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Chronic Wasting Disease:
A working hypothesis, the Agent and its Transmission

A Logical Causative Agent
PART Ia: TSE Testing Fallibility

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Abstract: **(Part Ia)** Transmissible Spongiform Encephalopathies (TSE) and in particular, Chronic Wasting Disease are devastating neuropathologic diseases caused by a unique, but unknown infective agent with high degree of refractivity to ordinary disinfectant and sterilization procedures. Four criteria are used to identify TSEs: 1) brain tissue vacoulation, 2) Scrapie Associated Fibrils, 3) abnormal protein resistance to proteinase-K digestion as detected by Western Blot and 4) immunostaining of diseased tissue, and more abbreviated ELISA tests. The fourth criterion is relegated to subset of third criterion, as both demonstrate the existence of the same PK-resistant tissue. All three of the primary diagnostic criteria are fallible in their ability to identify TSE disease. False positive and false negative results can be generated, as there is no "gold standard" of TSE diagnostic procedures. Various genera of bacteria have been demonstrated to contain and create PK-resistant proteins. Neurotropic, sterol ingesting bacteria of the Class Mollicutes, generally termed Mycoplasma and more specifically Spiroplasma bacteria can, and do, produce the defined TSE identification criteria including proteinase-K resistant proteins of the 25 to 30 kDa range, fibrils of 4 to 6 nm diameter, and will upon intracerebral inoculation of rodents, produce disease symptoms, such as central nervous system neuron vacoulation and the infectivity conditions generally associated with TSE disease and its prospective causal agents.

INTRODUCTION

Chronic Wasting Disease (CWD) is a Transmissible Spongiform Encephalopathy (TSE) affecting both wild and domestic cervidae, including elk, mule deer, black-tailed deer, white-tailed deer and white-tail hybrids. All TSE diseases are now grouped under the term of "Prion" diseases in recognition of the disease's destructive effect upon protective floating protein particles shielding nervous tissue cells. Prion diseases are fundamentally diseases of membrane tissue, although not necessarily neuronal tissue. Laboratory-created, transgenic, prion-deficient mice either failed to develop, or developed TSE disease much more slowly when inoculated with scrapie infectivity, while all produced antibodies against the inoculum (Prusiner SB, et al, 1993 and Weissmann C, et al, 1994).

A proteinaceous infective particle (or abnormal "Prion", PrP^{res}) has been widely postulated as the suspected causal agent, while lesser theories of slow acting virus, filamentous virus, retrovirus, viroids, virinos or a bacterial origin have waned. Excessive environmental manganese or deficient copper have been suggested to play a role in TSE pathogenesis (Purdy, 2000), while Kaneko (1997) suggests the necessary presence of a protein "X" associated with prions in order to initiate TSE infectivity

The unusual characteristics of TSE infections easily distinguish TSEs from more conventional infections. These include: unusually long incubation periods (from months to years); progressive central nervous system degeneration with characteristic thin section tissue lesions; the lack of an immune or inflammatory response; and unconventional biological and physical properties of the envisaged causal agent.

CWD was first recognized in 1967 as a clinical weight loss syndrome (deemed "chronic wasting") in wild mule deer held captive in Colorado Division of Wildlife research facilities and the Wildlife Disease Research Center managed by Colorado State University, both in Ft. Collins, Colorado.   In 1981, distinctive brain lesions were found associated with "chronic wasting" and was recognized as the pathological signs of a TSE. The clinical signature of CWD includes several non-specific, non-diagnostic symptoms, such as: weight loss, behavioral changes, excessive water consumption, salivation and urinating, together with erratic teeth grinding (Williams and Young, 1982). See *Figure 1.*
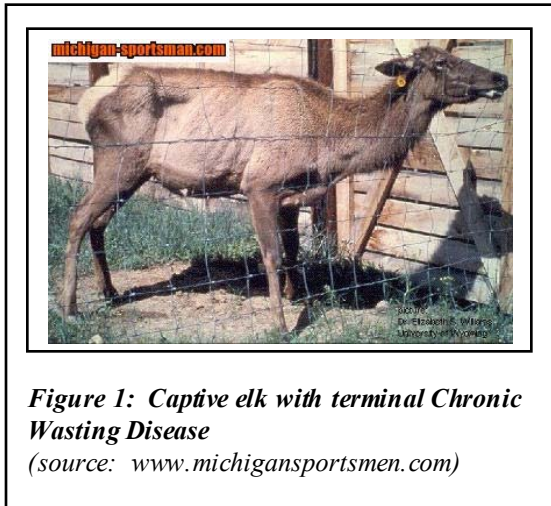

Figure 1: Captive elk with terminal Chronic Wasting Disease
(source: www.michigansportsmen.com)

Controversy surrounds the origins of CWD and whether it is a naturally occurring, sporadic disease in the wild, or may have been man-induced during research of co-habited deer and sheep at the government-operated research facilities. For the past three decades CWD has been erratically found within wild cervidae herds around limited regions of northeast Colorado and southeast Wyoming, and now more recently in northwestern Colorado, southern Wisconsin and southern New Mexico Domestic cervidae have been infected for at least 15 years but have recently sustained an expanded incidence of the disease. Infected domestic cervidae have been found in eight states, two Canadian provinces and in South Korea. Most, but not all disease occurrences of both wild and domestic cervidae have been traced back to a Colorado or Wyoming derivation. Poor data or limited background information prevents the remainder from being specifically tracked.

