IGeneX Inc.

NEXT GENERATION LYME TESTING

Broad Coverage Lyme Ab Assays
Lyme MultiSpecies ImmunoBlots

Webinar Presented by
Joseph J. Burrascano Jr. M.D.
Joined by Jyotsna Shah PhD for the Q&A

October 2019

Presenters

Joseph J. Burrascano Jr. M.D.

- Well-known pioneer in the field of tick-borne diseases, active since 1985
- Founding member of ILADS and ILADEF
- Active in physician education on all aspects of tick-borne diseases

Jyotsna Shah, PhD

- President & Laboratory Director of IGeneX Clinical Laboratory
- Over 40 Years of Research Experience in Immunology, Molecular Biology & Microbiology
- Author of Multiple Publications & Holds More Than 20 Patents
- Member of ILRAD as a Post-Doctoral Scientist
- Started the First DNA Sequencing Laboratory in E. Africa

Webinar Outline

I plan to present new information that will change forever your approach to Lyme

- A broad variety of diverse *Borrelia* strains and species are infecting our patients- will give case histories with discussion
- Outline the limitations of current serological tests and why
  - Will discuss narrow focus and insensitivity of ELISAs and two-tier testing
  - Will describe how a western blot is made and why this limits accuracy
- LATEST ADVANCEMENTS IN TESTING TECHNOLOGY
- Lyme ImmunoBlot
  - What is an ImmunoBlot?
  - What makes it superior?
  - Results of validation and clinical studies
- Broad-Coverage Lyme Antibody Assay (*NEW*)
- Recommendations on how to approach the laboratory diagnosis of Lyme

Is it really Lyme?

Why are my patients seronegative?

Seronegativity in Lyme

Study/Year	Sensitivity	Specificity
• Schmitz et al, 1993	66%	100%
• Engstrom et al, 1995	55%	96%
• Ledue et al, 1996	50%	100%
• Bakken et al. 1997	75%	81%
• Trevejo et al, 1999	29%	100%
• Nowakowski et al, 2001	66%	99%
• Bacon et al, 2003	68%	99%
• Coulter et al, 2005	18%	-
• Wormser et al, 2008	14.1%	-
MEAN TOTALS	**49.01%**	**96%**

1. Schmitz et al. Eur J Clin Microbiol Infect Dis. 1993;12:419-24
2. Engstrom et al. J Clin Microbiol. 1995;33:419-27.
3. Ledue et al. J Clin Microbiol. 1996;34:2343-50.
4. Bakken et al. J Clin Microbiol 1997; 35(3): 537-543.
5. Trevejo et al. J Infect Dis. 1999;179:931-8.
6. Nowakowski et al. Clin Infect Dis. 2001;33:2023-7.
7. Bacon et al. J Infect Dis. 2003;187:1187-99.
8. Coulter et al. ., J Clin Microbiol 2005; 43: 5080-5084.
9. Wormser et al. Clin Vaccine Immunol. 2008;(10):1519-22.

Seronegative Lyme?

THEY'RE NOT SERONEGATIVE- YOU ORDERED THE WRONG TEST!!

- You may be missing cases of other strains and species of Lyme *Borrelia* that are not being picked up by current tests
- Results of clinical cases were **eye-opening** to me and totally changed how I test my Lyme patients
- Surprisingly large degree of diversity!!
- The new IGeneX tests have multispecies capabilities that are crucial for a correct diagnosis

Case histories now follow…

Patient Series #1:

In an analysis of results from one client of IGeneX*

- 36 **Lyme ImmunoBlots** were performed
- Antibody testing detected ten cases of *B. mayonii*, six each of *B. spielmanii* and *B. californiensis*, five *B. garinii,* two *B. afzelii*, one *B. valensiana* and only **one case** of *B. burgdorferi* strain B31. Five cases could not be speciated.

All of these patients are US citizens; fifteen of them have travelled widely

- Americans travel, so it is important to be able to test them for all reasonable possibilities
- Many different species were found, supporting the need for broad spectrum testing

Of all 36, only a single case of *Bb* B31 was found!

Standard American serologies are based upon this single species.
The majority of these thirty-six patients would not have been correctly diagnosed if the IGeneX Lyme ImmunoBlots were not done.

