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Borrelia Invasion of Brain Pyramidal Neurons and Biofilm Borrelia Plaques in Neuroborreliosis Dementia with Alzheimer's Phenotype

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ABSTRACT

Dementia in Lyme borreliosis complex has been reported, mainly in post-mortem studies without available antemortem evidence of active Borrelia infection. Blanc in 2014 studied living patients with Lyme neuroborreliosis dementia and several dementia phenotype illnesses including an Alzheimer's Phenotype. Herein we report an additional case study of a longitudinal evolution of European neuroborreliosis over eight years from tick bite to mild cognitive disease, to advanced dementia to death with a brain Alzheimer's disease phenotype and concurrent Borrelia deposits in brain Alzheimer's disease sites at autopsy.

Intrathecal Borrelia specific Antibodies were detected by Commercial diagnostic laboratories (antemortem). Molecular autopsy tissue imaging was completed with borrelia specific DNA probes and an Immunomicroscopic detection histopathology method.

***Results:** Autopsy showed intact spirochetes, fragmented spirochetes, deposits of Borrelia-specific proteins inside plaque lesions and inside of neurons, and Borrelia DNA deposits in plaque and neuronal sites. Pure Alzheimer's disease (without Lewy bodies) was a routine neuropathological finding.*

CSF evidence for a brain compartment immune response is established here. Intrathecal antibodies to infection presented as oligoclonal total CSF IgG bands (n=twelve increase to n=13 bands) and separate Borrelia IgG Western blot band analysis in Cerebrospinal fluids (seven diagnostic Borrelia CSF antibody bands). Blood Western blot disclosed triple borrelia species infection; burgdorferi European type (eighteen bands), garinii (twelve bands) and afzelii (eighteen bands). Total Borrelia IgG antibodies in blood during life were two hundred-fold higher than normal range. Western blot of Cerebrospinal fluid prior to death disclosed 7 protein bands which were not represented in simultaneous blood Western blot studies, further validating the Intrathecal fingerprint of a separate brain compartment immune response to neuroborreliosis infection.

***Conclusion:** Borrelia protein antigenic stimulation of intrathecal Borrelia antibodies was caused by resident deposits of spirochetal protein deposits in plaques, in diseased neurons, and in neuropil brain sites, and in intact brain spirochetes. Deposits of Borrelia proteins inside neurons and Brain phagocytes and in Neuropil sites (invasosomes) confirm remnants of chronic brain infection.*

Keywords
Neuroborreliosis, Dementia, Alzheimer's, Intrathecal.

Abbreviations
DAB- Diamino Benzidine (brown chromogen); FISH- Fluorescence in Situ Hybridization technique; IHC – Immunohistochemistry technique; HS – Hippocampal Sclerosis; LD – Lewy; Body Dementia illnesses (of Blanc 2014); NBD - Lyme Neuroborreliosis Dementia (of Blanc 2014); VD- Vascular Dementia (of Blanc 2014); WB – Western Blot serology technique.

Introduction
Dementia due to European Neuroborreliosis with simultaneous neurodegeneration/brain atrophy was established by Blanc and colleagues and published in the Journal of Alzheimer's Disease in 2014 [1]. Thirteen dementia patients with notable intrathecal antibody formation to *Borrelia* infection demonstrated radiological evidence of brain atrophy and clinical recalcitrance to cure after three weeks of parenteral antibiotics. Only one patient died during Blanc's study and that patient autopsy disclosed Alzheimer's disease. No attempt was made to identify *Borrelia* microbes in that patient's brain. Herein we describe an additional case of intrathecal antibody positive *borrelia* dementia in which autopsy confirmed simultaneous Alzheimer's disease. Intact *Borrelia* spirochetes and their fragments, and *Borrelia*-specific protein and DNA deposits in plaques in diseased neocortical brain sites were microscopically confirmed. The spatial distribution of *Borrelia* infection overlapped with the distribution of Alzheimer's Disease-specific lesions. *Borrelia* communities in plaque form, *Borrelia* inside of diseased neurons, and *Borrelia* vascular invasion were documented by Fluorescence in situ Hybridization-(FISH)) and by antigen/antibody binding (immunohistochemistry) with *Borrelia*-specific antibodies. This is the first report of tissue confirmation by molecular diagnostic, methods of persistent brain *Borrelia* tissue infection in a patient with dementia during life. Our research methodology is easily adaptable to future autopsy studies of dementia patients and is within the reach of every hospital pathologist.

Intrathecal immune response to *borrelia* in Blanc's study of NBD-Neurodegenerative subtype provides for legitimacy of two pathways (infection and degeneration) to a dementia phenotype. (LNBD). Like the model of simultaneous Lewy Body disease/ Alzheimer's disease, a sound and validated system for two separate dementia ontogenies and provenance are combined in one brain [1.,2,3]. Blanc described mixtures of NBD with coexistent AD, LD, HS, VD, FTD phenotypes. We encourage future research to dissect these mixed categories. Separate, equal and co-incident may or may not be united by evidence in linkages of infection and neurodegeneration, as was previously proven in General Paresis by Noguchi and Moore in 1913.

Case presentation
This patient died with severe dementia at age 68. His past medical history was positive for erythema migrans in perineal skin in his mid-fifties followed by persistent arthralgias, joint stiffness, autonomic dysfunction with hyperhidrosis, 'transient global amnesia' and pronounced fatigue from age 56-60. He retired from medical practice. Dementia began at age 61 with cognitive and memory deficiencies, confirmed by neuropsychiatric testing. From 2003-2006 he developed dysarthria, agraphia, muscle spasms of the legs, photophobia and hyperacusis. Parenteral combination antibiotic therapy-pulsed with oral antibiotics was administered without durable curative effect from 2007-2009. He developed psychosis and died in 2010 in the seventh year of his dementing illness.

