Application of Nanoparticle Technology: urinary antigen test for Lyme disease

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Controversies surrounding the definition of chronic Lyme disease

- Chronic Lyme disease lacks an accepted clinical definition and the majority of patients have no objective evidence of infection
- Fatigue, pain, cognitive impairment and arthritis are common complaints and are not specific for Lyme
- Physicians are worried of side effects of long-term antibiotic therapy for which they feel there is little evidence of successful outcomes in controlled published trials

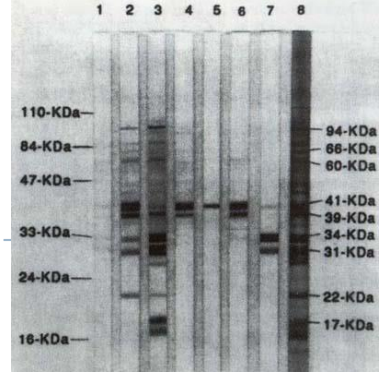
Controversies surrounding the definition of chronic Lyme disease – cont.

- "Four prospective, double-blinded, placebo-controlled trials have investigated the utility of prolonged antibiotics in patients with subjective 'post-Lyme disease syndromes'. With only one exception (fatigue) in one trial, no primary outcome measure favored treatment over placebo"
  Expert Rev. Anti Infect. Ther. 9(7), 787–797 (2011)

Issues with serologic testing

- **Serological tests** use Enzyme Immunoassay (EIA) and Western Blotting. The patient's serum is incubated with commercial bacterial antigen to detect antibodies in the serum which recognize the bacteria.
- **The drawbacks of serologic testing are false negatives in early stage disease** (Clin Infect Dis. 2006;43(9):1089-134) **and persistence of antibody titer even after successful treatment** (*JAMA*. 1994;271(6):460-463).
- Serological testing has been found to only be **60-77% specific** when coupled with symptomatic analysis (Cells 2013, 2, 607; J Clin Microbiol. 2005 43(10): 5080–5084).

Need for an Antigen Test



- **An antigen test may have an higher level of sensitivity and specificity for**
  1. **Early stage disease,**
  2. **Recurrent disease following therapy cessation, and**
  3. **Disease that persists in the face of therapy**
- **Current diagnostic testing for Lyme disease measures the presence of antibodies in the blood which react with Lyme bacteria antigens, but these tests do not measure the Lyme antigen itself.**

Antigen Test: Open Questions and Challenges

- **Lyme antigens** are bacterial proteins that may be shed in the plasma, urine, or joint fluid during active infection.
- Is it possible to reliably detect Lyme antigens in the urine of patients?
- **Which antigens** are most diagnostic in the following disease categories?
  1. **Early stage disease**
  2. **Failure of treatment**
  3. **Chronic active disease, and**
  4. **Successfully treated disease**

Antigen Testing: Which Body Fluid?

- Antigen testing would be most useful if samples could be collected at the time of tick exposure, and during the course of treatment.
- A **Urine Antigen Test** would be ideal because it is non invasive.
- Attempts at developing a reliable urine antigen test have failed because the antigen is
  1. Very low in concentration; below the detection limits of the experimental immunoassays.
  2. Masked by interfering substances in the urine.

Antigen Test

- We aim to develop a reliable and sensitive antigen test in urine by:
  1. Increasing test **sensitivity** -> **nanoparticle concentration**
  2. Verifying absolute **specificity** -> use of **monoclonal antibody and antigen competition**
  3. **Preserving antigens from degradation** -> **nanoparticle sequestration**

