Kenneth B. Liegner, M.D., P.C.
& Associates
Internal & Critical Care Medicine
Lyme Borreliosis & Related Disorders
592 Route 22 – Suite 1B
Pawling, New York 12564
Ph: 845 493-0274
FAX: 845 493-0279
October 30, 2021

A Proposal for a National Spirochetal Reference Laboratory (NSRL)

In the late 1980s, I was introduced to scientists from the Centers For Disease Control & Prevention (CDC) by Durland Fish, Ph.D. Durland, Director of Medical Entomology at New York Medical College at the time, had his office about ¼ mile away from my home office at 8 Barnard Road, in Armonk.

Durland knew that I was evaluating and treating persons who either did have or might have Lyme disease in the early days of the burgeoning epidemic of Lyme disease in Westchester County, New York.

Durland and I and the group from CDC, went out to dine together in a near-by Japanese restaurant. It was a convivial evening and CDC folks expressed an interest in receiving specimens from my patients. Specimens of blood, urine and, in some cases, cerebrospinal fluid, were forwarded to CDC, Fort Collins, Colorado, along with abstracted clinical data. Several score specimen sets were sent between 1989 and 1991.

Many of these specimens were forwarded by CDC to scientists Claude Garon and David Dorward at the National Institute of Allergy and Infectious Disease's (NIAID) Rocky Mountain Laboratory (RML) in Hamilton, Montana.

Garon, Dorward and Tom Schwan (also at RML) were in the process of developing a novel assay for Lyme disease based on the detection of Outer surface proteins shed as membranous blebs from the surface of Lyme spirochetes as an intrinsic part of the basic biology of the organism.[1] Their approach used an immune-gold labeling technique and required electron microscopy which was both resource and labor intensive.

The RML group published their work June of 1991 in the Journal of Clinical Microbiology entitled: "Immune Capture and Detection of *Borrelia burgdorferi* Antigens in Urine, Blood or Tissues of Infected Ticks, Mice, Dogs, and Humans".[2]

JOURNAL OF CLINICAL MICROBIOLOGY, June 1991, p. 1162–1170 Vol. 29, No. 6
0095-1137/91/061162-09$02.00/0

Immune Capture and Detection of *Borrelia burgdorferi* Antigens in Urine, Blood, or Tissues from Infected Ticks, Mice, Dogs, and Humans

DAVID W. DORWARD,* TOM G. SCHWAN, AND CLAUDE F. GARON

Laboratory of Vectors and Pathogens, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

Received 14 December 1990/Accepted 5 March 1991

Current biological and serological techniques for demonstrating infections by *Borrelia burgdorferi* can be inconclusive. In order to monitor Lyme borreliosis, we developed a rapid and sensitive assay for *B. burgdorferi* antigens in infected hosts. Polyclonal rabbit antisera were raised against membrane vesicles and an 83-kDa vesicle-associated protein band that was purified from in vitro *B. burgdorferi* cultures. Immunoglobulin G (IgG) antibodies were recovered from these sera and tested for a species-specific reaction with several geographically diverse *Borrelia* isolates by immunoblot analysis. Parlodion-coated electron microscope grids were activated with anti-vesicle $F(ab')_2$ fragments and then incubated with confirmed or experimental sources of spirochetal antigens. Such sources included cultured spirochetes; spirochete culture supernatants; samples of urine, blood, or serum from mice, dogs, and humans; triturates of *Ixodes* ticks; and bladder, spleen, liver, kidney, heart, or brain tissues from infected or control mice. Captured antigens were assayed by immune electron microscopy by using anti-83-kDa IgG antibodies and protein A-colloidal gold conjugates. The results indicated that *B. burgdorferi* appears to shed surface antigens which are readily detectable in urine, blood, and several organs from infected hosts. Such antigens were detectable in mouse urine at dilutions exceeding 10^{-6}. Intact spirochetes were frequently observed on grids incubated with blood, spleen, or bladder preparations, and *B. burgdorferi* was reisolated from the urinary bladders of all experimentally infected mice. These results indicated that *B. burgdorferi* antigens arise in a variety of host materials. Such antigens can be captured and identified with specific polyclonal antibodies, providing a sensitive assay for monitoring and studying Lyme borreliosis.