Methods
Autopsy, routine neuropathology and *Borrelia* forensic methods: Brain Autopsy was performed by a board-certified neuro pathologist. A second forensic type molecular analysis for DNA of *Borrelia* focused on brain tissue re-examination and utilized recut tissue unstained slides from three brain sites; right and left hippocampal tissue (site CA1) and cerebral cortex (Right frontal lobe).

Immunohistochemistry Method
Decoration (labeling) of *Borrelia* antigens in brain tissue sites IHC immunoperoxidase method Stage 1: Binding of Rabbit polyclonal antibodies to sites followed by Stage 2 incubation with anti-rabbit antibody linked to horseradish peroxidase; Stage 3; Chromogen deposition at sites of Bound Anti-*Borrelia* rabbit antibody (Di Amino Benzidine DAB-) resulting in a brown chromogen product deposition on specific tissue sites. *Ventana iView DAB kit [IHC Kit for detection of Rabbit antibodies] (product #: Roche Tissue Diagnostics; Catalogue 760-091* – according to Manufacturer's instructions with final Chromogen development (DAB); ABCAM Inc. www.abcam.com (Catalogue ab20950) *Borrelia* polyclonal antibodies: Harvested from rabbit immunized with total *Borrelia* proteins in a pure culture digestion *product.*

DNA probe/ FISH method
Fluorescence in situ DNA Hybridization (FISH) was performed as previously described by Sapi et al. (doi 10:1556/1886.2015.00049, European Journal of Clinical Microbiology and Infectious Disease; 2016) All control bacterial species validated for specificity of *Borrelia* hybridizations – with a *Borrelia burgdorferi* probe to Flagellin B 22 nucleotide sequence: Flagellin B open Reading frame (BBO 0147) were bacterially validated. *Probe Sequence: (nucleotide) TGG GAG TTT CTG GTA AGA TTAA; --- fluorescein isethionate label 5' position.*

Antemortem blood and CSF serology
Data from commercial and reference laboratories in the Netherlands and in Switzerland were entered a spreadsheet, and results were tabulated and graphically displayed for visual analysis of Cerebrospinal Intrathecal and peripheral blood antibodies to borrelia infectomes.

Digital Image Capture hardware and software
Digital images were captured with an Omax light microscope outfitted for Fluorescence Imaging and An Omax five-megapixel

camera (with a 0.5 reducing lens) through a trinocular head. Images were recorded in TIFF format at the highest permitted optical resolution allowed by the manufacturer supplied image capture software.

Results

Autopsy: Routine Neuropathology examination; Formalin fixed brain

On pathologic gross examination, the brain showed minimal pathology, with slight frontotemporal atrophy and relatively small hippocampi. Occipital and hippocampal tissue showed diffuse neuritic plaques, neurophil threads and tangle-like structures,and in the hippocampus gliotic changes were seen. Identical pathology was seen in the amygdala. There was no evidence of leukocytic infiltrates. Immunostaining: TDP43 staining: negative results. Alpha Synuclein Staining: negative results: Beta amyloid 1-24 plaques reactive, Congophilic angiopathy were noted. Autopsy Diagnosis: Braak Stage VI Alzheimer's disease without a Lewy body component.

Specialized Molecular Diagnosis in Previous Brain Autopsy Material: Molecular Detection of *Borrelia* Specific Proteins and Borrelia DNA in Brain

Figure Titles Summary:

Immunohistochemistry:

Borrelia proteins: Figure 1A *Borrelia* spirochete (spiral form) in brain, Figures 1B, *Borrelia* spirochete (non-spiral form) in brain, Figure 1C, Multiple deposits of *Borrelia* proteins in neurons and glia cells, Figure 1D, single neuron *Borrelia* protein deposits, Microglial round cell *borrelia* protein deposits, Figure 1E, Microglia cell *borrelia* protein deposits,1F, *Borrelia* proteins Only, deposits, configuration of plaque deposit.

Borrelia DNA deposits: 2A, Neuron with Cytoplasmic space *Borrelia*, Red color outlined DNA deposits from, 2B, *borrelia* spirochete in cortex (DNA probe FISH) Hybridization, 2C Multiple *Borrelia* spirochetes in Cortex (DNA probe FISH hybridization).

Biofilm communities of *Borrelia* in brain sites (FISH method : Image Data Results:

Figure 3A, Biofilm form *borrelia* with attached undulating spirochetal form transgressing the biofilm, Figure 3B, Biofilm *borrelia* with an undulating spirochetal form, figure 3C, Green biofilm Matrix contains DNA and internal spirochetes, figure 3D biofilm plaques of *borrelia* stained with Congo Red, and White DNA FISH positive *borrelia* particles shining through red stained sites (double stain technique).

I *Borrelia* antibodies in Blood and Cerebrospinal fluid (Western Blot): Figure 4A, Blood, Triple species *borrelia* antibodies, Bands arranged by Molecular mass of Protein Antigens, Figure 4B, Contribution of each *borrelia* species to observed Western blot bands in blood, Figure 4C, Cerebrospinal fluid *Borrelia* antibodies, with notable unique low molecular mass antigens (17,19,21Kda mass)(circle), Figure 4D Cerebrospinal intrathecal antibodies.

Discussion

Etiologic toxic protein co-travelers (pTau, Beta 1-24 Amyloid, ASN) in the dementia brain are invisible in Hematoxylin and Eosin (H&E) stained autopsy microscopy specimens but are disclosed in advanced Immunohistochemistry (IHC) labelling procedures. Etiologic *Borrelia* infection toxic protein products are invisible in H&E stained sections, too.

