DETECTION AND DIAGNOSIS OF PRIONS, THE CAUSATIVE AGENT FOR THE NEURODEGENERATIVE TRANSMISSIBLE SPONGIFORM ENCEPHALOPATIES (TSEs) IN SHEEP, CATTLE AND HUMANS – A REVIEW

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ABSTRACT

The technique to detect and diagnose infectious prions, the causative agent of transmissible spongiform encephalopaties (TSEs) at very early stages of infection where the disease can be controlled and even eliminated has been developed. The technique now in the field is being applied worldwide for the screening of cattle, sheep, rodents, humans and other animals. It has not only saved lives but also billions of dollars in agriculture by preventing the spread of infection in livestock. In the United Kingdom, over 2 million cattle have been destroyed due to prion infection. Prion has also been identified in the United States, and more recently in South Korea. While in Nigeria, many of our cattle and sheep have not been screened and no awareness exist for TSEs. Preliminary work is already underway. Also screening studies are just commencing at a very slow pace in Jos. Given the globalized nature of the present world, where infections move across continents at a rapid pace, it is very disturbing that the nation has no policy and is ill-prepared to respond to it should an epidemic of TSEs break out among its livestock or population. It is necessary to apply this ultrasensitive technique for detection and diagnosis of infectious prions to the screening of livestock, and the protection of Nigerian citizens from TSEs. The technique is also a sensitive tool for studying and uncovering the mechanism of potential drugs that can inhibit or slow down the spread of TSE infection. It will advance the frontiers on the study of the mode of infection and conversion of prions and has significantly already contributed to the new paradigm that has changed our knowledge on infections.

Keywords: Detection, Prions diagnosis, Neurodegenerative, Transmissible spongiform encephalopaties, Sheep, Cattle, Humans

INTRODUCTION

TSEs have been observed in humans, sheep and cattle (Figure 1). It has also been noted in deer, elk, mink, cats, rodents, exotic ungulates, other mammals, but not in dogs, rabbit, horses or birds. In humans it exists as CJD or vCJD, its variant form which resulted from humans infected by infected cattle. Infections can occur from ingestion or inoculation, or contact with infected surgical materials, and incubation time before the manifestation of symptoms can be from months to decades. TSEs derive their name due to the malfunction and death of brain neurons which exhibit spongiform pathology. Accumulation of plaques or amyloid (Figure 2).

But what is the causative agent? After eliminating bacteria, viruses or other microorganisms, Prusiner in his Nobel work purified the protein components from infected scrapie fibrils and cloned the gene (Prusiner *et al.,* 1984). Furthermore, others workers had cloned the mice and hamster prion gene (Chesebro *et al.,* 1985; Oesh *et al.,* 1985) and found no differences between the normal prion gene and the diseased prion gene. Thus the

gene responsible for TSEs was the host gene. A most convincing proof came with the cloning of the knock-out mice (mice which lack the prion gene) and the discovery that they did not develop TSEs (Chesebro, 1999).

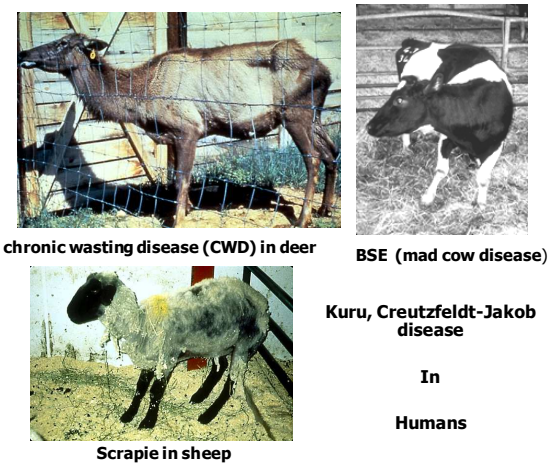

Figure 1: TSE (prion) diseases in animals

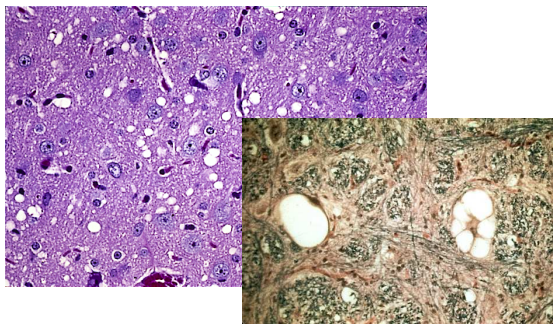

Figure 2: Malfunction and death of neurons in brain leads to spongiform pathology

