Pathology of Animal Transmissible Spongiform Encephalopathies (TSEs)

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Pathology is the study of the structural and functional changes produced by diseases or – more specifically – the lesions they cause. To achieve this pathologists employ various approaches. These include description of lesions that are visible to the naked eye which are the subject of anatomic pathology and changes at the cellular level that are visible under the microscope, the subject of histopathology. Changes at the molecular level which are identified by probes that target specific molecules – mainly proteins that are detected using immunohistochemistry (IHC). As transmissible spongiform encephalopathies (TSEs) do not cause visible lesions anatomic pathology is not applicable to their study. For decades the application of histopathology to detect vacuoles or plaques was the only means of confirming TSE disease. The subsequent discovery of the cellular prion protein (PrP^C) and its pathogenic isoform, PrP^{Sc}, which is a ubiquitous marker of TSEs, led to the production of anti-PrP antibodies, and enabled the development of PrP^{Sc} detection techniques such as immunohistochemistry, Histoblot and PET-blot that have evolved in parallel with similar biochemical methods such as Western blot and ELISA. These methods offer greater sensitivity than histopathology in TSE diagnosis and crucially they can be applied to analyze various phenotypic aspects of single TSE sources increasing the amount of data and offering higher discriminatory power. The above principles are applied to diagnose and define TSE phenotypes which form the basis of strain characterisation.

Key words: animal, TSEs, pathology, prion, PrP

Techniques and Applications of Pathology

Probably the oldest simplest definition of pathology is ‘the study of disease’. More specifically the aim of pathology is to describe the lesions that are caused by diseases. Some lesions can be large enough to be observed without any specialist equipment. Such lesions are designated as gross lesions and they are the subject of anatomic pathology.

The introduction of the microscope revolutionized pathology as it gave an insight of tissue changes that occur within gross lesions at the cellular level. Furthermore, it was made evident very quickly that cellular changes are not always

confined within the boundaries of gross lesions but they can also be observed in tissues that look normal macroscopically. This increases our ability to detect disease sooner and to identify more accurately the extent of the disease in an animal. The study of the microscopic lesions is the subject of histopathology.

The development of molecular techniques has given pathologists the opportunity to study disease-associated changes at the molecular level. Molecular changes can be detected even in the absence of gross or histological lesions and they are considered to be the first indicators of disease. Therefore, detection of molecular changes increases further

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Abbreviations: BSE: bovine spongiform encephalopathy; CWD: classical scrapie, chronic wasting disease; EM: Electron microscopy; FACS: fluorescent assorted cell sorting; IHC: immunohistochemistry; LP: lesion profiles; LRS: lymphoreticular system; PCR: polymerase chain reaction; PRNP: PrP gene; PrP^C: cellular prion protein; TME: transmissible mink encephalopathy; TSEs: transmissible spongiform encephalopathies

our ability to detect disease at the very early stages. The commonest molecular pathology technique is immunohistochemistry (IHC) where a specific protein can be detected in tissue sections using antibodies that have been raised against it. Immunocytochemistry is based on the same principle but it is applied on slide smears prepared from cell suspensions. Specific RNA or DNA molecules are detectable using *in situ* hybridization or *in situ* polymerase chain reaction (PCR).

Electron microscopy (EM) has increased the magnification and resolution of the pathology images and although EM is not used routinely for diagnostic purposes because of the expertise required and the costs involved it remains a very powerful research tool particularly when it is combined with IHC.

More recently technologies such as fluorescent assorted cell sorting (FACS), confocal microscopy, laser capture microscopy and microarrays are applied mainly in research pathology laboratories but some of these techniques are expected to revolutionise diagnostic approaches.

Currently immunohistochemistry, immunocytochemistry and to a lesser extent *in situ* hybridization using either light or fluorescent microscopy are the most widely used molecular pathology applications, particularly for diagnostic purposes^{1,2}.

Basic Biology of Transmissible Spongiform Encephalopathies (TSEs)

TSEs are neurodegenerative, incurable and fatal diseases of the nervous system. TSEs do not cause gross lesions but they are characterised by microscopic vacuolation in the brain which gives a spongiform appearance, giving rise to the term spongiform encephalopathies. Another hallmark feature of TSEs, although not always present, is the formation of amyloid plaques in the central nervous system (CNS)³.

