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Review Article

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Microbial DNA globular liquid crystal like deposits inside Lewy bodies in four Lewy dementia patients

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Abstract

Four autopsy brains demonstrated concurrent evidence of Diffuse Cortical Lewy Body Dementia and active Lyme neuroborreliosis in adjacent brain sites. Lewy bodies In DCLBD contain microbial DNA. Alpha Synuclein proteins, which intrinsically bind to human nuclear DNA also bind to microbial DNa deposits inside Lewy bodies in the cytoplasmic compartment of diseased neurons. This is the first report of an association between spirochetal brain infection and Lewy Body Dementia.

Introduction

Two patients with concurrent Diffuse Cortical Lewy Body dementia (DCLBD) and simultaneous brain borrelia infections were reported in 2016 in poster format on the F1000 Research website [1]. Two additional DCLBD patients were recently added to the present DCLBD study. Three new observations from Dna focused research of four DCLBD patients herein and which have no published precedent are as follows: 1. Borrelia spirochetes reside inside Lewy Brain neurons, 2. Borrelia proteins dwell inside Lewy neurons and inside Lewy Neurites, 3. Extracellular deposits of DNA from dead borrelia spirochetes dwell inside Lewy bodies. Alpha Synuclein (ASN proteins, are intrinsically and physiologically DNA binding proteins [2]. Studies of Parkinson's disease demonstrate that ASN binding to human DNA in the nucleus of neurons interact with nuclear histones, histone free DNA, and modulate the extent of folding of DNA and participate in the repair of fragmented DNA [3]. It is ironic that a focused search for ASN complexed to possible DNA content inside of Lewy bodies, based upon its nuclear DNA binding behavior, has never previously been deployed since Dr. Fritz Lewy's 1912 report.

Materials and Methods

Patient Material: Autopsy brain examination of four patients with clinical dementia profiles suggestive of Lewy Body Dementia based upon the decedent's clinical association of disturbing visual hallucinations and progressive dementia are presented here. Brain donation for this research study by the decedent's next of kin was only possible after the families were declined hospital autopsies and in each case a fee for service autopsy by a private practice pathologist for brain removal in a funeral home was completed. The brains were donated to the Dr. Paul H Duray MD Research Foundation for examination using borrelia specific DNA probes and borrelia specific immunohistochemistry to attempt to detect possible undiagnosed borrelia infections in brain.

DNA Probes: Advanced molecular diagnostics including FISH DNA hybridization with borrelia gene specific DNA probe bbO 0740 for detection of a Borrelia specific gene sequence utilized Fluorescence in situ Hybridization (FISH). The rationale for the choice of borrelia gene bbo0740 was provided by a Yale University primate borrelia infection study in which the bbo 0740 gene was prominently detected by advanced DECAL molecular studies of primate autopsy brain following laboratory induced infection with *borrelia burgdorferi* [4]. FISH method hybridization was accomplished under high stringency conditions with 100% dimethylformamide and heat denaturation of DNA probes layered over 3micron thick sections for 30 minutes at 70 degrees C and subsequent annealing of single strain patient Dna with single strand molecular beacon DNA probe at post-heat denaturation near room temperature. 2. The nucleotide sequence for the borrelia specific DNA probe bbo0740 the Borrelia DNA probe sequence is 5' attcaaagcaaattcgatgacatc 3'. A BLASTn supercomputer validation of this probe identified that 30 strains of *borrelia burgdorferi* group strains returned Dna sequence matches at the 100% identity level with no gaps. Synthesis and molecular validation of the borrelia burgdorferi group DNA probe for gene bbO 0740 was completed at Gene Link Inc, Hawthorne, New York. Validation of expected Probe hybridization with the oligonucleotide target sequence was established by melting curve analysis of the bbo 0740 probe/ hybridization products. Fluorochrome labels were attached to consecutive batches of probe bbo 0740 as follows: green color fluorochrome FITC probe bbo 0740 and red color Cy5 probe bbo 0740 were separately manufactured. Reactivity of DNA probe bbo 0740 was confirmed with FISH hybridization annealing of the probe with borrelia burgdorferi stain B31 American Type Culture Collection reagent 35210 smeared onto glass microscope slides. A Nonsense DNA Probe serves as a negative control reagent for FISH studies. This was designed by Ali Javed PhD, Director of Gene Link Laboratories, Hawthorne New York.