As a TSE, CWD is grouped via its diagnostic symptoms with several other TSE diseases, some more well known to the public, including Creutzfeldt-Jakob Disease and Kuru of humans, Scrapie in sheep and goats, Bovine Spongiform Encephalopathy ("BSE") of beef or dairy cows (a/k/a Mad Cow Disease), as well as, mink encephalopathy and several other more minor maladies. Some are thought to be spontaneous induced, others

under genetic control, and still others readily transmissible. All can be transmitted through direct intracerebral injection of infected tissue. A naturally occurring TSE with equally natural transmissibility, CWD presents a unique opportunity to examine a TSE disease in the wild, as well as in captive populations without man's substantial influence or interference in the genetic makeup of the infected species. As such, CWD represents a rare opportunity to achieve a better understanding of a unique form of disease, one potentially devastating to the cervidae industry and natural wildlife populations. Such information will be of great use for the full genre of TSE diseases, those capable of affecting domestic ruminants and those affecting man, himself.

IDENTIFYING TSE DISEASE

A critical aspect of defining any TSE disease relies on the use of cumulative, non-specific diagnostic procedures. Unfortunately, the majority of symptoms presented by a TSE disease are not individually diagnostic and must be objectively quantified and cumulatively applied to obtain a firm disease diagnosis confirmation.

As a matter of reliability and dependability, one would assume that the testing or analytical procedures, i.e. the assays, used to define and identify selected TSE disease recognition criteria would be beyond reproach so as to facilitate correct disease conclusions. Such is not the case with TSE diseases.

The author's earth science background has created an enduring appreciation of assays and assay procedures. Such assays must be infallible in order to define prospective geologic targets. Despite decades of dedicated research, special awards and many declarations of proof and success, the scientific community has failed to specifically identify a substantially provable TSE causal agent. Many theories still abound as to the true nature of the TSE pathogen. Competition for funding and the lopsided allocation of funding to those theories regarded as "award winning", "politically" or "popularly correct" have skewed research results toward theory conformity rather than true innovation in determining the authentic TSE causal agent.

Complicating the issue, a critical assessment of TSE diagnostic procedures reveals spurious behavior in assay procedures. Such aberrant testing behavior will inevitably lead to a myriad of incorrect conclusions. The inability to identify the TSE causative agent has materially hindered development of more specific and refined diagnostic procedures. This, in turn, has stifled the creation of symptomatic or prophylactic medicines and possible vaccines.

A critical review of TSE disease diagnostic procedures is fundamental to the understanding the agent recognition problems. In essence, three major processes are used for TSE identification. 1) Observation of brain vacuoles, i.e. "spongiform texture", 2) the presence of scrapie-associated fibrils ("SAF"), and, 3) the presence of proteinase-K resistant (PK^{res}) protein, also known by some as abnormal prion protein (PrP^{res}). The third criterion is regarded as the "gold standard" of disease verification by some practioners. But, the term "gold standard" is a misnomer as explained below.

Undesirably, each of the three individual identification steps has potential interference, and when taken individually can produce both false negative or false positive results. Formerly, all three criteria were generally required for a positive TSE identification, although more recently, shortened steps focused solely on identification of PK^{res} have come into vogue.

The first identification criterion, that of observing vacuoles in neuronal tissue is fairly distinctive, but is weak in its ability to be diagnostic of TSE or discern TSE at its early stages. Vacuoles are simply the residual locations of nerve tissue undergoing or having undergone cell death. At the latter stages of disease, vacuoles are easily identified via microscopic thin section observation, yet may not be present or difficult to recognize during earlier disease stages. This gives rise to the potential for early-stage, false negative results. Lamentably, vacuoles can also be found quite totally unrelated to TSEs, therefore may not then be independently indicative of a TSE disease and, hence, possibly yielding false positive results. Large numbers of vacuoles are generally not found in normal, healthy brain or nervous tissue.

The second, more evolved, criterion involves the visual identification of specific structures thought to be found only in TSE-infected brain and organ tissues. These structures are known as Scrapie-Associated Fi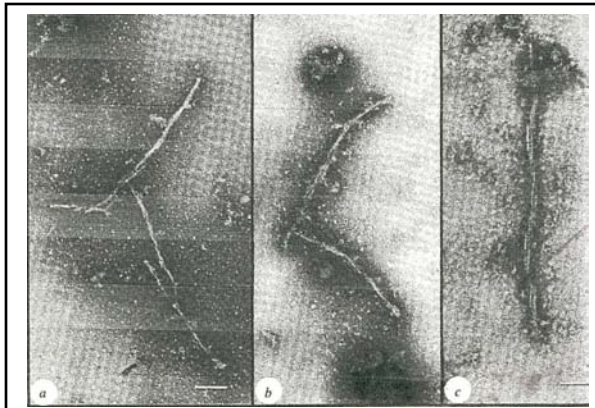brils or SAF's when found in scrapie, or CJD-associated fibrils (CJDaF's) when found in human disease. See *Figure 2.* Fibrils are characteristically present in diseased brain tissue and can also be found in the spleen, as well as other organs. These fibrils commonly possess a physical dimension of approximately four nanometers (nm = one-billionth of a meter) in width and approximately 150 to several hundred nanometers in length. Quite distinctive and excellent micrographic work by Mertz (1981) initially identified and qualified these unique fibrils, which have now become synonymous with TSE disease. SAF-like fibrils are not found in normal, healthy organ tissue although some other diseases such as Alzheimer's and other biological entities such as helical bacteria can produce similar looking fibular structures.

