

CWD Tuberculosis Found in Spongiform Disease Formerly Attributed to Prions: Its Implication towards Mad Cow Disease, Scrapie and Alzheimer's

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Abstract


The TSE'S or transmissible spongiform encephalopathies, include bovine spongiform encephalopathy (also called BSE or "mad cow disease"), Creutzfeldt–Jakob disease (CJD) in humans, and "scrapie" in sheep or goats (caprine spongiform encephalopathy). They remain a mystery, their cause still hotly debated. Current mad cow diagnosis lies solely in the detection of late appearing "prions", an acronym for hypothesized, geneless, misfolded proteins, somehow claimed to cause the disease. Yet laboratory preparations of prions contain other things, which could include unidentified bacteria or viruses. And the only real evidence that prion originator Stanley Prusiner had in his original paper that the disease agent behind "Scrapie" in sheep and goats was devoid of DNA or RNA– was based upon the fact that he couldn't find any. Furthermore, the rigors of prion purification alone, might, in and of themselves, have killed any causative microorganism and Heino Dringer, who did pioneer work on their nature, candidly predicts "it will turn out that the prion concept is wrong." Roels and Walravens as well as Hartly traced Mad Cow to *Mycobacterium bovis*. Moreover, epidemiologic maps of the origins and peak incidence of Mad Cow in the UK, suggestively match those of England's areas of highest bovine tuberculosis, the Southwest. The neurotoxic potential of bovine tuberculosis has for some time been well known. By 1911 Alois Alzheimer called attention to "a characteristic condition of the cortical tissue which Fischer referred to as 'spongy cortical wasting' in Alzheimer's disease (AD). But behind AD, Fischer suspected a microbe called Streptothrix which was constantly being mistaken and confused for tuberculosis. Our present investigation of the TSEs clearly shows cell-wall-deficient (CWD) tubercular mycobacteria present, verified by molecular analysis, ELISA, PCR and microscopy to cause spongiform encephalopathy.

Keywords: Prions; Scrapie; The Spongiform Encephalopathies; Alzheimer's disease; The etiology of Alzheimer's Disease; *Mycobacterium tuberculosis* Complex

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Introduction

With the transmissible spongiform encephalopathies (TSE's), including "scrapie" and "mad cow" in animals– mental and physical abilities deteriorate and myriad tiny holes appear in the brain's cortex causing it to appear like a sponge (hence spongiform). Such "spongiform change" is caused by intracellular vacuoles in neurons and glia. Yet through eons of evolution and as a survival strategy it is through the formation of just such intracellular vacuoles that TB and the mycobacteria, unlike most pathogens, have survived,

adopted, and multiplied either in the macrophages of the body or the neurons and glia of the central nervous system [1-3]. The very same intracellular vacuoles that eventually lead to sponge-like changes in the brain form in microglia within a few hours of an infection with fowl tuberculosis (*Mycobacterium avium*) – which shifts its morphology from thick short mycobacteria to the longer filamentous forms so often documented in the spongiform encephalopathies [1]. And a similar scenario is operative for *M. tuberculosis* and other mycobacteria, all of which thrive in these vacuoles, possibly creating a reservoir in the brain through which additional microglia and neurons can be infected.

Today, the TSE's are thought to be caused by "prions" characterized by neuronal loss, gliosis, spongiform change and intracellular vacuoles in neurons and glia— yet these very same changes are instigated by tuberculosis and its related mycobacteria. Microglia internalizes virulent *M. tuberculosis* more rapidly and efficiently than less virulent strains. Upon internalization, tubercle bacilli are found in sparse, but densely packed vacuoles (**Figure 1**).

Clinically what is seen in all the spongiform encephalopathies is a rapidly progressive dementia, and their most common clinical presentation in humans is CJD or Creutzfeldt–Jakob disease as well as in certain cases of Alzheimer's disease. In the opinion of experts, ample justification exists for considering a similar pathogenesis for Alzheimer's, Creutzfeldt–Jakob, scrapie and the other spongiform encephalopathies such as Mad Cow disease. In fact, Creutzfeldt–Jakob and Alzheimer's often coexist and at this point are thought to differ merely by time-dependent physical changes. One study linked up to 13% of all Alzheimer's victims as really having Creutzfeldt–Jakob disease [4].

In 1909, Sir Stewart Stockman, Chief Veterinary Officer of the Royal College of Veterinary Surgeons, while investigating a disease of sheep in Scotland known locally as "scrapy" or "scrapie", found acid-fast bacilli characteristic of fowl or cattle tuberculosis in lesions of animals dying from scrapie [5]. Stockman will later be mentioned as one of the historical figures in the discovery of prion disease, but the fact that he found acid-fast bacilli in scrapie, a premier finding for tuberculosis, would not.

By 1911 Alois Alzheimer noted in some Alzheimer's cases "a characteristic condition of the cortical tissue which Fischer has referred to as 'spongy cortical wasting'." Alzheimer was referring to the finding of his great rival, neuropathologist Oskar Fischer of Prague. Fischer named and was one of the first to describe such spongiform cortical atrophy [6]. But Fischer was investigating such Alzheimer's brain changes in the context of being from a microbe called Streptothrix, which was constantly being mistaken for and confused with tuberculosis. And just ten years before Oskar Fischer found Streptothrix-like forms in Alzheimer's cerebral plaque, Babès and immunologist Levaditi reported that once inoculated with tubercle bacilli beneath the dura mater of their brains, rabbits not only developed branched filamentous forms similar to Streptothrix, but rosettes that were identical to the "drüsen" that Oskar Fischer repeatedly reported in Alzheimer's plaque [7].

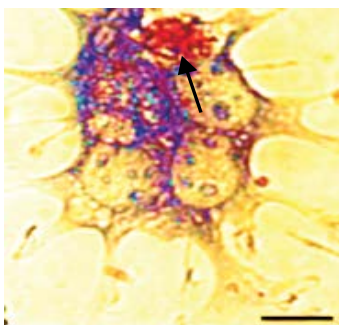


Figure 1 Microglia infected with *M. avium* mycobacteria are in one of the cytoplasmic vacuoles. (Arrow) Bar=5 μ m Ziehl-Neelson stain.

By 1936, George W. Dunkin reviewed Stockman's findings, confirming acid-fast organisms in the "scrapie" of sheep and goats. But unlike the bovine condition known as paratuberculosis (a subspecies of fowl tuberculosis or *Mycobacterium avium* found mainly in cattle), the acid-fast forms of TB confirmed by Dunkin in "scrapie" were longer and more slender than those encountered in paratubercular Johne's disease in cattle.

Not only is bovine tuberculosis (*Mycobacterium bovis* or *M. bovis*) capable of infecting all animals and men subject to the spongiform encephalopathies but by 1868 Jean-Baptiste Auguste Chauveau in France and Edwin Klebs in Prague proposed that eating infected meat could transmit bovine tuberculosis [9,10]. Subsequently Andreas Gerlach's experiments confirmed that either milk or flesh from tubercular cows could transmit tuberculosis to other animals to which they were fed [11]. Schliesser also saw meat from tuberculous animals as constituting a significant risk to humans if available for consumption [12]. With regard to bovine tuberculosis, anyone handling or consuming meat from an infected animal is at risk of contracting this disease. By the turn of the 20th century 25% of the many US deaths from TB in adults were caused by *M. bovis*.