**I do not believe these results reflect the actual percentage of non-B31 Bb in our patients. Most clients use IGeneX when standard tests are unexpectedly non-reactive.*

Patient Series #2

In another recent analysis of 39 client samples from American patients, antibodies measurements detected:

- Eleven cases of *B. spielmanii*, six *B. californiensis*, three *B. afzelii*, two *B. mayonii* and two Bb ss strain B31
- There were four cases in which the ImmunoBlot identified unspeciated European Lyme *Borrelia* and eleven in which unspeciated *Bb sl* were found

If standard western blotting were performed as the sole serologic test, only the two B31 cases and possibly some of the *Bb sl* cases would have been identified. The rest would have been incorrectly classified as negative (false negatives).

These results are being presented at a conference next year with publication to follow.

Patient Series #3

In yet another series in which speciation was recorded:

- A total of eleven clinical Lyme cases were studied
- Of the eleven, seven had antibodies to *B. spielmanii*, four to *B. californiensis*, and one each to *B. mayonii* and *Bb ss*

In this series as in the first one, the reason the total number of species is greater than the number of patients tested is because of co-infection with multiple species.

- In this series, two were seemingly co-infected with two species- one had antibodies to *B. spielmanii* plus *B. mayonii*, and one had them to *Bb ss* plus *B. californiensis*

If standard western blotting were performed, not only would the majority of the results have been falsely negative, the presence of co-infections would have been totally missed.

Patient Series #4

In an internal study to test the validity of the IGeneX Lyme ImmunoBlot, they tested **43 samples**, positive on Lyme ImmunoBlots, by Western blots prepared individually from the following eight species of Lyme *Borrelia: B. burgdorferi* B31*, B. burgdorferi* 297*, B. mayonii, B. californiensis, B. afzelii, B. garinii, B. spielmanii and B. valensiana*.

Results:

- When only a *B. burgdorferi* B31 western blot was performed, **only 14 of the 43** Lyme ImmunoBlot-positive samples were western-blot-positive: **missed 29 of the 43**
- However, when all eight western blots were performed, the remaining twenty-nine samples now were detected
- An alternate way of exploring this is to utilize the Lyme ImmunoBlot's discriminatory ability by reading selected bands
- Here, when only *B. burgdorferi* B31-specific bands were read on the Lyme ImmunoBlots, **30 samples were missed**
- However, when all the reactive bands for all the species were read, **all 43 samples were positive**
- This data clearly demonstrate the power and necessity of the IB's multispecies capability

Lyme Disease &
The Limitations of The Current Tests

Lyme Serologies

Most serologies (IFAs, ELISAs and western blots) are based upon ONE strain of ONE species

- Most commonly used strain is *Bb ss* strain B31
  - Is a lab strain collected from a tick- very different from clinical *Borrelia*
- One lab uses strain N40
  - Not commonly found across the USA- more prevalent in Europe
- IGeneX does add strain 297 to their western blots which is why it is the broadest and most sensitive western blot so far available

But NONE will reliably indicate antibodies to other species!

Two-Tier Testing

Very insensitive! Also only able to detect one species

- First tier is an ELISA made from a lysed lab strain
  - First-tier sensitivity at best is 70% *for Bb strain B31!*
  - If non-reactive, then testing ends and result is called negative
  - If positive, then the second tier is done
- Second tier- western blot or another ELISA
  - Combined sensitivity for this one *Borrelia* (B31) is 50%-60%

Western Blotting in Lyme

- Has long been the preferred Lyme serologic test
- But it has its limitations!
- Understanding the methodology is quite revealing….

Western Blot

How it is made is the basis of its limitations

In a western blot, *Borrelia* are grown in culture, then they are lysed and their protein antigens are then separated by size using electrophoresis.