GL Nonsense GL2 probe: 5' acaccgcttctccgaacgtgtcacgcgcggtgt 3'; Chromogen label Marina Blue 200 was attached for fluorescence detection of the hybridized probe in tissue sections. A BLASTn supercomputer validation of this probe disclosed no matches in the GenBank accessions.--Nonreactivity of the nonsense DNA probe in FISH with Smears of B31 *borrelia burgdorferi* was confirmed.

DNA intercalating fluorochrome stains Ethidium Bromide, Oligreen, DAPI, and Acridine orange were layered over unstained glass slides according to the manufactures' recommendations. Fluorescence microscopy with monochromatic light illuminations specific for each stain was completed to attempt to identify DNA deposits in cytoplasmic sites in Lewy neurons. Comparison of fluorescent DNA signals in native cell nucleus DNA with fluorescence in cytoplasmic DNA deposits was evaluated.

Protein detection studies for borrelia infection with immuno histochemistry were deployed to evaluate the presence of *borrelia burgdorferi* infections in four of four Lewy Body dementia autopsy brains. Monoclonal antibody CB 10 which uniquely binds to borrelia Outer surface protein OspA was gifted by Dr. Jorge L. Benach, University Professor and Dean of Research at the School of Medicine, State University of New York, Stony Brook. Validation of Monoclonal antibody CB10 was published in 1982 [4]. A red fluorochrome label was attached to MAB CB 10 (Biotium CF660R catalog 92243).

Protein detection studies for detection of tissue bound Alpha Synuclein proteins in Lewy bodies and in Lewy neurites were completed in Immunohistochemistry with Monoclonal antibody to Alpha Synuclein (Monoclonal purified clone 4F-1 (StressMarq reagent SMC-533S) according to the manufacturer's recommendations. All controls yielded expected positive and negative results.

Reagents: Antibody SMC-33S (murine Monoclonal) to Alpha Synuclein protein clone 4F1) purchased from StressMarq Biosciences, Victoria, BC, Canada was validated for its specificity and reactivity with Alpha Synuclein proteins purified as a separate reagent from Acro Biosystems Inc product ALN H52h8 (Alpha Synuclein (human)) lyophilized protein. Parallel testing with two additional Stress Marq antibodies to Alpha Synuclein proteins were completed (StressMarq SMC530S (Monoclonal antibody clone3C11 and StressMarq SPC800S polyclonal purified antibody). All ASN controls produced expected positive and negative results in validation studies. Antibody CB 10 (murine monoclonal) Benach and Coleman [5]. For detection of protein OSP A of borrelia burgdorferi was gifted to the author by Professor Jorge L. Benach PhD, Dean of Research, School of Medicine, State University of New York at Stony Brook, New York. This reagent was previously validated in the Benach Laboratory. Reactivity of CB10 MAB with strain B31 of *borrelia burgdorferi* (which contains abundant OspA protein in its outer membrane) was microscopically confirmed in the author's laboratory. Immunohistochemistry controls: (IHC) ASN protein from lyophilized powder (Acro Biosystems Inc product ALN H52h8 (Alpha Synuclein (human) lyophilized protein) was solubilized in phosphate buffered saline and smeared on glass slides for ASN control. This was utilized as a control for Immunohistochemistry studies to validate commercially available ASN antisera reagents. Borrelia spirochete controls Smears of pure culture *Borrelia burgdorferi* strain B31 (American Type Culture Collection ATCC 35210) were used as a positive control for monoclonal antibody CB10 which binds exclusively to the OSP A protein contained in Borrelia burgdorferi spirochetes in the outer surface membrane of intact spirochetes. Negative control for IHC was normal saline solution. Fluorescence in situ Hybridization (FISH) reagent molecular beacon DNA probe bbo0740 was custom designed, manufactured and validated at Gene Link Inc., Hawthorne, NY for detection of borrelia burgdorferi gene bbo 0740 in FISH hybridization. Reagent nonsense DNA probe GL2 bbo0740 was custom designed, manufactured and validated at Gene Link Inc., Hawthorne, NY for function as a negative control for Borrelia DNA probe FISH method studies of Lewy Body disease specimens.

FISH Method Controls: Borrelia spirochete controls Smears of pure culture *Borrelia burgdorferi* strain B31 (American Type Culture Collection ATCC 35210).

Results

The Mab-ASN immunohistochemistry results confirm that the microscopic threshold for a pathological diagnosis of Lewy body Dementia was satisfied based on counts of individual ASN positive Lewy body containing neurons . Lewy body numerical counts and Lewy neurite counts exceeded two or more in any 400x microscopic field of view. Figure 1.