While various theories continued to swirl around the cause of TSE's, the best epidemiologic maps of the peak incidence and prevalence of BSE or mad cow disease, done in the UK, it turns out, suggestively matched those of the highest prevalence of England's bovine tuberculosis in cattle, with a predominant distribution in the Southwest (**Figure 2**) extending to counties further north— the very area where BSE in the UK began [13,14].

The prion hypothesis

The TSEs or transmissible spongiform encephalopathies, are currently alleged to be caused by "prions." But the theory surrounding neurologist Stanley B. Prusiner's "prion", a word which he himself coined for gene-less proteins that were infectious, was under a rightful cloud of suspicion from its onset [15].

All living organisms, including germs, use their DNA and RNA to make copies of themselves and amplify themselves in the brains of those they infect. But prions supposedly did not have DNA or RNA. Therefore, Prusiner's theory was felt to be heretic, unorthodox, and contrary to accepted belief. And the only real evidence that he had in his original paper was that the disease agent behind "scrapie" was devoid of DNA or RNA— was that he couldn't find any. Not only is scrapie difficult to diagnose but it is always fatal, a degenerative disease that affects the nervous systems of sheep and goats. The name "scrapie" is derived from one of the clinical signs of the condition, wherein affected animals will compulsively scrape off their fleeces against rocks, trees, or fences. The disease apparently causes an itching sensation in the animals. Scrapie is the most common reportable disease in goats and sheep in the United States today.

Previous studies in which he had been involved in suggested to Prusiner that there may be similarities between the agents causing scrapie encephalopathy in goats and Creutzfeldt–Jakob disease (CJD), a rare but fatal disease in humans [16]. Yet Prusiner's initial 1980 scrapie paper, done with Hadlow, Kennedy,

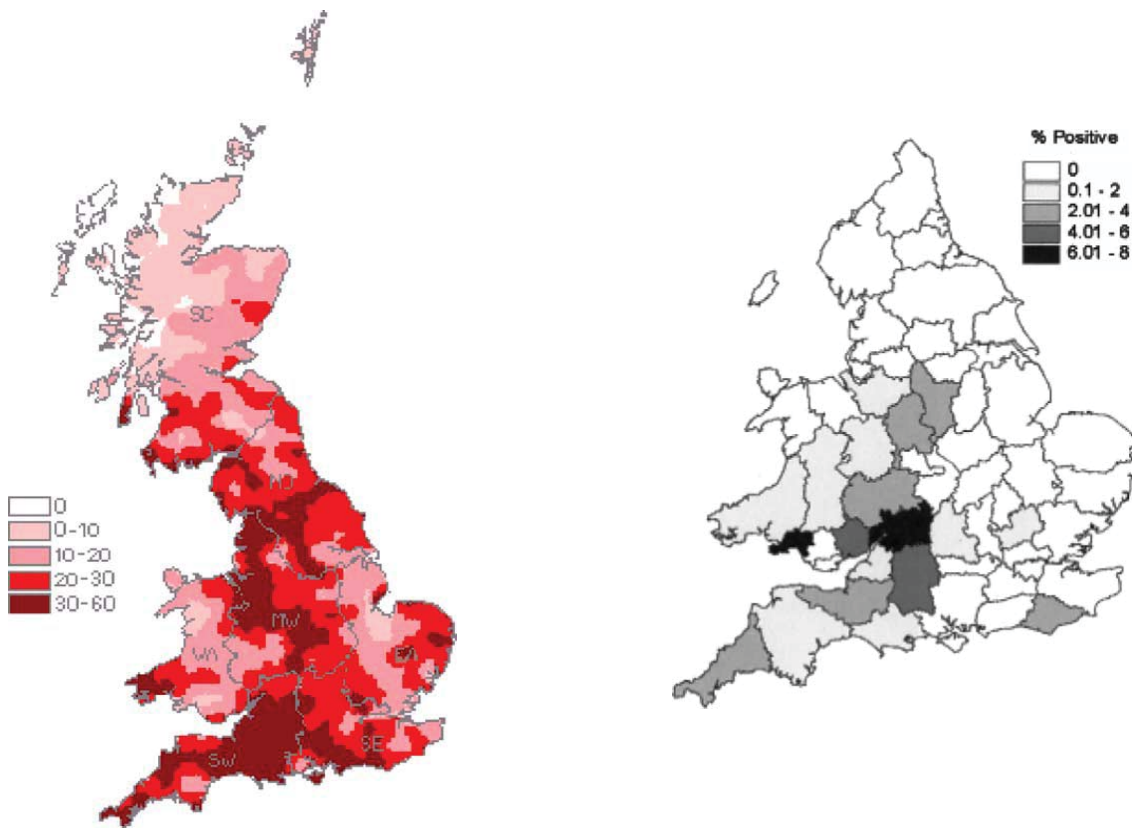


Figure 2 (Left) Mad Cow-positive (BSE) cattle in UK 1997; (Right) Bovine tuberculosis in UK 1999 — both with a predominant distribution in the Southwest, extending to counties further north, the very areas where BSE in the UK began.

and Race mentions absolutely nothing about prions, referring instead to “unconventional viruses”, “the CJD virus” and “the scrapie virus”.

All things considered, Prusiner’s idea wasn’t new. The thoughts of biologists in the 1930s who incorrectly said that viruses were only proteins and that ‘slow viruses’ might be gene-less had been proposed by and discarded in Britain by Griffith as early as 1967 [17]. To be certain, British mathematician J.S. Griffith was first to propose not only a self-replicating protein theory, but that proteins could be chemically identical but simply folded differently.

The finding that prions were proteins normally found in the body, including the brain of healthy controls, seemed to contradict the best evidence that they were infectious. The theory, like all questionable science, survived by finding a difference: prions from healthy animals were quickly claimed to be “cellular” protein; those from scrapie were “scrapie” protein. Scrapie protein aggregated into rods while cellular protein did not. Another so-called “critical clue” [18]: scrapie protein survived proteinase, while “cellular” did not. This still did not mean however that some virus or bacteria or mycobacteria did not cause the change being attributed to prions to begin with. Cow tuberculosis (*Mycobacterium bovis*), for example, both by virtue of its cell-wall-deficient, viral-like forms, and that it shares methyllysines with other mycobacteria is also protease-resistant [19]. Did this mean that tuberculosis was also geneless?

The amyloid proteins in Alzheimer’s, which as early as his 1982 Scrapie paper Prusiner was suggesting be linked to prions, were

also known at that time to be protease resistant, but not from prions [20].

By all logical estimates, the death-knell to the prion hypothesis should have occurred with Lasmézas’s 1997 interspecies transmission of mad cow in which more than half of injected mice had no detectable prions [21]. If this was not enough, then there was Baker and Manuelidis’s 2002 study on infectious neurons (microglia) with low prion levels in otherwise highly infectious material, which only served to support the concept that pathologic prions were the result of infection rather than being the actual infectious agent [22].