Infectious disease pathology is a neglected niche in the universe of academic medicine. *Borrelia* brain infection in Neuroborreliosis of Blanc is equally bona fide and menacing as *Treponema pallidum* brain infection in General paresis. Syphilitic dementias of the Nervous system exist as pure Paretic type and mixed Tabeo-paresis dementias. Neuroleptospirosis dementia in AIDS has been established. *Borrelia* pathogens and their brain infection sequalae may independently cause a dementia product separate and apart from other defined toxic human generated protein products: i.e. Amyloid, pTau, TDP43, toxic Type ASN.

No practicing Neurologist or Pathologist should fail in the accurate diagnosis of a patient with any category of brain spirochetosis associated dementia. Professional ignorance of neurospirochetosis illnesses (including categories as follows: dementing, or paretic, convulsive, psychiatric, hydrocephalic, meningitic) in year 2020 has heretofore limited the number of patient clinical and pathological cases submitted for detection of infectious microbe DNA in brain autopsy tissues.

Conclusion

Specialized techniques provided proof of *Borrelia* autopsy brain spirochetes, which were missed by a medical school faculty neuro pathologist in spite of the patient's medical history. A Molecular detection specialized for Borrelia signatures in tissue ("second look") investigation disclosed *Borrelia* DNA deposits, and *Borrelia*-specific protein deposits in brain in neocortical sites which overlapped with the distribution of lesions of conventional Alzheimer's disease (plaques and neuronal sites). *Borrelia* antigens deposited in the brain fully explain the origin of the patient's huge antemortem burden of intrathecal antibodies to *Borrelia* infection.

Our novel but simple methods for *Borrelia* mediated brain tissue injuries terminating in Lyme Neuroborreliosis Dementia are detailed in this report. These methods are within the reach of pathologists in community hospitals.

This is a call to action to embark on future studies of patients with *Borrelia*, and dementia. Antemortem measurement of intrathecal antibodies to *Borrelia* antigens is commercially available, and well within the reach of any practitioner. *Borrelia* tissue forensic diagnosis is now mandatory in the universe of dementia research.

The era of syphilis included Percutaneous Frontal lobe of Brain mini-core biopsy under Novocain local anesthesia, at the patient bedside, antemortem, as a diagnostic tool. In a future time, needle-mini-core biopsy of Frontal lobe of dementia brain, with follow up

(continued on Page 10)

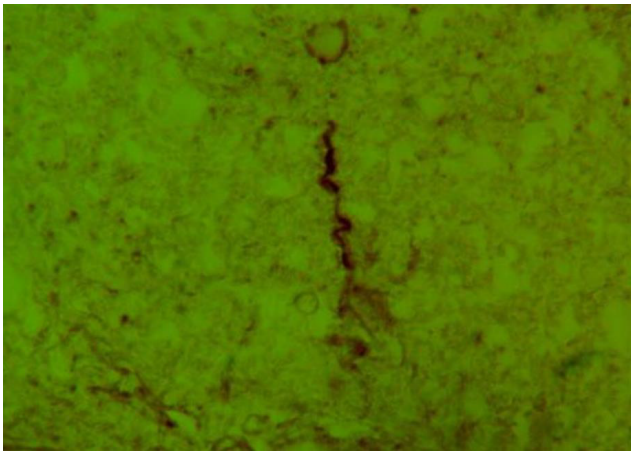

Figure 1A: An Immunohistochemistry demonstration of Borrelia specific proteins. A Coiled borrelia spirochete in Autopsy Alzheimer's disease Cerebral Cortex gray matter (brown color – Diaminobenzidine chromogen DAB. Reagent – ABCAM antibody to immunized rabbit with whole cell sonicated borrelia spirochetes in pure culture.

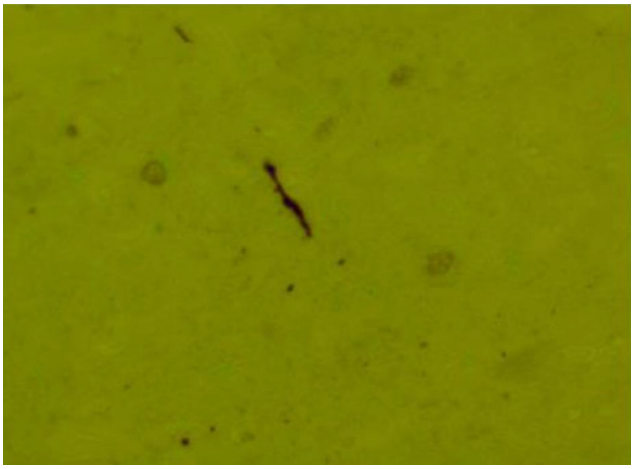

Figure 1B: Immunohistochemistry demonstration of Borrelia specific proteins. A non-coiled borrelia. Spirochete in neuropil of cerebral cortex Autopsy Alzheimer's disease gray matter (brown color- Diaminobenzidine chromogen DAB). Reagent – ABCAM, antibody raised in rabbit after immunization with whole cell lysate of sonicate borrelia spirochetes in pure culture.