Figure 2: **Electron micrograph of SAF from a) CJD hamster brain, b) CJD human brain, and, c) Scrapie sheep brain. Magnification: a) = 86,000x, b.& c.) = 133,000x , bar = 100 nm. (source: Mertz, 1983, Nature V306, P474)**

The third criterion for the recognition of TSE disease is that of locating proteinase-K resistant proteins (PK^{res}). PK^{res} material is generally not found in normal, healthy mammalian nervous tissue. The presence of PK^{res} is the cornerstone of the currently popular Prion theory of TSE diseases, as promulgated by Dr. Stanley Prusiner of the University of California at San Francisco, for which he has received the Nobel Prize for Medicine. According to Prusiner (1999) prion diseases are aberrations in protein

conformation whereby a normal cellular glycoprotein (PrP^c), which forms a floating protective antioxidant coating over neuronal and other organ cells, is converted into abnormal prion protein (PrP^{res}) without a nucleic code directing the creation of adjacent new, abnormal but conformable PrP^{res}. PrP^{res} is thought to be the transmissible agent and is regarded as a pathogenic product common to all TSEs. As presented herein, however, PrP^{res} is not necessarily synonymous with PK^{res}.

Under the "gold standard" test of animal infectivity, either PrP^{res} or PK^{res} are identified via digestion of suspect diseased tissue via proteinase-K. Proteinase-K (PK) is a powerful protein-dissolving enzyme, active in strong detergents in a pH range of 2.0 to 12.0 (optimum pH: 7.5 to12.0) and, at the proper concentration has the ability to digest most proteins, both native and denatured, in generally less than 2 hours (McKinley, 1983). Only a few unusual proteins can survive PK treatment, with PK itself and PrP^{res} being the two most well known. Under a codified regimen of detergent dissolution and PK digestion, followed by Western Blot gel electrophoresis and immunostaining, a TSE-diseased tissue will reveal the residual presence of suspected abnormal prion protein (PrP^{res} or other PK^{res}) of a molecular weight, generally in the 20 to 35 kiloDalton (kDa) range.

PrP^{res} or PK^{res} determination may also use less-involved immunohistostaining or immunochemical techniques. Initially, under earlier TSE identification protocol, four selection criteria were used to identify TSE diseased tissue. Under the old four criteria system, the chemical immunohistoassays were considered as separate and distinct from the Western Blot test. However, given that immunochemistry is designed to specifically respond to PK^{res} or PrP^{res}, such a differentiation is unwarranted. While still regarded by some investigators as its own separate criterion, immunohistostaining (IHS) detection for the presence of PrP^{res} is a sub-type of the third criterion, that of PK^{res} identification.

Effectively, IHS is a micro-procedure whereby a chemical stain hitches a ride on a serum soup composed of poly- and/or monoclonal antibodies specifically created to react to or attach to the desired material, in this case the PrP^{res} (or PK^{res}) that may be present in suspected TSE-infected brains. Generally, it can be assumed that the response to IHS in TSE-infected tissue is, in fact, a response to the same PK resistant proteins identified in the Western Blot test for which the staining antibody was intimately created.

Recently, several new, more rapid, Enzyme-Linked ImmunoSorbent Assays (ELISA) have evolved around detecting the presence of PrP^{res} material. ELISA tests rely on the affinity of a contrived antibody or pool of antibodies together with a chemical marker to seek out and attach to the desired disease agent, in this case presumed to be PrP^{res}, making for rapid identification via visual or photometric means without excessive sample preparation. ELISAs have greatly enhanced the productivity of TSE diagnostic workers.

Prior to the year 2000, most TSEs were identified with the cumulative positive results derived from at least three of the four initial ID criterions. A bit subjective, it was generally accepted that three of the four criteria being positive would result in a positive TSE diagnosis. One or two positive results would suggest the presence of a TSE, but

would be relegated to a "suspect" status, in need of additional scrutiny.   The complete lack of any positive criteria was indicative of a negative response to TSE, and hence was reported as a negative. However, one must recognize that the lack of all four criteria does not necessarily eliminate a TSE disease, but in actuality represents only the failure to identify any outward TSE disease-related symptoms. In a known disease environment, a serious problem of eliciting false negatives can be present, particularly at the very early stages of disease, when infection is present but prior to the development of abnormal prions or PK^{res}. Ultimately, the failure to properly identify the putative TSE causal agent has rendered all TSE diagnostic procedures potentially fallible as they are seemingly focused on a prospective symptom of disease (prions) rather than the underlying causative agent.

PK Resistance

Bacterial Interference

The corner stone of current TSE identification lies with immunohistostaining and, now more recently, ELISA tests for the detection of PrP^{res} proteins confirmed by Western Blot, and followed up with microscopic tissue examination to identify SAFs and vacuoles.   Does this cornerstone and its subsequent building blocks form a reliable foundation upon which to gauge all TSEs?

No, unfortunately, like the TSEs themselves, the procedures used to evaluate TSE are far from well understood. The foundation of TSE detection, the presence of proteinase-K resistant proteins is thought to be a rare phenomenon, but unfortunately, it is not exclusive to TSE disease. Other natural processes can and do fabricate the elusive PK^{res}.