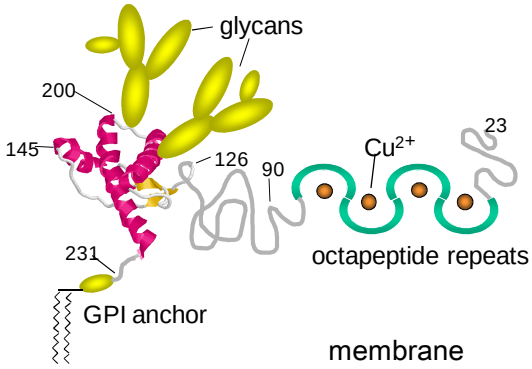

Figure 3: Normal prion protein: (normal; PrP-sen or PrP^C). *Sensitive to proteases; Soluble in detergents; In diverse tissues, cell types; Apparent cellular roles; Adhesion; Differentiation; Neuritogenesis; Synaptogenesis; Cell survival; Resistance to oxidative stress; Essential for TSE diseases.*

MATERIALS AND METHODS

A comprehensive search was made from the internet, various journal articles and textbooks reports on the detection and diagnosis of prions, the causative agent for the neurodegenerative transmissible spongiform encephalopaties (TSEs) in sheep, cattle and humans in various parts of the world. Such articles were assembled, studied and synthesized into this review.

RESULTS AND DISCUSSION

Normal Role of Prions (PrP-sen): How then does the normal prion protein differ from the diseased prion protein, since they are the same protein from the same host gene? The normal prion protein is highly sensitive to protease digestion, and is therefore designated as PrP-sen. It is soluble in detergents and present in diverse tissues and cell types. Its apparent cellular roles include adhesion, differentiation of cells, neuritogenesis, synaptogenesis as well as cell survival. It is also known to play key roles in the homeostasis of metals most especially copper in the central nervous system. Also altered sleep patterns and circadian rhythm activity regulated by the penal gland has been observed in mice that lack prions such as the knock-out mice (Tobler *et al.,* 1996; 1997). Normal PrP has also been shown to have super-oxide dismutase activity, thus making it a powerful anti-oxidant for the central nervous system (Brown *et al.,* 1999). Other roles in which PrP has been implicated includes signal transduction in neuronal cells (Mouillet-Richard *et al.,* 2000), and the activation of lymphocytes (Li *et al.,* 2001) (Figure 3).

Infectious and Diseased Prion (PrP-res): However, diseased prions associated with TSEs are generally designated as PrP-res, because they are highly resistant to protease digestion. Their capacity to form insoluble aggregates and polymers enable them to form amyloids, spongiforms and plaques in the brain. They are always found within the region of neuropathology and are always associated with infectivity.

