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研究報告の概要

本稿では，1990 年代にウシ海綿状脳症（BSE）が英国で流行したことを受けて，ヒト及び動物において伝染性海綿状脳症（TSE）を検出するために開発された様々な技法について焦点をあてている。脳組織中で典型的な病変及び異常プリオンタンパク PrP^{Sc} の蓄積を検出する従来の組織学的及び免疫組織学的方法に加えて，感受性の高いげっ歯類に疾患を実験的に伝播させることも確認手段としての機能を果たしてきた。さらに，ウェスタンブロット法も組織抽出物中の PrP^{Sc} 検出において高感度であることが実証されている。しかしながら，早急にハイスループットスクリーニングに適した手法が必要性であったことから，ウシ及び小型反芻動物（ヒツジ，ヤギ）の屠畜後に TSE を診断する，いわゆる「迅速な検査」が導入された。これらの多くは，感染動物又は非感染動物の脳組織を検討した大規模試験（対象は数百万検体）で妥当性が確認された後に，EU 保健・消費者保護総局（European Directorate General for Health and Consumer Protection）の承認を受けている。検査法の大半は ELISA 法であり，プロテイナーゼ K による PrPSc 消化とその後の変性及び特異的抗体による検出に依存している。現在，発症前での早期診断が可能となり得る屠畜前検査法が必要となっている。蛋白折りたたみ異常反復増幅法（Protein Misfolding Cyclic Amplification：PMCA）と呼ばれるアプローチ法は有望であると考えられていたが，最近では当初考えられていたほど特異的ではないことが明らかになっている。ヒトにおいて輸血により vCJD が伝播し得ることが示されていることから，プリオン研究分野では信頼性の高い血液検査法の開発が最優先事項となっている。様々な戦略が実施されているものの，いずれも未だ満足のいく結果にはつながっておらず，当該分野は依然として活発な研究領域である。

報告企業の意見	今後の対応
著者は，これまでに開発された動物及びヒトにおける TSE 検出法について詳しく調査している。本研究分野は，大規模な調査に基づいたスクリーニング方法の開発に成功したと考えられるが，依然として，血液における迅速な生前診断の必要性は高い。これが可能になれば，血液ドナーのスクリーニングにも使用でき，血液由来製剤の安全性も高まるであろう。	今後も引き続き，血漿分画製剤の製造に適用できるプリオンスクリーニング検査の有用性の情報収集に努める。

使用上の注意記載状況・その他参考事項等

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Review article

Progress and limits of TSE diagnostic tools

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Abstract – Following the two "mad cow" crises of 1996 and 2000, there was an urgent need for rapid and sensitive diagnostic methods to identify animals infected with the bovine spongiform encephalopathy (BSE) agent. This stimulated research in the field of prion diagnosis and led to the establishment of numerous so-called "rapid tests" which have been in use in Europe since 2001 for monitoring at-risk populations (rendering plants) and animals slaughtered for human consumption (slaughterhouse). These rapid tests have played a critical role in the management of the mad cow crisis by allowing the removal of prion infected carcasses from the human food chain, and by allowing a precise epidemiological monitoring of the BSE epizootic. They are all based on the detection of the abnormal form of the prion protein (PrP^{Sc} or PrP^{res}) in brain tissues and consequently are only suitable for post-mortem diagnosis. Since it is now very clear that variant Creutzfeldt-Jakob disease (vCJD) can be transmitted by blood transfusion, the development of a blood test for the diagnosis of vCJD is a top priority. Although significant progress has been made in this direction, including the development of the protein misfolding cyclic amplification (PMCA) technology, at the time this paper was written, this objective had not yet been achieved. This is the most important challenge for the years to come in this field of prion research.

TSE diagnosis / PrP / blood test / PMCA

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1. INTRODUCTION

Humans have a long history of contact with animals affected by a transmissible spongiform encephalopathy (TSE), without apparent problems. Scrapie in sheep has been described since the 18th century and the available epidemiological data do not indicate a detectable risk for humans under natural conditions. The huge epizootic of bovine spongiform encephalopathy (BSE) in Great Britain that was first detected in 1986 (over 200 000 confirmed cases to date) and, above all, the announcement in 1996 of possible transmission of BSE to humans in the form of the variant Creutzfeldt-Jakob disease (vCJD), created enormous concern among European consumers and triggered the first European-wide mad cow crisis. In 1999, testing of at-risk populations was introduced in some European countries and subsequently identified cases of BSE in countries previously believed to be untouched by the epizootic (Germany, Italy, Spain). This realisation triggered a further mad cow crisis at the end of 2000 and prompted the European authorities to take a whole series of measures to stop the spread of the epizootic, and to protect consumers from possible contamination by BSE. In particular, there was a total ban on meat and bone meal (held to be the main reason for BSE propagation in cattle) in livestock feed. Consumer protection was essentially ensured by removal of organs most likely to contain prions (specified risk material), and the implementation of systematic testing of all cattle aged between 24 and 30 months, depending on the carcass category and country[1]. The BSE epizootic has clearly receded since 2001 in Western Europe, but the situation is less clear in Eastern Europe.