PROBLEMS:

- Antigens are derived from cultured lab strains, not clinical specimens
  - Most use only *Bb ss* strain B31 (IGeneX adds strain 297)
  - Variability in culture conditions and age can affect test performance
  - Test result may be affected by other laboratory variables such as reagent concentrations and temperature
- Will not reliably pick up strains and species that are unlike B31
- Possibility to react with similar but non-*Borrelia* antigens, resulting in false positives
  - Other spirochetes, viruses, autoantigens

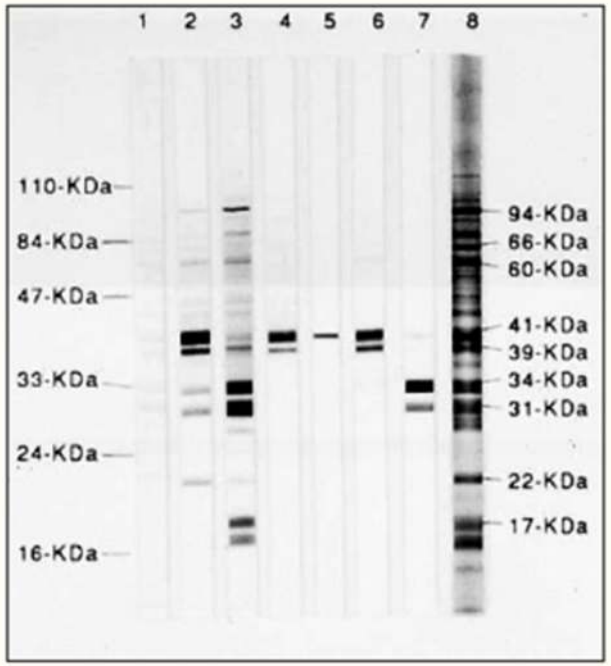
Lyme Western Blot - How Bands are Identified

IDENTIFICATION IS MIGRATION-DEPENDENT

- If *Borrelia*-specific antibodies are present and bands appear, their location is used to identify the antigen, meaning that this identification is migration-dependent.

PROBLEMS:

- Migration is not an exacting process and the variable location of the bands can make identification of individual antigens very difficult- may not line up
- Also, some nonspecific or unimportant proteins may co-migrate with important *Borrelia* proteins, and the WB cannot distinguish these
- Is why epitope testing is needed to determine the significance of band 31



Significant Lyme disease antibodies detected on Western blot test, including 31- and 34-kilodalton bands. Courtesy of IGeneX, Inc.

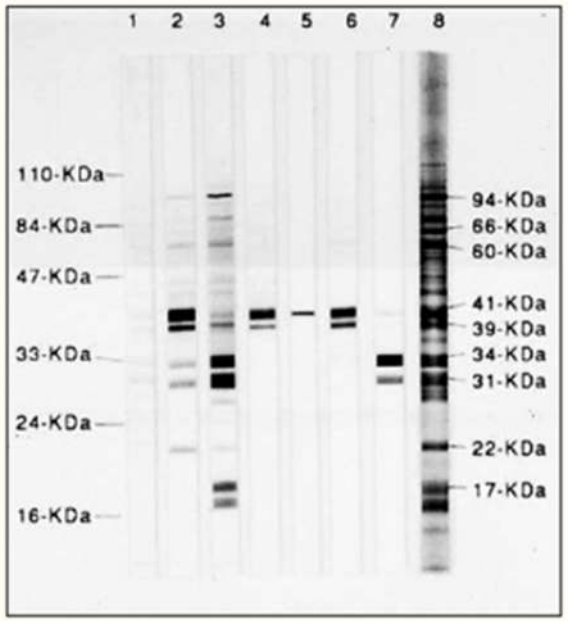
Lyme Western Blot – Scoring the Results

SCORING IS BASED UPON BAND INTENSITY

- If *Borrelia*-specific antibodies are present, a band will appear
- The test is then scored based upon the intensity of the band

PROBLEMS:

- How dark must a band be to be called positive? Indeterminate? Because protein content of any given culture can vary, the band intensity can vary, potentially causing false positives and false negatives.
- What does a broad band mean?
  - More active culture?
  - Multiple co-migrating antigens?
  - Stronger host reaction?
  - ??



Significant Lyme disease antibodies detected on Western blot test, including 31- and 34-kilodalton bands. Courtesy of IGeneX, Inc.

Replacing the Western Blot

The result of these limitations is suboptimal sensitivity, reduced specificity and narrow strain and species coverage.

THE IMMUNOBLOT ADDRESSES THESE PROBLEMS

- **Significantly increases sensitivity and specificity and broadens coverage** as compared to the Western Blot

Lyme ImmunoBlots

What is an ImmunoBlot?