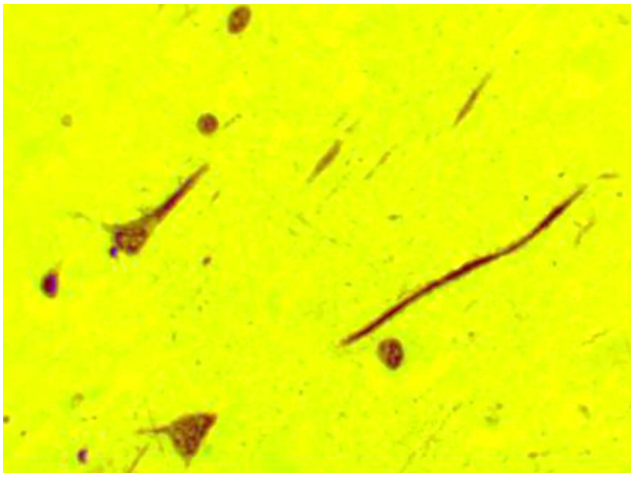

Figure 1C: Immunohistochemistry demonstration of Borrelia specific proteins. Human brain cortical neurons from Alzheimer's disease gray matter cerebral cortex stain Brown color for detection of intraneuronal deposits of borrelia proteins (brown color – Diaminobenzidine chromogen DAB).
Note: Discrete deposits as particle like areas in the Soma region of neurons and as diffuse non particulate deposits along the axons of nerves. Rounded cell profiles with brown color deposits are activated Microglia phagocyte cells. No spirochetes are imaged in this field of view.

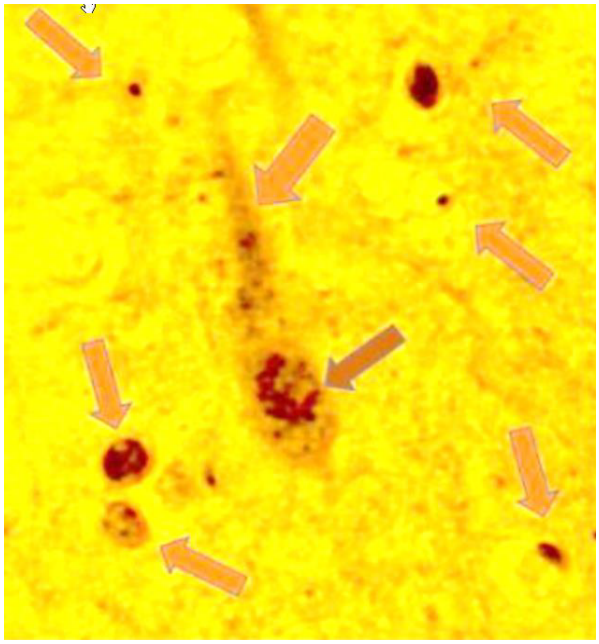

Figure 1D: Immunohistochemistry for demonstration of Borrelia specific proteins. Deposits of brown particulates of borrelia proteins surround the nucleus region in the soma of a pyramidal neuron and also reside in the axonal region of the neuron. Additional brown particulate deposits are present in round microglial brain macrophages.

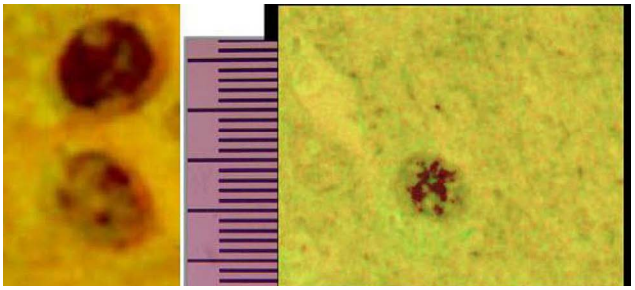

Figure 1E: Immunohistochemistry demonstration of Borrelia specific proteins. Deposits of brown particulates reside in the cytoplasm regions of brain Microglial cell macrophages.

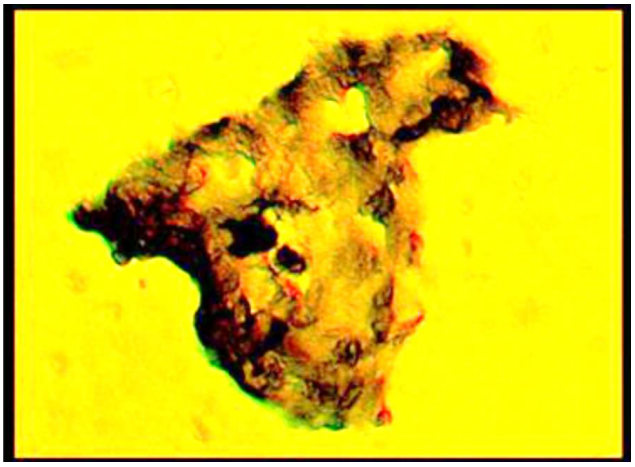

Figure 1F: Immunohistochemistry demonstration of Borrelia specific proteins. Cell free deposits of brown particulates and diffuse non particulate borrelia protein deposits reside in a biofilm community of borrelia specialized forms and borrelia protein rich extracellular matrix (glue-like extracellular matrix material) which invests around and through the biofilm community. A single spiral form represents a borrelia planktonic type spirochete in the biofilm. Note that empty spaces with a golden color course through the biofilm community. These empty spaces represent the water channels of a healthy biofilm community which provide nutrients, and which also serve as a waste removal sewer-like system. The biofilm community has a "coral-like" architecture with empty spaces and solid zones.

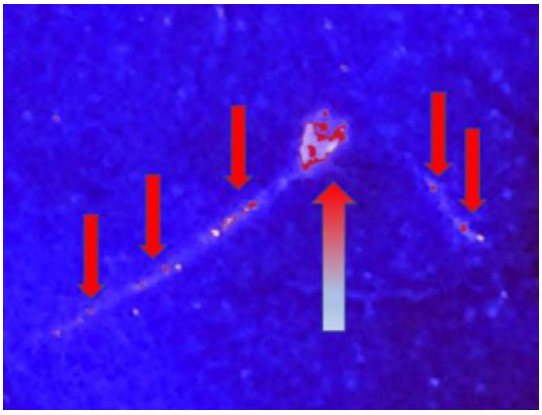

Figure 2A: DNA probe hybridization to borrelia DNA and RNa sites inside of a pyramidal neuron in the cerebral cortex of Alzheimer's disease by Fluorescence in Situ Hybridization method with a Flagellin B DNA borrelia sequence shared by European borrelia species and North American borrelia species. Red color is from Chromogen Cy5 which is attached to the DNA probe. Note : punctate signals in narrow axon sites and signals in triangular soma region where the nucleus resides.