The RML group evaluated specimens of urine and serum from 51 of my patients, who had all been well-characterized. In correspondence from Dr. Garon, he had indicated that he hoped to collect additional specimens from patients of other Lyme-treating clinicians including Joseph Burrasano, Jr, Paul Lavoie, Dorothy Pietrucha and John Drulle. I do not know whether specimens from those clinicians' patients were ever studied using RMLs methods.

Garon provided for me a print out summarizing the results of testing using their direct detection antigen-capture method in the urine of my patients and antibody results in blood on three different Lyme antibody assays developed at CDC, Fort Collins.

Comparison of RML Urine Test vs.CDC ELISA-Son.& Fla.

ELISA Son. & Fla. data= Ex. 12/11/90
P=Positive & N= Negative

RML +'s= 51
ELISA T.Q.+'s=26
ELISA Son.+'s=23
ELISA Fla.+'s= 8

SUMMARY

#	RML	ELISA-T.Q.	ELISA-Son.	ELISA-Fla.
5	P	P	P	P
5	P	P	P	N
3	P	P	N	N
37	P	N	N	N
1	P	N	P	P
21	N	N	N	N
9	N	P	P	N
1	N	N	P	N
2	N	P	N	N
2	N	P	P	P

The striking finding was that 37 out of the 51 patients showed positive RML antigen-capture assays in urine while corresponding bloods were dead negative by antibody testing.

The patients whose specimens had been tested demonstrated a impressive array of clinical presentations and many of them had been previously intensively treated with antibiotics. One of them, who gave a history of a rash historically compatible with erythema migrans evolved pulmonary hypertension and subsequently died in a Cardiac Care Unit. Despite the history of probable erythema migrans rash, standard tests for Lyme disease had been negative in her case while under my care. We reported our experience as an Abstract at the Fifth International Conference on Lyme Borreliosis in Arlington, Virginia in 1992.[3]

This experience was one of the early indicators to me that standard methods of testing for Lyme disease might be missing the diagnosis in many patients, including those who were seriously ill.

The National Institutes of Health (NIH) licensed the technology to Richard Tilton, Ph.D. who was Director of North American Laboratory Group and later BBI Clinical Laboratories. He had the license for a year or two while he tried to adapt the technology a high through-put method that would be suitable for commercial use. Despite a great deal of effort and expense (he confided in me that he had spent on the order of one million dollars in this effort) he was unable to successfully adapt the test method and the license reverted back to the NIH where, as far as I know, it was never again utilized.

