June 28, 2015

Dear Dr. MacDonald,

Please find this letter in response to your request for my opinion with respect to your findings and autopsy report submission for PATIENT IDENTIFIER C.W.B, JR.: DATE OF BIRTH APRIL 28, 1925. After careful review of your micropictographs and your report findings, I definitively concur with your conclusions that tissues sampled in your report contained Borrelia burgdorferi sensu lato and Borrelia miyamotoi organisms.

Methods used in the report:

As you know I am familiar with the FISH (in situ hybridization) technique and have used several of the same molecular beacons (DNA / protein targets) that you have used in this case to definitively and directly identify the various Borrelia contained and presented in the numerous case images.

It is my opinion that the FISH assay used in the production of images contained in this report is perhaps not only the most powerful and precise tool with respect to the identification of specific DNA or protein sequences of the organism of interest, but also allows for the direct visualization of the organism itself, in this case Borrelia, contained in tissue samples, and in this case further verifies the Borrelia in their in situ hallmark forms.

I know Dr. MacDonald 's probes have been validated by himself and other researchers to detect only the organisms of interest. In this case the beacon probes were specifically designed to bind and emit fluorescence only if hybridized (matched up and bound to) protein sequences of Borrelia burgdorferi sensu lato (specifically genospecies B. burgdorferi, B. afzelii and B. garinii), which are the causative agents of Lyme disease, as well as Borrelia miyamotoi, another tick borne Borrelia organism. I know the DNA/protein sequences underlying these probes to be validated not to occur in other organisms.

Further adding to the certainty of Dr. MacDonald 's findings is the fact that these particular molecular beacon probes are designed to bind only to fully complementary sequences found in the specimen. A mismatch of a single base-pair will not allow hybridization (binding) to take place and therefore fluorescence is absolutely dependent on an exact probe / sample match.

Additionally, I know that in development of beacons used in this case, proper measures were taken in order to adjust the signal-to-noise ratio to eliminate possible auto-fluorescence and background noise, thus assuring fluorescence only emanates from stable probe / target matches. While some conditions such as temperature, pH, and the type of salts used, can affect any such molecular assay, Dr. MacDonald 's use of appropriate side by side controls assures

that what is demonstrated in the images of this report are, without doubt, the Borrelia of the genospecies and subspecies associated with the specific probes used.

Images:

As to the report images, they also demonstrate hallmark morphology and architecture of Borrelia found in both CSF and in brain tissue. While the report does not contain microscope objective magnifications or image processing magnifications, nevertheless it is evident that the Borrelia have appropriate size and shape of the known multiple forms of Borrelia found in vivo / ex-vivo.

Specifically the intact spirochetal forms range from 5-30 × 0.2-0.3 μm as demonstrated in the case images. Unlike the spiral forms found in artificial tissue culture medium, some Borrelia in this case present as typical long cylinders, which are uncoiled versions, as they were exposed to various bodily fluids and tissues in vivo.

Case images further demonstrate a typical inner compartment of the spirochetes that fluoresces a central bright green core dense with DNA as tagged by the BB 0740 inner membrane target beacon. A pallid green outer compartment is also demonstrated with typical variable thickness noted due to shedding of blebs containing DNA from the outer cell membrane and occurring along the spirochete cylinder. The unique thin endoflagella of the Borrelia wind longitudinally along the spirochete cylinder and are clearly demarked by the intense green fluorescence provided by a perfect match of the BB 0147 molecular flagellar beacon with the flagella DNA.

Other morphological forms of Borrelia are clearly demonstrated in the case images as well, which are typically found in ex-vivo environment. In the brain tissue (hippocampus) sections one can see round bodies (cysts) which when living are less motile and biofilm-like colonies of Borrelia.

Some of what Dr. MacDonald describes as "small fragments of ***Borrelia burgdorferi*** Spirochetes" are in fact protein or DNA fragments specific to Borrelia burgdorferi. Some of the small rounded structures with an intense green central DNA core are actually Borrelia cysts and not solely transverse cross sections of Borrelia as produced by tissue monolayer preparation. Cross sections or transversely cut spirochetes are however also imaged and the inner cell membrane can be demonstrated by the small fluorescent rings (tagged by the inner membrane beacon BB 0740) with surrounding less intense fluorescence.

Borrelia cysts are known to originate by transverse constrictions on single spirochetes forming a string of pearls that later break in to individual cysts. The formation of these round bodies or cysts can be seen in the higher magnification images of spirochetes contained in these case images. Several infective cyst units are produced by transverse divisions which usually starts at

the ends of the spirochete, and are demonstrated particularly well in the first case image as a bright rounded bleb at one end with several bright fluorescent blebs and incongruous fluorescence seen along the length of cylindrical spirochete.

Multiple case images of both the Borrelia burgdorferi sensu lato (fluorescent green) and the B. miyamotoi (fluorescent red and yellow) demonstrate well colonies of Borrelia surrounded by an adherent polysaccharide-based matrix or biofilm. In this case, biofilm-like colonies are clearly demonstrated in the hippocampus region, an area of the brain associated with memory and with atrophy in some dementia patients. Again, as these probes are designed to fluoresce only when bound to fully complementary sequences found in the specimen and are further designed to eliminate all possible background fluorescence, the pallid green, red and yellow fluorescence film surrounding distinct spirochetes and round body forms of Borrelia in the case images must represent as stated in this case "**Extracellular matrix material** composed in part by **Extracellular DNA** from once living but now dead ***Borrelia*** spirochetes".

Lastly, I must remind you that I am not a medical doctor and nothing contained herein either expressly stated or implied is intended or constitutes a medical diagnosis, nor implies specific cause of death. However, as a microbiologist and after careful review of the FISH molecular procedure used to derive images contained in the autopsy report and after careful review of all case images, it is my expert opinion that tissues sampled and presented in your report, with upmost certainty, did in fact contain species Borrelia burgdorferi sensu lato and Borrelia miyamotoi organisms in their various morphological forms. I can ratify the validity of the images which appear in Dr. MacDonald's report and concur that Dr. MacDonald's Molecular Beacons used for FISH method imaging of Borrelia in diseased tissues in this case are absolutely specific to their respective targets and are in my opinion the gold standard for identifying Borrelia in ex-vivo tissue.

Submitted Respectfully,

Dr. Jennifer Souders, D.PT., ATC.