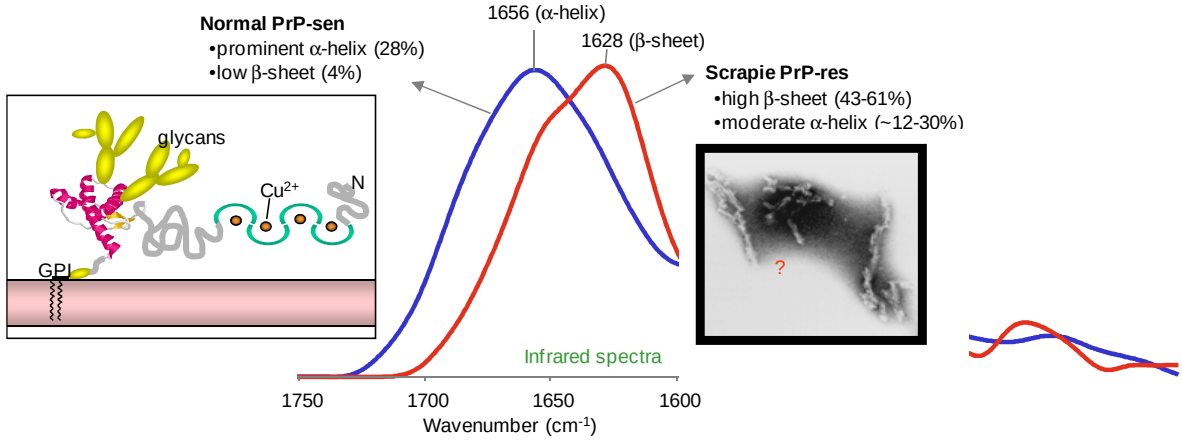

Figure 4: Conformational difference between normal and TSE-associated PrPs with no known chemical modification; conversion involves refolding from α-helix + random coil to β-sheet

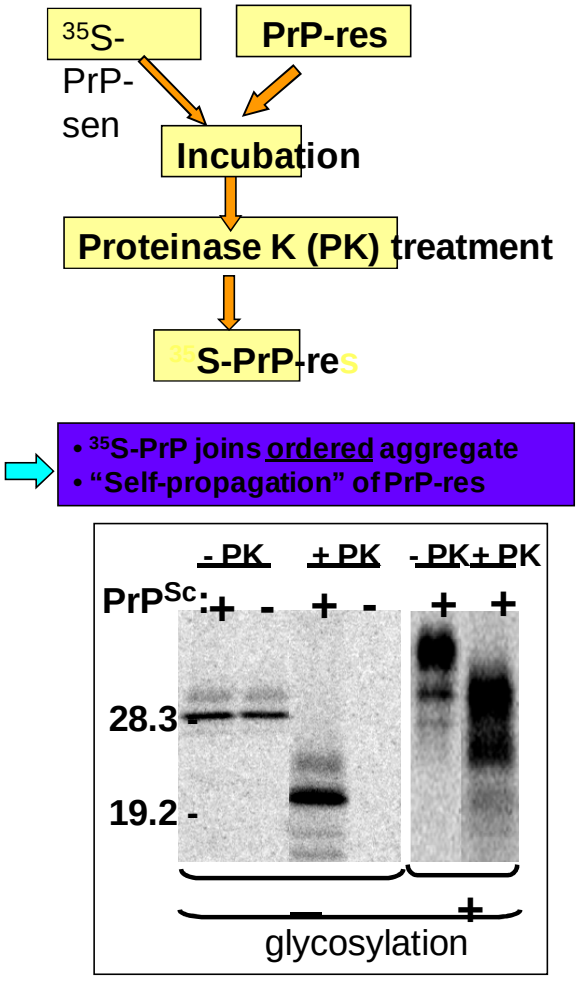

Figure 5: PrP-res-induced conversion of PrP-sen into a PrP-res-like protease-resistant form (Kocisko *et al.*, 1994)

They are not only located in the brain but are also found in lymphoid tissue. Yet, their covalent structure is indistinguishable from normal PrP-sen. PrPres isoforms are co-noted with subscripts to indicate the infected specie: PrP-sc for scrapie or sheep, PrP-CJD for humans, PrP-BSE for cattle, etc. PrP-res differ mainly from the normal PrP-sen by conformational differences (Figure 4).

Normal PrP-sen contains 28% alpha helix, and 4% beta-pleated sheet, while the abnormal and infectious PrP-res contain 43 – 61 % beta pleated sheet. PrP-sen therefore absorbs at 1656 cm^{-1} wavelength while PrP-res can be identified at 1628 cm^{-1} due to its higher beta sheet content.

PrP Conversion: Mode and Mechanism of Infection: One of the intriguing problems of molecular biology and biochemistry is to show how PrP-sen is converted to PrP-res. When 35-S-PrP-sen is incubated with PrP-res and treated with proteinase K, the product is 35-S-PrP-res, indicating the conversion of PrP-sen to PrPres (Kocisko *et al.,* 1994). Also continuous amplification of infectivity when crude brain haemogenates is used as a source of PrP-res has also been observed (Saborio *et al.,* 1999; 2001) (Figure 5). Furthermore, structural assessments of the various domains of PrP-sen upon conversion to PrP-res oligomers are well known and have been illustrated in figure 6. It can be seen that the octarepeat region which normally binds four copper atoms remain exposed to proteases and can easily undergo alteration. Independent deletion mutagenesis studies have shown that the N-terminus of PrP facilitates prion propagation and PrP-res formation (Flechsig *et al.,* 2000; Supatta *et al.,* 2001).