Originally it was believed that the causative agent of TSEs was a virus. However, no viral particles were ever detected and it was shown that infectious tissues would retain infectivity after exposure to inactivation treatments that would kill conventional viruses. Meanwhile, experimental evidence accumulated supporting a theory, first suggested in 1967, that the major or single component of the infectious agent could be a cell membrane bound protein^{4,5}. According to this theory, known as the prion theory, a naturally occurring protein, prion protein or PrP, can under certain circumstances alter its configuration from its normal cellular structure, PrP^C, and adopt a pathogenic tertiary conformation, designated PrP^{Sc} (C denotes the cellular form and Sc denotes pathogenic conformer)^{6,7}.

PrP^C has a mainly a helical composition^{8,9} whilst PrP^{Sc}

has a mainly beta sheet structure^{10,11}. It is believed that the beta sheet composition can adopt numerous distinct tertiary conformations which account for the different strains of the agent^{12,13}. This has been a very significant development as it provided an explanation for the existence of different 'strains', a major point of debate for the researchers who were advocating that an independent genome is a prerequisite for an organism that presents as multiple strains. Due to its enriched beta sheet structure compared to PrP^C, PrP^{Sc} is more resistant *in vitro* to digestion by proteinase K and also to cellular digestion^{14,15}, a feature which can be exploited by diagnostic tests¹⁶.

The presence of PrP^{Sc} in the brain, either by transport after peripheral exposure (bovine spongiform encephalopathy (BSE), classical scrapie, chronic wasting disease (CWD), transmissible mink encephalopathy (TME)) or by *de novo* stochastic generation (most likely atypical scrapie, H- and L- type BSE), acts as a seed that provides a template of self-propagation by converting endogenous PrP^C to PrP^{Sc}. Therefore, unlike other pathogens, which retain their own genome when they invade and infect different hosts, PrP^{Sc} always adopts the sequence of the host PrP^C¹⁷. The pathogenicity is determined by the different tertiary conformations PrP^{Sc} can adopt¹⁸.

TSE Diagnosis

The primary testing methods now used routinely for large scale disease screening rely on the detection of PrP^{Sc}. However, histopathology is still used for the confirmation of TSEs in surveillance and research projects^{19–21}. Using histopathology, identification of vacuoles in the grey matter of brain or spinal cord sections stained with haematoxylin and eosin was the first diagnostic criterion of TSEs^{3,22}. Identification of amyloid plaques in similar sections is another diagnostic hallmark of TSEs but unlike vacuolation which is considered to be ubiquitous the presence of plaques is not³. Histopathology is one of the few approaches that is not reliant on an antibody raised against PrP.

The discovery of PrP^C and PrP^{Sc} and the ability to raise antibodies that can bind to them gave rise to the development of reliable IHC techniques that identify PrP^{Sc}^{23–25}. As PrP^C and PrP^{Sc} are both present in tissue sections and antibodies raised specifically against PrP^{Sc} are not widely available, specific detection of PrP^{Sc} is achieved by carefully adjusting the titre of the anti-PrP antibody and the exposure time so that only PrP^{Sc} is identified. Specific pretreatment is required to enhance the detection of PrP^{Sc} by facilitating antigen retrieval^{26,27} but the exact mode of action is unknown. This is in contrast to other methods such as Western blot or ELISA

where the sample is treated with a protease for a specified time during which the protease sensitive PrP^C is destroyed whilst the relatively protease resistant PrP^{Sc} is retained and subsequently detected by the anti-PrP antibody¹⁶). Tissue sections can also be treated with protease, but it is believed that the agent only affects the free surface of the section and it may help in general antigen retrieval rather than specific elimination of PrP^C. Application of protease for extended periods, to reach the deeper layers of the section, usually digests most or the whole of the section with detrimental effects on PrP^{Sc} detection and tissue morphology. It has also been reported that streptomycin may be used to amplify immunohistochemical detection of PrP^{Sc}²⁸). The majority of laboratories that apply immunohistochemistry to detect PrP^{Sc} currently use no protease or streptomycin pretreatment.