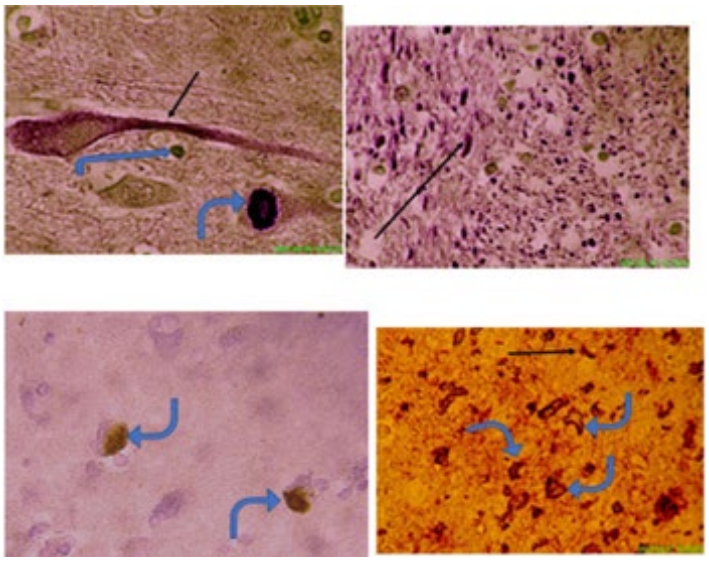

Figure 1: Lewy bodies and Lewy neurites bind alpha synclein protein-patients ABCD. Arrows designate Lewy bodies, and which also appear as threadlike longitudinal profile or dot like cross sectional profiles. Purple color Lewy neurites which are also stained in brown color with Monoclonal antibody Mab-ASN to Alpha Synuclein protein deposits inside the Lewy bodies.

Microscopic Detection of Borrelia Spirochetes in Cerebral Cortex

FISH hybridization studies with Borrelia gene probe bbO 0740 demonstrate intact borrelia fluorescent spirochetes (green fluorescence and yellow fluorescence) representing annealed microbial DNA probes with live chromosomal DNA inside living spirochetes after Hybridization to DNA of gene bbo 0740 genes in chromosomes of *Borrelia burgdorferi* spirochetes was photographed. Many cortical neurons containing borrelia spirochetes also contained a Lewy Body structure adjacent to spirochetes in their cytoplasm. Note: Each borrelia spirochete carries sixteen copies of the linear chromosome where gene bbo0740 is resident at chromosome locus 782795-782773. Total nucleotides in chromosome = 910724 (Figure 2).

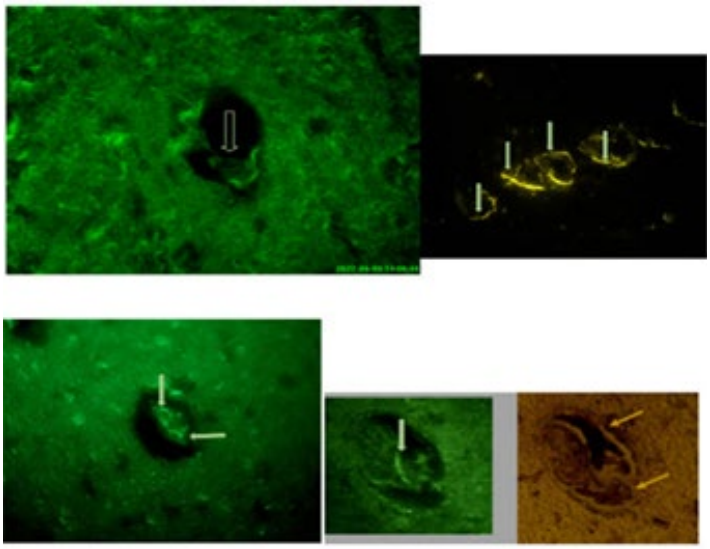

Figure 2: Borrelia spirochetes invade Neurons-patients A,B,C,D. Arrows designate intact live borrelia spirochetes which are stained in FISH method with Fluorochrome FITC (green or yellow fluorescence) which are labeled with DNA probe bbo 0740 which hybridizes uniquely with Borrelia chromosomal DNA.