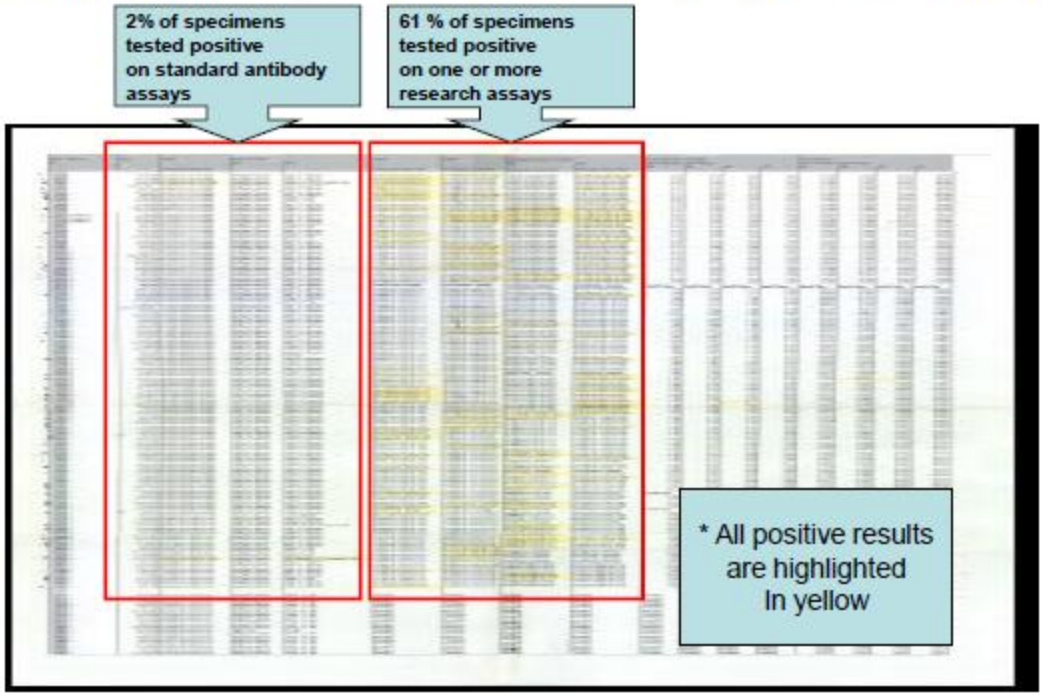
#######

In the early 2000s, due to fortuitous circumstances, I had the opportunity to send about 140 frozen cerebrospinal fluid samples on my patients to Pat Coyle's research lab at the State University of New York at Stony Brook. That lab had been involved in a CDC-funded research study measuring shed borrelia Outer surface protein (Osp) antigens OspA and OspC and borrelia-specific IgG and IgM immune complexes in cerebrospinal fluid of patients with suspected central nervous system Lyme disease.[4,5] These were compared to standard antibody assays in the same spinal fluid run at the same time.

The laboratory supervisor provided me with a spread-sheet summarizing all of the results obtained on my patients.

On the spread-sheet, she highlighted all positive results in yellow. This demonstrated that some 62% of specimens tested positive on one or more of the four research assays (e.g. OspA or OspC antigen detection or IgG or IgM borrelia-specific immune complexes) in CSF whereas only some 2% of the same specimens tested positive using standard antibody assays for Lyme disease in the spinal fluid.

Print-out of Results of Research Assays on CSF: OspA & OspC Antigen Capture, IgG & IgM Borrelia-specific Immune Complexes versus Standard CSF Antibody Assays



I am unaware of any publications on this work that was done at Stony Brook on their own specimens having ever been published and it seems as though the project came to an abrupt end for reasons that were never explained.

If the findings on testing on Stony Brook's own spinal fluid repository of specimens was similar to the findings on my patients' spinal fluids, it suggests that the incidence of nervous system Lyme disease may be, arguably, as much as 30 times higher than disclosed by standard antibody methods.

Having had access to these two 'cutting-edge' direct detection methodologies both at the Rocky Mountain Laboratory and at SUNY Stony Brook's research laboratory and caring for many patients who clinically seemed to have Lyme disease but whose standard tests were either negative or inconclusive, well, I found it frustrating to know that advanced methods had already been devised that might enable more certain diagnosis but that these were not available to practicing clinicians or their patients.

######

Fast forward to 2015 when specimens from three patients with early Lyme disease with erythema migrans were studied using methods developed at the National Institute of Standards and Technology (NIST) using Multiple Reaction Monitoring (MRM) Liquid Chromatography & Triple Quadripole Tandem Mass Spectrometry (LC-MS/MS) to detect membranous outer surface protein A.[6,7]

Published in final edited form as:
Anal Chem. 2015 November 17; 87(22): 11383–11388. doi:10.1021/acs.analchem.5b02803.

Quantification of *Borrelia burgdorferi* membrane proteins in human serum is a new concept for detection of bacterial infection

Crystal S. F. Cheung[‡,#], **Kyle W. Anderson**[‡,#], **Kenia Y. Villatoro Benitez**[#], **Mark J. Soloski**[¶], **John N. Aucott**[¶], **Karen W. Phinney**[‡], and **Illarion V. Turko**[‡,#,*]

‡

#

¶

Abstract

The *B. burgdorferi* spirochete is the causative agent of Lyme disease, the most common tick-borne disease in the United States. The low abundance of bacterial proteins in human serum during infection imposes a challenge for early proteomic detection of Lyme disease. To address this challenge, we propose to detect membrane proteins released from bacteria due to disruption of their plasma membrane triggered by the innate immune system. These membrane proteins can be separated from the bulk of serum proteins by high-speed centrifugation causing substantial sample enrichment prior to targeted protein quantification using multiple reaction monitoring mass spectrometry. This new approach was first applied to detection of *B. burgdorferi* membrane proteins supplemented in human serum. Our results indicated that detection of *B. burgdorferi* membrane proteins, which are $\approx 10^7$ lower in abundance than major serum proteins, is feasible. Therefore quantitative analysis was also carried out for serum samples from three patients with acute Lyme disease. We were able to demonstrate the detection of ospA, the major *B. burgdorferi* lipoprotein at the level of 4.0 fmol of ospA/mg of serum protein. The results confirm the concept and suggest that the proposed approach can be expanded to detect other bacterial infections in humans, particularly where existing diagnostics are unreliable.