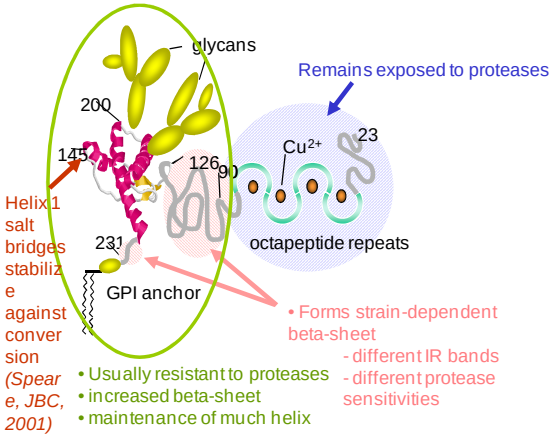

Figure 6: Fates of domains of PrP-sen upon conversion to PrP-res oligomers

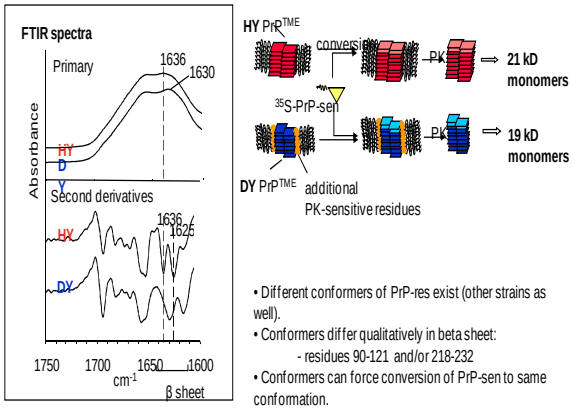

Figure 7: Hyper and Drowsy strain-dependent conformers of PrP-res in hamsters

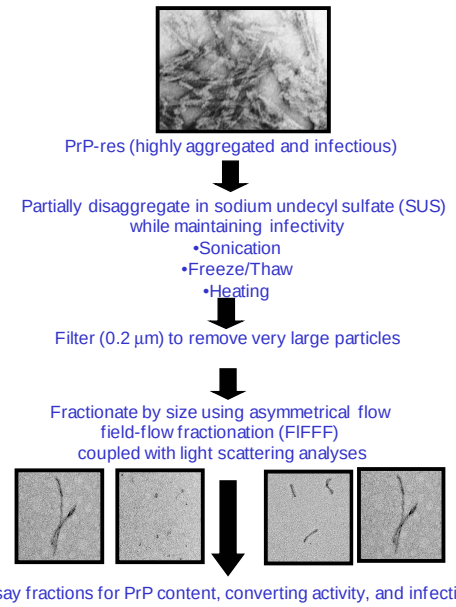

Figure 8: Fragmentation & fractionation of infectious PrP-res preps (Jaysilveira *et al.,* 2005)

However, residues 90 to 231 which include the Helix 1 salt bridges are unaltered and stabilized against conversion. This region is also known to form strain dependent beta sheets identified by various infra-red bands to reflect strain specific conformations.

In general, PrP-sen conversion reaction to PrP-res showed that it can be stimulated by sulfated glycans, chaperone proteins, partially unfolding detergents and temperature increase to 65^0C (Wong *et al.,* 2000). It is inhibited by disulfide bond reduction and requires PrP-res as multimers for seeding the process. Additionally, there appears to be specific bonding of PrP-sen to the PrP-res polymer (DeBurman *et al.,* 1997). This specificity is thought to be responsible for the strain specificity observed in infected animals. In addition, the binding of PrP-sen to PrP-res precedes the conversion to the proteinase K resistant state because the product remains associated with pre-existing PrP-res (Baron *et al.,* 2002; Baron *et al.,* 2003). The conversion process is virtually irreversible without denaturants in vitro and is consistent with an autocatalytic polymerization mechanism (Vorberg and Priola, 2002).