When Yale’s Laura Manuelidis and Sotirios Botsios revisited this subject in 2016 they outright stated that CJD and Scrapie require agent-associated nucleic acids for infection, therefore turning the entire original prion hypothesis on its head [23]. Manuelidis called for a re-opening of the entire prion concept to find out just which genetic material was involved, and its origin. To Manuelidis this was likely to be a virus, although she admitted the fundamental mystery remained. In fact, to many dissenters, some other, not as yet identified pathogen such as a virus, bacteria, or mycobacteria caused “prions” to misfold — thus damaging the brain.

Cell-Wall-Deficient Mycobacteria

Long before the prion theory was proposed, scientists searched unsuccessfully for the virus or viruses that might cause TSE, including Dr. Stanley Prusiner. And even today, some speak in

terms of the TSEs (transmissible spongiform encephalopathies) being caused by actively replicating infectious particles of viral-size and density. But “viral-sized” does not necessarily mean virus.

Tuberculosis is an infectious disease that is found throughout the world and which can have a dramatic impact on the economy and on human and animal health. In humans, the principal cause of infection is *Mycobacterium tuberculosis*, but the animal-specific pathogen, *M. bovis*, is arguably the most important zoonotic agent in human history [24].

Tuberculosis due to *M. bovis* has a worldwide distribution, and represents one of the most important public and veterinary health problems [25]. It is of greatest concern in cattle even though it can occur in humans and many domesticated and wild animal species including goats, swine and sheep. *M. bovis* is an important factor in human tuberculosis [26]. Furthermore, in at least one form of tuberculosis in sheep and goats — paratuberculosis, the wool or hair is often damaged and easily shed, not unlike in scrapie. Signs are rarely evident until two or more years after the initial infection, which usually occurs shortly after birth. And once symptoms appear, paratuberculosis is progressive and affected animals eventually die.

Of all the bacteria, the preferred and predominant form of the mycobacteria is their cell-wall-deficient “L” forms which are crucial to their survival in the body. And it is here that diagnosis becomes most problematic [27]. Mycobacteria are difficult to detect, especially in their viral-like, obscure state. Similar to prions, mycobacterial L-forms escape destruction by the body’s immune system, and are seemingly indestructible. Tubercular L-forms, no matter their exact mycobacterial species, are remarkably different from L-forms of other bacteria in their resistance to chemical and physical agents [28,29]. And the smallest and most resistant to such environmental stresses are their filterable tiny DNA containing L-granules — although other polymorphic viral-like and staph-like coccoid forms also form [30]. Yet genetic analysis by *16S rRNA* 16S ribosomal RNA confirms them as tubercular. Despite what is referred to as prions “heterogeneity with respect to size” — this is also a well-known characteristic of polymorphic CWD mycobacteria, with their diminutive L-granules mimicking resistant “small proteinaceous particles” in every way. So the fact that CWD tuberculosis is protease resistant or “resistant to inactivation by most procedures that modify nucleic acids” comes as no surprise without the need to label them “protease-resistant proteins.”

It is in the relatively dormant state of cell-wall-deficiency that the mycobacteria, not unlike prions, develop enhanced resistance to harmful factors and a long life— a survival strategy that has allowed the mycobacteria to become one of the most successful pathogens on the planet. But the downside is that such dormant cell-wall-deficient or “L-forms” also become among the most difficult to cultivate and identify, especially in their early non-cultivable or so-called “invisible” stage [31]. Therefore to find them in the living organism (*in vivo*) takes mandatory novel strategies including special growth techniques to enrich and revive them to an actively growing, colony-forming state. To Zhang this meant the resuscitation of dormant *Mycobacterium*

tuberculosis by phospholipids or specific tubercular protein peptides [32]. However, the same CWD growth stimulation can occur without the use of mycobacterial elements, such as the use of growth stimulants which create nutrient starvation or hypoxic conditions for *M. tuberculosis in vitro* [33]. With either method, CWD mycobacteria — otherwise non-cultivable, will grow, often transitioning from ovoid or egg-shaped to rod shaped cells. In fact, the recovery of the ability of mycobacteria to cultivate has traditionally been associated with the formation of the same rod-shaped, filamentous and granular forms that make CWD tubercular forms indistinguishable from prions [34]. We used a mycobacterial Cell-Wall-Deficient growth stimulant without any mycobacterial components.

Amyloid: the common denominator in all Spongiform Encephalopathies

“It is an astounding finding, because we never would have dreamed that amyloid and prions are the same”, proclaimed Stanley Prusiner [35]. But previous to this and throughout the history of medicine amyloid was usually considered the deposition that took place due in the course of chronic inflammatory disease (with genes), mainly tuberculosis— its usual precipitating cause.

Hass’s study at Cornell proved a direct correlation between amyloid deposition and the mycobacteria by injecting *M. bovis* into rabbits and following *M. tuberculosis* in humans. He concluded that the only infectious disease which served as an apparent cause of amyloidosis was tuberculosis [36]. All of the 21 human subjects with amyloid in Hass’s investigation had chronic pulmonary tuberculosis.

In addition, in a 50-year study based upon autopsy, Schwartz saw amyloidosis, primary and secondary, in the brain and elsewhere as a by-product of underlying infectious tuberculosis, either reactivating itself or being reactivated by a host of traumatic, chemical, biologic or physical insults [37]. Microscopically, in the brain, Schwartz found plaques and amyloid degeneration of nerve fibrils. When Schwartz injected 22 guinea pigs with *M. tuberculosis*, all but four came down with amyloidosis. His uninfected controls, with the exception of one, showed no amyloid. He thereby confirmed Hass, whose large series of rabbits showed that three out of four inoculated with bovine tuberculosis had amyloid disease within 15 months [36]. Hass’s amyloid uniformly showed a principal protein fraction.

The amyloid issue surfaced again when in 1978, Researcher Pat Merz, in breakthrough work, identified tiny fibrils in the brains of scrapie infected mice not present in well controls. Prion purists refused to admit that their prion rods were related to Merz’s find, citing her entities as longer fibrils and claiming that Merz stated plainly that her scrapie-associated fibrils (or SAF) were not amyloid and therefore could not be prion rods — the term Prusiner used for amyloid fibrils. Actually Merz said that her scrapie associated filaments were amyloid-like on more than one occasion and other workers in the field suggested that the two entities in Merz and Prusiner’s papers were identical [15]. Delgado saw such fibrils in either case as typical features of amyloid [38].

Meanwhile, by 1994, de Beer, studying the relationship between a major rise of serum amyloid and having tuberculosis, saw a

rapid descent in amyloid in patients treated with anti-tubercular drugs [39]. As an offshoot of de Beer's work, Tomiyama dissolved β -amyloid plaque with rifampin, a first line drug in the treatment of TB, and one of the few agents, to this day, that is able to dissolve amyloid plaque [40].