Butler (1991) found proteinase-K resistant proteins of 40 kiloDaltons (kDa) molecular weight in the outer membrane of a porcine pathogen, *Mycoplasma hyorhinis*. Additional non-immunological, cross reactive PK resistant proteins of 46 kDa were also found in *M. pneumoniae, M. orale, M. aginini* and *M. salivarium.*

At least one PK resistant protein at the 18 kDa molecular weight range and perhaps more bands of PK^{res} material of bacteriological origin was found in Leptospria by Nicholson and Prescott (1993).   Bastian, et al (1987) found that GT-48 stain of *Spiroplasma mirum* yielded PK^{res} protein bands of 28, 30, 66, and 76 kDa upon Western Blot analysis. The two lower PK^{res} bands mimic those considered diagnostic of PrP^{res} (prions) found in TSE disease. Not surprisingly, the ME-7 antibody used for the detection of SAF proteins in TSE-infected brains (Rubenstein, 1986) reacts positively against all four of Bastian's PK^{res} Spiroplasma proteins at 28, 30, 66 and 76 kDa (Bastian, 1987).   Brodeur (1997) found a proteinase-resistant, low molecular weight protein of 22 kDa in the outer membrane of *Neisseria mengingitidis*, the causal agent in some meningitidis diseases.

The seeming abundance of PK^{res} material, particularly in bacteria of the Class Mollicutes-Genera Mycoplasma and Spiroplasma, suggests that PK resistance maybe not be an unusual trait of Mollicutes or even other morphologically spiral bacteria.   Very few

studies have specifically attempted to find natural PK^{res} materials and Mollicutes like TSE disease are very poorly understood. The following is excerpted from Bove (1988) and from www.zmbh (2002) (see References)

> Mollicutes are bacteria with the smallest known genomes. This class presently comprises the six eubacterial genera Acholeplasma, Anaeroplasma, Asteroleplasma, Mycoplasma, Spiroplasma and Ureaplasma (however, the term mycoplasma has been frequently used to denote any species included in the class Mollicutes). The common characteristics are the complete lack of a bacterial cell wall, osmotic fragility, colony shape and filterability through 450-nm pore diameter membrane filters. The relatively close genetic relationship of these genera was measured by comparative sequence analysis of the 5S and 16S ribosomal RNA (rRNA). The rRNA sequence analyses also revealed that the Mollicutes are not at the root of the bacterial phylogenetic tree, but rather developed by degenerate evolution from gram-positive bacteria with a low mol% G+C (guanine plus cytosine) content of DNA, the Lactobacillus group containing Lactobacillus, Bacillus, Streptococcus and two Chlostridium species. The Mollicutes lost during the process of evolution a substantial part of their genetic information. This is reflected by significantly smaller genome sizes as low as 600 kbp and extending to 2300 kbp as compared with 2500-5700 kbp long genomes of their ancestor bacteria. The loss of coding capacity could probably be tolerated because of the parasitic life style of the Mollicutes. They have never been found as freely living organisms. In nature Mollicutes depend on a host cell, respectively on a host organism. For instance, Mycoplasmas and Ureaplasmas are parasites in different vertebrates, from which they obtain essential compounds such as fatty acids, amino acids, precursors for nucleic acid synthesis and cholesterol, a compound normally not found in bacteria

> Insect vectors transmit the plant pathogenic mollicutes. Many varieties of insects carry Mollicutes, particularly Spiroplasmas, and deposit these organisms on plant surfaces where other insects pick them up. New acholeplasma, mycoplasma and Spiroplasma species have been identified in insect hosts or on plant surfaces. Some Mollicutes are pathogens of animals, some produce diseases in humans. Mollicutes are also frequent contaminants of animal cell cultures.

Spiroplasmas, a division of Mollicutes, are not at all very well understood. These unique bacteria were discovered in 1972 when helical microorganisms were envisioned by phase microscopy from the sap of plants infected with corn stunt disease. The taxonomy of the genus *Spiroplasma* actually began in 1973, when Saglio, et al, published a description of the cultivated filamentous mycoplasma-like agent isolated from tissues of citrus plants affected with Stubborn Disease. Other Spiroplasmas, discovered earlier and studied under various aliases, then turned up. One was from *Drosophila* (sex-ratio organism) and

two were from ticks (strain 277F and the suckling mouse cataract agent "SMCA"). Of the all unnamed Spiroplasmas, only one (strain 277F) was readily cultivable but that particular one may have been originally derived from plant Spiroplasma (First Internet Conference on Phytopathogenic Mollicutes, Invited Lecture, *Spiroplasma* Taxonomy).

The proteinaceous makeup of Spiroplasma is critical for understanding of these unique bacteria. Wroblewski, (1977) noted that Spiralin protein of a molecular weight of 26 kDa is a major protein of the *Spiroplasma citri* membrane and speculated that spiralin-like proteins are probably present in all Spiroplasma. Le Henaff and Fontenelle (2000) determined that spiralin is a "classical" lipoprotein (i.e. is triacylated) and composed of acylated S-glyceryl cysteine.