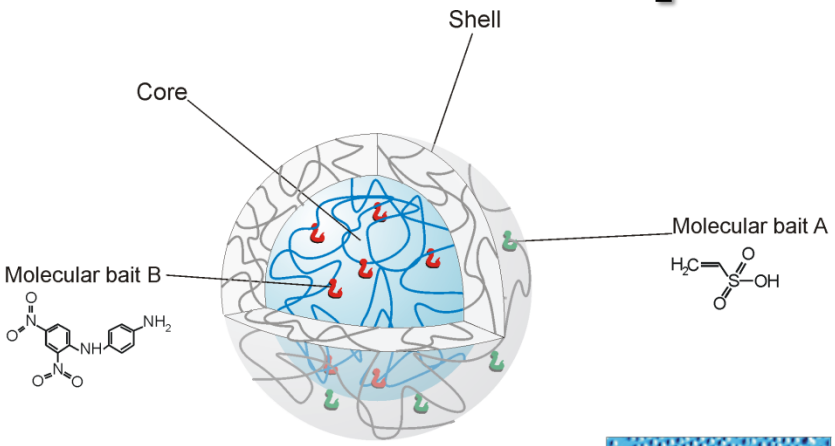
Why OspA?

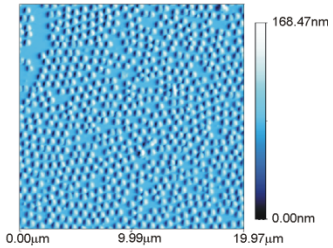
- 31-kDa lipoprotein localized on the surface of *B. burgdorferi*
- OspA antibodies are detectable in patients in early and late stage disease *(Infect. Immun. 1995, 63:2228-2235)* or antibiotic resistant Lyme (*Arthritis Rheum. 1999 42:1809-1812)*
- *B. burgdorferi* expresses high levels of OspA in the mammalian host in an inflammatory environment (*Infect. Immun. 2003, 71,7; 4003-4010)*
- Lipidated OspA has inflammatory properties *(stimulation of neutrophils, The Journal of Immunology, 1997, 4838*)
- OspA and surface lipoproteins provide protective shielding against mammalian host innate immunity (*Mol Microbiol*. 2008; 69(1): 15–29)
- OspA has been found in Lyme patient urine, cerebrospinal fluid and synovial fluid

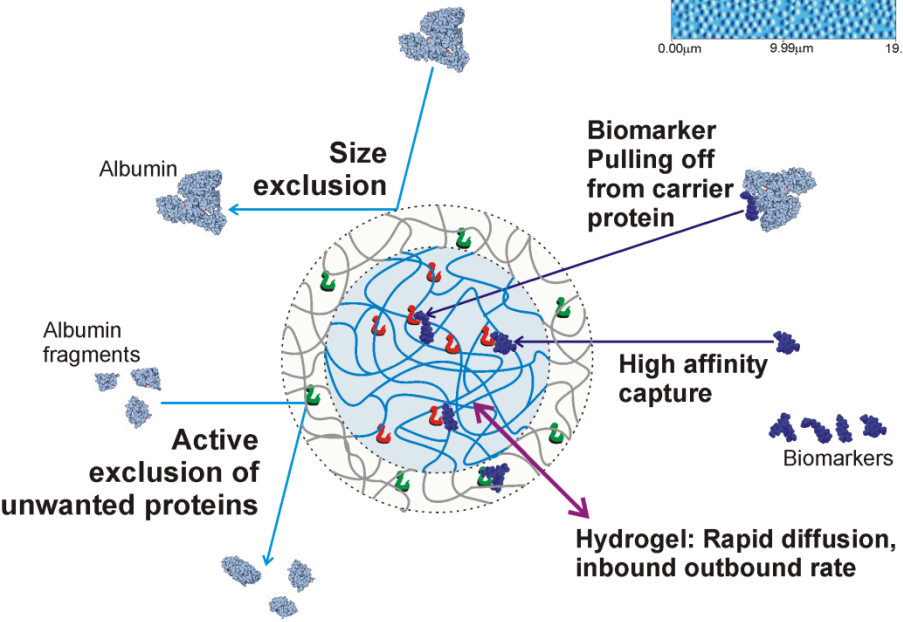
Nanoparticle-Enhanced Antigen Test

- **Nanoparticle technology can overcome low abundance and interfering substance drawbacks of previous antigen tests**
  1. Harvest and massively concentrate antigens while protecting the antigens against degradation
  2. Excluded unwanted contaminants