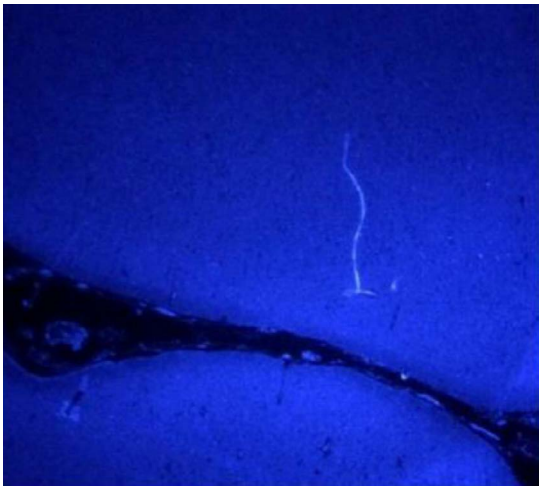

Figure 2B: DNA probe hybridization to borrelia DNA and RNA sites inside a single elongate borrelia spirochete residing in Alzheimer's disease Cerebral cortex by Fluorescence in Situ Hybridization method with a Flagellin B DNA borrelia sequence shared by European and North American borrelia species. Blue - white color is from FITC fluorochrome attached to the DNA PROBE illuminated with 500 nm monochromatic light. Blue color from non-fluorescent cerebral cortex tissues which do not manifest borrelia spirochetes or punctate fragments of Borrelia DNA which often appear inside of biofilm borrelia communities.

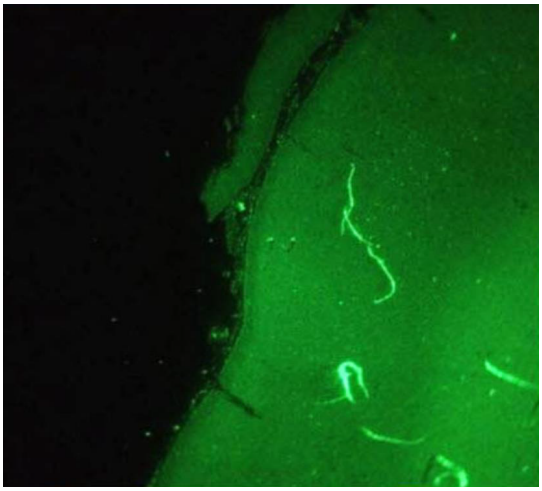

Figure 2C: DNA probe hybridization to borrelia DNA and RNA sites inside multiple borrelia spirochetes. Residing in Alzheimer's disease cerebral cortex by Fluorescence. In situ Hybridization method with a Flagellin B DNA borrelia sequence shared by European and North American borrelia species. Green color fluorescence is from FITC fluorochrome attached to the DNA probe and illuminated by 540 nm monochromatic light. Non-fluorescent green color represents the cerebral cortex tissues which do not contain borrelia spirochetes or punctate fragments of borrelia DNA.

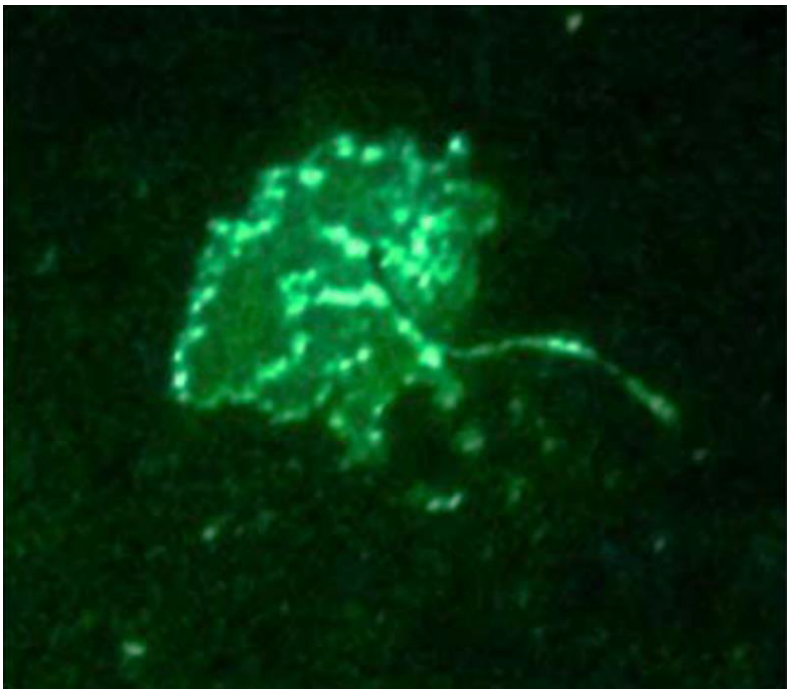

Figure 3A: DNA probe hybridization to Borrelia DNA and RNa. Multiple sites of bright signal fluorescence represent membrane bound borrelia DNA in spirochetal fragments and a wavy spirochetal form piercing a biofilm borrelia community in Alzheimer's disease cerebral cortex inside of an amyloid coated plaque. Note : Punctate signals represent Specialized "coccoid" or Granular live borrelia spirochetes inside the biofilm; and less intense green fluorescence of Extracellular Borrelia DNA/RNA in the ECM extracellular matrix investment.