Microscopic Detection of Borrelia Specific Protein Deposits in Lewy Bodies

Immunostains for borrelia specific Outer Surface protein A (OSP A) with Monoclonal Antibody CB 10, 3. carrying a red chromogen (Biotium CF660R)uniquely bind to Outer Surface protein A (OspA) deposits common to all borrelia burgdorferi group SL spirochetes. Borrelia Protein OSP A deposits (red color) inside of Lewy body structures in cortical neurons and Lewy body neurites also carrying Alpha Synuclein protein deposits are displayed from four patients (Figure 3).

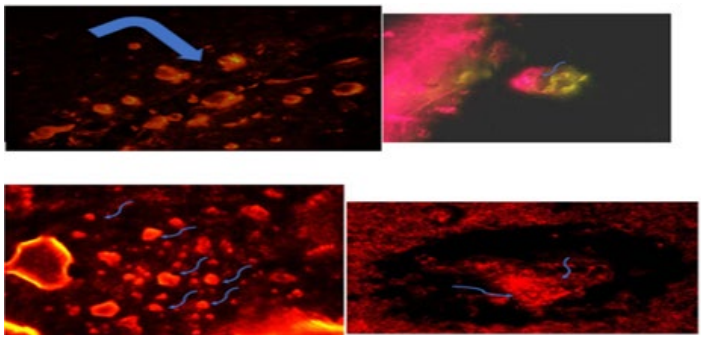

Figure 3: Borrelia protein deposits inside Lewy bodies-patients A,B,C,D. Arrows designate Borrelia specific globular protein OspA deposits which reside inside Lewy bodies (redorange color) (arrows) and which also appear as Lewy neurites in longitudinal (threadlike) or cross sectional (dot-like) signals.

Microscopic Detection of Microbial DNA inside of Lewy Bodies with FISH Method

Lewy bodies contained DNA of *Borrelia burgdorferi* group gene bbo 0740 DNA by FISH hybridization with DNA probe bb0740-Cy5 label-red color (Figure 4).

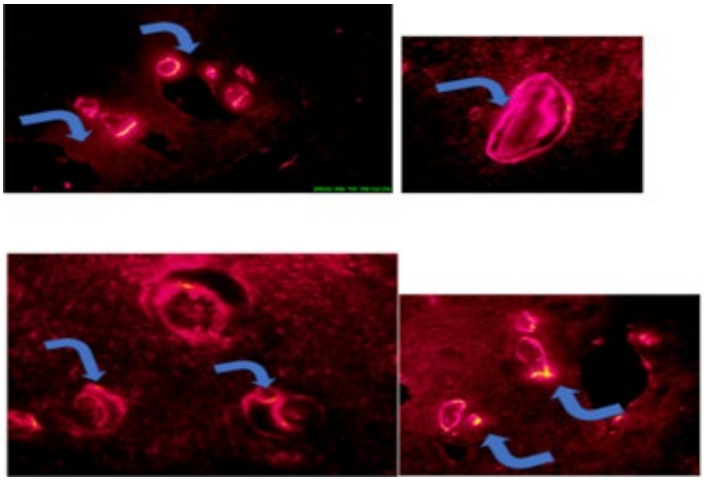

Figure 4: Borrelia DNA deposits inside Lewy bodies-patients A,B,C,D. FISH method DNA probe hybridization uniquely anneals to Borrelia DNA globular silhouette liquid crystal like deposits associated with Lewy neurons. Globular configuration Lewy bodies (red color) stain with red fluorochrome Cy5 attached to DNA probe bbo0740 which uniquely hybridizes with Borrelia Dna deposits inside Lewy Neurons.

Note: Borrelia DNA probe bbo 740 gene DNA Hybridization (FISH method) is only positive inside of Lewy bodies (red color) in the fields of view above. No hybridizations are seen in adjacent cerebral cortex tissues. DNA acquires fluorescence because the single strand DNA probe becomes chemically integrated into denatured double strand DNA target sites as opposed to the mechanism of DNA intercalating stains of non-denatured DNA molecules (below) (Figure 5).