Although arguments have accumulated since 1996 to confirm a link between BSE and vCJD [10, 11], fewer people are affected by vCJD than might have been feared (201 as of August 2007, with 163 in the UK and 22 in France) all of them carrying the Met/Met genotype at codon 129 of the prion protein (PrP). It is possible, however, that affected carriers of other genotypes (Val/Val and Met/Val) may appear in the future. Furthermore, a risk of secondary transmission within the human species is now clearly identified, following the detection in Great Britain of four cases linked to blood transfusion [41, 56, 84].

In the late 1990s, there was a pressing need for rapid and sensitive diagnostic methods to identify animals infected by the BSE agent in order to define the extent of the epizootic and avoid transmission to humans. Surprisingly successful rapid tests have been developed since the mid-1990s and, as we shall see, have proven to be very useful.

The development of a blood test for the diagnosis of vCJD is now a priority, first to make blood transfusions safe, and secondly to identify affected individuals early so that treatment (which at present does not exist) can be initiated before neuroinvasion and onset of the first clinical signs. At the time this paper was written, this objective had not yet been achieved.

2. THE CURRENT STATUS OF TSE DIAGNOSIS

Before the mad cow epizootic, the diagnosis of prion diseases was not a public health or economic issue. For humans and live animals, it was essentially based on the analysis of clinical signs and post-mortem histological analysis.

2.1. Conventional methods

Historically, techniques used to diagnose TSE were designed to detect, in appropriate tissue samples, lesions characteristic of TSE, or disease associated forms of the prion protein (PrP^{Sc}), or the transmissible agent itself.

2.1.1. Histological and immunohistological techniques

Histological detection of lesions typical of TSE (spongiosis, astrogliosis, amyloid plaques), essentially in the tissue of the central nervous system, is the reference method

[1] This does not hold for the United Kingdom since, between 1996 and 2005, cattle over 30 months were not eligible for human consumption (certainly the more efficient protection), and consequently not systematically tested. The same active surveillance scheme as in other European countries has been in place since November 2005.

for confirming a clinical diagnosis [23]. It is very specific, since it allows direct observation of the signs of the disease, notably symmetrical spongiform lesions, but is less sensitive than other techniques [24, 83]. The sensitivity of microscopic observation can be increased by immunohistochemical techniques that use antibodies specific to PrP to detect accumulation of PrP^{Sc} in amyloid deposits [79, 80]. This technique's efficiency depends greatly on sample preparation and on the nature of the antibodies used. Although these methods are ill-suited to rapid, routine analysis, they are excellent for confirmation. They are also effective for the analysis of samples of lymphoid tissues (tonsils, Peyer's patches, lymph nodes) and can be utilised, for example, in preclinical diagnosis of scrapie in sheep [2, 79] and chronic wasting disease [73], i.e., diseases characterised by marked replication of the prion in the lymphoid organs during the presymptomatic phase. The same observation has been reported for the diagnosis of vCJD and retrospective examination of over 8 000 tonsil and appendix samples by immuno-histochemistry (IHC) identified one case of vCJD in Great Britain in a person presenting no clinical signs [32, 33].

2.1.2. Experimental infection tests

The most sensitive and specific method of diagnosing TSE is unquestionably experimental infection in laboratory animals. The animal is injected (usually by the intracranial route) with a homogenate prepared from the potentially infected tissue and is watched for the appearance of clinical signs. After the death of the experimentally infected animal, disease development is confirmed using classic techniques (histology, immunohistology, Western blot). For obvious practical reasons, these experiments are generally performed in rodents (mice, hamsters, bank voles). Recently, the availability of transgenic mice that overexpress the same PrP as that of the donor species has significantly increased the efficiency of experimental transmission and shortened incubation periods [71]. However, these methods are too labour-intensive and time-consuming for use in routine high-throughput screening.

