the multiplexing method used for Syphilis testing
Syphilis Topics

- Syphilis Incidence
- Serologic Tests
  - Manual
  - Automated
- Testing algorithms
  - Classic or “Traditional”
  - Reverse
  - European
- BioPlex® 2200 Syphilis Test
  - A new, combined approach
1984 – Teaching “Aerobics”
Etiology of Syphilis

- Venereal syphilis is caused by spirochete bacterium, *Treponema pallidum (TP)*, subspecies *pallidum*, *Nichols strain*
- Thin (0.2 μm) spirochete 6-20 μm in length with 10-13 coils
Syphilis - Infectious Stages

Primary Syphilis
- Painless Chancre
  - Spontaneously resolves
  - 9-90 days

Secondary Syphilis
- Diffuse Rash, Swollen Glands
  - Spontaneously resolves
  - 6 wks – 6 mo

Latent Syphilis
- Asymptomatic
  - Spontaneously resolves
  - 2 – 4 yr

Tertiary Syphilis
- Cardiovascular, Cutaneous Diseases, Neurosyphilis
  - Spontaneously resolves
  - 3 – 20 yr

Infectious Stages

Non Infectious Stages
CDC - US Syphilis Prevalence (rates per 100K people)

Avg. Prevalence (Positivity Rate): 5%

Rate per 100,000 population
- <=2.9 (n=13)
- 3.0-5.0 (n=14)
- 5.1-7.3 (n=13)
- >=7.4 (n=14)
Primary and Secondary Syphilis: Distribution of Cases by Sex & Sexual Behavior, 2015

- Men who have sex with men only (n = 12891)
- Men who have sex with men and women (n = 1338)
- Men who have sex with women only (n = 3178)
- Men without data on sex of sex partners (n = 4140)
- Cases with unknown sex (n = 27)
- Women (n = 2298)
Laboratory Testing
Cassette Players Don’t Rewind Themselves
Direct Organism Detection: Fluid from Lesion

- Dark Field Microscopy
- DFA-TP
  - Direct Fluorescent Antibody – Treponemal pallidum
- DFAT-TP
  - Direct Fluorescent Antibody Tissue – Treponemal pallidum
SeroLogic Assays
Non-Treponemal Assays

- Detects antibodies to lipoprotein material from damaged cells, cardiolipin from Treponemes and other bacteria
- Not specific for *T. pallidum* infection
  - May be positive in anti-phospholipid antibody syndrome (APS); not directed against causative agent of syphilis
- Classically used for screening test and monitoring disease response to treatment
  - In reverse sequence algorithm; used as a second line test
Non-Treponemal Assays

• **RPR- Rapid Plasma Reagin**
  - Anti-phospholipid (cardiolipin) antibody
    • Phospholipids with charcoal beads
  - Screening test
  - May become negative in late stage disease
  - Will decline (slowly) with successful treatment
  - Macroscopic test
  - Not used on CSF
  - Can be semi-automated (ASI) or fully automated (Gold Standard AX100 & Bio-Rad BioPlex 2200)

• **VDRL- Venereal Disease Research Laboratory Test**
  - Similar to RPR
  - Flocculation, test with freshly prepared liposomes of cardiolipin, lecithin and cholesterol
  - Microscopic test
  - Used for CSF
Syphilis Serology, 2

- Both non-treponemal tests are titered following a positive screening test
- Prozone effect may occur
  - Common in flocculation tests
  - May result in false negative test results in high antibody titer, due to interference in forming of antigen-antibody lattice
RPR

Non reactive

Reactive
VDRL Slide
Syphilis: Treponemal Tests

- FTA-ABS
  - Immunofluorescent procedure using a non syphilitic (Reiter) strain of *T. pallidum* to absorb out non specific treponemal antibodies
  - Not titered
  - Remains positive in >85% of individuals throughout life
  - False positives (beaded pattern?) associated with rheumatic diseases and pregnancy
FTA
Treponemal Assays, 2

- **TP Agglutination Assays**
  - Agglutination assay with Treponemal antigens coupled to red blood cells (HA-TP, MHA-TP) or latex (TP-PA) particles
  - May be more specific than FTA; fewer false positives, especially in pregnant women
- **ELISA/chemiluminescent (CIA) assays**
- **Multiplex bead immunoassays (MBIA)**
TP-PA

Reactive

Non-reactive
ELISA / Chemiluminescent Assays

- Treponemal specific assays
- Automated/Semi Automated platforms
- High volume testing
- Objective interpretation
- Screening test
  - Blood Supply
  - Reverse Sequence Algorithm
Save the Manuals!!!
Knowledge Check

• What species causes Syphilis?
Knowledge Check

• Which assay is used to monitor syphilis treatment?
Algorithms
Classic Syphilis Testing Algorithm

- **Nontreponemal test (RPR or VDRL)**
  - **Reactive**
    - **Treponemal test (EIA, TP-PA, TPHA, or FTA-ABS)**
      - **Reactive**
        - Syphilis (new case or previously treated case)
      - **Nonreactive**
        - **Biologic false positive**
  - **Nonreactive**
    - No syphilis or very recent infection; testing concludes

*If the nontreponemal test is reactive qualitatively, a titer is then quantitated.

**Biologic false positive (BFP) results of nontreponemal tests can occur in the setting of older age, autoimmune disease, intravenous drug use, recent vaccination, or certain infections.
Reverse Sequence Syphilis Algorithm

Nonreactive
- Not syphilis or early syphilis (repeat if suspected)

EIA
- Equivocal
  - Repeat EIA
    - Equivocal or reactive
      - RPR test
        - Nonreactive
          - Not syphilis
        - Reactive
          - TP-PA or FTA-ABS test
            - Nonreactive
              - Not syphilis
            - Reactive
              - Syphilis
                - Late latent or treated?
FIGURE. Composite results of syphilis testing algorithms using treponemal tests for initial screening and likely interpretations* — four laboratories, New York City, October 1, 2005–December 1, 2006†

- **Initial EIA treponemal test (N = 116,822)**
  - 6,587 (6%)
  - 110,235 (94%)

  **Rapid plasma reagin (RPR) test (n = 6,548)**
  - 2,884 (44%)
  - 3,664 (56%)

  **Syphilis, old or new. Treatment usually indicated unless previously treated. Retreatment indicated if titer has increased four fold or more.**

  **Probably old treated syphilis. Treatment might be indicated if not previously treated.**

  **If false-positive screening treponemal test result suspected, or if not previously treated, retest with a different treponemal test.**

  **Second treponemal test** (n = 2,512)
  - 2,079 (83%)
  - 433 (17%)

  **Treatment indicated, unless a history of treatment exists.**

  **No treatment, or a third treponemal test can be used to resolve the discrepancy between the two treponemal test results.**

* One laboratory provided limited interpretation of the test results; the other three summarized the results without interpretation. No formal recommendations exist regarding the interpretation of results derived from testing algorithms using treponemal tests as the initial test.