Using antiserum, Townsend and Archer (1983) purified Spiroplasma proteins and determined that a single 55 kDa protein was unique to Spiroplasmas together with a lesser abundant protein in the 25 kDa range. Additional protease digestion of this exclusive 55 kDa protein yielded residual peptides of 27 kDa and 28 kDa while in particular the SMCA strain of *Spiroplasma mirum* cleaved to produce an additional 21 to 23 kDa resistant product. Townsend and Plaskitt (1985) found that spiralin protein of a 25 kDa mole weight was localized in the plasma membrane and as extra-cellular strands.

Clearly, spiralin protein, a major building block of Spiroplasma bacteria, has +/- 25-26 kDa mole weight protein, and at least a portion of which is PK-resistant protein of its own accord (Brenner, 1997). Additional PK-resistant band widths are found in numerous varieties of mycoplasma bacteria.

Since spiralin and perhaps other PK-resistant proteins are a major component of the Spiroplasma wall membrane, then selected *Spiroplasma spp*. can and do respond similarly to: 1) PK-resistant Western blots and 2) associated immunohistochemistry preformed upon TSE tissue. An affirmative ELISA response can be expected from spiralin, but is yet to be tested. Effectively, two of the four original TSE diagnostic criteria: PK resistance and positive immunostaining can be met by Spiroplasma as well as other mycoplasmas.

Scrapie-Associated Fibrils

TSE disease characteristics

Scrapie-Associated Fibrils (SAF) are archetypical of TSE disease.   Numerous studies have documented SAF presence, which has continually been re-confirmed in all versions of TSE disease. A chronological review of pertinent data is appropriate:

According to Merz (1981) abnormal fibril structures, were first observed using negative stain techniques in sub-fractions of brains from scrapie-affected animals. SAF was observed in all combinations or strains of scrapie agent, and strains or species of host examined, regardless of their histopathology, or, in particular, the presence or absence of amyloid plaques. SAF consist either of two or four filaments. They are morphologically

dissimilar to the normal brain fibrils--microtubules, neurofilaments, glial filaments, and F actin. However, SAF did bear a resemblance to amyloid.

Merz (1983) observed that the fibrils isolated from brains with senile dementia of Alzheimer type were 4-8 nm in diameter, narrowing every 30-40 nm and apparently composed of two 2-4 nm filaments. The fibrils from a Gerstmann-Straussler Syndrome brain (a variety of CJD) were 7-9 nm in diameter, narrowing every 70-80 nm and with a suggestion that they are composed of two 3-5 nm filaments. Fibrils isolated from 87V scrapie-affected mouse brains were 4-8 nm in diameter with a twist every 15-25 nm presumably composed of two 2-4 nm filaments. The fibrils from the scrapie brains were usually observed in pairs

Further, abnormal fibrils were observed in synaptosomal preparations of scrapie-infected brain, but never in control animals. SAFs are present in CJD brain fractions in the experimentally transmitted disease, as well as, in a few naturally occurring human cases of CJD. SAF are also present in spleen extracts of animals experimentally infected with scrapie or CJD. This close association of SAF with these two diseases and two different organ tissues (brain and spleen) known to contain titers of infectivity, suggest that the SAF are: (1) a unique pathological response to the disease or (2) the infectious agent of these diseases (Merz, 1983)

Diringer (1983) was able to fractionate fibrils from scrapie-infected golden hamsters visually similar to SAF discovered by Merz. Such fibrils under high magnification were found to be short rods composed mainly of two helically twisted filaments 50 to 300 nm long and 4 to 6 nm in width possessing a molecular weight of 26 kDa. Infectivity measurements were directly proportional to visual fibril density. Additionally, a scrapie agent infectivity size range of about 20 nm to 55 nm was found.

In 1985, Kascsak isolated and purified three different scrapie strains each having different morphology, sedimentation rate and protein composition. SAF from three scrapie agents were distinguishable from each other by their sensitivity to proteinase-K digestion. SAF co-purified with infectivity. SAF appeared to be a unique class of structures, which are related but specific for each individual scrapie strain.

Bode (1985) found, through a modified ELISA technique used on SAF proteins obtained from hamster, mouse, and from patients who died of Creutzfeldt-Jakob disease, that the antisera predominantly detected five bands in a Western blot analysis with apparent molecular weights of 26K, 24K, 20K, 18K and 16K. By gel electrophoresis these antigens seemed to be identical in mouse, hamster and man, but the amount of material in the various bands varied according to host or agent.

This time using 4 scrapie strains, Kascsak (1986) found that SAF proteins were antigenically distinct from those of paired helical filaments or amyloid isolated from patients with Alzheimer disease. Distinct Western blot profiles were demonstrated for SAFs isolated from animals infected with each scrapie strain. Differences seen among SAFs were independent, at least in part, of host species or genotype, implying that certain

specific structural and molecular properties of SAFs are mediated by the strain of scrapie agent. Bastian, (1987) found 4 to 5 nm width, fibrillar proteins in the synaptosomal-mitochondrial fraction of scrapie infected sheep brains arranged in twisted, intertwined collections.

Narang, in1988 and again in 1993 describes "tubulofilamentous' particles in CJD brains under electron microscopy. Sodium docecyl sulphate detergent treatment revealed twisted fibril cores, which were identified as scrapie associated fibrils. Treatment of the tubulo-filamentous particles with enzymes demonstrated that each larger tubule possessed an outer coat of protease-sensitive material, a middle coat of nuclease-sensitive material and an inner protease-resistant protein identical in morphology to the Merz SAFs.