Nanoparticle structure







PARTICLE ATTRIBUTES

- Particles can be produced in large quantities (gram quantities)
- Stored lyophilized and easily reconstituted before use
- Stable at room temperature indefinitely
- Low cost
- Uniform in size (~0.7 micron)
- High reproducibility among batches

Smart Hydrogel Particles: Biomarker Harvesting: One-Step Affinity Purification, Size Exclusion, and Protection against Degradation

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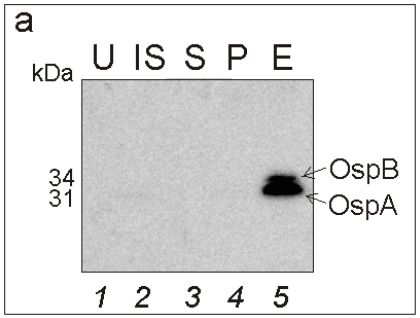
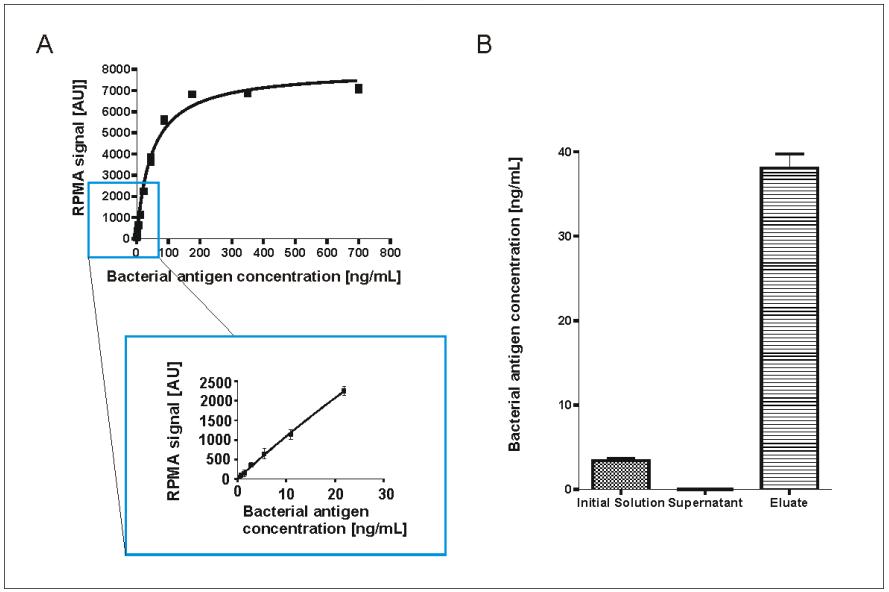
Nanoparticle concentration of *Bb* protein

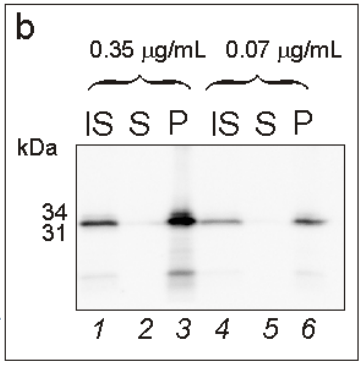
- *B. burgdorferi* protein spiked in human urine: the test does not cross react with human proteins present in urine

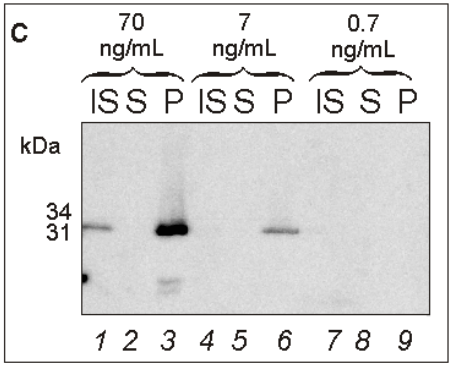


- Reverse phase protein array analysis: sensitivity <90 pg/mL



- B. burgdorferi proteins spiked in synthetic urine: sensitivity of western blot analysis: 700 pg





(Biomaterials, 2010. **32**(4): p. 1157-1166)