† Using a convenience sample of 116,822 specimens. The four laboratories used different testing algorithms. Data shown are a composite of results from all four laboratories.

§ Enzyme immunoassay.

‡ Reactive with EIA treponemal test but nonreactive with RPR test.

** Using *Treponema pallidum* particle agglutination or fluorescent treponemal antibody tests.
5 Laboratories using reverse sequence algorithm from 2006-2010

56.7% of specimens reactive on the EIA/CIA were non reactive on an RPR/VDRL

31.6% of these discordant specimens were nonreactive using a second treponemal assay (TP-PA/FTA)
• Traditional screening algorithm is recommended

• Reverse sequence may be used if a laboratory has appropriate equipment
  – Discordant specimens should be tested using the TP-PA instead of the FTA-ABS as the third level
  – Studies have been published since 2011 supporting the use of the automated Treponemal Specific Assays as the first line of testing
Three Algorithms

I. Traditional

Nontreponemal test, eg, RPR

-  +

Treponemal test, eg, TPPA, EIA, CIA

+  -

Serodiagnosis  BFP

II. Reverse

Treponemal test, eg, TPPA, EIA

-  +

Quantitative nontreponemal test

+  -

Serodiagnosis  Syphilis unlikely

A second and different treponemal test

+  -

Serodiagnosis  Syphilis unlikely

III. ECDC

Treponemal test, eg, TPPA, EIA

-  +

A second and different treponemal test

+  -

Serodiagnosis  Syphilis unlikely
- Compared classical, reverse and European algorithms
- Classical algorithm: 76% accuracy
- Reverse algorithm: 99.9% accuracy
- European algorithm: 99.6% accuracy
Performance of Automated Treponemal Specific Assays

- Park, et al., 2016. JCM, 54:163-167
- Compared 6 different automated syphilis IgG assays to FTA-ABS, n= 615
  - Architect, Centaur, Cobas, HISCL, Immunoticles, and Mediace.
  - Agreement: 98-99.8%
  - Sensitivity: 96.8-99.4%
  - Specificity: 98-100%
- Discrepant specimens were false positives or from past, treated individuals
Performance of Automated Treponemal Specific Assays

- Compared Mediace RPR vs Mediace TPLA
- N= 24,681
- Reverse algorithm found 190 screen positive, traditional algorithm found 30 screen positive
- 140/190 were confirmed by RPR and/or TPPA
- Reverse algorithm detected 110 more true positives than traditional at a cost of more false positives
Knowledge Check

• What are the 2 US Syphilis Testing Algorithms?
But, Is There a Way to Have Both Treponemal and Non-Treponemal Results Simultaneously?
Can Replaying a Song Be as Easy as Selecting Repeat Option?
Finally...Auto Playing...Bluetooth
But, Is There a Way to Have Both Treponemal and Non-Treponemal Results Simultaneously?

YES!!!
BioPlex 2200 Syphilis Total and RPR Assay

Multiplex Treponemal and RPR Dual Assay
BioPlex Syphilis Total and RPR Assay

- Random Access Multiplex Analyzer
- Beads
  - Treponemal fusion protein rTP47/rTP17
  - Cardiolipin
  - Internal Standard
  - Serum Verification
- Conjugate
  - Monoclonal anti IgG and Monoclonal anti IgM
- Results
  - Total Anti Treponemal
  - Total RPR (Cardiolipin)
  - RPR Titer
Intended use

• An initial qualitative test for syphilis diagnosis
  – Not intended for screening blood or plasma donors
• Second qualitative step in either the standard or reverse algorithm
• Dilutions can be run to determine an RPR (Cardiolipin) end point titer up to 1:64 (1:2048)
# BioPlex Test Result Interpretation

<table>
<thead>
<tr>
<th>BioPlex Syphilis Total</th>
<th>BioPlex RPR Total</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive</td>
<td>Reactive</td>
<td>Presumptive evidence of infection</td>
</tr>
<tr>
<td>Reactive</td>
<td>Non reactive</td>
<td>Primary or latent infection or previously treated or untreated syphilis. Recommend additional testing consistent with clinical findings</td>
</tr>
<tr>
<td>Non reactive</td>
<td>Reactive</td>
<td>Presumptive evidence of early and/or incubating infection. Possible cross reactivity with other spirochetes/related antigens. Recommend additional testing consistent with clinical findings</td>
</tr>
<tr>
<td>Non reactive</td>
<td>Non reactive</td>
<td>No serological evidence of infection. Early or incubating infection cannot be excluded.</td>
</tr>
</tbody>
</table>
Bio-Rad In-House Data

• Zheng, et al., ECCMID Conference, Amsterdam, 2016
  – BioPlex RPR Clinical sensitivity
    • 92.7% (n= 41) for untreated syphilis
    • 82.7%  (n=81) for treated syphilis
  – BioPlex Treponemal assay clinical sensitivity
    • 97.6% (n=41) for untreated syphilis
    • 95.1% (n=81) for treated syphilis
• Zheng, et al., 2017 APHL Conference
  – Compared BioPlex results to a comparator result consisting of Diasorin LIASON treponemal assay, BD Macro-Vue RPR and Fujirebo SERODIA-TP-PA
  – Positive agreement: 98.7% (n=541)
  – Negative agreement: 98.5% (n=675)
OK, so I can have both results simultaneously. Which Algorithm should I use?
“The Lady or the Tiger” Ending. You decide!!!

- You can follow the classical algorithm and use the RPR results as the primary
  - Or, follow the reverse algorithm and use the treponemal result as the primary
  - If the reverse is chosen, a 3rd level of testing (TP-PA) will be needed for discordant specimens
Syphilis Testing Summary

• CDC has recommended the traditional algorithm

• Automated treponemal specific tests offer increased sensitivity, possibly at a cost of reduced specificity
  – The CDC and ECDC reverse algorithms use these assays as their initial test

• The BioPlex System provides both treponemal and non treponemal results in a single test with rapid turnaround time. Each lab can choose which algorithm they should follow
Acknowledgements

• Special thanks to
  – Thomas S. Alexander, Ph.D., D(ABMLI)
    • Immunologist
      – Summa Health, Akron, OH
    • Professor of Pathology
      – Northeast Ohio Medical University
      – Rootstown, OH
Teaching “Zumba”

Wireless Music
Questions?