Whether in humans or animals, it is impossible to address tuberculosis without speaking of its cell-wall-deficient or "L-forms" which immediately start to form in the body's macrophage and microglial defenders once under attack and continue to propagate thereafter [41-43].

The purpose of this work was therefore to establish whether central nervous system CWD tubercular infection could result and account for in the clinical and histopathological changes that we associate with transmissible spongiform encephalopathies (TSEs).

Materials and methods

Transmissible Spongiform Encephalopathy

Fragments from the brains of two goats with advanced clinical damage to the central nervous system and clinical signs of scrapie were fixed in 15% formalin (1 month). Laboratory diagnostics already performed had ruled-out rabies or listeriosis. Brain fragments, after being washed with formalin in sterile water were homogenized and incubated on a mycobacterial growth stimulant (1:4) containing 0.1% chlorhexidine for 48 hours, allowing for the growth and eventual isolation of cell-wall-deficient (CWD) tubercular/mycobacterial forms [44-46]. This was then planted in test tubes with a solid mycobacterial nutrient media containing agar, asparagine, peptone, glutamate, and the elements K, Mg, Zn, Mn, Fe and S and P and incubated at 37°C for an additional 10 days. A repeat observation and mycobacterial probe was conducted after 5 years of preservation in formalin in Brain 1 and after 3 years in the case of Brain 2.

Microscopy

Histological sections of the cortex and other sites of the brain were prepared by standard technique and stained with Hematoxylin-Eosin stain, Ziehl-Neelsen, and modified immunoenzyme technique (MIET) [45,47]. MIET staining procedure included fixing at 65°C for 2 hours followed by the suppression of endogenous peroxidase with 3% H₂O₂. Staining by Kinyoun carbol-fuchsin was done for 5 minutes, followed by decolorization with 3% acid alcohol for 70 seconds, counterstained by 0.3% methylene blue for 2 minutes. Further conjugates of affinity purified antibody to *M. bovis* were drawn and then placed in Phosphate-buffered saline (PBS) with 0.02% Tween 20 for 1½ hours followed by emersion into a substrate solution consisting of 10 mg. 3,3'-diaminobenzidine (Fluka Sigma-Aldrich) in 2 ml of DMSO and 10 ml of deionized water with 10 μ l of 33% H₂O₂ for twenty minutes.

At each stage, smears were washed with deionized water with 0.02% of Tween 20. MIET (modified immunoenzyme technique) stained slides showed either classical acid-fast (AF) tubercular bacilli stained in red, or non-acid-fast (NAF) cells or antigens to mycobacteria in brown. Other microflora and tissue cells were stained blue. Results of microscopy were recorded using an Olympus 51BX microscope.

Cell-Wall-Deficient (CWD)

Tubercular reference standards used included isolates from the 1st International standard PPD for *Mycobacterium bovis*, isolates from the blood of a TB patient, isolates from the lymph node of a tubercular cow, isolates from embryonic (fetal) bovine heart tissue, and CWD forms of *M. bovis* BCG and *M. bovis* Vallée. That all CWD isolates were from the genus Mycobacteria was confirmed both by the existence of common antigens with reference strains of *M. bovis*, *M. avium*-paratuberculosis complex and *M. tuberculosis* as well as PCRs with primers for 16S rRNA, MPB70 – a major protein antigen of *Mycobacterium bovis*, and primers for *M. avium*-paratuberculosis complex.

SDS-PAGE electrophoresis and Western blotting

Mycobacterial CWD isolates were washed three times with a 0.5% phenol solution suspended in stock buffer and warmed for 5 minutes at 99°C. Lysates of isolates were separated by SDS-PAGE electrophoresis in 15% of PAGE (Laemmli, 1970).

Polymerase Chain Reaction (PCR)

The identification of isolates was carried out by 16S rRNA mycobacterial primers, MPB70 complex tuberculosis-bovis primers and primers for *Mycobacterium avium*-paratuberculosis complex (Pramytech). Suspensions of isolates in lysing buffer were warmed for 5 minutes at 95°C. The DNA was isolated on columns with a sorbent (IBOH NANB) and amplification was carried out on a C1000 Touch™ Thermal Cycler (BioRad) according to standard protocols. An electrophoresis of amplicates was then carried out in 2% agarose gel and results obtained from a Molecular Imager® GelDoc™ XR+system. (BioRad).

ELISA

ELISA of the sonicate isolates from both brains were done with the rabbit Antisera to sonicates of *M. tuberculosis* H37Rv, *M. bovis* Vallée and *M. avium* 1603.

Results

Spongiform changes found in tubercular cortex

Multiple brain thin cuts were performed at the level of the cerebral cortex and the pons in two tubercular goats which before dying demonstrated the clinical signs of advanced transmissible spongiform encephalopathy (scrapie). Spongiform changes caused by intracellular vacuoles, accompanied by thrombotic vessels, proliferation of cerebral vessels, perivascular cuffing, and lymphocyte infiltration were all in evidence. In addition to porous spongiform changes there was also a proliferation of heavily vacuolated glia (**Figure 3**).

Cell-Wall-Deficient tubercular forms

At microscopy, some of the smears of a brain stained with Ziehl-Neelsen (ZN) stain yielded various cell-wall-deficient mycobacterial forms. Among these were partially acid-fast spherical shapes (**Figure 4a-4c**) which were greater in number in Brain 1, and rarer in Brain 2. In one such case, a partially acid-fast sphere (spheroplast) is actually seen connected to a partially acid-

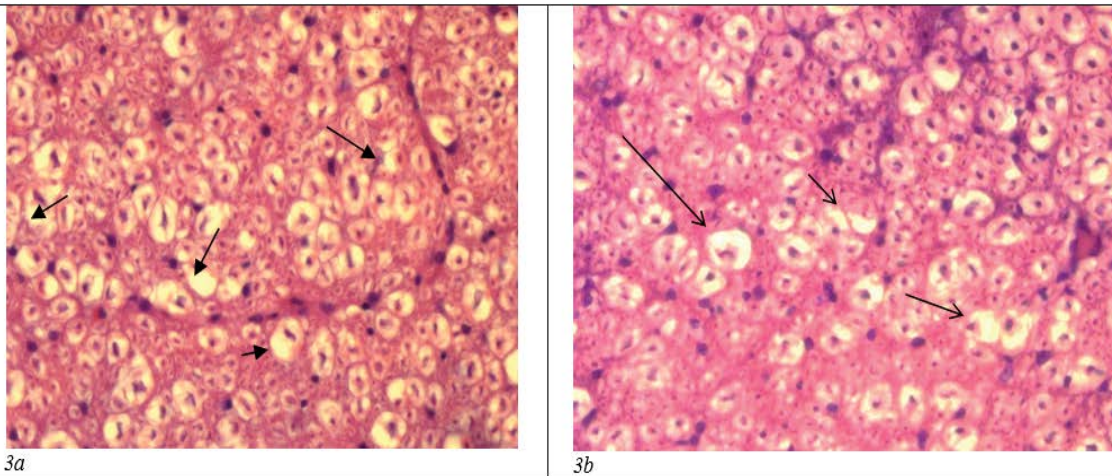


Figure 3 Thin histologic slices from both brains. (a) from Brain 1. (b) is from Brain 2. Sponginess in both brains noted. Haematoxylin-Eosin, 10X 100.