RECOMBINANT MULTI-SPECIES IMMUNOASSAY

- It is a serological test for infection, but it is fundamentally different from other serologies
- The difference is that it uses pure, specifically created recombinant proteins as the test antigens, and not proteins from cultures of lab strains
- Can design these recombinant antigens to identify specific *Borrelia* biomarkers
- Can even be genus, species and strain-specific
- Result is ability to increase sensitivity without sacrificing specificity
- Also allows for identification of a broad range of *Borrelia* species and strains

Lyme ImmunoBlot

Overcomes WB Limitations

Using antigens derived from **recombinant proteins**:

- Significantly increases real-world sensitivity
  - Are able to include antigens from a variety of clinically important species and strains
  - Are able to include antigens from geographically diverse species and strains
  - No tradeoff between sensitivity and specificity
  - Renders western blots totally obsolete and should replace them

Lyme ImmunoBlot

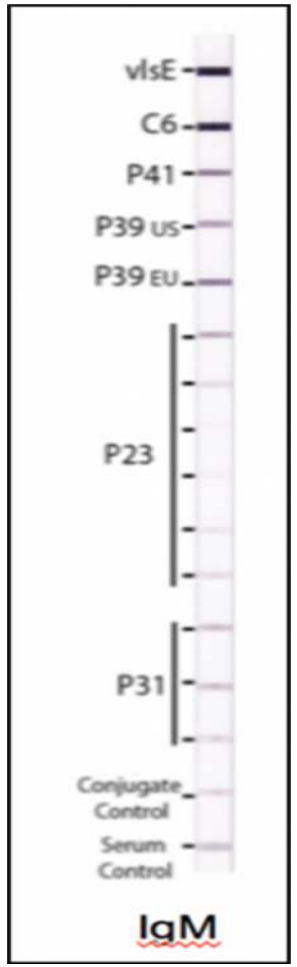
Overcomes WB Limitations

Using antigens derived from **recombinant proteins**:

- Significantly increases specificity
  - Much less likely to cross react with viruses
  - Much less likely to cross react with non-*Borrelia* bacteria
  - Much less likely to cross react with autoantigens
  - Specific for Lyme *Borrelia*
    - Will not cross react with Relapsing Fever *Borrelia*

How the Lyme ImmunoBlot is made?

- In the ImmunoBlot, specific, important recombinant proteins are created
- Then a **precise amount** is sprayed directly onto the membrane at **specific locations**
- Because quantity and location are controlled, results are far more consistent and accurate



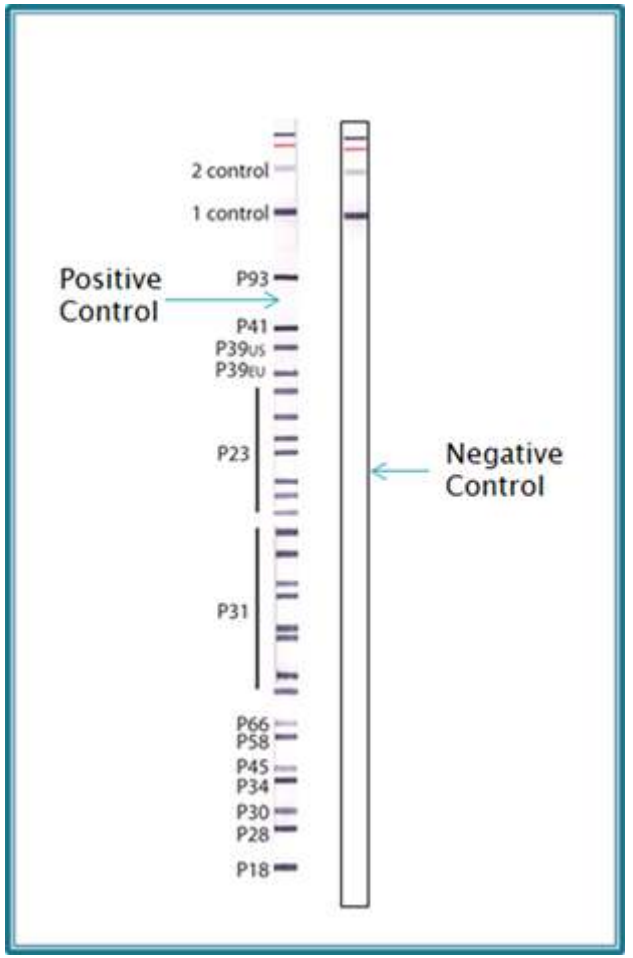
How the Lyme ImmunoBlot is made?