TSE Disease and Strain Characterisation

Because prions do not have their own independent genome, methods such as PCR or other techniques that are used widely in strain typing of bacteria, viruses or other organisms cannot be applied to TSEs. However, different TSE strains, can be identified by studying the disease phenotype in a specified host^{29,30}). Phenotype is the composite metabolic, physiological and physical characteristics, both normal and pathological, exhibited by a host as a result of its interaction with the environment inside and outside the organism. Disease phenotype includes all the pathological characteristics caused by a disease in a host. By definition, the phenotype is host specific and includes a very large number of parameters which may evolve over time as new techniques are applied. For practical purposes in TSEs the parameters that are currently considered to define phenotype are incubation period, clinical signs, and quantitative aspects of vacuolation which give rise to lesion profiles, IHC deposition patterns and Western blot characteristics^{31–33}). For the purposes of this review different phenotypes in the original host are considered as different TSE strains ie BSE, H- type BSE and L- type BSE in cattle, and classical and atypical scrapie in sheep. However, after experimental transmission of a TSE to wild type or transgenic rodents a single or multiple phenotypes may emerge in a single mouse line, leading to the laboratory definition of a strain. According to this definition, classical scrapie includes several strains as various distinct phenotypes can be identified in mice^{33–35}). In contrast every time a source of atypical scrapie has been bioassayed in mice a single phenotype has been detected suggesting that atypical scrapie is attributed to a single strain^{36,37}). It must be emphasized that, particularly in rodents, the phenotypes are not simply host specific but mouse line specific^{30,33,38}).

This means that phenotypes between different mouse lines are not directly comparable even within wild type mice due to polymorphisms in the murine PrP and other genetic factors^{34,39–41}). TSE strain characterisation using mouse bioassays is considered part of the phenotype of a TSE but this complete phenotype is only available in a minority of cases and it is not usually included in the phenotype of every TSE case identified in the field.

Classification of phenotypes requires more detailed data than simple disease diagnosis. Therefore more elaborate pathology interpretation approaches have been developed.

For histopathology the density and distribution of vacuolation in the brain can be assessed semi-quantitatively at specified neuroanatomical areas and plotted to produce graphs, termed lesion profiles (LP)^{42,43}). Although LP approaches have been used for disease classification in the original hosts they are very elaborate and cannot be applied routinely, not least because for practical and financial reasons the only brain areas that are regularly sampled for surveillance purposes are brain stem and cerebellum. LP are used widely in experimental studies, particularly in rodents, and are considered disease/strain specific^{42,44}). However, because lesion profiles were originally optimised to detect a specific mouse adapted classical scrapie strain (ME7) in CBA or C57BL/6 mice exceptions can occur⁴⁵) as the methodology has been used relatively unchanged since it was developed⁴²) to every other TSE strain in any mouse line. This method is semi- quantitative and it requires that the profiles are applied to animals dying during the clinical phase of the disease. At least five clinically positive animals should contribute to a lesion profile for it to be considered robust, although this is not always possible if attack rates are low^{33,46}).

The application of IHC revealed a variety of different PrP^{Sc} deposits classified morphologically as intraneuronal, intragial, granular, punctate, coalescing, plaques etc^{24,38,47}). It was observed that each TSE strain/host combination produces a unique IHC signature. These signatures can be assessed semi-quantitatively at predetermined specific neuroanatomical areas to produce graphs similar to lesion profiles^{48,49}). As these IHC profiles are semi quantitative approaches the same restrictions regarding the clinical status of the animals and the number of animals that have been mentioned for the LP apply. Alternatively the distribution of the different PrP^{Sc} types can be used to produce high definition neuroanatomical maps that are strain specific^{33,50,51}). The later approach is very powerful, as it does not require semi-quantitative assessment in predetermined brain areas. As a result it can be applied successfully to single animals from the preclinical stages to allow TSE characterisation when adequate hallmarks of the specific TSE are recognised. It can

also be used to identify TSE strain coinfection provided that the signature of each TSE is expressed to an extent that can be identified by the reader⁴⁶.

Histoblot is a technique that allows application of a protease to eliminate PrP^C and preserve PrP^{Sc} in tissue sections. Frozen tissue sections are mounted on nitrocellulose membrane and are subsequently treated and developed in the same way as a Western blot^{52–54}. This method can also be applied to paraffin embedded tissue sections (PET-blot)^{55,56}. Pathology laboratories usually prefer PET-blots as they archive tissues in paraffin blocks. Although histo- or PET- blots are useful in specific applications, the resolution is significantly reduced compared to IHC as it is not possible to differentiate different PrP^{Sc} types, with the possible exception of plaques, and therefore only the extent of distribution of PrP^{Sc} in the brain can be determined³⁸. Histo- or PET- blot is also applied to individual mice, but because it has a lower resolution than IHC, neither histo- or PET- blot can be used to identify coinfection in single animals.