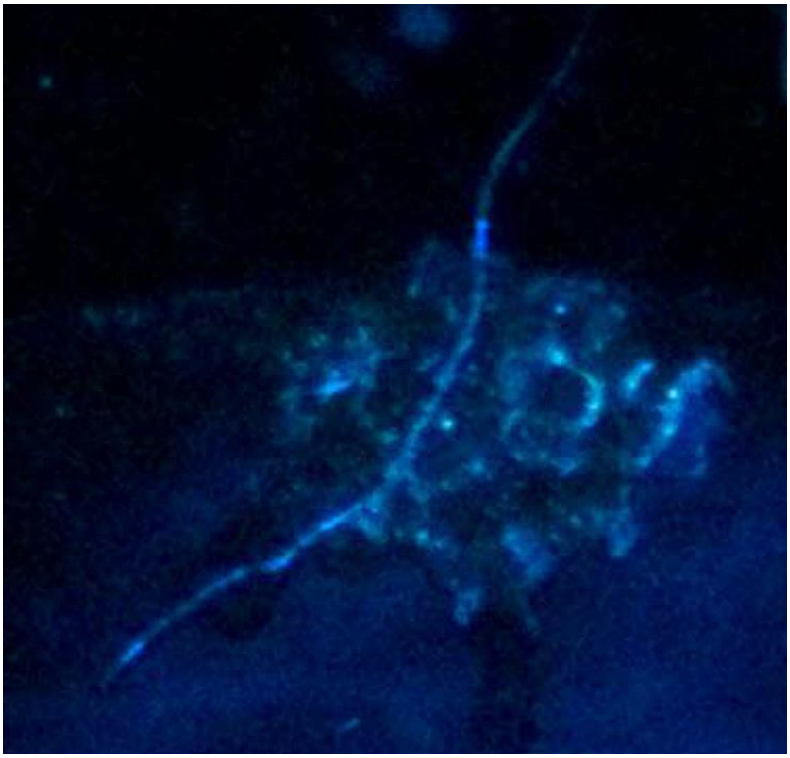

Figure 3B: DNA probe hybridization to Borrelia DNA and RNA dwelling inside of a biofilm community Of Borrelia microbes resting at the surface of cerebral cortex in an Alzheimer's disease brain Tissue section. Note: the brain cortex signal in lower half of the field of view is non-fluorescent

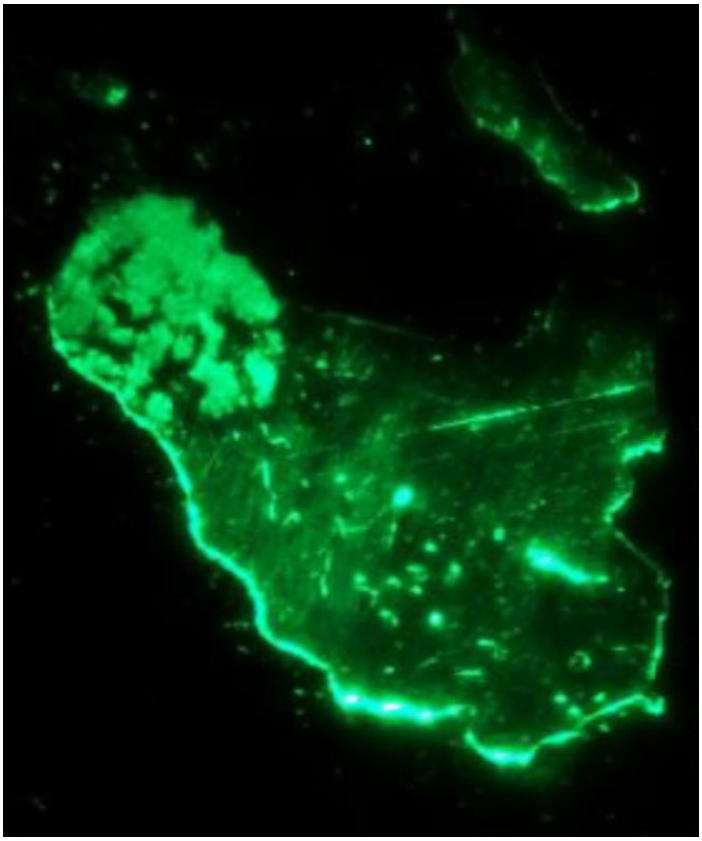

Figure 3C: DNA probe hybridization to borrelia DNA and RNa inside of a biofilm borrelia community residing inside of an Amyloid plaque in the cerebral cortex of an Alzheimer's disease brain tissue section. Extracellular Borrelia DNA fills the Extracellular matrix investment which surrounds living membrane Bound Borrelia specialized microbes with Specialized "coccoid" or "granular" profiles.