Precise Amount

- Banding intensity is no longer culture-dependent
- Positive bands are more clearly displayed
- Reduces false positives and false negatives

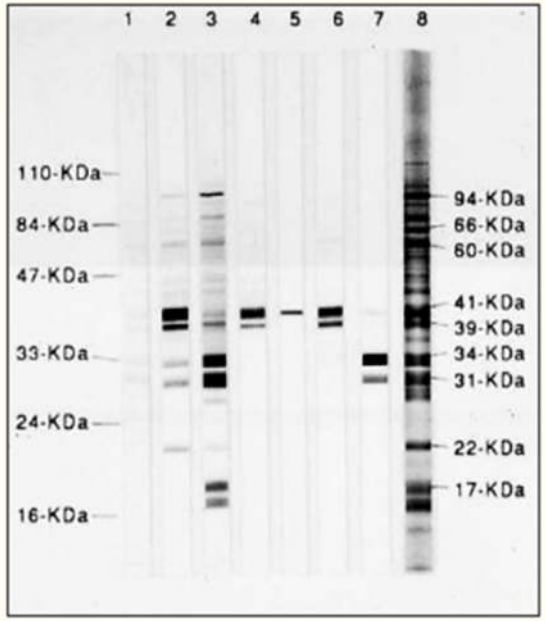
Specific Locations

- Band locations are no longer migration-dependent
- Know exactly what each positive band represents
- No longer an issue with co-migration
- No longer need confirmatory epitope tests



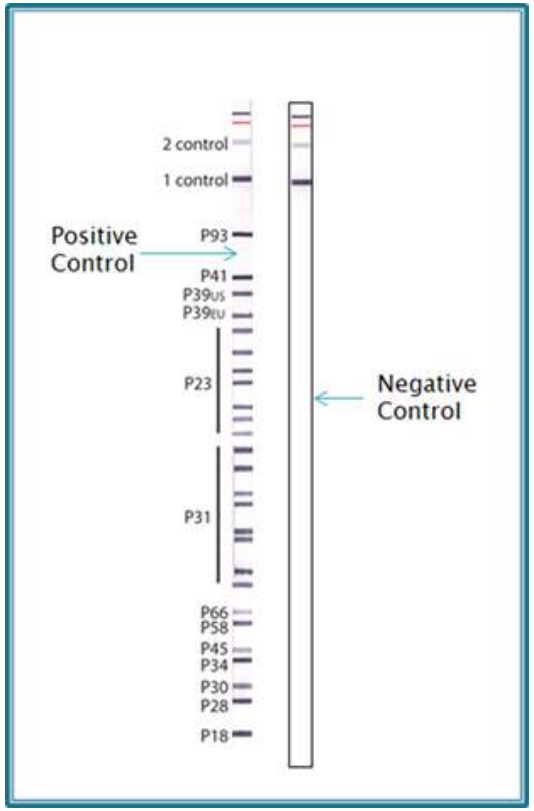
Comparison

Western Blot



Significant Lyme disease antibodies detected on Western blot test, including 31- and 34-kilodalton bands. Courtesy of IGeneX, Inc.

IGeneX Lyme ImmunoBlot



The IGeneX ImmunoBlot – More Inclusive

The **IGeneX** ImmunoBlot:

- Includes all *Borrelia*-specific antigens covering North American and European species
  - not just *Bb ss* B31 or 297
- Increases *real-world* sensitivity
  - Antigens from multiple species
  - Antigens from multiple strains

The IGeneX ImmunoBlot – Incredibly Complete

EIGHT *Borrelia* strains and species are included:

- *B. burgdorferi* B31
- *B. burgdorferi* 297
- *B. californiensis*
- *B. mayonii*
- *B. afzelii*
- *B. garinii*
- *B. spielmanii*
- *B. valaisiana*