Several other factors have been observed to influence the rate of conversion of PrPsen to PrP-res (Korth *et al.,* 2000; Saborio *et al.,* 2001). Correlations between PrP sequences show that the closer the sequences between PrPsen and PrP-res, the higher the conversion rate. Thus the more the similarities in structure between species, the higher the rate of conversion and inter-species transmissibilities of TSEs. It is therefore reasonable that the highest rate of conversion is obtainable within the same species (Prusiner *et al.,* 1990).

One of the most fascinating aspects of TSEs is the existence of different strains in the same species. In hamsters, PrP-res have two or more strains which manifest with different symptoms of TSE. These strains are distinguished by intro-red spectroscopy. The 1636 cm-1 conformer is referred to as PrP-Hyper because these TSE infected hamsters are hyperactive before their death. Another hamster strain has a slightly different conformation at 1630 cm-1 wavelength and is noted as PrP-

drowsy because these TSE infected hamsters are usually drowsy before their death (Figure 7).

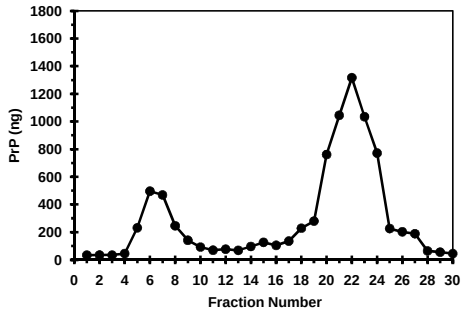

Figure 9: Infectivity in flow field flow fractionation of partially disaggregated PrP-res

There have been concerns as to whether PrP-res infect PrP-sen as a monomer and whether particle size affects infectivity. Studies applying asymmetrical flow field-flow fractionation (FIFF) coupled with light scattering analysis led to generation of fractions of PrP-res which were in turn used to assay for converting activity and infectivity (Silveira *et al.,* 2004). These results indicated that particle size was important in conversion and infectivity. While large fibrils were not the most infectious particles, surprisingly smaller fibrils from monomers to pentamers had the lowest capacity to induce infectivity (Figure 8). The most infectious particles were 17 to 27nm in diameter; they were 500 - 600 Daltons which indicates a mass equivalent of 25 molecules of PrP, although part of this mass could be due to bound detergent molecules. These most infectious particles were found to be roughly spherical and slightly elongated in structure. They were associated with the highest level of specific converting activity. Therefore large amyloid fibrils or spongiform plaques are much less infectious per unit protein than the smaller but most infectious particles (Figure 9).

Prions and Protein Folding Diseases: The transmissible spongiform encephalopathies (prion diseases) are not the only protein folding diseases, and are not the only neurodegenerative diseases. They are however unique because they are the only ones that are transmissible.

Their infectious causative agent is a non-living protein, and is the first known particle that can transmit infection without recourse to DNA or RNA. They have therefore changed our conventional understanding of infectious diseases. Other protein misfolding diseases that are not transmissible include Alzheimer's disease, type II diabetes, arnyotropic lateral sclerosis, Parkinson's disease, sickle cell anaemia, cystic fibrosis, Huntington's disease, spinocerebellar ataxis, etc. However while some of this protein misfolding diseases are neurodegenerative, non are transmissible as is noted for the prion diseases. Despite their low rate of propagation interspecies, that they are transmissible at all from one species to another is a challenging problem confronting the world. The interspecies rate of transmission is as illustrated significantly threatening mankind.

Death through Prion Infection: Besides infecting cattle, sheep, rodents and humans, death through TSEs has been described as the worst way of dying. Young women once infected, go into menopause despite their age (Max, 2006). Also infected individuals are unable to sleep, and at best go into a state of stupor, where they still retain full consciousness. There is a gradual decay of muscular coordination, which affects both sight and speech. Max who interviewed patients with CJD in Europe also noted a rapid loss of weight that resembles those of AIDS patients. Young infected males are also known to become sterile with the progression of the disease. It is possible that these changes may be associated with a dysfunctional circadian rhythm where normal PrP function is lost.