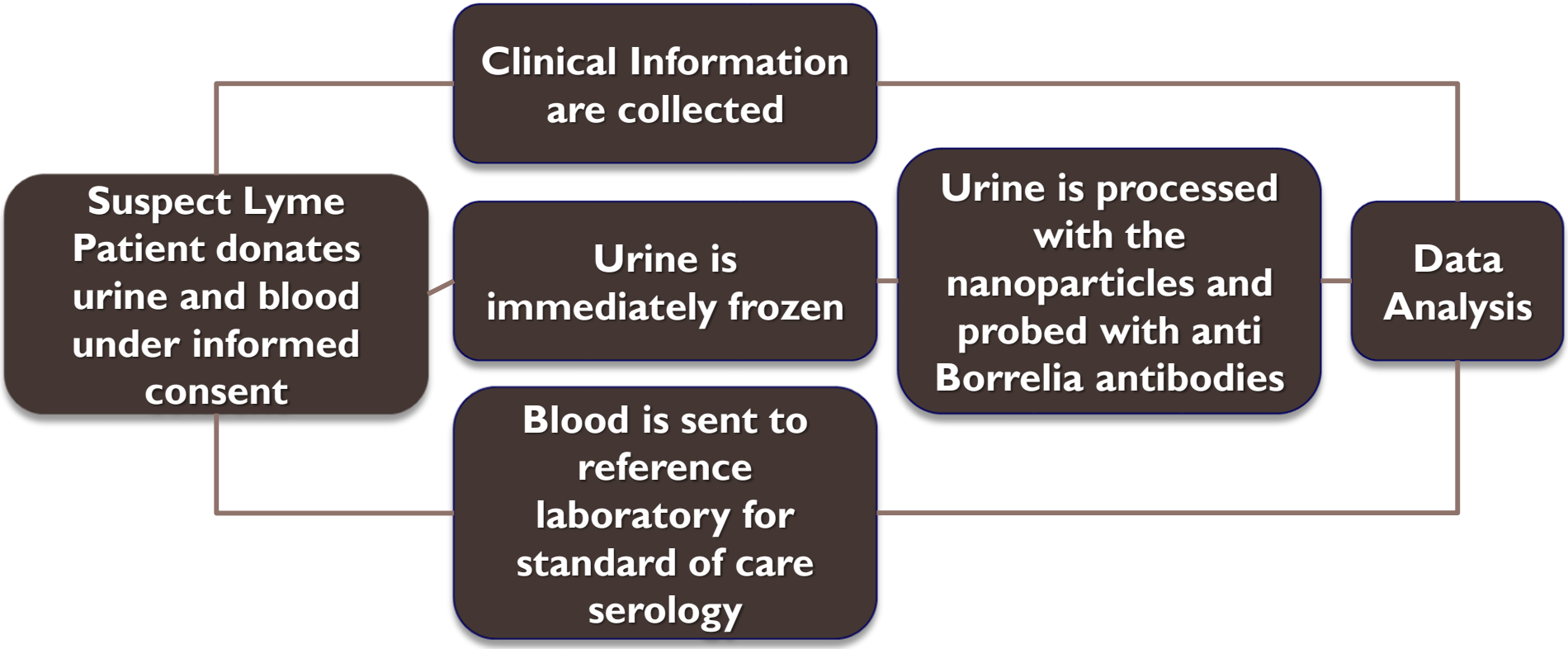
Nanotrap Lyme Urinary Assay Trial

- Bait-containing hydrogel **nanoparticles**, combined with standard immunoassays, have been shown to attain **sufficient analytic sensitivity to directly detect *B. burgdorferi* antigens in human urine** (Biomaterials, 2010. **32**(4): p. 1157-1166)
- **Eligibility**: all comers, patients undergoing standard of care diagnosis and therapy for all stages of Lyme disease, or patients suspected of contracting Lyme disease
  - Frekko Primary Care, MD 78
  - Internal Medicine of N.VA, Dr Shor, VA 112
  - CareID Dr Poretz, VA 27

Lyme Urinary Antigen Test: Research Goals of the Trial

1. Harvest and measure *Bb* antigen level in urine at all stage of disease including early stage pre treatment, post treatment, recurrent disease and successfully treated disease
2. Correlate the urinary antigen level and type with Lyme serology of the same patient
3. Determine how early antigens are shed into the urine following onset of infection
4. Determine which antigens are lost following successful treatment, and which antigens persist following treatment failure
5. Specifically, is OspA associated with early stage disease or does it become elevated during persistent or recurrent disease?