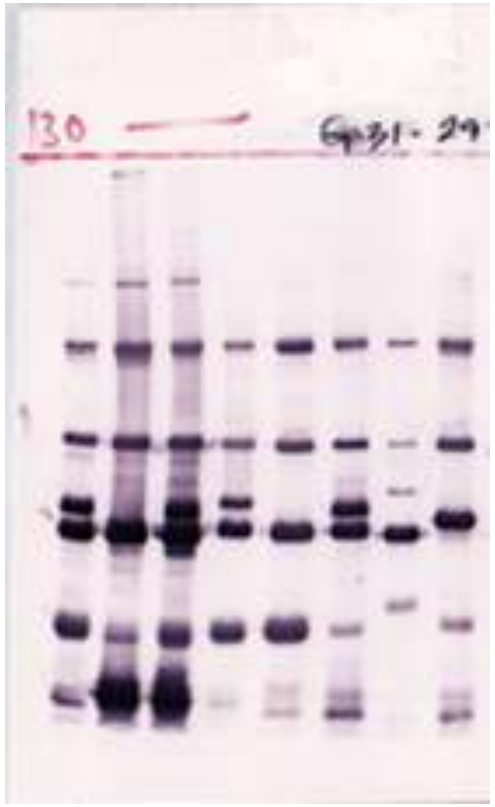
The IGeneX ImmunoBlot – IgM & IgG

- If you were able to find tests for all of these, between the IgM and IgG, you would need **SIXTEEN** different tests to do what this one set of Lyme Immunoblots will do!
  - But would not even begin to cover all the geographic subgroups
- Widens the scope of the IGeneX ImmunoBlot
- Increases its diagnostic usefulness
  - Recognition of broadening range of *Borrelia* species and strains
  - People and their pets travel

Western Blot

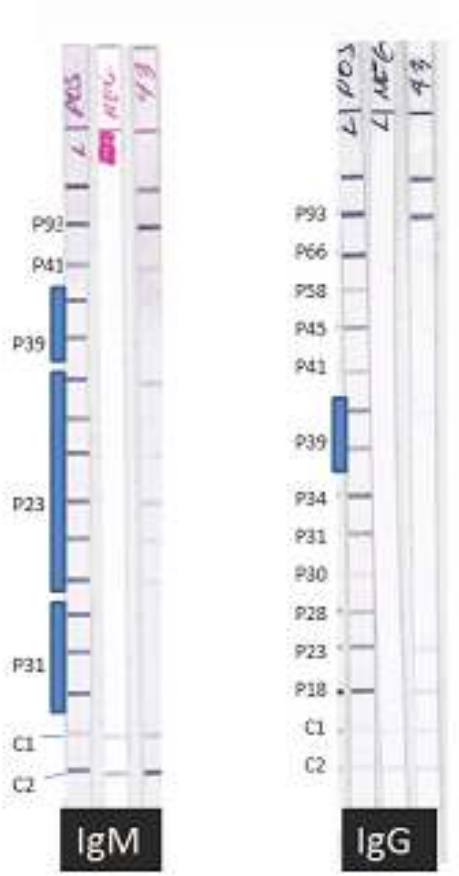
Mixture
297 and B31

Western blot Panel



WB required to include
B. burgdorferi sensu lato Group

ImmunoBlot



ImmunoBlot- Validation

Western Blot and ImmunoBlot - Reading Criteria

Western Blot- CDC criteria for positivity:

- **IgM WB-** two of the three antigen bands 23, 39 and 41 kDa
- **IgG WB-** five of the ten antigen bands 18, 23, 28, 30, 39, 41, 45, 58, 66 and 93 kDa

Western Blot- IGeneX In-House criteria for positivity:

- **IgG and IgM WBs-** positive if two from the following six bands are present: 23, 31, 34, 39, 41 and 93 kDa

Exceptions:

- A WB is indeterminate if only bands of 31 and 41 kDa are present on IgG and/or IgM or 31 and 93 kDa on IgM (recommend epitope testing to confirm origin of the 31 kDa band)
- An IgM WB is considered negative if only bands of 41 and 93 kDa are present.

IgM ImmunoBlot criteria for positivity- positive if two out of the five bands of 23, 31, 34, 39 and 41 kDa are present

IgG ImmunoBlot criteria for positivity- positive if two out of the six bands of 23, 31, 34, 39, 41 and 93 kDa are present.