Microscopic Detection of DNA inside of Lewy Bodies with DNA Intercalating Stains (independent of DNA Probe hybridization)

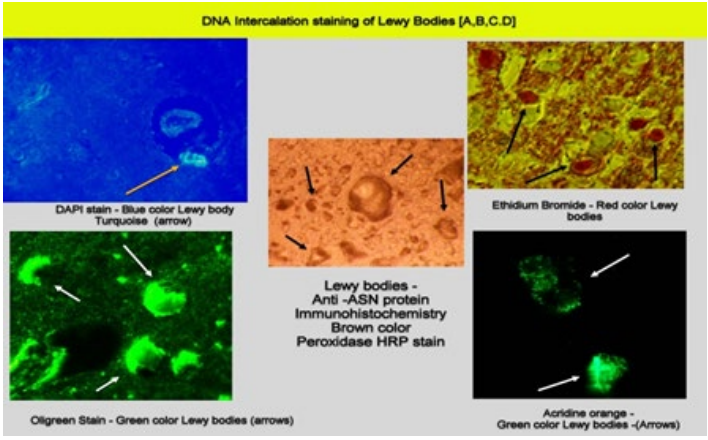

Figure 5: DNA Intercalation staining of Lewy bodies (A,B,C,D). DNA stains with Intercalation reagents DAPI, Ethidium bromide, Oligreen, and Acridine Orange. Non-denatured DNA is present inside Lewy bodies as verified with four intercalation DNA stains. Compare with Figure 4. which demonstrates in FISH method, that after DNA denaturation , the DNA of borrelia microbes hybridizes with the fluorescent label Cy5 red DNA probe bbo 740 (red color signal) in Figure 4.

Lewy bodies were stained separately with four DNA intercalating stains which produced positive results showing a rounded globular fluorescent liquid crystal like silhouette inside cytoplasmic Lewy bodies. Ethidium bromide stains demonstrated high signal Ethidium bromide dye staining in Lewy bodies and staining of DNA inside Lewy Neurites. Oligreen stains demonstrated high signal staining of Lewy Bodies and Lewy neurites. DAPI stains demonstrated high signal DNA staining of Lewy bodies and Lewy neurites. Acridine orange stains demonstrated high signal DNA staining of Lewy bodies and Lewy neurites. The magnitude of fluorescence of DNA content in Lewy bodies with DNAstain intercalators exceeded the intensity of staining of native DNA bound to human chromatin proteins in nuclei in human neurons.

Discussion

FISH probe studies are positive for borrelia spirochetes inside neurons which also contain Lewy bodies in their cytoplasm (Green color DNA probe bbO 0740). This is a first of kind observation. In four patients' autopsies, Fluorescence in Situ DNA Hybridization with a borrelia burgdorferi group SL DNA probe for the gene bbO 0740 demonstrate single intact borrelia spirochetes residing adjacent to or inside of cortical brain neurons which contained Lewy Bodies. Intraneuronal *borrelia burgdorferi* infections are microscopically confirmed with this DNA hybridization evidence.

Globular liquid crystal like DNA deposits from borrelia spirochetal DNA originating from dead borrelia spirochetes resides inside the Lewy bodies as homogeneous globular deposits inside Lewy bodies in four Cortical Lewy Body dementia patients. (Red color DNA signals with probe bbo 0740). Extracellular globular Borrelia specific DNA deposits occupy entire Lewy bodies in four patients. This evidence is separate proof of concept that Lewy bodies contain infectious microbial DNA.

DNA intercalating stains (Ethidium Bromide, Oligreen, DAPI, Acridine orange) confirm that Lewy bodies in four patients contain globular deposits of DNA which match the profiles of Borrelia specific DNA deposits in FISH hybridizations above.

Borrelia burgdorferi Protein OSP A dwells inside of Lewy bodies in Cortical Lewy Body Dementia.

Immunohistochemistry with a *borrelia burgdorferi* Monoclonal Antibody to Outer Surface Protein CB10 (OSPA) demonstrates that borrelia specific protein OSP A is present inside Lewy bodies in four LBD autopsy brains. The protein Immunohistochemistry positive results for positive decoration of Lewy bodies separately and independently confirm intracytoplasmic borrelia infections in Lewy neurons.

Neurotropism of Borrelia spirochetes explains the ability of borrelia to invade individual neurons.

Data from Livengood and Gillmore [6] documents the neurotropisms and neuroinvasive activity of live borrelia burgdorferi spirochetes when in contact with human cortical brain neurons in tissue culture.