Contact: Maria Crisostomo
US Sr. Product Manager
Email: maria_crisostomo@bio-rad.com
Phone: (510) 741-4693
ANA Screen - Outline

- Antibody, autoantibody & antigen
- Autoimmunity & autoimmune diseases
- 5 primary categories of autoimmune diseases
- Test methods used for autoimmune diagnostic testing
- Autoimmune testing algorithm
- Test performance
Autoimmune Testing
Antibody vs. Autoantibody vs. Antigen

- **Auto**: Self
- **Antibody**: a blood protein produced by our immune system to protect against foreign invaders
- **Autoantibody**: a blood protein produced by our immune system but mistakes our own tissues & cells as foreign invaders

*Autoimmune disorders in a nutshell.*

*Beatrice the Biologist*
What is an Autoimmune Disease?

- Immune means resistant to a foreign invader

- An autoimmune disease is a disorder whereby the body mistakes its own tissue and cells for a foreign invader
Why do people get autoimmune disease?

- Environmental Trigger
- Genetics Predisposition

Immune System

- Autoimmune Response
- Normal immune response

Variable clinical presentation
Autoimmune Diseases*

• ~80 autoimmune diseases have been described; most of these diseases are rare (low prevalence)

• Common diseases include autoimmune thyroid disease, rheumatoid arthritis and celiac disease

• Every patient is unique and presents with different clinical symptoms and autoantibody profile

• Systemic Lupus Erythematosus (SLE) or Lupus is the prototypical systemic rheumatic disease known as “The Disease of a Thousand Faces”

Lupus – Butterfly Rash

A Disease of a thousand faces
Autoimmune Disease Burden*

- Affect up to 8% of the US population
- Is responsible for $100B in annual direct healthcare costs in the US

Gender Bias*

Knowledge Check

• What is an autoantibody?
Knowledge Check

• What is an autoantibody?
  • A blood protein produced by our immune system but mistakes our own tissues & cells as foreign invaders
Knowledge Check

- What is an autoimmune (AI) disease?
Knowledge Check

• What is an autoimmune (AI) disease?
• An autoimmune disease is a disorder whereby the body mistakes its own tissue and cells for a foreign invader
Knowledge Check

• What triggers an autoimmune disease?
Knowledge Check

• What triggers an autoimmune disease?
  – Environmental trigger
  – Genetic predisposition
Examples of Autoimmune Diseases

- Thyroid
  - Hashimoto’s thyroiditis
  - Grave’s disease

- Stomach/Intestinal
  - Atrophic gastritis
  - Autoimmune Hepatitis
  - Celiac Disease

- Adrenal
  - Addison’s Disease

- Pancreas
  - Type 1 Diabetes

- Circulatory
  - Wegener’s Granulomatosis

- Muscle
  - Dermatomyositis
  - Polymyositis

- Kidney
  - SLE

- Skin
  - Scleroderma

- Joints
  - Rheumatoid arthritis

- Eyes & Mouth
  - Sjogren’s Syndrome

- Mixed Connective Tissue Disease

Autoimmune Diseases

Systemic

Organ Specific
Autoimmune Testing Disease Categories

- Typical Autoimmune Test Menu Offering

![Venn Diagram showing Systemic, Gastrointestinal, Vasculitis, Organ-specific, and Anti-Phospholipid Syndrome categories]

- Systemic
- Gastrointestinal
- Vasculitis
- Organ-specific
- Anti-Phospholipid Syndrome

Bio-Rad Autoimmune
Knowledge Check

• Q: What are the five typical autoimmune disease categories used by IVD manufacturers?
Common Test Methods

- **Solid Phase**: Glass Side
- **Detection**: Epifluorescent Microscope
- **Result**: Subjective

- **Solid Phase**: Microwell
  - **Detection**: Spectrophotometer
  - **Result**: Objective

- **Solid Phase**: 8 µm magnetic Bead
  - **Detection**: Flow Cytometer
  - **Result**: Objective
What is a Testing Algorithm?

• A testing algorithm is a sequence of tests used in combination to improve the accuracy of the laboratory diagnosis of the targeted disease based on testing of serum or plasma specimens.

• A testing algorithm can also be described as tiered testing (i.e. first tier, second tier, etc.), whereby the result of the initial test (first tier or screening test) will determine if a subsequent test (second tier or confirmatory test) will be used to confirm the initial test result.
Let’s take a look at typical ANA testing algorithm

**ANA Screen or 1\textsuperscript{st} Tier Testing**

- **(-)**: No further testing required
- **(+)**: Perform 2\textsuperscript{nd} Tier Testing

- *dsDNA* (+)  *Chromatin* (+)  *SS-A* (+)  *SS-B* (+)  *Sm* (+)  Sm/RNP  RNP  Jo-1  Scl-70  Ribosomal P  Centro-mere

* = Lupus biomarker
Knowledge Check

- What is a testing algorithm?
Knowledge Check

• What are the 3 most commonly test methods used for autoimmune diagnostic testing?
# Test Performance: Sensitivity vs. Specificity

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Definition</strong></td>
<td>Proportion of patients with a disease who test positive</td>
<td>Proportion of patients without the disease who test negative</td>
</tr>
<tr>
<td><strong>100% (1.0) Means</strong></td>
<td>The test correctly identify every person who <strong>has</strong> the target disorder</td>
<td>The test correctly identify every person who <strong>does not have</strong> the target disorder</td>
</tr>
<tr>
<td><strong>Statistical Outcome</strong></td>
<td>True Positive</td>
<td>True Negative</td>
</tr>
<tr>
<td><strong>Ideal Test Result</strong></td>
<td>Negative Test Result</td>
<td>Positive Test Result</td>
</tr>
<tr>
<td><strong>Test Interpretation</strong></td>
<td>They are definitely <strong>not positive</strong> → They <strong>DON’T</strong> have it</td>
<td>They are definitely <strong>not negative</strong> → They <strong>DO</strong> have it</td>
</tr>
<tr>
<td><strong>The Rule</strong></td>
<td>Rule Out (SnOut)</td>
<td>Rule In (SpIn)</td>
</tr>
</tbody>
</table>
Test Performance: Sensitivity vs. Specificity

- Sensitivity = If you got it, we detect it

- Specificity = If you don’t got it, we won’t detect it
Test Performance: Sensitivity vs. Specificity
Knowledge Check

• What is assay sensitivity?
Knowledge Check

• What is assay sensitivity?
  – Ability to detect targeted disease in patients with targeted disease
    • If you got it, we detect it
Knowledge Check

• What is assay specificity?
Knowledge Check

• What is assay specificity?
  • Ability to not detect targeted disease in patients without targeted disease
    – If you don’t got it, we won’t detect it
More topics

- Bio-Rad Autoimmune History
- Multiplex Instrument and Workflow
- Multiplex ANA Panel
- MDSS: Medical Decision System Software
- ANA Testing Algorithms
- Summary
EMBRACING TRADITION
The Clear Choice in IFA Testing