Ruberstein and Merz (1991) found that SAF derived from scrapie-infected spleen tissue was virtually indistinguishable from infected brain tissue. Further, since brain tissue undergoes various gross microscopic thin-section degenerative changes while spleens do not, if the SAF was derived from the pathologic changes, then the SAF in spleen should have originated from the brain. However, the earliest detection and maximum yield of infectivity and SAF is found in the spleen although several hundred fold levels of SAF, abnormal PK^{res} and infectivity where later found in the brain. Hence, they concluded that SAF and abnormal PK^{res} appear to be a direct result of agent replication rather than pathologic changes.

Nanang (1992) speculated that PrP molecules aggregate to form SAF, which are wrapped around with a single stranded DNA, which then acquires an outer undetermined protein coat. The creation of SAF deprives the cell membrane of needed PrP eventually producing cell disruption.

Guiroy et al, (1993b) isolated abnormal fibrils in CWD-infected elk brain tissue. The isolate fibrils resembled those found in scrapie-infected hamster brains, were immuno-reactive to scrapie antibodies and possessed proteinase-K resistant protein bands of 26 to 30 kDa mole weight, confirming that CWD and scrapie were closely related TSEs.

Bacterial Interference

Fundamentally, the presence of SAF is intimately associated with TSE disease. While considered diagnostic, one must reflect on the nature of the fibrils and the procedures through which they are identified. Certainly the microscopic determination of their presence after detergent and proteinase digestion seems to be definitive for two sizes of particles, 1) fibers, tubules or rods from 20 to +100 nm in width, and 2) thin, sometimes helical filaments 3 to 6 nm in width. The SAF were not artifacts of preparation, but are affected by preparation actions. Interestingly, length appears to be a function of physical preparation procedures, the less sample homogenization, the greater the particle length, hence the particles can be broken apart by agitation. Additionally, chemical preparation also seems to affect particle width. The smaller particles generally appear after specific steps of sonic or detergent treatment, suggesting their release from within the larger width

particles.

Knowing the morphology and nature of SAFs, one can then attempt to find comparable natural structures, simply through comparative morphology. Are there any potential causative agents that could produce the presence of SAF within diseased tissue?

In the mid-1970's Spiroplasma, wall-free bacteria were first recognized as a newly discovered variety of Mollicutes, a cell-wall-less (membrane bound) parasitic bacterium containing the smallest of living, self-replicating genomes. Both Stalheim (1978) and Williamson (1974) found that osmotic shock and non-ionic detergents can induce a Spiroplasma cell membrane breakdown (normally +/- 100 nm width), which then release long, flexuous fibrils 3-4 nm in width, some helical in form. Williamson suggested the presence of 3 or 4 individual intertwined 4 nm filaments producing a 12-15 nm ribbon used for cell form stability. In 1979, Bastian compared photomicrographic images of spiral CJD inclusions with recently discovered Spiroplasma bacterial images, and discovered that the visual comparison was striking in both size and shape similarities.

Townsend in 1983 observed via thin section, and then proved via detergent cell wall breakdown that 80 to 100 nm wide *Spiroplasma melliferum* (BC-3, honey bee stain), when subjected to detergents and agitation released internal fibrils arranged in helically twisted ribbons 3 to 6 nm in diameter and that the fine fibrils were composed of 55 kDa mole weight protein together with a lesser 26 kDa protein. Bastian (1984) micrographs of *Spiroplasma mirum* (GT-48, tick strain) from pure broth cultures showed abundant bleb forms and occasional distinctive, and sometimes crescent, tubule-like organisms enclosing fine sinuous filaments.

Clearly, at least one natural agent, our ubiquitous Spiroplasma, has been found to create fibrils virtually identical to those found in TSE disease. As such, Spiroplasma has now been shown to: 1) contain 26 kDa PK-resistant proteins (responding positively to Western Blot tests), 2) responds affirmatively to IHC "prion" staining, and now 3) can form 4-6 nm fibrils. Three of the four TSE diagnostic criteria are met by Spiroplasma.

Neuropathology

The neuro-degenerative pathology of TSE is undisputed. All TSEs are neuropathologically similar. In particular, Chronic Wasting Disease like other TSEs is characterized by intraneuronal vacuolation, spongiform change of nerve tissue, and astrocyte (star-like cell) embellishment. Abnormal findings consist of (1) extensive vacuolation in neuronal processes, within myelin sheaths, formed by splitting at the major dense lines or within axons; (2) degenerated neurites (dendrites, axonal preterminals and sheathed axons containing degenerating mitochondria and pleomorphic, electron-dense inclusion bodies); (3) prominent flooding with astrocytes; (4) amyloid plaques; and (5) giant neuronal self-destructing vacuoles. Other findings include activated macrophages and occasional spheroidal structures containing electron-dense fibrillar material of unknown origin, abundant structures suggestive of degenerating microtubules entrapped in filamentous masses, vacuoles and myelin figures (Guiroy, 1993a). Liberski (1989)

noted that multiple forms of electron-dense inclusion bodies were found as early as 2 weeks post-inoculation of hamsters with 263K scrapie. Inclusion numbers increased with the incubation period, and their highest density was observed at the terminal stage of disease. A distinct feature of TSE disease is that subsequent passages of the disease through an identical host species, generally produces a more virulent, shorter-incubation pathogen (Hadlow, 1987).