Detection and Diagnosis: By the application of an ultra-sensitive means of detecting infectious prions at ag and fg levels, infections can be controlled at a much earlier and less threatening stage, thereby intervening with drug inhibitors to prolong the life of patients, as well as prevent the spread of infection to healthy life stock, thereby saving the world agricultural industry billions of dollars (Atarash *et al.,* 2007;

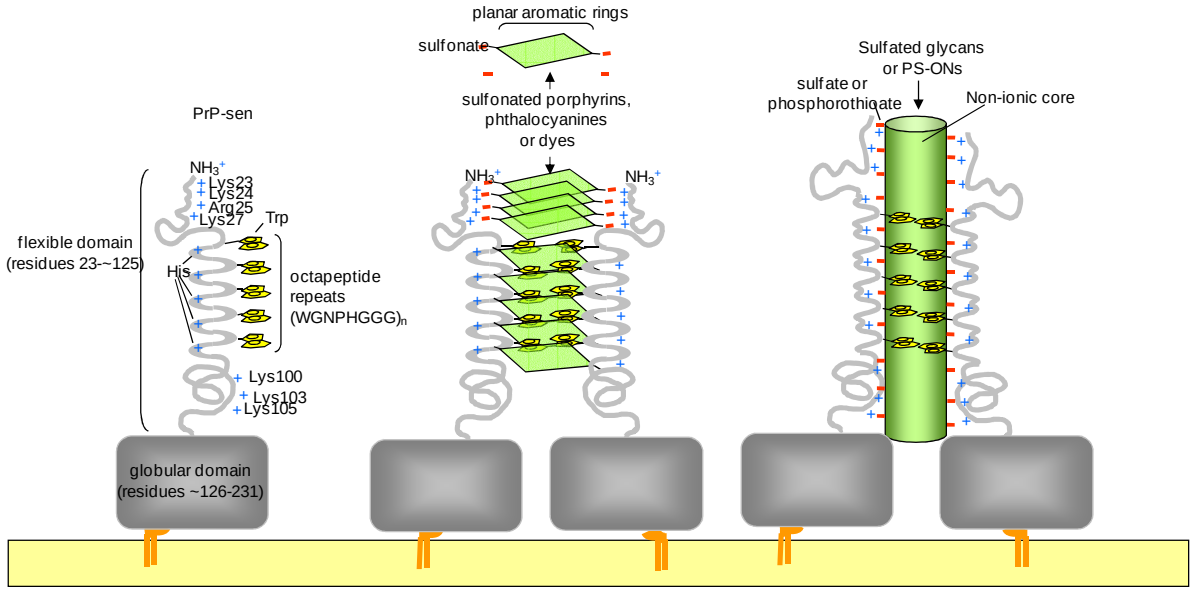

Figure 10: Stacked amphipath model of inhibitor interactions with PrP-sen

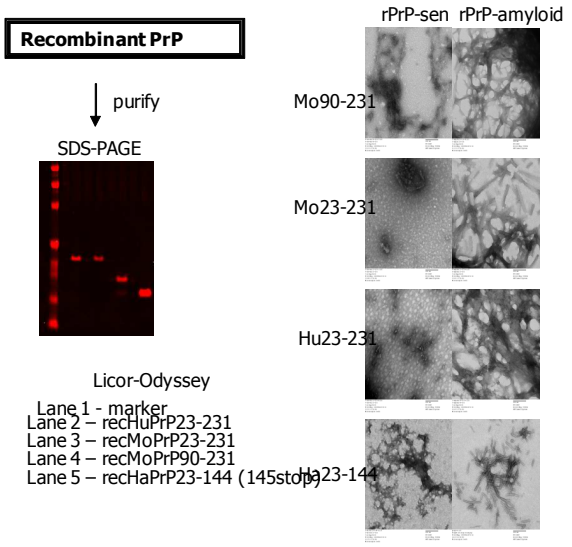

Figure 11: electrophoresis of recombinant prion isoforms

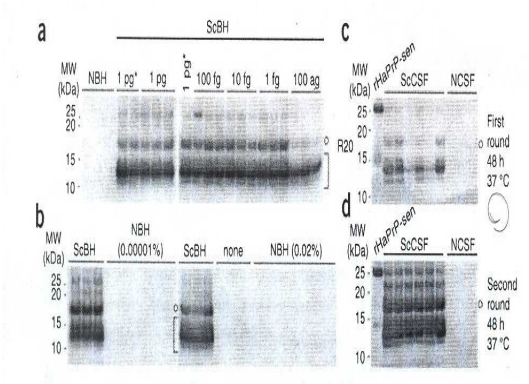

Figure 12: QUIC reactions seeded with brain homogenates and CSF samples from normal or scrapie-affected hamsters (Atarashi *et al.*, 2007; 2008).