Since pioneering autoimmune diagnostics with the introduction of the Kallestad HEp-2 IFA slides, Bio-Rad has been delivering high quality products to clinical laboratories worldwide.
Multiplex Immunoassay System Overview
Example: BioPlex 2200 System

- Fully automated, random access, multiplex testing platform

- Increased efficiencies through labor savings, faster TAT and improved workflow
  - Up to 100 samples per hour
  - 45-64 minutes time to first result

- High reproducibility using Luminex® technology for bead identification and analyte detection

- Consolidation of testing from multiple platforms

- Track line connectivity available: Abbott, Siemens, and InPeco
Lean Laboratory Workflow

“The 7 Wastes”

1. Over Production
   Excessive preparation of reagents

2. Waiting Time
   Batch-intensive processes

3. Transportation
   Unnecessary movement of specimens

4. Over Processing
   Pre-analytical sample preparation

5. Overstock
   Sizeable storage of supplies

6. Excess Motion
   Frequent user interaction with manual or semi-automated methods

7. Scrap
   Unnecessary repeats due to poor quality methods

The BioPlex® 2200 Solution

1. Ready to Use
   Liquid, ready-to-use reagents, minimize the waste of supplies

2. Random access
   Continuous flow of patient samples improves turnaround time

3. Comprehensive menu
   Multipleplex panels support platform consolidation

4. Primary-tube sampling
   Use of primary sample tubes frees laboratory personnel

5. Multiplexed chemistry
   Reagents, calibrators and controls utilize less space

6. Walkaway
   Complete automation requires minimal user intervention

7. Quality Assurance
   Integrated QC technologies enhance quality and accuracy
BioPlex 2200 Track Line Connectivity Option

- BioPlex TLC Option
  - Streamlined laboratory workflow with connectivity
    - Achieve LEAN efficiencies for infectious disease, autoimmune & vitamin D testing
    - Multiplex testing + track line connectivity = optimized efficiency
Multiplex IA system benefits

- STAT capability
- Automated primary tube sampling
- Ready to use reagents
- No need to batch samples
- Multiplex detection of up to 12 analytes
- Small sample size: 3 – 40 µL
- High assay precision
- Multiplexed, liquid calibrators and controls
- Internal quality control beads processed with every patient sample: ISB, AVB, SNB, etc.
Autoimmune and Specialty Menu
9 Kits – 27 Assays

- ANA Screen
- Vitamin D
- Celiac IgA
- Celiac IgG
- Antiphospholipid IgA
- Antiphospholipid IgG
- Anti-CCP
- Vasculitis

Expanding Infectious Disease Menu
9 Kits – 24 Assays

- Lyme'
- HIV Ag-Ab
- Syphilis Total & RPR
- EBV IgG
- EBV IgM
- HSV-1 & HSV-2 IgG
- ToRC IgM
- ToRC IgG

BioPlex 2200
Autoimmune Menu

BioPlex 2200
Infectious Disease Menu

- MMV IgM: in development for OUS
New Assays and Future Menu

• BioPlex 2200
  – Newest FDA Approved Assays
    • Syphilis Total & RPR
    • ToRC IgM
    • HIV Ag-Ab (5th Generation)
  – Lyme Total Assay (Launched in 2019)
    • Multiplex Assay: Proteins & proprietary peptides
    • American & European strains
    • Improved sensitivity for detection of early Lyme disease; improved Lyme IgM specificity
Multiplex ANA Screen Assay
Multiplex ANA Screen Assay

- dsDNA
- Chromatin
- Sm
- SmRNP
- RNP (A<sup>r</sup> & 68<sup>r</sup>)
- Ribosomal P
- SS-A (60 & 52<sup>r</sup>)
- SS-B<sup>r</sup>
- Jo-1<sup>r</sup>
- Scl-70<sup>r</sup>
- Centromere B<sup>r</sup>

- Autoimmune Screen (reports POSITIVE if any of the antibodies are detected)
Assay Calibration

• dsDNA is a **quantitative** assay, calibrated against WHO Wo/80 Standard
  – Negative: ≤4 IU/mL
  – Indeterminate: 5-9 IU/mL
  – Positive: ≥10 IU/mL

• All other assays are **semi-quantitative** results, expressed in terms of AI (Antibody Index)
  – Negative: <1.0 AI
  – Positive: ≥1.0 AI

• All calibrators are constructed by making serial dilutions of pooled positive serums.
Why Did We Choose Current Kit Configuration?

12 Analytes

- SS-A/Ro, SS-B/La, Jo-1, RNP, Sm are standard commercial offerings because of their high level of disease specificity.
- Anti-SS-A 52 is often seen in Myositis patients.
- Anti-Ribosomal P and Anti-Sm are very specific to SLE.
- Anti-dsDNA and Anti-Chromatin are somewhat specific for SLE.
- Anti-Scl-70 is extremely specific for scleroderma.
- Anti-Centromere B is required for the differential diagnosis of CREST.
- Anti-Sm/RNP confirms the presence of Sm (SLE) and RNP (MCTD). Allows detection of additional antibodies against the complex.
Anti-SS-A / Ro

- HEp-2 IFA is less sensitive in detecting SS-A than the BioPlex 2200 and EIA.
- Prevalence in clinical study
  - >80% of patients with Sjögren’s Syndrome
  - 33 to 52% of patients with SLE
  - >10% of other patients with Systemic disease
- Positive Agreement: 92-95%
- Negative Agreement: 98-100%

Two beads coated with:
SS-A 60 affinity purified antigen
SS-A 52 recombinant antigen

IFA pattern: Speckled

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested
Anti-SS-B / La

– Prevalence in clinical study
  • >80% of patients with Sjögren’s Syndrome
  • 13 to 27% of patients with SLE
  • <10% of other patients with Systemic disease
– Positive Agreement: 83-100%
– Negative Agreement: 98-99%

IFA pattern:
Speckled

One bead coated with:
SS-B recombinant antigen

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested
Anti-Sm

– One of the American College of Rheumatology (ACR) criteria for diagnosis of SLE

– Prevalence in clinical study
  • 15 to 42% of patients with SLE
  • <10% of other patients with Systemic disease, except MCTD

– Positive Agreement: 71-88%
– Negative Agreement: 97-100%

**One bead coated with:**
Sm affinity purified antigens

**IFA pattern:**
**Speckled**

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested
Anti-RNP

- Nearly all **MCTD** patients demonstrate anti-RNP activity
- Prevalence in clinical study
  - 94% of patients with MCTD
  - 22-48% of patients with SLE
  - <10% of other patients with Systemic disease
- Positive Agreement: 83-90%
- Negative Agreement: 97-98%
  - Antibody most commonly seen in healthy people