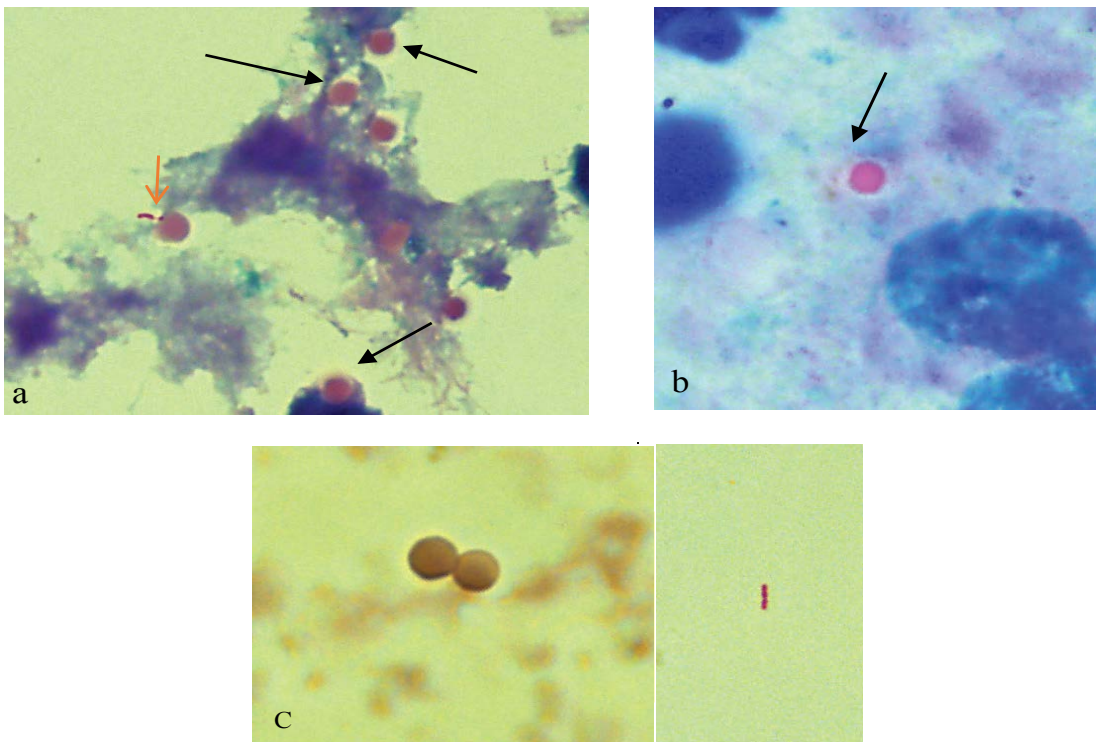


Figure 4 (a) In brain 1, there were red spherical shapes (black arrows). Also (red arrow), a red rod was connected with one of the spheres, Z-N, 10 × 100; (b) Is from brain 2, with a similar spherical shape Z-N, 10 × 100; (c) From brain 1, shows brown (non-acid-fast) mycobacterial spherical shapes and a red-brown (partially acid-fast) rod, MIET, 10 × 100.

fast (red color) rod (pink arrow in **Figure 4a**). MIET stained these spheroplasts brown, indicating non-acid-fast (NAF) mycobacterial forms (**Figure 4c**). Also seen in **Figure 4c** is one of the occasional acid-fast rods seen (**Figure 4c**).

MIET staining of brain specimens also revealed brown rods (**Figure 5**). Such rods were more prevalent in Brain 2. The rods found in both brains had almost identical morphology (**Figure 5**); as well as having a double rod configuration of similar morphology. Double rods with similar morphology were also found in tubercular

embryonic (fetal) cardiac smears obtained from a tubercular cow (**Figure 5c**).

Antibodies to *M. bovis* reacted with autopsied tissue from both animals with advanced scrapie, making possible the visualization of other commonly encountered tubercular cell-wall-deficient (CWD) forms and mycobacterial antigens. In **Figure 6a**, the white arrow points to a neuron while various CWD non-acid fast mycobacterial inclusions (brown) in the cytoplasm can be seen in **Figures 6a-6e**. Such brown inclusions in the cytoplasm (**Figures**

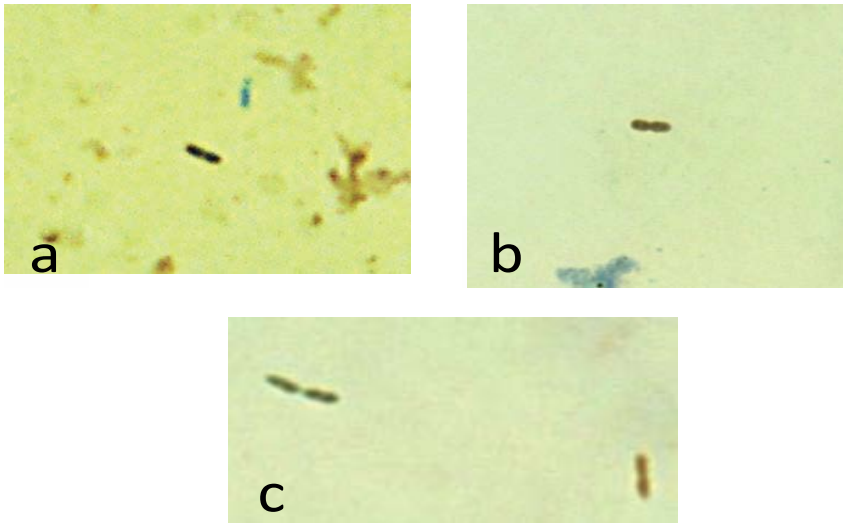


Figure 5 (a) – from brain 1, (b) from brain 2, (c) embryonic (fetal) cardiac tissue from a tubercular cow. Brown “double” rods, of identical morphology, all fields. MIET, 10 × 100.

6a-6e) are often surrounded with a brown web-like syncytial network (6e). **Figure 6b** shows brown fibrils similar to scrapie-associated-fibrils (SAF).

Fragments of brain tissue were washed in sterile water and homogenized. Upon PCR, primers for both 16sRNA and bovine tubercular MPB70 gave positive reactions (**Figures 7**).

The rods of bovine tuberculosis are often spindle-shaped. After repeated subcultures, the color of the cells maintained a green shade that is connected with the weak synthesis of tubercular antigens. Isolates from both scrapie brains were polymorphic, yet shared common forms between themselves and CWD tubercular isolates from other sources. In smears, it was not unusual to see CWD mycobacterial forms in the same field that were long and short bipolar rods, thin granular rods, thick “empty” rods, and spindle-shaped forms (**Figure 8**). These same forms were found also in CWD isolates from tuberculin PPD [44], as well as from humans and cows sick with tuberculosis [44]. Very characteristically, as seen in **Figure 8**, are the forms of two rods seemingly bound together, with a septum in between – a configuration found in isolates from our brain segments, the tuberculins, and the blood of a tubercular patient, possibly representing a process of division. Such tubercular septation can create two daughter cells [48,49]. Again, the rods of the bovine tubercular bacillus are often spindle-shaped (**Figure 8**).