Application of Disease Phenotype to Define TSEs and TSE Strains in Small Ruminants

Classical Scrapie

Classical scrapie is considered to be the archetypical TSE and is known since 1732^{57,58}. The sheep PrP gene (*PRNP*) is highly polymorphic, and some codons have been identified which can susceptibility to scrapie and can also affect disease phenotype. An association between incubation period and *PRNP* genotype has been reported^{59,60}, and LP also appear to be *PRNP* dependent⁴³. It has been suggested that in sheep it may be possible to identify different scrapie strains in the original host using a single phenotypic characteristic, IHC⁴⁷. However, that study involved experimental scrapie sources and not enough sheep were included to assess the effect of the PrP genotype on the IHC characteristics. Subsequently studies have indicated that there is an association between *PRNP* genotype and IHC patterns^{61–63}. It has been proposed that, at least under natural conditions, sheep may be exposed to various strains and specific *PRNP* genotypes may allow certain strains to propagate preferentially. In addition, the phenotype of a single strain may be influenced by the PrP background⁶¹. Therefore, it is believed that because of this interplay between classical scrapie strains and *PRNP* genotype it is not possible, at least in naturally occurring cases, to accurately discriminate classical scrapie strains by analysing the phenotype of individual sheep. The existence of classical scrapie strains has been established based on the different phenotypes that can emerge from different sources

after bioassay in wild type rodents or transgenic mice which express or overexpress a farm animal *PRNP* transgene whilst murine *PRNP* has been ablated.

In classical scrapie the agent is also distributed outside the nervous system, mainly in the lymphoreticular tissues where it is readily diagnosed using IHC, Western blot or other related techniques but it does not cause any histological lesions and is considered that it does not interfere with the function of the lymphoreticular system (LRS)⁶⁴.

Atypical Scrapie

Until a report in 2003, only classical scrapie cases had been reported in small ruminants⁶⁵. That report described five cases of ovine TSEs that were distinct from any classical scrapie case known at the time. There were differences in the distribution and intensity of vacuolation, the distribution and the different types of PrP^{Sc} deposits identified by IHC, and a strikingly different Western blot profile that included four bands instead of the three bands that are usually associated with animal TSEs. This new phenotype was designated Nor98 and mouse bioassays showed that it transmits only to transgenic mice and produces a phenotype that is distinct from any classical scrapie phenotype identified in these mice^{36,37}. This novel disease attracted a lot of interest as it raised serious questions regarding our knowledge of TSEs and our ability to detect them reliably, as retrospective studies showed that atypical scrapie cases preexisted but they had not been diagnosed as the tests used at the time were not optimised for detecting this disease^{66,67}. Subsequently atypical scrapie was studied extensively and the phenotype of the disease in the original host^{31,68,69} and in transgenic mice^{36,37} was described in detail. Knowledge of the distribution of the agent in the nervous system in these cases led to the suggestion that during diagnostic sampling the cerebellum should be collected in addition to the obex. This simple modification has increased significantly the sensitivity of detection of atypical scrapie during surveillance schemes. Unlike classical scrapie, in atypical scrapie the agent is not distributed in peripheral tissues systematically which, in conjunction with its limited distribution in the obex and widespread distribution in the cerebral and cerebellar cortex, have led to the belief that this TSE occurs sporadically^{70,71}. This is supported by epidemiological data indicating that it is not infectious under natural conditions⁷², although it can be transmitted experimentally via the oral route⁷¹.

BSE in Small Ruminants

BSE in cattle was first discovered in the UK in 1985. The diagnosis of the disease was initially based on the identification of spongiform changes in the brain⁷³. At that time no

other TSE was known in cattle and studies to define the disease phenotype in this species were initiated. One of the most important parameters was to characterise the strain of the agent by defining the disease phenotype in mice that had been challenged with bovine BSE cases⁷⁴). This was subsequently crucial information for demonstrating that BSE was associated with the variant CJD cases that were identified in 1996, as the phenotype of the two diseases in mice was indistinguishable, implying that they were caused by the same strain^{75–77}).

The BSE epidemic was attributed to meat and bone meal supplementation of proprietary cattle feed⁷⁸). As small ruminants were fed the same contaminated food supplements it was rational to assume that BSE might also affect sheep and goats, and subsequent experimental challenges proved this to be the case. Studies were initiated to identify the phenotype of BSE in small ruminants and develop tools that allow its detection and differentiation from scrapie in the field⁷⁹). This EU wide effort has resulted in the identification of naturally-occurring BSE in two goats^{80–82}).