2008). Nigeria requires this ultra-sensitive technique to ensure that its cattle and sheep which serve as the key source of protein for its population ore completely screened. It need not wait until it experiences an epidemic the method is also being applied at hospitals in the developed world to prevent infected surgical tools from being used inadvertently on healthy people. PrP-res are very insoluble and sticky and are not inactivated by radiation or by conventional heating. It also is known to bond to metals and is extremely stubborn to destroy. Detecting PrP-res on such tools will require their immediate disposal for new and uninfected ones. We have commenced experiments with rodents at the University of Nigeria Nsukka, as work with cattle and sheep would require more hands and capital.

As there are no practical treatment known, this technique is based on the conversion process, and can be applied to screen drugs and inhibitors of conversion. Hundreds of compounds that inhibit the conversion of PrP-sen to PrP-res, that can serve as potential therapeutic agents for TSE treatment are regularly being identified using aspects of an ultra-sensitive technique on prion detection. Once to be useful potential inhibitors, they are then tested in TSE infected deer cattle, hamsters, sheep, and other lower animals before clinical trials in CJD infected humans. So far at least two inhibitors of conversion are

being tested in CJD patients — pentosan polysulfate and quinacrine. Quinacrine is used also as an anti-malarial drug and is not expected to have life threatening side effects. A stacked amphipathic model has been proposed as a mechanism for inhibitor binding and action on the PrP-res molecule (Figure 10). The compounds are thought to stack at the N-terminal octarepeat region between residues 23-1 25, the same site necessary for PrP-sen conversion to PrPres (Supattapone *et al.,* 2001).

Conclusion: Finally, to develop an ultra-sensitive means of detecting infectious prions and diagnosis of TSEs requires a reasonable understanding of the cell-free conversion process. A clean and steady supply of PrP-sen is obtained by generating recombinant forms of the protein from bacteria. To assure their purity they are subjected through SDS-PAGE electrophoresis. Figure 11 shows the various recombinant PrP proteins when subjected to electrophoresis, and their purity. Their capacity to generate amyloid was also tested as is evident in figure 11. RecHuPrP231 refers to human recombinant Prp-sen full length 23 – 231. Mo and Ha refers to mouse and hamster recombinant PrP segments, respectively. Satisfied with the purity and spectroscopic characterization of the recombinant PrP segments its effect on the cell free conversion reaction with Pr Pres from brain haemogenates of sheep, as well as cerebrospinal fluid of sheep is tested (Figure 12). ScBH represents scrapie infected sheep brain homogenate, while NBH designates normal brain hemogenate used here as control. Also ScCSF represents scrapie sheep cerebrospinal fluid, while NCSF stands for the Normal Cerebrospinal fluid used here as control. MW indicates the molecular weight markers which helped to identify the molecular weight of the various PrP fragments present after the reaction have been subjected to proteinase K digestion, and Western blotting. Of significant note is the complete absence of the 17kd band in either the normal brain homogenate control or the normal cerebrospinal fluid control. However the 17kd band is clearly evident even at 1fg and 20ag levels of infected cerebrospinal fluid (ScCSF) or infected scrapie brain homogenate (SCBH) (Atarashi *et al.,* 2007; 2008). These results showed possibility of detecting infectious prions at minute quantities, and that TSEs can be diagnosed at the very onset of infection, when conversion from PrP-sen to Pr P-res is still very low. Under such intervention, drug inhibitors could be used to shoal an otherwise infectious and presently incurable disease. Also billions of Naira could be saved from preventing the spread of infection to yet to be infected livestock. It should be noted that since PrP-res and PrP-sen have the same primary and covalent structure, the body's immune system does not recognize the infectious PrP-res as foreign. Therefore PrP-res is able to evade the body's cellular and humoral immune response. However monoclonal antibodies directed to various sites of conversion have become useful tools for western blotting as well as obtaining information on drug design and action against TSEs as mankind is confronted without question by the most challenging paradigm which has changed the way we see infectious diseases.

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