With SDS-PAGE electrophoresis, both brain isolates had identical polypeptide profiles (**Figure 9**, lane 1 and 2) which were, in turn, almost identical to both the profile for PPD tuberculin PPD (Lane 3) and that of a CWD *M. bovis* isolate (Lane 4).

In addition, Western blot revealed three amino acid (40-70 kDa) fractions from brain isolates reacted with antiserum to *M. bovis*, as well as similar fractions of isolates from standard PPD tuberculin of *M. bovis* (**Figure 10**). Similar triple fractions were also found in a typical MTB sonicate.

Upon ELISA, isolates from both brains reacted to the antisera of *M. tuberculosis*, *M. bovis* and to a lesser extent *M. avium*

(**Figures 11 and 12**).

The subsequent formalin-resistance of the CWD tubercular forms taken from our brain specimens, even after 3 to 5 year exposure to 10% formalin were of particular interest. Brain samples (3-4 Gms) taken from formalin solutions under sterile conditions were placed in 100 ml of sterile water. These were then homogenized in 100 ml of sterile water. After 12 hours these were again homogenized with 10 ml of mycobacterial growth stimulant, incubated for 48 h at 37°C, and a 300 µl aliquot of this was then planted in test tubes containing slanted semisolid mycobacterial growth medium. These test tubes were incubated at 37°C and after two days, smears from both specimens began to yield growth but visible colonies were not present. But under blind subculture at 24 hour intervals both series (Brain 1 and Brain 2) yielded small, transparent, merging colonies, the smears of which showed the same polymorphic rhabdoid spindle-shaped forms (**Figure 13a**) designating bovine tuberculosis as originally seen. And despite the large time interval of 3 to 5 years separating these assays from those of the original, they still reacted with the antibody of *M. bovis*, and the same mycobacterial PCR primers (**Figure 14**) and gave the characteristic brown-color for non-acid-fast bacilli with MIET stain.

Discussion

Spongiform and other pathological changes similar to BSE (Mad Cow Disease) were found in goats with the clinical symptoms of scrapie, who fell ill with signs of a central nervous system lesion in their brain. Yet in assays of both experimental animal’s brains, partially acid-fast tubercular cell-wall-deficient (CWD) spheroplasts, rods, and other mycobacterial CWD forms — along with the existence of common tubercular antigens were found [50]. Other known infectious diseases yielding similar brain lesions were excluded.

Besides this, net-like structures reacted with the antibody to *M. bovis* which were either the result of tubercular antigens or actual CWD tubercular forms developed a net-like syncytial structure

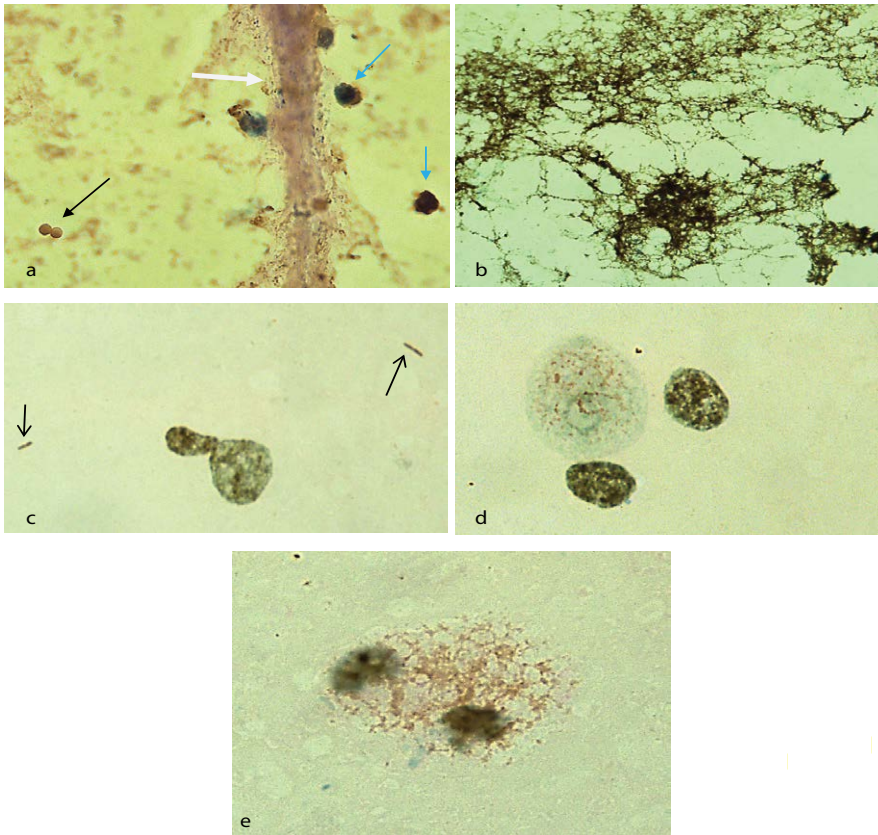


Figure 6 (a) is from brain 1. The long brown structure (white arrow) is a neuron, while the black arrow points to two non-acid fast brown spheroplasts. Also present are two large CWD mycobacterial forms with dark brown cytoplasm (blue arrows). (b) shows scrapie associated fibrils in brain 1, which also proved to be tubercular on molecular analysis. (c) From Brain 2 shows brown tubercular CWD forms infiltrating two cells as well as two brown rods (black arrows). (d) From Brain 2 shows the same brown NAF tubercular inclusions. Also from Brain 2, (e) shows 2 large cells surrounded by a web-like syncytial structure, again showing signs of mycobacterial infiltration. MIET, 10 × 100.

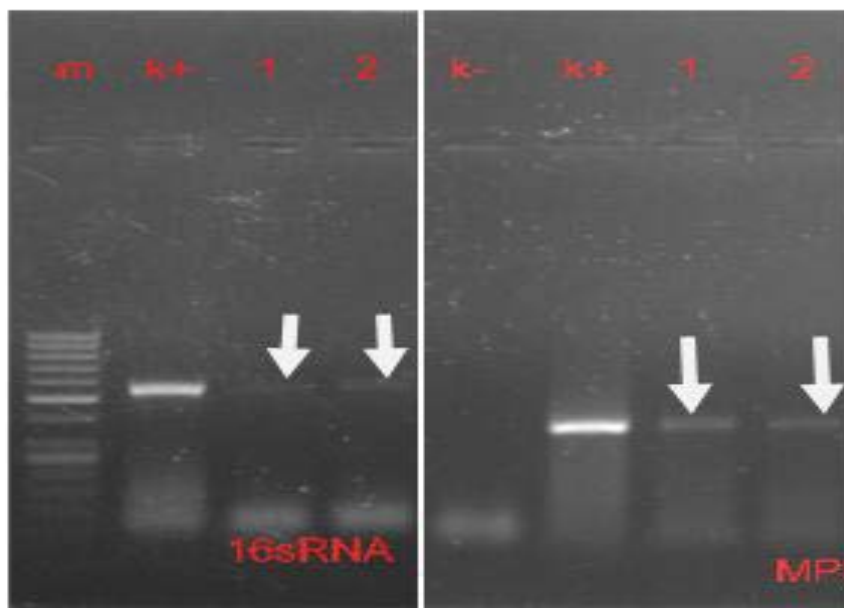


Figure 7 PCR of DNA from homogenates of Brain 1(1) and Brain 2 (2). k – Negative control, k+ positive control.