IFA pattern: **Speckled**

Two beads coated with:
- Recombinant antigen RNP-68
- Recombinant antigen RNP-A

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested
Anti-Sm/RNP

– Nearly all **MCTD** patients demonstrate anti-Sm/RNP activity
– Sm/RNP assay detects Sm, RNP and specific Sm/RNP antibodies
– Prevalence in clinical study
  • 94% of patients with MCTD
  • 23-45% of patients with SLE
  • <10% of other patients with Systemic disease
– Positive Agreement: 80-95%
– Negative Agreement: 98-100%

IFA pattern: **Speckled**

One bead coated with: **SmRNP purified antigen**

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested
Anti-Ribosomal P

- May be present in *lupus sera* even when other ‘ANA-positive” antibodies are not detected.
- Many labs do not report a cytoplasmic pattern (not true ANA).
- Prevalence in clinical study
  - 9 to 30% of patients with SLE
  - <10% of other patients with Systemic disease
- Possibly added to the ACR criteria for diagnosing SLE. May correlate to CNS involvement.
- Positive Agreement: 82-86%
- Negative Agreement: 98%

**IFA pattern:**

*Cytoplasmic*

One bead coated with: Ribosomal P affinity purified antigens

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested
Anti-dsDNA

- Frequently used to confirm a diagnosis of SLE and to track disease activity.
- High avidity antibodies have been shown to be associated with disease activity & renal involvement.
- Prevalence in clinical study
  - 28 to 45% of patients with SLE
  - 12% of patients with MCTD
  - <10% of other patients with Systemic disease
- Positive Agreement: 77-86%
- Negative Agreement: 97-100%

IFA pattern: Homogeneous

When present, can easily mask speckled and centromere patterns.

One bead coated with:

dsDNA synthesized by PCR
Anti-Chromatin

- Most common autoantibody in patients with SLE and Drug Induced Lupus
- Typically positive when dsDNA is positive
- Prevalence in clinical study
  - 37 to 73% of patients with SLE
  - >80% of patients with MCTD
  - 8-14% of other patients with Systemic disease
- Positive Agreement: 39-62%
- Negative Agreement: 91-98%

IFA pattern:
Homogeneous

When present, can easily mask speckled and centromere patterns.

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested

One bead coated with:
Purified nucleohistone antigens
Response of dsDNA to Disease Status

Patient A

Patient B
Anti-Centromere B

- Historically associated with the limited form of scleroderma: CREST syndrome.
- Prevalence in clinical study
  - 27% of patients with scleroderma
  - 3 to 12% of patients with SLE
  - <10% of other patients with Systemic disease
- Positive Agreement: 97-100%
- Negative Agreement: 97-99%

IFA pattern:
- Centromere

Easily masked by other IFA patterns.

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested

One bead coated with:
- Recombinant Centromere B antigen
Anti-Scl-70

- Strongly associated with scleroderma
- Reported as a “mixed pattern”, although it is only a single autoantibody
- Prevalence in clinical study
  - 16% of patients with scleroderma
  - 2-3% of patients with SLE
  - <10% of other patients with Systemic disease
- Positive Agreement: 53-77%
- Negative Agreement: 97-98%

IFA pattern:
Mixed

One bead coated with:
Recombinant Scl-70 antigen

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested
Anti-Jo-1

- Strongly associated with polymyositis
- Rarely seen in other patients—very high specificity
- Usually missed by HEp-2 IFA
- Prevalence in clinical study
  - 17% of patients with polymyositis
  - <2% of other patients with Systemic disease, except MCTD
- Positive Agreement: 55-100%
- Negative Agreement: 100%

IFA pattern:

Cytoplasmic

One bead coated with: Recombinant Jo-1 antigen

Cohort: 1000+ Retrospective & prospective ANA+ & ANA- serum samples; BioPlex & EIA tested
<table>
<thead>
<tr>
<th>Pattern</th>
<th>ANTIBODY</th>
<th>Targeted Connective Tissue Diseases</th>
<th>Prevalence Non-Disease</th>
<th>Prevalence Non-Disease</th>
<th>Prevalance--Other Rheumatic Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SLE (Study #1) SLE MCTD Sjögren's Syndrome Scleroderma Polymyositis Blood bank --U.S</td>
<td>Blood bank outside U.S. (study #3)</td>
<td>Rheumatology Clinic (study #1)</td>
<td></td>
</tr>
<tr>
<td>Specified</td>
<td>SS-A (60 &amp; 52)</td>
<td>52% 33% 13% &gt;80% 23% 42%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>SS-B</td>
<td>27% 13% &lt;2% &gt;80% 5% &lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td></td>
<td>Sm</td>
<td>42% 15% 31% &lt;2% 5% 8%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td></td>
<td>SmRNP</td>
<td>45% 23% &gt;80% &lt;2% 7% &lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td></td>
<td>RNP (A &amp; 68)</td>
<td>48% 22% &gt;80% &lt;2% 9% 8%</td>
<td>3%</td>
<td>2%</td>
<td>7%</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>Chromatin</td>
<td>73% 37% &gt;80% 12% 14% 8%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>3%</td>
</tr>
<tr>
<td></td>
<td>dsDNA</td>
<td>45% 28% 12% 6% 9% &lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>Mixed</td>
<td>Sci-70</td>
<td>3% 2% 7% &lt;2% 16% &lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td>Cent</td>
<td>Centromere B</td>
<td>12% 3% 7% &lt;2% 27% &lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>3%</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Ribosomal P</td>
<td>30% 9% 7% &lt;2% &lt;2% &lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
</tr>
<tr>
<td></td>
<td>Jo-1</td>
<td>&lt;2% &lt;2% 7% &lt;2% &lt;2% 17%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
<td>&lt;2%</td>
</tr>
</tbody>
</table>
### SLE Patients Followed Over Time

<table>
<thead>
<tr>
<th>Draw Date</th>
<th>dsDNA</th>
<th>Chrom</th>
<th>Sm</th>
<th>Ribo P</th>
<th>SS-A</th>
<th>SS-B</th>
<th>SmRNP</th>
<th>RNP</th>
<th>Cent B</th>
<th>Scl-70</th>
<th>Jo-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/9/2003</td>
<td>2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>3.3</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>10/3/2003</td>
<td>1</td>
<td>0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>2.9</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>10/30/2003</td>
<td>1</td>
<td>0.4</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>2.3</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>11/6/2003</td>
<td>1</td>
<td>0.4</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>2.3</td>
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### Patients Followed Over Time

#### Sjögren’s Syndrome

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Advantages of the BioPlex 2200 for Tracking Patients

- Ability to track a specific autoantibody relating to a specific CTD, high sensitivity.
- Track specific autoantibodies relating to clinical status.
- Consistency of result over time; controls interchangeable between lots of reagents.
- IFA Titer is subjective to many variables.
- Positive IFA relates to many autoantibodies, some of which have unknown clinical utility.
Multiple Reporting Options

- Total assay screen: if any assay in the ANA kit is positive a qualitative positive result is reported
- Individual assay results
- Multiple beads can be used to provide an individual assay result
- “Add-On” testing from previously run samples
- Design custom reporting panels
- Medical Decision Support Software (MDSS) – is an optional laboratory tool that associates patient antibody results with predefined autoantibody patterns that are associated with specific Connective Tissue Diseases.
Total ANA Assay Screen and/or Individual Results

- BioPlex ANA Screen will simultaneously detect levels of the 11 antibodies listed below.
- If one or more of the individual antibodies is present at an abnormal level, then the ANA Screen will be positive.
- If levels of all 11 antibodies are within normal limits, then the ANA Screen will be negative. Individual antibody results are only reported if ordered by the physician.
How does MDSS work for ANA Screen?