Clinical Trial Design

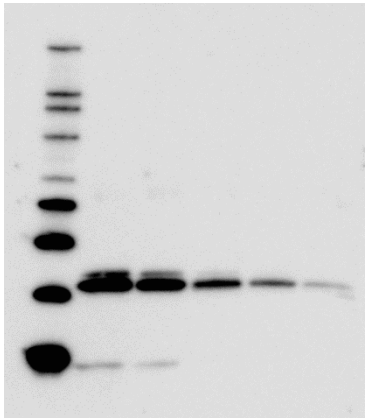


Clinical Trial Accrual to Date

- ***151*** patient urine samples have been collected, transferred to GMU, and are being analyzed to date by GMU.
- **52** of 151 are positive by serology (LabCorp or Quest). These include patients that underwent up to three courses of treatment over a period of time from 6 months up to two years, 17 had persistent symptoms in the face of treatment
  - **8** patients presented with a recent tick bite and EM rash and donated urine prior to treatment and became serology positive (**TRUE POSITIVE**)
  - **82** of the patients with a negative serology were listed as suspect of having Lyme because of persistent symptoms
  - **17** patients were not suspected of having Lyme and they have a negative serology and are untreated

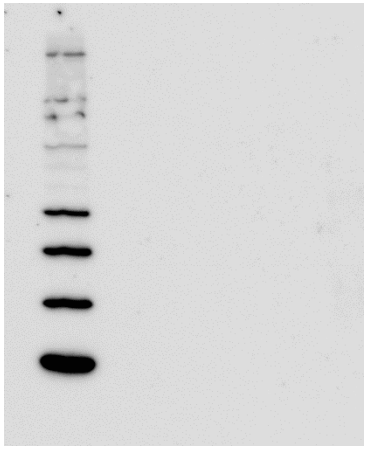
Qualification of reagents

- **Antibody and antigen selection and specificity validation was conducted in our CAP/CLIA certified laboratory** (7223012) **following CLSI guidelines**
- Specificity of primary and secondary antibody was determined by dose response of nanoparticle captured Borrelia antigen spiked in human urine followed by competition with cultured Borrelia antigens
- Secondary antibody sources were screened against normal serum and was adsorbed with human serum to achieve completely negative background staining in human urine processed by nanoparticles



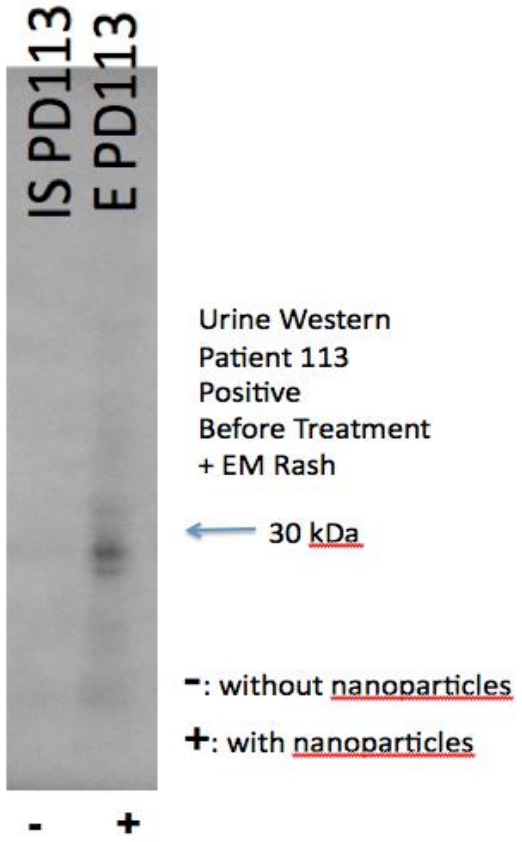
Qualification of reagents

- True negatives were verified as being negative by western blot in patients with no history of tick bite, no symptoms, no evidence of bands in western blot. False urinary positives were not detected in normal asymptomatic healthy individuals.
- The monoclonal antibody antigen epitope was definitively sequenced and shown to be specific for all the major strains of *B. burgdorferi*