2.1.3. Western blotting

Western blotting has been used to detect PrP^{Sc} in tissue extracts for 20 years now [6]. Since all samples also always contain PrP^{C}, the protease-sensitive prion protein, they are systematically treated with proteinase K. After denaturation of the tissue extract by heating with sodium dodecyl sulfate (SDS), it is analysed by polyacrylamide gel electrophoresis (PAGE) and the denatured protein is transferred to a solid support and detected with an enzyme-labelled antibody. The specificity of Western blotting stems, among other things, from the fact that proteolysis with proteinase K characteristically alters the molecular weight of the PrP^{res}, because of the partial degradation of the N-terminal part of the protein. As a consequence, in addition to the residual signal observed, the gel bands shift in a manner typical of PrP^{res}. This technique has enabled highly sensitive detection of PrP^{Sc} in various tissues from vCJD patients [81]. Western blotting is also commonly used to characterise prion strains. The characteristics of the molecular pattern (size and relative intensity of the bands) of the three glycoforms of PrP^{res}, and the reaction of certain antibodies directed against the N-terminal part of the PrP can, in some cases, be used to identify the molecular signature of the prion strain [75, 76].

2.2. Rapid tests for post-mortem diagnosis of TSE

2.2.1. General characteristics

None of the methods mentioned above are really suited to high-throughput screening, and cannot be automated. After the 1996 mad cow crisis, and the fear of possible transmission to humans, it became clear that there was a need to develop new simpler and faster diagnostic tests for large-scale epidemiological studies, and more accurate assessment of the characteristics of the epizootic, or for routine testing to warrant safety of animal meat, for instance, of all cattle before they enter the food chain or industrial circuits. A new generation of so-called "rapid" diagnostic tests emerged, all based on the immunological detection of PrP^{Sc}, the only identified reliable marker of TSE.

It has long been perfectly apparent that antibodies can distinguish between the different conformations of the same protein, and the abnormal form of the prion protein is known to differ from the normal cellular form by its conformation, which contains a much higher proportion of β sheets, and less α helices [57, 58]. Yet, despite several promising publications [17, 37, 54, 87], there are, as yet, no clearly identified antibodies that, under practical conditions, specifically recognise PrP^{Sc} with satisfactory affinity in its native form. This is not the least of the paradoxes encountered in this field of research, but is beyond the scope of the present review.

In the absence of antibodies that specifically recognise PrP^{Sc}, it was necessary to resort to indirect approaches to distinguish between PrP^{Sc} and PrP^{C} in tissue extracts, which is generally present at a higher concentration. In almost all the rapid tests developed hitherto, this distinction is based on the distinct biochemical properties of the two forms of the protein. Most tests utilise the relative resistance of PrP^{Sc} to degradation by proteolytic enzymes, particularly proteinase K. Other tests are based on the aggregation properties of PrP^{Sc} when extracted using detergents. Note that the extraction of PrP (PrP^{C} or PrP^{Sc}) is an indispensable step in all tests, because it is hard to envisage detecting PrP without extracting it from its neighbouring membrane structures. This is generally achieved by treating a tissue homogenate with one or more detergents. Lastly, all the rapid tests include a step in which PrP^{res} is denatured, to permit its detection by antibodies that recognise PrP^{C} or denatured PrP (whether from PrP^{C} or PrP^{Sc}).

2.2.2. *European validation campaigns*

In May 1999, the Directorate General XXIV (Consumer Policy and Consumer Health Protection) of the European Commission validated, under very strict conditions (blind testing in a limited time overseen by a European Commission representative), three tests (from Enfer Technology Ltd (Newbridge, Ireland), Prionics (Zurich, Switzerland), and CEA (Saclay, France)) that were suitable for rapid industrial development.

The Prionics test uses an industrialised format of Western blotting that enables large-scale analysis [49, 65], and was the first rapid test used in large-scale epidemiological studies, first in Switzerland and then in France [12].

A diagnostic test developed by the CEA since 1998 is another example of a rapid test based this time on a conventional immunoenzyme approach (enzyme-linked immunosorbent assay, ELISA). This test is now marketed by Bio-Rad (Hercules, CA, USA, TeSeE tests). In the first step of the test, PrP^{res} is selectively purified using proteinase K, centrifugation, and denaturation. In the second step, the solubilised and denatured PrP^{res} is measured by a two-site (so-called sandwich) immunoassay that uses two monoclonal antibodies [27].

Enfer Technology Ltd developed an ELISA [46] in which PrP^{Sc} is directly immobilised on a solid support in the presence of proteinase K, denatured, and then detected using a polyclonal antibody directed against a peptide sequence characteristic of PrP.

The evaluation of these three tests was performed on more than 1 600 brain stem samples from uninfected animals (1 000 animals from New Zealand) and from animals at the clinical stage of the disease (300 animals from the UK). Brain homogenates were also diluted to test the analytical sensitivity of the tests [46]. A fourth test (from Wallac, Bucks, UK) gave unsatisfactory results and was subsequently re-evaluated in a substantially different format in 2001. The Enfer Technology, Prionics, and CEA tests were found to have 100% sensitivity and specificity on the series studied. Later work demonstrated that the CEA test, and its industrial version developed by Bio-Rad, were as sensitive as intracerebral inoculation tests in conventional RIII mice [20, 27]. It was also shown that rapid tests can also detect the accumulation of PrP^{res} in nerve tissue before the appearance of clinical signs [3, 26].