Validation Samples

			Expected Result	
Source	Samples	n	Positives	Negatives
CDC	Patient Samples	42	17	25
Proficiency Samples (NYS & CAP)	PT Samples	20	9	11
Proficiency Samples	Autoimmune (22 Rheumatoid arthritis)	42	0	42
New York Biologics	Viruses	46	0	46
New York Biologics	RPR (+)	28	0	28
Total Samples		178	26	152

Improved Overall Sensitivity

Samples	Positives	Lyme Western blots							Lyme ImmunoBlots					
		Lyme WB (in- house)			Lyme WB (CDC)				Lyme IB (in-house)			Lyme IB (CDC)		
		IgM	IgG	G+M	IgM	IgG	G+M		IgM	IgG	G+M	IgM	IgG	G+M
CDC - Set 1	5*	2	4	4	2	3	4		3	5	5	2	4	4
CDC - Set 2	12*	7	8	9	7	5	9		11	10	12	8	5	10
PT Samples	9**	9	6	9	9	6	9		9	6	9	9	6	9
Total Positives	26	18	18	22	18	14	22		23	21	26	19	15	23
Sensitivity (%)		69.2	69.2	84.6	69.2	53.8	84.6		88.5	80.8	100.0	73.1	57.7	88.5

Improved Overall Specificity

Samples	Negatives	Lyme WB (in- house)			Lyme WB (CDC)			Lyme ImmunoBlots					
								Lyme IB (in-house)			Lyme IB (CDC)		
		IgM	IgG	G+M	IgM	IgG	G+M	IgM	IgG	G+M	IgM	IgG	G+M
CDC - Set 1*	5*	0	1	1	0	0	0	0	0	0	0	0	0
CDC - Set 2*	20*	0	1	1	0	0	0	0	1	1	0	0	0
PT Samples	11	0	0	0	0	0	0	0	0	0	0	0	0
Autoimmune disease	42	0	0	0	0	0	0	0	0	0	0	0	0
Viral Infections	46**	3	3	6	2	0	2	1	1	2	0	0	0
RPR +	28	5	1	6	1	0	1	0	2	2	0	1	1
Total False Positive		8	6	14	3	0	3	0	4	5	0	1	1
Total True Negative	152	144	146	138	149	152	149	151	148	147	152	151	151
Specificity %		94.7	96.1	90.8	98.0	100.0	98.0	99.3	97.4	96.7	100.0	99.3	99.3

*Western blot results were provided by CDC; **Out of 46 samples with antibodies to viruses, 11 had antibodies to CMV; 24 to EBV, 7 to HSV; and 4 to HCV. **Only two samples with antibodies to EBV were positive by Lyme IB. RPR– rapid plasma reagin test for syphilis; PT – proficiency test; G+M – Positive for both IgG and IgM.

Lyme ImmunoBlot – Excellent Accuracy

- **Published study** of 178 well-defined samples, comparing the IGeneX ImmunoBlot to a Western Blot
  - Superior sensitivity (100% using in-house criteria)
  - Superior specificity (>97%)
  - Superior positive predictive value
  - Superior negative predictive value
  - There was no cross reactivity with the two common Tick-Borne Relapsing Fever species *B. hermsii* and *B. coriaceae*

ImmunoBlot- Clinical Studies

Improved Sensitivity – Early Lyme

Comparison of Lyme ImmunoBlots with MarDx Test Using Well Characterized CDC Samples (CDC Criteria)										
Patients with:	n	ELISA	**Lyme Immunoblots**				**Western blots**			
			IgM	IgG	IgM+IgG	Sensitivity	IgM	IgG	IgM+IgG	Sensitivity
Early Lyme Acute (Stage 1)	15	7	9	2	11	73.3%	3	0	3	20%
Early Lyme Convalescent (Stage 1)	15	11	13	6	14	93.30%	10	5	12	80%
Neurological Lyme (Stage 2)	9	9	9	7	9	100%	9	5	9	100%
Lyme arthitis (Stage 3)	10	10	3	10	10	100%	1	10	10	100%
Total	49	37	34	25	44	89.8%	23	20	34	69.4%

Comparison of Lyme ImmunoBlots (In-House Criteria vs CDC Criteria Using Well Characterized CDC Samples									
Patients with:	**Lyme Immunoblots (In-house criteria)**					**Lyme ImmunoBlots (CDC criteria)**			
	n	IgM	IgG	IgM+IgG	Sensitivity	IgM	IgG	IgM+IgG	Sensitivity
Early Lyme Acute (Stage 1)	15	10	7	14	93.3%	9	2	11	73.3%
Early Lyme Convalescent (Stage 1)	15	13	7	15	100.0%	13	6	14	93.3%
Total	30	23	14	29	96.7%	22	8	25	83.3%