The method was able to detect OspA, the major *B. burgdorferi* membranous lipoprotein to a level as low as 4 femtomoles/mg of serum protein. Furthermore, the authors indicated that by choosing suitable surface membrane liporoteins, other strains of borreliae could be detected and differentiated.

It is plausible that this technology could be adapted to other spirochetal infections including varying strains of relapsing fever borreliosis,[8] syphilis[9] and possibly the endemic treponematoses[10] for which improved diagnostics are sorely needed.

The cost of the equipment needed to carry out the methods disclosed in the NIST publication may exceed the budgets of privately owned commercial laboratories, but already exists at the National Institute for Standards and Technology, where scientists pioneered this 'proof of concept'.

A National Spirochetal Reference Laboratory (NSRL) is feasible. The technology already exists. All that is lacking is the will to bring it to fruition. Suitable membrane target lipoproteins would need to be selected for differing spirochetal infections and the performance characteristics of the NIST method defined with well-characterized patients in differing stages of illness. Clearly, there is more work to be done.

Patient advocacy through the legislative process can transform this long-delayed 'dream' of accurate diagnosis by means of high throughput semi-quantitative direct antigen detection in to a reality.

Can we have a practical measure of 'borrelial load' analogous to measures of 'viral load which has informed and transformed H.I.V. care?

It is within our power to shape our future.

Very truly yours,

Kenneth B. Liegner, M.D.

Member, Treatment Panel, N.I.H. State-of-the-Art Conference on Lyme Disease, March 1991, Bethesda, MD.

Co-Chair, Treatment Poster Discussion Section, Fifth International Conference on Lyme Borreliosis, May/June 1992, Arlington, VA.

Participant, N.I.A.I.D. Consultations on Chronic Lyme Disease, February & October, 1994, Rockville, MD.

Member, Program Committee, 7th International Conference on Lyme Borreliosis, San Francisco, CA., Spring 1996.

Presenter to Infectious Diseases Society of America Lyme Disease Review Panel, July 30, 2009, Washington D.C.

References:

1 Garon CF, Dorward DW, Corwin MD. Structural features of Borrelia burgdorferi--the Lyme disease spirochete: silver staining for nucleic acids. Scanning Microsc Suppl. 1989;3:109-15. PMID: 2482525.

2 Dorward DW, Schwan TG, Garon CF. Immune capture and detection of Borrelia burgdorferi antigens in urine, blood, or tissues from infected ticks, mice, dogs, and humans. J Clin Microbiol. 1991 Jun;29(6):1162-70. doi: 10.1128/jcm.29.6.1162-1170.1991. PMID: 1864935; PMCID: PMC269963.

3 Liegner KB, Garon G, Dorward D. Lyme Borreliosis (LB) Studied with the Rocky Mountain Laboratory (RML) Antigen Capture Assay in Urine. In: Program and abstracts of the Fifth International Conference on Lyme Borreliosis, Arlington, Va., May 30-June 2, 1992. Bethesda, Md.:Federation of American Societies for Experimental Biology, 1992:A18
(Note:The Abstract is not easily accessible, so it is provided, below)