In 2002 and 2004, 15 new tests were evaluated by the Directorate General for Health and Consumer Protection by a similar

procedure, albeit on fewer samples[2]. Nine of the tests were approved for the post-mortem diagnosis of BSE: Prionics-Check LIA and Prionics-Check Prio-Strip (both from Prionics), TSE Kit Version 2.0 (Enfer), CDI-5 (InPro, San Francisco, USA), Ceditect BSE (Cedi, Lelystad, The Netherlands), HerdChek BSE Test Kit (IDEXX, Westbrook, USA), Speed'it BSE (Institut Pourquier, Montpellier, France), Beta Prion BSE EIA (Roboscreen, Leipzig, Germany) and PrionScreen (Roche, Basel, Switzerland).

Most of these new validated tests work in an ELISA format except Prio-Strip (lateral flow technology). However, three are based on markedly different principles. InPro's CDI-5 and Cedi's Ceditect BSE detect PrP^{Sc} by taking advantage of the fact that its immunoreactivity increases upon denaturation due to the unmasking of cryptic epitopes [63, 64]. HerdChek BSE from IDEXX is unique in two aspects, in that it does not use proteinase K digestion, and uses an aggregate specific capture ligand on a dextran polymer (Seprion ligand technology, Microsens Biotechnologies, London, UK) of PrP^{Sc}, which after denaturation is detected using an anti-PrP antibody.

Today, virtually all testing of cattle is done with the tests from Bio-Rad, Prionics, IDEXX, and Enfer. Some of these tests have also proved effective in diagnosing chronic wasting disease in wild ruminants [31].

From 2002 to 2004, five tests validated for the post-mortem diagnosis of BSE in cattle were provisionally approved for the post-mortem diagnosis of TSE in small ruminants[3]: TeSeE (Bio-Rad), TSE Kit (Enfer), CDI-5 (InPro), Prionics-Check LIA and Prionics-Check Western (both Prionics). Between 2004 and 2005, the European Commission specifically assessed nine tests for application to small ruminants, and recommended eight of them: TeSeE and TeSeE sheep/goat (Bio-Rad), TSE post-mortem test (IDEXX), Prionics-Check Western SR and Prionics-Check LIA SR (both from Prionics), Enfer TSE test Version 2.0 (Enfer), CDI-5 (InPro), Institut Pourquier Scrapie ELISA test (Institut Pourquier). It should be noted, however, that only the first three of these effectively detect atypical scrapie (Nor98) in brain stem samples. In practice, almost all testing on small ruminants is now done with the Bio-Rad, Prionics, IDEXX, and Enfer tests.

Note too that all results recorded using the rapid tests are confirmed in national reference laboratories, essentially using histopathology, immunohistochemistry, and Western blotting.

2.2.3. *Large-scale use of rapid tests*

Between 1st of January 2001 and 31st of December 2006, nearly 60 million tests on cattle within the European Community (almost 90% at the slaughterhouse) detected over 4 800 cases of BSE, approximately 1 170 at the slaughterhouse and about 3 700 in at-risk animals collected in rendering plants[4]. Over the same period, passive surveillance detected only 2 361 cases of BSE.

Rapid tests have therefore contributed significantly to consumer protection, first by providing a basis for confidence in meat safety, and secondly, because they led to the withdrawal of over 1 000 infected carcasses from human consumption. In addition to the increased safety they provide, these large-scale analyses have detected BSE in

[2] The evaluation of five rapid tests for the diagnosis of spongiform encephalopathy in bovines (2nd study), 27 March 2002, http://ec.europa.eu/food/food/biosafety/bse/sci_advice_en.htm, and scientific report of the European Food Safety Authority on the evaluation of seven new rapid post mortem BSE tests, 16 November 2004, http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620780462.htm [consulted January 2008].

[3] Scientific report of the European Food Safety Authority on the evaluation of rapid post mortem TSE tests intended for small ruminants, adopted on 17 May 2005, http://efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620780483.htm [consulted on January 2008].

[4] Reports on the monitoring and testing for the presence of TSE in the EU, 2001–2006, http://ec.europa.eu/food/food/biosafety/bse/annual_reps_en.htm, and monthly report of Member States on BSE and TSE, http://ec.europa.eu/food/food/biosafety/bse/mthly_reps_en.htm [consulted on January 2008].