Spongiform changes to brain tissue can be formed by several processes both pre- and post-mortem. Various neurological diseases, toxins, heavy metals, chemical excesses or deficiencies have all been implicated. While several potential pathogens can certainly create all or a variable portion of the symptoms described by Guiroy (1993a) and others, can these symptoms found in virtually all TSE disease be created specifically by Spiroplasma? The answer is affirmative.

Select versions of Spiroplasma have a distinct affinity for neuronal cells. Elizan, (1972) found that mice inoculated with *Spiroplasma mirum* (SMCA) developed prominent microcystic encephalitis localized in the subpial and subependymal zone and within deep gray matter with locally prominent astrocytes.

Tully (1982) inoculated suckling rats with either *Spiroplasma mirum* GT-48 or SMCA and found that a fatal dose due to septicemia approached 10^9 organisms for SMCA, but that lesser dose of 10^7 to 10^8 organisms showed a high incidence of ocular cataracts. The GT-48 strain required only 10^4 to produce pathological septicemia results. A third strain TP-2 initially mimicked SMCA but became more pathogenic for rats and showed less propensity for cataracts as it was passaged and purified in artificial media.

Utilizing rabbit-tick-derived strain, GT-48, Tully (1984) intracerebrally inoculated one-day-old neonatal rats. Rats receiving the largest challenge dose (300 organisms) in seven days had larger numbers (10^7 to 10^9) of Spiroplasmas in the brain and smaller numbers in the spleen (10^1 to 10^4). Since only two rats out of ninety receiving the 300-organism dose survived more than 14 days, apparently a lethal septicemia dose of GT-48 is easily attained. Those given an order of magnitude less (30 organisms) showed moderate to abundant brain Spiroplasma (10^4 to 10^9) at 21 days but a much lesser spleen presence and only a few infection deaths. Undoubtedly *S. mirum* is pathogenic.

In conjunction with Tully (1984), Bastian (1984) evaluated infected brain material of GT-48 inoculated rats. Histopathology at 14 days post intracranial inoculation revealed microcystic encephalitis with Spiroplasmas recognized as filaments, crescents and membrane blebs. Surprisingly, at and after 25 days post inoculation electron microscopy showed little inflammation, some neuronal vacuolization, but widespread dilation of neuronal processes and an apparent non-existence of detectable Spiroplasma organisms despite assay titers approaching 10^4 organisms per gram of brain material. Immunostaining using GT-48 antisera showed marked localization to superficial and deep gray matter (cerebral cortex and basal ganglia), yet no visible GT-48 organisms. Cerebellum staining was in close association with Purkinje and granular cells. Despite positive immunostaining Spiroplasma organisms were not detected in any +25 day

biopsies. The authors postulated that the membrane bleb form of Spiroplasma was cloaked within the host neuropil.

In 1987b Bastian demonstrated that intraperitoneal and subcutaneous inoculation with the vertebrate virulent GT-48 strain of *Spiroplasma mirum* alleviated short term mortality but produced alopecia (localized hair loss) and a reduction in body weight. Titers were found in both spleen and brain tissue, initially building up in the spleen but then later exceeding in the brain but without vacuolar encephalopathy as found in previous intracranial inoculation. A significant development of cataracts (15 out of 38 rats) both unilateral and bilateral appeared in contrast to prior studies where cataracts were not found with the GT-48 strain. The migration from peripheral tissue into central nervous system indicated that Spiroplasma GT-48 is neurotropic.

Humphrey-Smith and Chastel (1988) point out that while most Spiroplasmas are non-pathogenic, antibodies for some strains have been found in humans including at least one with amyotrophic lateral sclerosis, a neurological disorder known as Lou Gehrig's disease, which is linked with gene mutant copper-zinc dismutase enzyme toxicity.

Select strains of Spiroplasma are able to survive up to nine months in intracerebrally inoculated mice and are associated with significant runting syndrome and an increase incidence of mortality and neurological symptoms generally without the appearance of antibody mimicking and immunological tolerance (Chastel, 1991). Humphrey-Smith (1992) found distinct Spiroplasma mitochondrial pathogenicity in both *in vitro* and *in vivo* situations cultivated at 38°C.

In 1998, Kern, documented the only known case of a Spiroplasma infection of a human, describing the etiology of a rapidly progressing unilateral cataract associated with severe anterior uveitis (inflammation of the iris) in a 27th-week Caesarean-delivered, premature baby, then at 4 months of age. While cell cultures were negative, polymerase chain reaction ("PCR") and DNA sequencing identified a positive marker for Spiroplasma. Prior to birth, recurrent maternal vaginal infections presumably due to mycoplasmas were treated with erythromycin and the baby sustained a bout of pneumonia of unknown cause at age three months.