104

LYME BORRELIOSIS (LB) STUDIED WITH THE ROCKY MOUNTAIN LABORATORY (RML) ANTIGEN CAPTURE ASSAY IN URINE. Kenneth B. Liegner, MD*, Armonk, NY, USA and Claude Garon, PhD and David Dorward, PhD, NIAID RML, Hamilton, MT, USA. METHOD: 70 subjects living in a Lyme epidemic area and having known or suspected LB were studied between 1988 and 1992. Sera and urines were shipped to the CDC, Fort Collins, CO, where sera were tested by an ELISA method. The CDC shipped aliquots of urine from these patients to the NIAID RML for testing with a newly developed assay designed to detect B. burgdorferi (Bb)-specific antigen. RESULTS: Antigenuria was present in the urine of 42 patients whereas standard ELISA techniques by CDC and other reference laboratories yielded positive results in 31 patients. Combining ELISA and RML results, 57 patients tested positive by one or the other method. Comparing RML to ELISA there was concordance of negative results in 12 and of positive results in 18. Antigenuria was present in 21 cases in which ELISAs were negative and absent in 15 cases in which ELISAs were positive. Western blots were performed in 32 cases and 14 were either diagnostic or suspicious; 10 of these showed antigenuria, half from ELISA negative patients. PATIENT CHARACTERISTICS: Of the 42 antigenuria positive patients, many had received lengthy and repeated courses of oral and/or parenteral antibiotics prior to testing. They had an extraordinarily diverse range of symptom complexes including classic LB presentations but also one case neurologically indistinguishable from classic relapsing/remitting multiple sclerosis, one case of fatal "primary" pulmonary hypertension developing 3 years after occurrence of an untreated rash compatible by history with erythema migrans, one case of hives, pyrexia, and polyarthralgias, a case resembling primary anti-cardiolipin syndrome with ophthalmoplegia, seizures, white matter UBOs on MRI, and high titre ACLA, and two cases with PMR-like presentations. Two women had stillbirths. One previously treated seropositive, antigenuria negative patient showing no evidence of active infection at the time of testing, had developed global cerebellar atrophy. In many subjects symptoms resolved wtih antibiotics, reoccurred with their discontinuence, and remitted with reinstitution of treatment, suggesting chronic persisting infection. Chronic fatigue, cognitive dysfunction, and memory impairment were common as well as mood swings, anxiety, depression, irritability, and sleep disturbance in individuals without such symptomatology prior to their LB. Most patients responded favorably to antibiotics but treatment often had to be prolonged to be successful. Cure seemed unattainable with available methods in some patients, but they were kept relatively well and functioning with maintenance antibiotic treatment. CONCLUSIONS: Study results using this direct antigen detection method suggest seronegative LB is not rare and lend support to the hypothesis that Bb can persist in the human host despite intensive antibiotic treatment.

4 Coyle PK, Schutzer SE, Deng Z, Krupp LB, Belman AL, Benach JL, Luft BJ. Detection of Borrelia burgdorferi-specific antigen in antibody-negative cerebrospinal fluid in neurologic Lyme disease. Neurology. 1995 Nov;45(11):2010-5. doi: 10.1212/wnl.45.11.2010. PMID: 7501150.

5 Coyle PK, Schutzer SE, Belman AL, Krupp LB, Golightly MG. Cerebrospinal fluid immune complexes in patients exposed to Borrelia burgdorferi: detection of Borrelia-specific and -nonspecific complexes. Ann Neurol. 1990 Dec;28(6):739-44. doi: 10.1002/ana.410280603. PMID: 2285261.

6 Rebman AW, Yang T, Aucott JN. Symptom heterogeneity and patient subgroup classification among US patients with post-treatment Lyme disease: an observational study. BMJ Open. 2021 Jan 13;11(1):e040399. doi: 10.1136/bmjopen-2020-040399. PMID: 33441353; PMCID: PMC7812114.

7 Cheung CS, Anderson KW, Benitez KY, Soloski MJ, Aucott JN, Phinney KW, Turko IV. Quantification of Borrelia burgdorferi Membrane Proteins in Human Serum: A New Concept for Detection of Bacterial Infection. Anal Chem. 2015 Nov 17;87(22):11383-8. doi: 10.1021/acs.analchem.5b02803. Epub 2015 Nov 4. PMID: 26491962; PMCID: PMC4809138.

8 Tabuchi N, Murakami N, Fukunaga M. [Identification of the immunogenic outer membrane proteins of relapsing fever Borrelia]. Yakugaku Zasshi. 2013;133(12):1417-23. Japanese. doi: 10.1248/yakushi.13-00203. PMID: 24292191.

9 Lewinski MA, Miller JN, Lovett MA, Blanco DR. Correlation of immunity in experimental syphilis with serum-mediated aggregation of Treponema pallidum rare outer membrane proteins. Infect Immun. 1999 Jul;67(7):3631-6. doi: 10.1128/IAI.67.7.3631-3636.1999. PMID: 10377149; PMCID: PMC116554.

10 Mitjà O, Šmajs D, Bassat Q. Advances in the diagnosis of endemic treponematoses: yaws, bejel, and pinta [published correction appears in PLoS Negl Trop Dis. 2013 Nov;7(11). doi:10.1371/annotation/20cc3a69-d7d3-49d2-bea4-1759e95a1e0910.1371/annotation/20cc3a]. *PLoS Negl Trop Dis*. 2013;7(10):e2283. Published 2013 Oct 24. doi:10.1371/journal.pntd.0002283