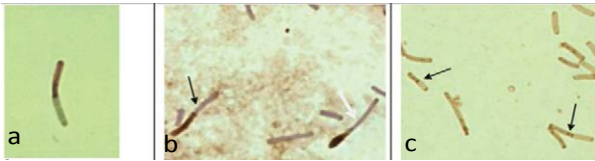


Figure 8 (a) An isolate from a Brain 1 showing a partially AF rod (red-brown color) shared with formation of a NAF rod (brown color). Cells with similar morphology are found in isolates from PPD tuberculin (b), and from the blood of a TB patient (c) black arrows. (b) shows the formation of a rhabdoid bipolar rod forming a spindle-shaped cell (white arrow).

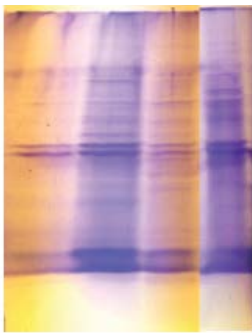


Figure 9 SDS-PAGE electrophoresis 15%: (1) Isolate from Brain 1. (2) Isolate from Brain 2 (3) Isolate from the PPD tuberculin, (4) CWD *M. bovis* BCG.

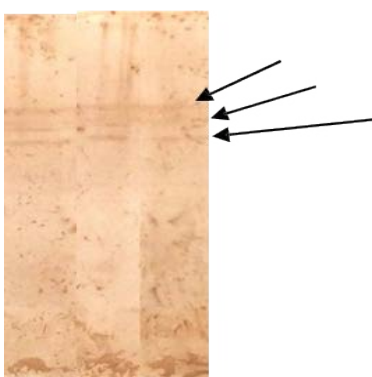


Figure 10 SDS-PAGE electrophoresis 15% and western blot with bovine antiserum to *M. bovis* (1) Isolate from Brain 1, (2) Isolate from the brain 2. (3) Isolate from PPD tuberculin.

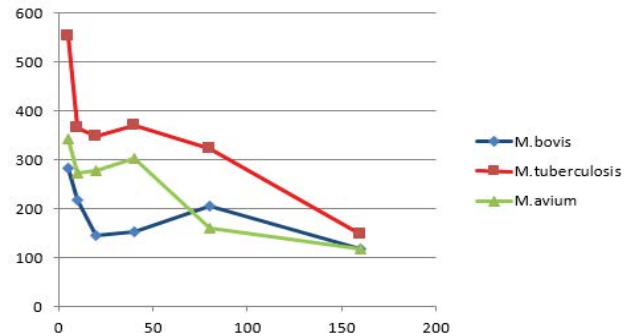


Figure 11 ELISA of sonicates of the isolates from brain 1 with antiserum to *M. tuberculosis* H37Rv, *M. bovis* Vallée, and to *M. avium* 1603. Abscissa axis - dilutions of antisera, ordinate axis - OD 450 nm.

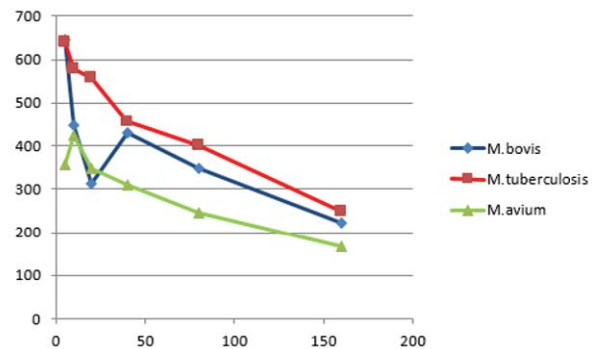


Figure 12 ELISA of sonicates of the isolates from brain 2 with antiserum to *M. tuberculosis* H37Rv, *M. bovis* Vallée, and to *M. avium* 1603. Abscissa axis - dilutions of antisera, ordinate axis - OD 450 nm.

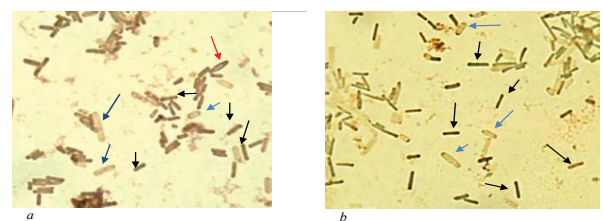


Figure 13 (a) Growth of seeds of Brain 1, (b) Growth of seeds of brain 2. Long and short bipolar rods, sometimes with granules (black arrows), thick "empty" rods (blue arrows), In (a) a spindle-shaped form (red arrow). MIET, 10 × 100.

animals with scrapie were caused by acid-fast or partially acid-fast tubercular bacilli [5,8].

similar to scrapie associated fibrils (SAF). Yet on the grounds of morphology, partial acid-fastness, the existence of antigens in common with MTB, and PCR/ELISA results, it was obvious that again, we were dealing with cell-wall-deficient and classical tubercular forms. Furthermore, our results simply confirmed studies done at the turn of the 20th Century, first by Stockman and later by Dunkin that spongiform changes in the brains of

Natural transmission of scrapie in the field seems to occur via the alimentary tract in the majority of cases, and scrapie-free sheep flocks can become infected on pastures where outbreaks of scrapie has been observed before. These findings point to a sustained contagion in the environment, notably in the soil [51]. Detectable "infectious" prions (PrP^{Sc}) have been reported in the feces of sheep both in the terminal and the early preclinical stages of the disease, suggesting the prions were likely to be shed into

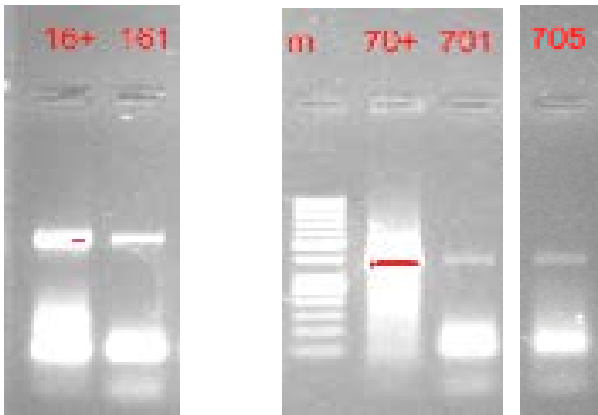


Figure 14 PCR with DNA of isolates from brain. (16+) positive control for primers 16 s RNA, (161) from Brain 1 (growth in a test tube No. 1), (165) from a Brain 2 (growth in a test tube No. 5), (70+) positive control for primers of MPB 70, (701) - isolate from Brain 1 (growth in a test tube No. 1), (705) from Brain 2 (growth in a test tube No. 2).

the environment throughout the course of the disease. But there is the precise same ability for transmission with tuberculosis and the mycobacteria through the alimentary tract as well as the sustained contagiousness of the *Mycobacterium tuberculosis complex* and related mycobacteria in the soil [52].