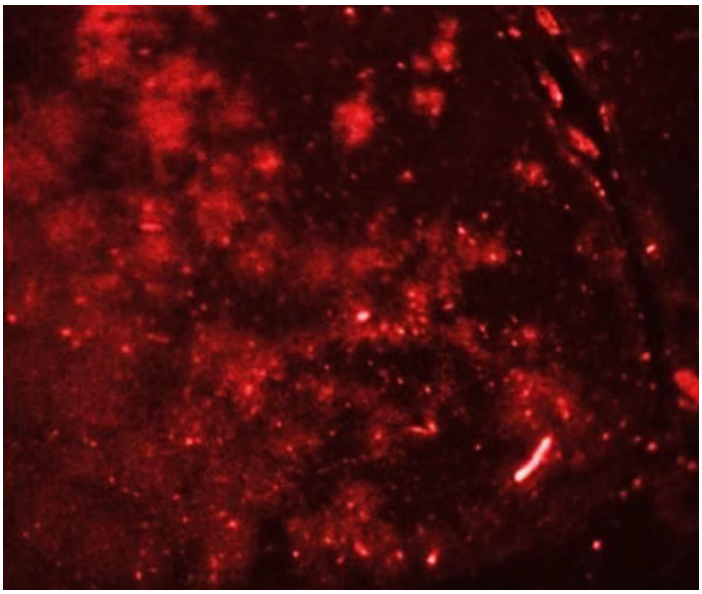

Figure 3D: Dual stain preparation (Congo Red Stain for Amyloid and DNA probe hybridization for Flagellin B Borrelia DNA carrying a Cy5 red fluorescent label. Non-fluorescent Red signal is a Congo Red stain which demonstrates many Amyloid positive plaques which are confluent with one another in the hippocampus of the brain in an Alzheimer's disease patient. Bright signal red and red-white fluorescence emanates from the live membrane bound borrelia DNA in living specialized morphology borrelia which resides inside the many plaques.

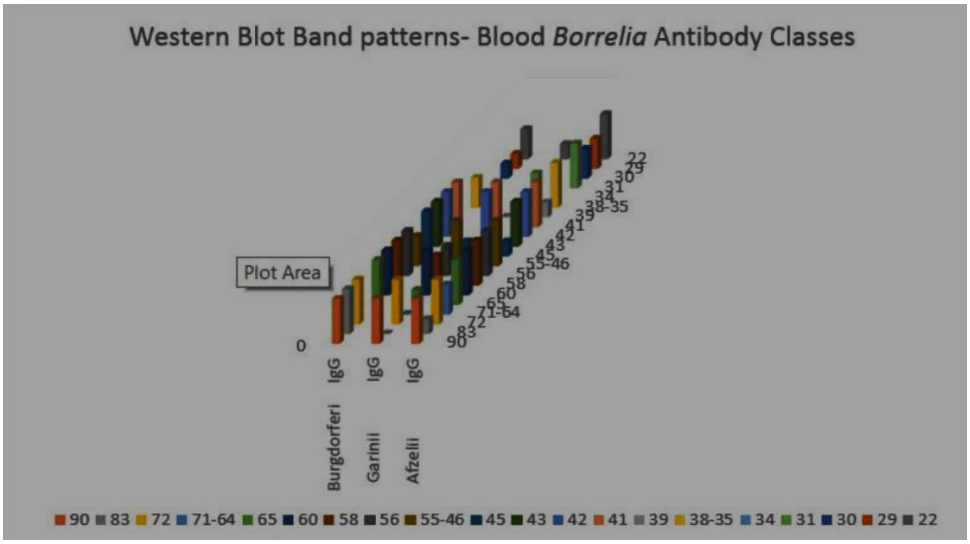

Figure 4A: Blood: Graphic display of a family of human antibodies which are specifically bound to an array of borrelia structural proteins from each of three species of European borrelia which were represented ***by Blood Western Blot studies of a patient with Triple Species simultaneous neuroborreliosis and*** Alzheimer's disease. By the criteria of the German Borreliosis society, Western blots demonstrating more than 5 positive Western Blot reactive bands are diagnostic of past borrelia infection (with IgG reactive bands) and recent borrelia infection (with IgM reactive bands. This panel displays an IgG western blot with up to 18 highly reactive bands in the immunoblot (Western Blot) membrane.

This data represents a patient with chronic active Neuroborreliosis, and Autopsy verified Alzheimer's disease which overlaps clinically and pathologically.

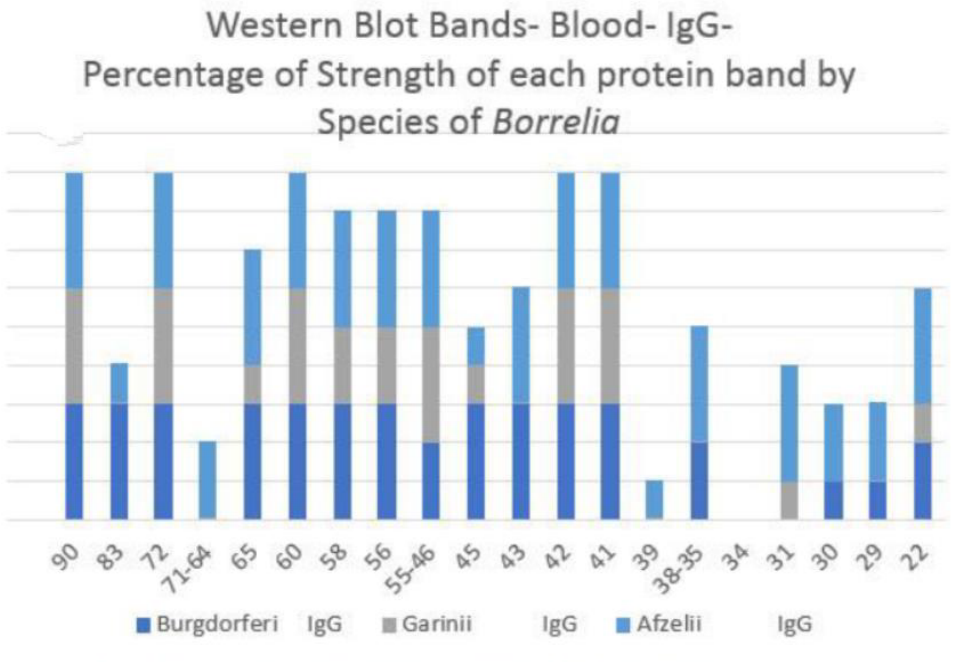

Figure 4B:Blood: Graphic display of Cerebrospinal fluid Western Blot study with IgG patient antibodies which represents the Brain compartment Immune Response (Intrathecal Antibodies) to borrelia infection in the brain. In a patient with autopsy verified Alzheimer's disease and Autopsy verified neuroborreliosis involving brain sites of Alzheimer's disease. The pattern of IgG antibodies in the Blood differs considerably from the Cererospinal fluid Antibody pattern demonstrated in Figure 4C (next image)