Although it appears that the PRNP genotype is related to susceptibility, the phenotype of the BSE in affected sheep seems to be uniform, and genotype independent^{83–85}). Some disease characteristics, such as clinical signs, are indistinguishable between classical scrapie and BSE⁸⁶). Similarly, vacuolation cannot be used to differentiate between these two TSEs unless LP are employed⁴³) – an impractical solution, as mentioned earlier. PrP^{Sc} profiles or distribution patterns on their own also did not offer a reliable means for discrimination for field cases as only a small part of the brain is sampled during surveillance projects. Partially the problem is the wide phenotypical variance within classical scrapie which makes it very difficult to know when you have compiled an exhaustive library of phenotypes, especially given the often limited material available from field cases.

Western blot of tissues from small ruminants affected with BSE after experimental challenge showed that the PrP^{Sc} retained the BSE characteristics observed in cattle, i.e. a lower molecular weight unglycosylated band compared to classical scrapie and a different glycoprofile ratio⁸⁷). It must be noted that Western blot is the least host-affected parameter during interspecies transmissions and therefore it is generally accepted that if the Western blot properties are retained then the same agent has overcome the transmission barrier and has propagated true in the new recipient. Consequently, changes in the Western blot properties indicate fundamental changes in the strain characteristics of the agent.

In addition to the Western blot two other methods that would allow discrimination between the two TSE entities in small ruminants were developed, discriminatory Western

blot and discriminatory IHC. They are both based on the same principle that scrapie and BSE prions are cleaved at different sites during normal *in vivo* processing producing a core fragment that it is longer in scrapie compared to BSE. Therefore antibodies can be used to exploit that difference. One antibody binds to the C-terminus fragment that is retained to both prions, producing a signal in both classical scrapie and classical BSE; the other binds to the N-terminus fragment that is preserved only in scrapie producing a signal that is detectable in classical scrapie but is lost in BSE. This principle can be applied to Western blot⁸⁸), PET-blot⁸⁹) and IHC⁸⁵) using either nervous, LRS or any other tissue that may become available during the course of a surveillance programme.

L-type BSE

As with BSE, the identification of L-type BSE in cattle raised the issue of what the diagnostic criteria would be in small ruminants if they are susceptible, and experimental transmission studies were designed to characterise the phenotype of the disease in sheep^{90–92}).

An association between susceptibility and a genotype has been noticed, which is the same as for classical BSE. However, in the susceptible genotypes, the disease phenotype is stable, but none of the contributing characteristics (clinical signs, LP, WB or IHC) resemble any previously known TSE in this species and does not appear to be influenced by PrP sequence⁹²). Mouse bioassays also support this view, and indicate that the strain characteristics of L-BSE have been retained on cross-species transmission.

Interestingly, in discriminatory Western blot L-BSE appears as scrapie-like as it retains N-terminal antibody signal whilst in D-IHC it is clearly identified as BSE-like.

The main clinical presentation of L-BSE affected sheep is cataplexy, a syndrome that is not normally associated with TSE. Therefore, it is likely that these sheep would not be identified as TSE suspects through passive surveillance.

However, current rapid tests will detect L-BSE cases in all genotypes providing reassurance that they can be identified through active surveillance schemes.

H-type BSE

As with L-type BSE the emergence of H-type BSE in cattle questioned our capacity to identify this disease in small ruminants if it occurs. To characterise the disease in sheep, experimental transmissions were undertaken, and revealed that sheep are not completely resistant to this TSE although no clinical signs were identified prior to culling five years post inoculation. A limited amount of PrP^{Sc} was identified in a small number of challenged sheep, and revealed two

different patterns. One had only florid aggregates and no diffuse PrP^{Sc}; the other had diffuse PrP^{Sc} deposits in addition to the aggregates. It is not clear if the different presentations correspond to two different phenotypes or if the second presentation reflects a more advanced disease stage. None of these cases showed LRS involvement (own unpublished data).

Conclusions

Pathology initially offered the only means of diagnosing TSEs and still plays an important role in disease confirmation and strain typing. Although new methods are emerging for TSE diagnosis and some of these may be incorporated in the list of characteristics that are necessary to define TSE strains pathology will continue to play a vital role in identifying and characterizing TSEs either in the original host or in experimental rodents.

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