The length of time it takes for sheep or goats to develop scrapie is usually 18 to 24 months, a slow course which is not unusual in the progression of CWD tuberculosis. By 1996, Insanov warned that an infection with cell-wall-deficient forms of TB in children not only made standard treatment less effective but created a disease with a gradual, insidious onset and a slow accumulation of cerebral damage [53]. This made CWD tuberculosis more difficult to diagnose, because its slow burn into nervous systems, allowed months to years before its full spectrum of damage became obvious. Yet in this same study Insanov also showed that cell-wall-deficient forms in tuberculosis meningitis accounted for an incredible 87.6% of the tuberculosis found in children with TB meningitis and 87.3% of cases of TB meningitis in adults.

The original cause of cerebral tuberculosis, as well as other forms of the disease, can be as a result of exogenous infection or because of the activation of a latent tuberculous infection that crossed the placenta. We found evidence for this placental cross when the same tubercular rhabdoid, rod-like forms in our scrapie victims also appeared in the embryonic fetal tissue from a tubercular pregnant cow (**Figure 5**). In addition the lack of characteristic lung and lymph node involvement in our subject animals pointed towards a more systemic trans-placental infection.

Once a pathogen like tuberculosis has its cell wall disrupted, it become plastic, having the ability to assume many forms. Some are so tiny that they pass through a 22- μm filter, the so-called “viral stage” of a bacteria or mycobacteria. It is known that CWD TB can form various forms, including those with a size less than 0.22 μm (millimicrons) [33,54]. Because of this Cell-Wall-Deficient forms of MTB can penetrate not only the placenta but the blood-brain barrier, and get into the brain. Calmette noted that after

such infection with cell-wall-deficient tuberculosis, quick fetal death could occur. But even if the child were born alive, with no apparent ill-effects [55], their disease almost invisible – such stealth CWD forms of their dormant tuberculosis could spring back at any time to the diseases virulent forms.

Coming as no surprise, a similar and parallel failure to detect “prions” in the uterine and gestational tissues from a gravida with Creutzfeld-Jakob Disease has been reported (**Figure 15**) [56].

Brieger, working at Cambridge University, demonstrated mainly pleomorphic stealth growth when he inoculated tuberculosis directly into the amniotic sac of lab animals [57]. The outer layer of the amniotic sac is part of the placenta. Rapidly, cell-wall-deficient granules formed that did not stain with traditional acid-fast stain. Within three days, other cell-wall-deficient forms such as the long branching fungal filaments of TB appeared – easily mistaken for so-called “Scrapie Associated Fibrils” (SAF). Brieger repeated the study using fowl tuberculosis in chicken embryos. His results were similar [58]. Cell-wall-deficient forms appeared, again not identifiable by traditional stains or cultures.

Tubercular cell-wall-deficient forms, such as we isolated in scrapie are easily as resistant and long lasting as prions. Yet their proper isolation requires special mycobacterial growth stimulants and nutrient mediums [44-46]. The mechanism for the viability of tubercular CWD forms, even in the face of otherwise lethal thermal and chemical assault is a matter of active research, but just how this happens is still as yet undetermined. Our brain samples, which yielded such forms easily, withstood 3 to 5 years of storage in 10% formalin, comparable to the vaunted viability of prions. The formalin and high heat issues are no small part in the history of prions. Prusiner cited his predecessor, UK veterinarian Lain Pattison throughout his works saying at one point that Pattison was one of the first to suggest that the “scrapie agent” might be a basic protein “based on the results of his attempts to purify the infectious pathogen” [59]. Actually during the 1960s Pattison, even before mathematician Griffith, was astonished by the scrapie agents surprisingly high resistance to formalin as well as high heat [60] – properties which he and his collaborator



Figure 15 The emergence of a typical, familiar classical tubercular rods (arrows) from STEALTH pleomorphic (multi-shaped) cell-wall-deficient tubercular growth [50].

Katherine Jones were certain that were closely similar to those of 'encephalitogenic factor', which Pattison believed to be 'a basic protein' [61]. But in reality, studies performed since Pattison suggest that formalin also does not effectively inactivate *Mycobacterium tuberculosis* or related mycobacteria in formalin-fixed tissue [62,63] – certainly reflected by the fact that our brain samples continued to yield CWD tubercular forms – easily withstanding 3 to 5 years of storage in 10% formalin.

As life expectancy increases, with waning immunity, so too does the probability of acquiring an active TB infection of the CNS and elsewhere. Therefore Alzheimer's most comprehensive 1911 paper was entitled: "*On certain peculiar diseases of old age*" [64]. Although Alzheimer's disease, like Parkinson's disease, is characterized by extracellular A β plaque formation and intraneuronal tau protein inclusions – there is significant hesitancy to use the term "prion" to describe their occurrence. This is primarily because in contrast to CJD and Kuru, the transmission of Alzheimer's and for that matter Parkinson's has not been demonstrated between individuals and most experimental prion work on this subject have used transgenic animals that overexpress disease protein [65]. Transgenic mice, which have a foreign gene deliberately inserted in their genome, overexpress amyloid beta protein and are an incomplete and limited model for Alzheimer's disease [66]. On the other hand tuberculosis and in particular its CWD forms were an integral part in conceptions regarding Alzheimer's from its inception to the present. By 2013 Mawanda and Wallace pointed to two prime suspects for Alzheimer's amyloid-beta deposition: "especially

chronic infections like tuberculosis and leprosy" [67].

Recently, Alteri showed that during active infection, *M. tuberculosis* produces outer fringelike bacterial amyloid structures called "fimbriae" or "pilli" on its surface that also bind Congo red dye, a property associated with human amyloidosis [68]. Jordal reconfirmed that mycobacteria like TB produce a level of amyloid not previously described for any other bacteria. Not only was amyloid found present in tubercular mycobacterial biofilms, but in the actual cell envelopes of the mycobacteria, as well as coating the spores they produce [69].

There is no known disease which better fits into what is occurring in Mad Cow, scrapie and the rest of the spongiform encephalopathies – including its human counterparts than the *Mycobacteria tuberculosis complex* and its blood-brain barrier penetrating, viral-like, cell-wall-deficient forms. It is for these reasons that future research needs to be aimed in this direction.

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Conflict of Interest

The authors have no conflict of interest, financial or otherwise with regard to this study. Procedures involving experimentation on animal subjects were done humanely and in accord with either the guide of the institution in which the experiments were done.

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