• Each “unknown” sample is compared to a pre-established database of over 1,400 characterized sera/plasma.

• Results of MDSS analysis fall into one of the following general outcomes:
  – Negative
  – No Association
  – Association with Disease

• When results are associated with a disease, MDSS will propose a maximum of two disease classifications based upon the similarity of the current analysis to the stored results.
MDSS Functionality- ANA Screen

A

B

C

No Disease

Disease State “SLE”

Disease State “SS”

Patient result
Indication

- **Associates** patient antibody results with pre-defined profiles **correlated** with the following systemic autoimmune diseases:
  - Systemic Lupus Erythematosus (SLE)
  - Mixed Connective Tissue Disease (MCTD)
  - Sjögren’s Syndrome (SS)
  - Scleroderma (Systemic Sclerosis)
  - Polymyositis
How to Order an ANA Screen with MDSS result

- Autoimmune sample BioPlex™ 2200 ANA Screen
  - ANA Screen or Individual Tests
    - MDSS not available
      - Add on features available
  - ANA +
    - Qualitative Screen with MDSS comment
  - ANA 11
    - All 11 autoantibody Results with MDSS Comment
    - Add on features available
1. All antibody levels for systemic autoimmune disease are below pre-established cutoffs.

2. Antibody levels show no association with MDSS defined patterns for systemic autoimmune diseases.

3. Antibody levels show association with systemic autoimmune disease. Consider SLE.

4. Antibody levels show association with systemic autoimmune disease. Consider Sjögren's syndrome (primary or secondary) or SLE.

5-16. Total of 16 possible outputs.

Disclaimer: MDSS outputs of “Negative” or “No association” do not rule out autoimmune disease. Patients with Rheumatoid Arthritis may result in an SLE association from MDSS, thus MDSS associations from patients with RA should be interpreted with caution.”
Possible Test Groups

• **ANA Screen** – Qualitative Screen: Positive or Negative Result, is dependent upon the presence or absence of 11 specific clinically relevant antibodies
  – Add-on specific autoantibodies: Encouraged to order all 11
  – Available with MDSS comment: ANA +

• **ANA Screen with Reflex** - If ANA test result is Positive, it will be automatically reflexed to report all 11 autoantibodies (dsDNA, Chromatin, Sm, Ribosomal P, SS-A, SS-B, SmRNP, RNP, Scl-70, Centromere B and Jo-1)

• Any of the **eleven antibodies** can be ordered individually
ANA TESTING ALGORITHMS
EXAMPLES
Connective Tissue Disease Algorithm

CTDC / Connective Tissue Diseases Cascade, Serum

Cyclic citrullinated peptide antibodies, IgG
- Positive result supports the diagnosis of rheumatoid arthritis
- ≤20 U
- >20 U
  - STOP
  - No further testing required

Antinuclear antibodies (ANA)
- ≤3.0 U
- >3.0 U
  - STOP
  - No further testing required

DNA double-stranded (dsDNA) antibodies with reflex, IgG
- ≤30-75 IU/mL
- >75 IU/mL
  - CRITH / DNA Double-Stranded (dsDNA) Antibodies by Cribbidae lucillae IFA, IgG, Serum
  - Positive result supports the diagnosis of SLE
  - Negative result unable to confirm borderline positive result obtained by EIA

Antibody to extractable nuclear antigen evaluation
- This test includes the following IgG antibodies, which are automatically performed:
  - SS-A/Ro
  - SS-B/La
  - Sm
  - Scl 70
  - Jo-1

Centromere antibodies, IgG
- >1.0 U

Ribosome P antibodies, IgG
- >1.0 U

Positive result supports the diagnosis of SLE and may indicate the presence of central nervous system involvement

Clickable PDF

NOTE: Positive results are not diagnostic for any CTD and should be interpreted within the clinical context of the patient.
Another take-away

• Note: positive results are not diagnostic for any CTD and should be interpreted within the clinical context of the patient
Connective Tissue Disease Algorithm

Test Directory

Test Code 10547

ANA Multiplex, with Reflex to dsDNA
This assay may be useful in diagnosing and monitoring connective tissue diseases.

Includes
If ANAchoice® Screen is positive, then ds-DNA will be performed at an additional charge (CPT code(s): 86225)

Methodology
Immunoassay (IA)

Reference Range(s)
ANAchoice® Screen Negative

Alternative Name(s)
Systemic Lupus Erythematosus (SLE), Antinuclear Antibody Screen
Connective Tissue Disease Algorithm

Test Directory

ANA Multiplex with Reflex to 11 Antibody Cascade

Test Code 19946

The ANAchoice® Cascading Reflex provides physicians with a cost-effective and medically justified approach to evaluating a patient with suspected rheumatologic disease. Eleven antibodies associated with specific rheumatologic disease entities are analyzed and resulted in sequential tiers until positive findings are reported. The test is not intended for the work-up of autoimmune hepatitis or other non-rheumatologic diseases.

Clinical Significance

ANA Multiplex with Reflex to 11 Antibody Cascade - The ANAchoice® Cascading Reflex provides physicians with a cost-effective and medically justified approach to evaluating a patient with suspected rheumatologic disease. Eleven antibodies associated with specific rheumatologic disease entities are analyzed and resulted in sequential tiers until positive findings are reported. The test is not intended for the work-up of autoimmune hepatitis or other non-rheumatologic diseases.

Test Details

Includes

ANA Multiplex with Reflex to 11 Antibody Cascade begins with an ANAchoice® Screen.

If the ANAchoice® Screen is positive, it will reflex the following five antibodies at an additional charge: dsDNA (CPT code(s): 86225), Sm/RNP (CPT code(s): 86235), Sm (CPT code(s): 86235), and Chromatin (CPT code(s): 86235).

If any of those five antibodies are positive, the cascade stops and the results are reported.