Superior in all Stages

Patients with:	n	2-tier Serological Testing for LD (ELISA follwed by Western blots)				Lyme ImmunoBlots		
		IgM	IgG	G+M		IgM	IgG	G+M
Early Lyme Acute (Stage 1)	15	20.0%	0.0%	20.0%		66.7%	46.7%	93.3%
Early Lyme Convalescent (Stage 1)	15	66.7%	33.3%	80.0%		86.7%	46.7%	100.0%
Neurological Lyme (Stage 2)	9	100.0%	55.6%	100.0%		100.0%	77.8%	100.0%
Lyme arthitis (Stage 3)	10	10.0%	100.0%	100.0%		30.0%	100.0%	100.0%
Total	49	46.9%	40.8%	69.4%		71.4%	63.3%	98.0%

ImmunoBlot Summary

- Capable of detecting antibodies to all of the currently known, clinically relevant Lyme *Borrelia* strains and species
- Validated to be superior to the Western Blot with regards to sensitivity, specificity and positive and negative predictive values
- Detects all stages of the disease
- Replaces two-tier testing
- Will not cross react with Relapsing Fever *Borrelia*

IGeneX Introduces a New Test!

The IGeneX **Broad Coverage Lyme Antibody Assay**

Single serological test that covers clinically relevant *Bb sl* species

- **Replacement for the ELISA**
  - Far broader and more inclusive (multispecies capability)
  - Far more sensitive
    - Overall sensitivity greater than 90%
- And just as specific as the two-tier test!

IGeneX Broad Coverage Lyme Antibody Assay

- **Cost-effective replacement for the ELISA**
  - Includes both IgM and IgG in the one test
  - Therefore, this single test replaces both the IgM ELISA and the IgG ELISA
- Simple yes-no result
  - no complicated interpretation necessary
- Rapid turnover

New Tests: Summary

- The **Broad Coverage Lyme Antibody Assay** offers a more sensitive, highly specific and broad-spectrum alternative to both the ELISA and the Western Blot
  - Includes multiple *Bb sl* species
  - Very cost-effective
  - Replaces the Two-Tier test scheme with a better test
- The **Lyme ImmunoBlots** also include broad, multi-strain and multi-species coverage, but can also identify species
  - Extremely useful in selected early Lyme cases
  - Provides information not available anywhere else
  - **Allows the clinician to identify patterns of presentation and treatment response associated with specific species of Lyme *Borrelia***

Points on Testing

- **Not every patient has detectable antibodies, especially in late and complicated cases**
- **Serologies are but one tool in our diagnostic toolbox- Suggest a test panel approach**
  - I often measure total serum immunoglobulins and IgG subclasses- if significantly **low**, serologic tests may be less sensitive. If IGs are **high**, then worry about false positives.
  - Patient may be immune-suppressed
  - Antibodies may be bound in immune complexes
  - Co-infections may cloud the picture
- **Early Lyme-** is the rash really an EM? Or no rash? Summer “flu”?
  - The Lyme ImmunoBlot is sensitive even in early Lyme and may be extremely useful here
- Maybe it is not Lyme - consider **Relapsing Fever**
  - **IMMUNOBLOTS AND BROAD COVERAGE Ab ASSAYS ARE AVAILABLE FROM IGENEX FOR THE TICK-BORNE RELAPSING FEVERS TOO!**

Recommended Test Panels

- **Early Disease (EM)**
  - Lyme/TBRF ImmunoBlots
  - Lyme IgX SPOT
  - Skin PCR
- **Active/Disseminated Disease**
  - Lyme and TBRF ImmunoBlots
  - Blood and Serum PCR
- **Late/Chronic Disease**
  - Lyme and TBRF ImmunoBlots
  - Lyme IgX SPOT
  - Blood and Serum PCR
- **Immune Integrity Assessment**
  - Lyme and TBRF ImmunoBlots
  - Lyme IgX SPOT
  - Blood and Serum PCR

NOW TIME FOR QUESTIONS