Preliminary Results – Nanoparticles are necessary to detect Bb antigens in the urine

- ***B. burgdorferi*** **antigen can be detected in the urine of patients with clinical diagnosis of Lyme disease prior to treatment (early stage disease)**
- **The antigen can only be detected if the urine is processed with the nanotrap nanoparticles**
- **Approximate antigen concentration is 1-10 pg/mL in true positive cases**



True positives: very early stage disease prior to treatment

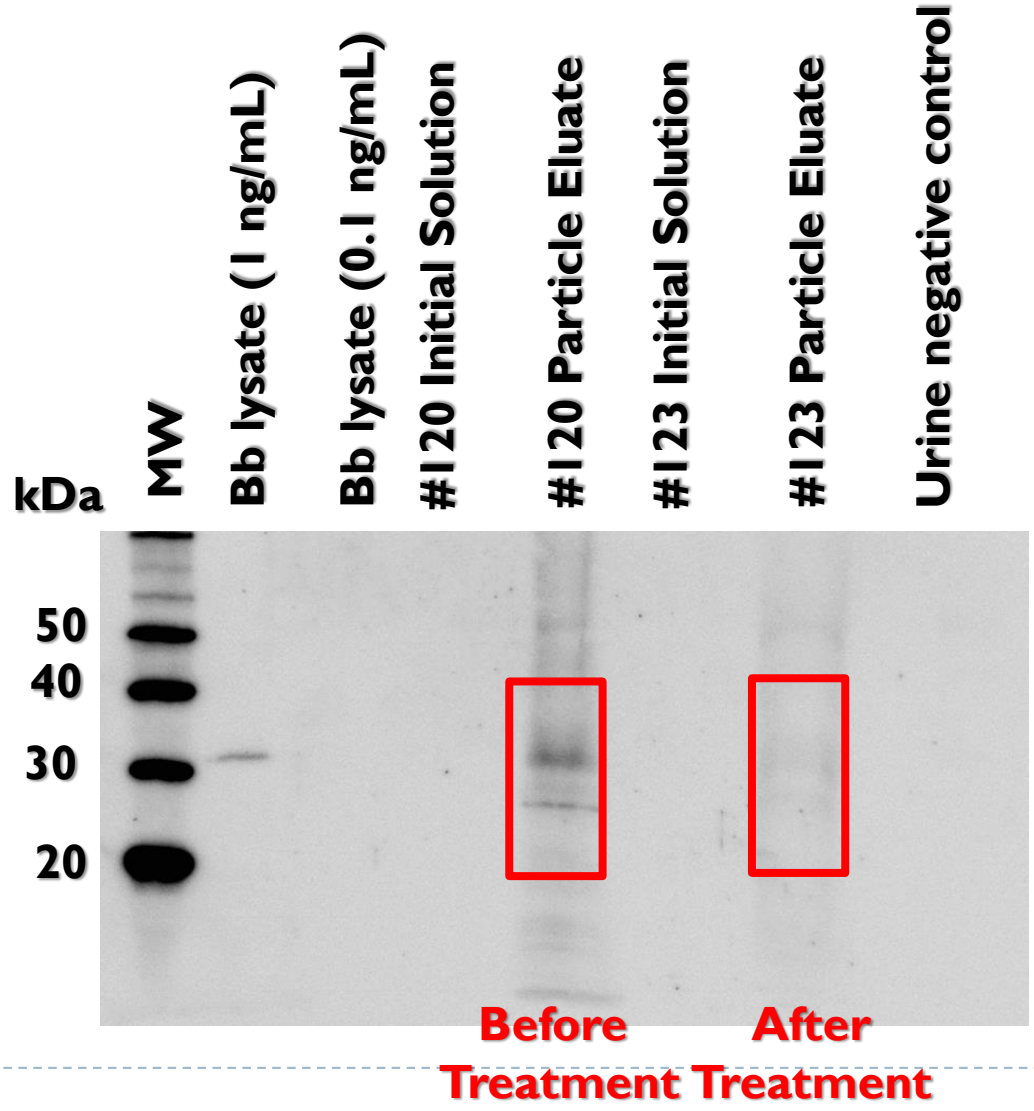
- In all 8 true positive patients enrolled in the trial so far we could identify a band at ~30 kDa with OspA