If all five of those antibodies are negative, four additional antibodies will be reflexed at an additional charge: SSA (CPT code(s): 86235), SSB (CPT code(s): 86235), Scl-70 (CPT code(s): 86235), Jo-1 (CPT code(s): 86235).

If any of those four antibodies are positive, the cascade stops and the results are reported.

If all four of those antibodies are negative, the following two additional antibodies will be reflexed at an additional charge: Ribosomal P (CPT code(s): 83516) and Centromere B (CPT code(s): 86235).

The cascade stops upon the first positive antibody found in a group. It is possible that antibodies in subsequent groups are also positive, but will not be added or reported. Please contact your local Quest Diagnostics Laboratory if you are interested in adding this additional testing.
Utility of Antinuclear Antibody Screening by Various Methods in a Clinical Laboratory Patient Cohort

Kiaoli Deng,1 Brian Peters,2 Michael W. Ettore,3 Judy Ashworth,2 Lynn A. Brunelle,2 Cynthia S. Crowson,1,4 Kevin G. Moders,4 and Melissa R. Snyder2,5

Background: Antinuclear antibody (ANA) testing is routinely performed during evaluation of patients with a suspected connective tissue disease (CTD), yet the question of which method is most appropriate remains controversial. The purpose of this study was to evaluate the clinical utility of ANA testing by an enzyme immunoassay (EIA), an immunofluorescence assay (IFA), and a multiplex immunoassay (MIA) in a routine laboratory population.

Methods: Samples (n = 1000) were collected from specimens submitted for ANA testing by EIA (Bio-Rad). All samples were subsequently analyzed by IFA (Zeus) and MIA (Bio-Rad). The sample cohort was weighted to represent the routine testing population. Diagnostic information was obtained by chart review.

Results: For the diagnosis of a CTD, ROC curve analysis demonstrated no significant differences between IFA (area under the curve 0.81) and EIA (0.84) (P = 0.25), with overlap of a single point for the MIA. When normalized to a specificity of approximately 90%, the sensitivities of the MIA, EIA, and IFA were 57%, 67%, and 56%, respectively. By varying the clinical cutoff, the IFA could achieve the highest sensitivity of 94%; however, the corresponding specificity was only 43%. In contrast, a strongly positive EIA had a specificity of 97%, although, at this cutoff, the sensitivity was only 40%.

Conclusions: Although the overall diagnostic performance of the IFA, EIA, and MIA were not statistically different, the clinical sensitivity and specificity varied dramatically based on the positive/negative cutoff. Knowledge about the performance characteristics of each method will significantly aid in the interpretation of ANA testing.
Methods

• Samples (n=1000)
  – One of the largest studies ever conducted to study ANA performance by method
  – All of the testing was performed by the Mayo Laboratories

• Samples were identified with ANA EIA (Bio-Rad)
  – The samples needed to be chosen and categorized before starting the study
  – Using one of the study methods could have introduced bias if only two methods were compared
Methods (continued)

• Sample identification ANA EIA (Bio-Rad)
  – Negative samples (n=273) randomly chosen with results ≤1.0 U
  – Weak positive samples (n=225) 1.1 – 2.9 U
  – Positive samples (n=250) 3.0 – 5.0 U
  – Strong positive samples (n=252) ≥6.0 U

• All samples subsequently tested by:
  – IFA = HEp-2 (Zeus) titered 1:40 – 1:640
  – MIA = BioPlex 2200 ANA Screen (Bio-Rad)
Results

• 580 samples were ≤1:40 by IFA
  – 515 (88.8%) of these samples were also negative by EIA
  – 65 (11.2%) were positive by EIA
Results (continued)

- 271 samples were IFA 1:80 to 1:160
  - 205 (75.6%) had a negative EIA result ≤1.0 U
  - 66 (24.4%) had a positive EIA result ≥1.1 U
- 149 samples were IFA ≥1:320
  - 36 (24.2%) had a negative EIA result ≤1.0 U
  - 61 (40.9%) had positive EIA results of 1.1 – 5.9 U
  - 52 (34.9%) had strong positive EIA results of ≥6.0 U
- 515 samples were negative by both IFA and EIA
  - 490 (95.1%) were negative by BioPlex ANA
  - 25 (4.9%) were positive by BioPlex ANA
- The strongest correlation was between the EIA and the BioPlex 2200 ANA Screen
Specificity, Sensitivity, and Likelihood Ratios

Table 2. Sensitivity and specificity of EIA, IFA, and MIA at various cutoffs.\(^a\)

<table>
<thead>
<tr>
<th>Test</th>
<th>Cutoff</th>
<th>Specificity, %</th>
<th>Sensitivity, %</th>
<th>Positive LR</th>
<th>Negative LR</th>
<th>Specificity, %</th>
<th>Sensitivity, %</th>
<th>Positive LR</th>
<th>Negative LR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFA</td>
<td>1:40</td>
<td>43</td>
<td>94</td>
<td>1.66 (0.088, 3.13)(^b)</td>
<td>0.14 (0.00, 125.11)</td>
<td>27</td>
<td>96</td>
<td>1.33 (0.81, 2.18)</td>
<td>0.13 (0.00, 128.5)</td>
</tr>
<tr>
<td></td>
<td>1.80</td>
<td>62</td>
<td>84</td>
<td>2.19 (0.85, 5.41)</td>
<td>0.26 (0.00, 21.17)</td>
<td>42</td>
<td>93</td>
<td>1.59 (0.79, 3.20)</td>
<td>0.18 (0.00, 18.76)</td>
</tr>
<tr>
<td></td>
<td>1:160</td>
<td>77</td>
<td>70</td>
<td>3.07 (0.92, 10.24)</td>
<td>0.39 (0.02, 6.45)</td>
<td>55</td>
<td>85</td>
<td>1.87 (0.72, 4.85)</td>
<td>0.28 (0.01, 6.34)</td>
</tr>
<tr>
<td>EIA</td>
<td>1.1 U</td>
<td>80</td>
<td>74</td>
<td>3.67 (1.32, 10.23)</td>
<td>0.32 (0.00, 195.82)</td>
<td>34</td>
<td>97</td>
<td>1.48 (0.84, 2.59)</td>
<td>0.09 (0.00, 140.5)</td>
</tr>
<tr>
<td></td>
<td>3.0 U</td>
<td>93</td>
<td>58</td>
<td>8.32 (2.03, 34.14)</td>
<td>0.45 (0.03, 7.66)</td>
<td>61</td>
<td>88</td>
<td>2.24 (0.82, 6.11)</td>
<td>0.20 (0.01, 6.81)</td>
</tr>
<tr>
<td></td>
<td>6.0 U</td>
<td>97</td>
<td>40</td>
<td>13.37 (1.48, 120.73)</td>
<td>0.62 (0.17, 2.22)</td>
<td>84</td>
<td>58</td>
<td>3.75 (0.52, 27.14)</td>
<td>0.50 (0.10, 2.36)</td>
</tr>
<tr>
<td>MIA</td>
<td>≥1+</td>
<td>87</td>
<td>67</td>
<td>5.28 (1.27, 22.00)</td>
<td>0.38 (0.02, 6.56)</td>
<td>69</td>
<td>86</td>
<td>2.73 (0.85, 8.78)</td>
<td>0.21 (0.01, 5.28)</td>
</tr>
</tbody>
</table>

\(^a\) Sensitivity was determined in patients with a diagnosis of a CTD (n = 76, weighted; n = 227, unweighted). Specificity was calculated in all patients without a confirmed CTD (n = 924, weighted; n = 773, unweighted).