Bastian and Foster (2001) found the first direct evidence of an association of TSEs with a bacterium. Initially, three oligonucleotide primers specific to Mollicutes 16S rDNA were utilized via PCR and DNA sequence analysis to systematically study diseased CJD brain tissue and scrapie-infected tissue. Preliminary studies of two CJD-brains produced PCR products with 96% to 99% homology to *Spiroplasma mirum*, while control brains had no response. Additionally, and separately, various Mycoplasma and Spiroplasma controls produced recognized PCR products. Continued testing of refined primers toward a Spiroplasma model yielded no PCR products from Mycoplasma and a 276-bp PCR product in 7 out of 8 Spiroplasma species tested. A third CJD-brain tested using the new primers yielded a +99.5% homology to *Spiroplasma mirum*. A GenBank blast search of the product sequences failed to find any sequences over 20-bp in a human gene, hence the defined products were seemingly of non-human origin. Subsequently, sixty-five brains

(50 controls and 13 CJD positives) were subjected to the newly refined primers. All 13 positive brains produced the predicted 276-bp PCR products, while none of the 50 controls had a response, strongly suggesting the presence of foreign bacterial rDNA within diseased tissue.   That DNA signature was most assuredly Spiroplasma bacteria.

Five of nine scrapie-infected tissues had similar responses with at least two scrapie brains having a +99% homology with *S. mirum*. Normal sheep brains produced no products. The failure to identify 100% of the scrapie-brains was attributed to the diversity of the scrapie agent and the likelihood of over 20 individual scrapie strains.   Interestingly, according to USDA personnel one of the detected scrapie positives was very early in its incubation period (Bastian, PC, 2002).

Conclusively, Spiroplasma are neurotropic, have an appetite for sterol, an affinity for gray matter and have been positively identified in diseased brain tissue.   Several strains have a documented history of producing neuronal degeneration in vertebrates.   The neonatal human symptoms described by Kern (1998) mimic the conditions of SMCA-strain Spiroplasma infections in neo-natal laboratory animals, which show cataracts, posterior eye inflammation, encephalitis and death. The rat brain degeneration described by Bastian (1984) mimics the symptoms found in all TSE diseases.   Vacoulation in neuronal processes, mitochondrial degeneration, and variably shaped inclusion bodies are all possible products of Spiroplasma infection. Astrocytes have been documented. Only amyloid plaques have yet to be identified within Spiroplasma infections, however, one must recognize that Spiroplasma vertebrate pathology studies has only scratched the surface of possible disease manifestations. Conclusively, all four of the required TSE diagnostic criteria are capable of being met by Spiroplasma bacteria.

DISCUSSION

As demonstrated, all three of the three (or four of four) major criteria used for TSE identification, those in use by virtually all major TSE diagnosed labs is subject to error. All labs identify TSE disease by cumulatively: 1) defining the presence of PrP^{res} via Western Blot or IHC, 2) identifying SAF, and 3) observing spongiform vacuoles.   Each of the three individual criteria is subject to, and can be compromised by interfering agents Further, all criteria are effectively identifying middle to late-stage symptoms of TSE disease, hence, are incapable of detecting the presence of suspected disease prior to specific manifestation of disease symptoms.

The implications of such findings are somber. The inability to define disease at its early stages precludes these particular tests, as currently used, from effectiveness in a live-animal disease-monitoring program. A strong likelihood of high rates of false negatives at the initial stages of disease can be expected. Additionally, post mortem monitoring is potentially subject to grave errors derived from false negatives.   This is further compounded by occasional false positives from interfering elements.

The use of the term "Gold Standard" test to describe any specific, supposedly superior test regime for TSE's is almost farcical, and is most certainly misleading.   No test

currently being used for TSE diagnostics can be compared to any gold standard. One might actually conclude the use of the term "gold standard" test is to hide the facts of fallibility to the unsuspecting public.

Perhaps a short history lesson might be useful. Why is any test called the "Gold Standard"? Supposedly, this is a test with which to compare all other successive tests for accuracy and infallibility. A "gold standard" test is actually a veiled reference to the gold mine assay business, the author's prior expertise. The gold industry is an industry, which has been plagued by literally hundreds of poorly or hastily created gold assay procedures implemented over tens of decades. Some dubious assay procedures were created by well wishing individuals, others created by nefarious soles. The spurious results from these deviant procedures created many woes for companies and investors, wasting abundant time and resources. Assay results were not reliable, nor in most cases even reproducible. However, for centuries only one, tried and true assay procedure, invented some 500 years ago is still being used today, the gold "fire assay", i.e. the gold "Standard" test, to which all tests must be compared. A test, that is totally reliable and virtually infallible if performed correctly. Categorically, no such test exists for TSE disease.

While dozens of potential interference factors can affect any number of the diagnostic criteria, only two agents are known to positively express all four criteria. Abnormal prions are thought to be the common denominator, however, as documented herein, Spiroplasma has also been shown to: 1) contain 26 kDa PK-resistant proteins yielding positive Western Blot results, 2) respond affirmatively to PrP^{res} immunohistochemistry staining, 3) form 4-6 nm SAF-like fibrils and; 4) demonstrate vertebrate neurotropic and neuropathologic tendencies virtually identical to those expressed in TSE diseases. All four of the four TSE diagnostic criteria are met by Spiroplasma. Conclusively none of the TSE diagnostic tests are fully diagnostic only of TSE disease, but may, in fact, identify Spiroplasma bacteria or potentially even other unrecognized interfering agents. Much more analytical work will be necessary prior to any "Gold Standard" being met. Spiroplasma warrants much closer evaluation as a potential agent of TSE disease.

A follow-on paper: **Chronic Wasting Disease - A Logical Causative Agent, Part 1b** "**The Quest for an Agent**" will address detailed information regarding the explicit role of Spiroplasma in the development of TSE disease.

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