Pure cultures of *borrelia burgdorferi* which were co-cultured in the laboratory with live human cortical brain neurons demonstrate cytoplasmic invasion in less than 24 hours of co-incubation borrelia spirochetes which invade the cytoplasm of live human neurons and glial cells. Borrelia protein A (OspA) is found in the outer surface membrane of the spirochete. In the life of the borrelia spirochete, small units of borrelia OSP A are continually released into its external milieu as minute liposome units or as large bleb units [7]. The release of OSP A protein from living borrelia resident inside the diseased neurons in these patients explains an inside of the human neuron source of a unique borrelia specific protein in the cytoplasmic compartment of Lewy Body Containing neurons. An intracellular borrelia infection provides ongoing release of OSPA proteins inside the cytoplasm space from self-renewing borrelia outer surface membrane sites of OSPA protein. OSP A borrelia proteins are released from the surface of once living but now dead borrelia spirochetes dwelling inside the cortical neurons which contain Lewy bodies. Live borrelia spirochetes in vitro and in vivo spontaneously release of OSP A proteins from their "slime layer" of surface membranes of the microbe into the milieu. OspA protein release does not compromise the viability of the borrelia. Over time, the mass of the released OspA protein may exceed the dimensions of the individual live spirochete because the outer membrane tissue site of protein release is continually replenished Co-Localization of Alpha Synuclein proteins inside Lewy bodies with non-human DNA intraneuronal deposits inside Lewy bodies

is a new paradigm, Alpha synuclein is a DNA binding Protein. It is not mere coincidence that Lewy bodies, which universally contain Alpha Synuclein excess protein deposits, demonstrate co-localization of extracellular DNA deposits inside Lewy bodies and inside Lewy neurites. The cytoplasmic compartment of Lewy neurons contains DNA deposits defined here. Borrelia specific DNA probe FISH hybridizations visualize non-human DNA from dead borrelia spirochetes and DNA chromosomal labelling in intact live spirochetes.

The marriage of Alpha Synuclein binding to DNA, establishes ASN as an example of a DNA binding protein. Therefore, the microscopic evidence herein of an overlap (co-localization) between zones of Alpha Synuclein reactivity in Lewy bodies in DCLBD lesions and *borrelia burgdorferi* DNA deposits inside those Lewy bodies is not without precedent from other research documenting ASN as a DNA binding protein with Human DNA. Results herein are a potential challenge to the present dogma that ASN is necessarily and intrinsically toxic to human neurons. A possible scenario is offered here is that intra-cellular and/intraneuronal infection by borrelia burgdorferi inside Lewy bodies might support Cortical Lewy body Dementia as an infection-initiated neurodegeneration illness.

Conclusion

Lewy bodies and Lewy Neurites contain previously unrecognized deposits of non-human Borrelia DNA in four patients. Alpha synuclein protein deposits show a microscopically circumscribed profile inside the Lewy bodies which overlies the domains of DNA deposits in the Lewy bodies. ASN is complexed to extracellular Borrelia gene DNA which also shows a microscopical "Lewy body profile" inside the diseased neuron. The variable size of the Lewy bodies as defined microscopically by immunostains to proteins of ASN mirrors the size and topographies of globular liquid crystal like deposits of DNA deposits confirmed by FISH DNA hybridizations of dead and dying cortical brain neurons. Borrelia spirochetes visualized inside the neurons in four Lewy body Dementia patients are capable of penetrating living neurons in a manner which mirrors the neuroadherence and neuroinvasion of neurons by borrelia burgdorferi spirochetes which are co-cultured with human cortical neuronal cells in tissue culture systems. This unique discovery of the existence of intraneuronal cytoplasmic extracellular Borrelia DNA contained inside of Lewy Bodies in four DCLBD patients might be used as a conceptual model for the study of all categories of Lewy bodies including Parkinson's disease Lewy bodies to interrogate for possibility that additional infectious agents might leave behind discrete zones of extranuclear cytoplasmic DNA deposits inside injured human neurons

Ethical Considerations and Conflict of Interest Statement

The author declares that he has no conflicts of interest and no commercial interests in this matter. Acknowledgement: The autopsy brain specimens were donated by the legal next of kin of each patient and granted permission to the author to publish the research results. Strict patient confidentiality has been maintained to protect the identities of the participants in this study. This research was approved by the Institutional Review board of the Dr. Paul H. Duray Research Foundation. The Generosity of Dr. Jorge L. Benach PhD, Dean of Research, School of Medicine, SUNY Stony Brook, School of Medicine, Stony Brook, N.Y., his colleague James Coleman, manufactured, validated, and gifted Murine monoclonal Antibody CB 10 to this author is gratefully acknowledged. The expertise of Dr. Ali Javed and Gene Link Inc. Hawthorne, NY in his design and manufacture and validation of the DNA probes utilized in this study is gratefully acknowledged. The Lindorf Family Foundation's support of this research activity is gratefully acknowledged.

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