\(^b\) LR data in parentheses are 95% CIs.
Cutoff Influences Sensitivity and Specificity

- **ANA IFA**
  - 1:40 to 1:160 result have a low positive LR
- **ANA EIA**
  - 1.1 U cutoff has a higher sensitivity than 1:160 IFA
- **BioPlex 2200 ANA Screen**
  - Same specificity as 1:320 ANA IFA
  - Higher positive LR than ANA EIA at 1.1 U cutoff
Predictive autoimmunity using autoantibodies

Dolores Pérez, Boris Gilburd, Óscar Cabrera-Marante, Jose A. Martínez-Flores, Manuel Serrano, Laura Naranjo, Daniel Pleguezuelo, Luis Morillas, Ora Shovman, Estela Paz-Artal, Yehuda Shoenfeld* and Antonio Serrano

Predictive autoimmunity using autoantibodies: screening for anti-nuclear antibodies

DOI 10.1515/cclm-2017-0241
Received March 19, 2017; accepted April 21, 2017

Abstract

Results: At 3 years of follow-up, 312 (76%) subjects were positive for autoantibodies by IIF and 99 subjects continued to be negative. A diagnosis of autoimmune disease was found in most of the subjects (87%).
Summary

• 3-year follow up of 411 subjects without a clear diagnosis of autoimmune disease

• Subjects were initially positive by BioPlex ANA and negative by HEP-2 IFA (<1:160)

• At 3 years follow-up:
  – 312 (76%) were positive by HEP-2 IFA
  – 99 (24%) continued to be negative by HEP-2 IFA
  – 87% developed a diagnosis of autoimmune disease
Positive Predictive Value by Disease

Table 5: The positive predictive value (PPV) of the antibodies detected by BioPlex ANA Screen for the diagnosis of autoimmune diseases.

<table>
<thead>
<tr>
<th></th>
<th>PPV, %</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A) SLE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNP-A</td>
<td>37.0</td>
<td>27.73–47.29</td>
</tr>
<tr>
<td>Ro60</td>
<td>37.7</td>
<td>30.45–45.58</td>
</tr>
<tr>
<td>Ro52</td>
<td>24.1</td>
<td>16.75–33.28</td>
</tr>
<tr>
<td>RIB-P</td>
<td>75</td>
<td>47.41–91.67</td>
</tr>
<tr>
<td>La</td>
<td>23.1</td>
<td>14.60–34.25</td>
</tr>
<tr>
<td>Sm</td>
<td>84.6</td>
<td>64.27–94.95</td>
</tr>
<tr>
<td>Sm-RNP</td>
<td>66.7</td>
<td>51.49–79.19</td>
</tr>
<tr>
<td>Chrom</td>
<td>79.7</td>
<td>67.42–88.33</td>
</tr>
<tr>
<td>dsDNA</td>
<td>68.1</td>
<td>52.75–80.48</td>
</tr>
<tr>
<td>(B) SS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Topo I/Scl-70</td>
<td>37.9</td>
<td>21.30–57.64</td>
</tr>
<tr>
<td>CenB</td>
<td>57.4</td>
<td>43.27–70.50</td>
</tr>
<tr>
<td>(C) SSj</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ro60</td>
<td>37.1</td>
<td>29.88–44.97</td>
</tr>
<tr>
<td>Ro52</td>
<td>24.1</td>
<td>16.75–33.28</td>
</tr>
<tr>
<td>La</td>
<td>42.5</td>
<td>32.14–53.58</td>
</tr>
<tr>
<td>(D) RA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNP-A</td>
<td>15</td>
<td>8.91–23.85</td>
</tr>
</tbody>
</table>

PPV for the diagnosis as (A) systemic lupus erythematosus, (B) systemic sclerosis, (C) Sjögren’s syndrome and (D) rheumatoid arthritis.
### Table 7: The predictive value of BioPlex ANA Screen when more than one antibody is positive.

<table>
<thead>
<tr>
<th>Number of positive autoantibodies</th>
<th>Autoimmune diseases after 3 years of follow-up (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>77 (128/166)</td>
</tr>
<tr>
<td>Two</td>
<td>88.6 (78/88)</td>
</tr>
<tr>
<td>Three or more</td>
<td>96.8 (152/157)</td>
</tr>
</tbody>
</table>
Discussion

- ACR recommends that laboratories using methods other than ANA IFA should provide comparative data of their method to their clients
- This study fulfills the ACR recommendation
- The statistical weighting is intended to represent the laboratory’s testing population not the general population
- Discrepant results for samples are not unexpected
- The different methods have different antigen presentation
Discussion (continued)

- Approximately 5% of IFA / EIA double negative samples are positive on the BioPlex ANA Screen
  - The number of samples was too small to address this discrepancy
  - Likely caused by differences in antigen presentation
- No statistically significant difference in the clinical utility for IFA, EIA, or BioPlex 2200 ANA (AUCs and ROC analysis)
- Depending on the cutoff used by the laboratory, differences in the sensitivity and specificity were noted
• ANA EIA demonstrated the highest positive LR of all three methods
• ANA IFA showed modest positive LR
• BioPlex 2200 ANA had a positive LR of 5.28
• Positive results on EIA and IFA increase the likelihood of a CTD more significantly than that of a sample only positive by IFA
• None of the three methods include sufficient sensitivity such that a negative result conclusively excludes a diagnosis of a CTD
And, don’t forget…

- Positive results are not diagnostic for any CTD; should be interpreted within the clinical context.
- Should always pair clinical history + lab test results.
- Pre-test probability ↑ with # of clinical symptoms.
- As pre-test probability ↑, the test PPV ↑.
- If patient has a pos ANA test, the autoantibody specificity should be determined; aids in diagnosis.
- Test PPV & LR ↑ when multiple autoantibodies are pos.
- Ensure test method has excellent lot-to-lot precision; samples from same patient retested months apart.
Questions?