Patient ID Number	Presence of Urinary Lyme Antigen at ~30 kDa	Serology	EM Rash	Treatment
23	√	IgM+/IgG+	Y	N
25	√	IgM+/IgG-	Y	N
108	√	IgM+/IgG-	Y	N
113	√	IgM+/IgG-	Y	N
118	√	IgM+/IgG+	Y	N
120	√	IgM+/IgG-	Y	N
139	√	IgM+/IgG+	Y	N
140	√	IgM+/IgG+	Y	N

*True positive: positive serology, history of recent tick bite and Erythema Migrans rash

Bb proteins are not detectable in urine of successfully treated patients

- Patient #120: EM rash, ELISA and IgM positive, before treatment, *specific gravity = 1.005*
- Patient#123: same patient, asymptomatic, **after treatment**, *specific gravity = 1.005*



Examples of Correlation of Nanoparticle Urinary Antigen Test with Clinical Data

Patient ID#	Nanoparticle Urinary Antigen Test	Clinical Symptoms	IgM WB	IgG WB	Treated	Notes
4	-	-	-	-	-	
5	-	-	-	-	-	TRUE NEGATIVES
138	-	-	-	-	-	
117	-	-	-	-	-	
13	+	+	+	-	+	
14	+	+	*ND*	+	+	
15	+	+	+	-	+	SYMPTOMATIC, POSITIVE SEROLOGY
24	+	+	+	-	+	
119	+	+	+	-	+	
118	+	+	+	+	-	
23	+	+	+	+	-	
120	+	+	+	-	-	
123	-	-	+	-	+	Reverts to negative after treatment
105	+	+	-	-	+	SYMPTOMATIC, NEGATIVE SEROLOGY
116	+	+	-	-	-	

Clinical Trial Results, Pilot Data

- Sensitivity of antigen detection in the urine achieved is 1 picogram per milliliter
- For all patients tested to date with early stage symptomatic disease who became serology positive, OspA antigen was detected in the urine
- For all patients with persistent symptomatic disease but uncertain serology, antigen was detected and verified by competition (21/21)
- Patients who were asymptomatic following treatment were noted to revert from positive to negative
- Examples of true negatives (no symptoms and no history of tick bite and negative serology) were negative but the true false negative and false positive rate is unknown and awaits proper evaluation of interfering substances and concurrent non Borrelia infections

Clinical Trial Results, Pilot Data

- *Bb* antigen OspA is present in the urine of patients at early stage disease
- For patients with later stage disease, the presence of antigen in the urine correlates more closely with the presence of symptoms than it does with serology or number of treatments
- Hypothesis: *Bb* antigen is shed in the urine only in presence of active infection producing symptoms

Current Completion and Future Ongoing Goals

- **Milestone 1: complete clinical trial by March 2014**
- **Milestone 2: open commercial testing under full CAP CLIA compliance in Summer of 2014**
- **2015 goals:**
  - **Extend panel of measured analytes to other *Bb* antigens (e.g., OspC, VlsE, DbpA, DbpB, Erp proteins)**
  - **Extend panel of measured analytes to Lyme Borrelia complex antigens (Babesia, Bartonella, etc.) *(Journal of Chronic Fatigue Syndrome, 2003, 11: 51-68)***
  - **Extend panel to host immune response factors indicative of active disease**

Acknowledgments

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