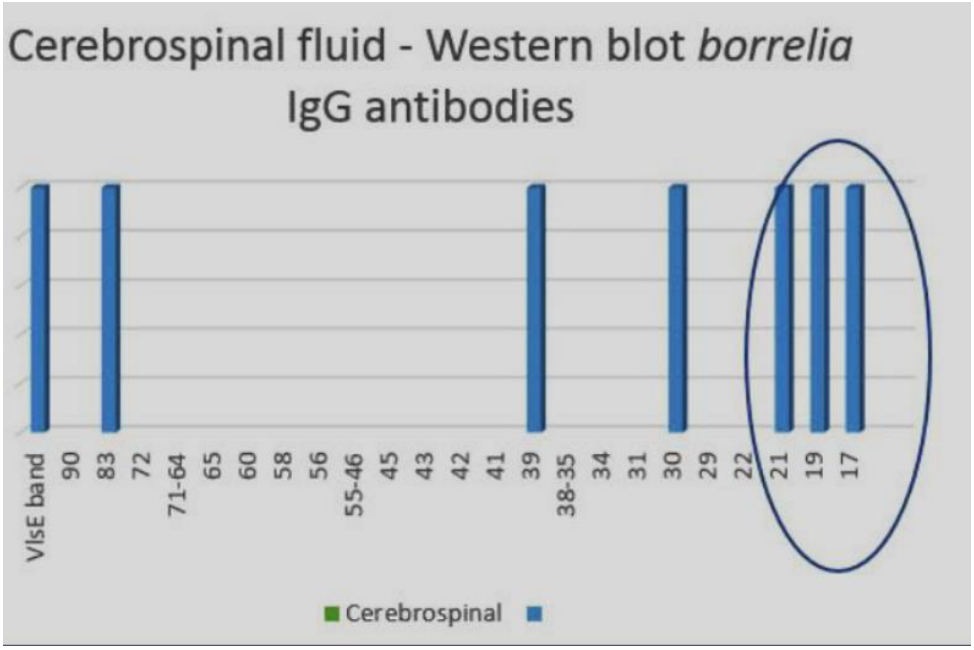

Figure 4C: Cerebrospinal fluid : Graphic display of Western blot cerebrospinal antibodies in the Alzheimer's/ neuroborreliosis patient from Figure 4A and 4B above. Note the strength of the immune response by virtue of the intensity of staining of patient CSF antibodies to stripes of dried borrelia specific proteins embedded in a membrane and arranged in a ladder-like array from High molecular weight proteins (VLSE an 83 kilodalton mass (to the extreme left) and extremely low molecular weight proteins 21 kilodalton 19 kilodalton and 17 kilodalton mass (to the extreme right position on the blot membrane).

This evidence is elegant proof of concept that this patient with Alzheimer's disease and active brain sites of neuroborreliosis produced the antibodies entirely within the brain compartment (Inrathecal - Antibodies) - proof of an "inside of the brain" separate immune response to infection.

application of Borrelia specific DNA probes, under the informed view of a microscopist, might provide astonishing new insights to diagnostic insights of pathogenesis in the dementia conundrum.

Acknowledgments

Dr. Judith Miklossy referred this patient's surviving spouse to the University of New Haven ***Borrelia*** Research Laboratory (Dr. Eva Sapi) for assistance in commencing ***Borrelia*** focused molecular studies of autopsy brain tissues in year 2012. This report is the product of an eight-year professional collaboration to assist the family's efforts to prove the validity of a ***Borrelia*** pathogen contribution to the decedent's dementia illness.

Dr. Eva Sapi and her fellows provided laboratory support to Alan MacDonald MD over the seven-year term of this research effort. She independently validated and published the exact Fluorescence In situ Hybridization methods (FISH) which are reported here.

Dr. Eva Sapi's research group has independently studied tissues from our patient R.E.(MD) with advanced microscopic technologies (Atomic Force Microscopy) and further completed successful PCR DNA amplification of *Borrelia* specific amplicons derived from autopsy tissues of the patient reported here. She will report these confirmatory studies separately.

Proof of intrathecal IgG synthesis by oligoclonal IgG band analysis of cerebrospinal liquor (12 bands increasing to 13 bands over the course of illness in our patient) and measurement of Q IgG = 2.9 was completed by Klinisch chemisch laboratorium (KCL), Universitair Medisch Centrum St Raboud, Nijmegen, 6500 HB, the Netherlands.

The original R.E.(MD) patient brain autopsy and the diagnosis of Braak Stage VI Alzheimer's Disease (without an associated ASN or TDP43 component) were rendered: Department of Neuropathology Universitair Medisch Centrum St. Raboud, Nijmegen, 6500 HB, Netherlands. Autopsy Accession S-10-00259. We gratefully acknowledge the help provided by Dr. M.C.P. Braat, PhD, and Minnie Elias -VanDer Lande for medical records retrieval.

Histology preparation of tissue recut slides from the original brain autopsy paraffin blocks was contracted through McClain Laboratories, Smithtown, New York.

Manufacture of the DNA probes used in this study, the purification, and the validation by Melting Curve Analysis of Probe/Target DNA oligomer annealing products were completed at Gene Link Laboratories, Hawthorne, New York, under the expert direction of Dr. Ali Javed, PhD.

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(Limited to "***Borrelia* dementia only**", PubMed Citations):

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