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Bovine spongiform encephalopathy: epidemiology and diagnosis

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Bovine spongiform encephalopathy (BSE) belongs to the group of of so-called "prion diseases". These fatal neurodegenerative diseases are characterised by long incubation periods, and in the clinical phase by the presence of amyloid deposits, in the central nervous system, of an abnormally folded form of a host protein, PrP. BSE emerged in the UK in 1986, probably through feeding cattle with animal proteins from infected carcasses, possibly those of scrapie-infected sheep. Not only BSE was exported to other countries in Europe via MBM and live cattle, but it was most probably the origin in humans of a variant of Creutzfzeld-Jacob disease (vCJD), first described in Great Britain in 1996. vCJD incidence is regularly increasing, most cases being among UK citizens. Due to a very difficult clinical diagnosis, BSE incidence in countries outside UK has been and still is most probably underestimated. Some countries (Germany, Spain, Italy) have ignored indigenous BSE cases until 2001, when EU directives implemented systematic tests for over 30 months cattle entering the food chain. Present tests approved by the EU rely upon detection of abnormal PrP (proteinase K resistant) in the brain of cattle after slaughter. These tests provide a reasonable precautionary measure for protection of human health (the best precaution being the removal of SRM from carcasses), but cannot help much the eradication of BSE. Control and eradication of BSE will depend on specific and sensitive diagnostic tests that could be applied on live animals early in infection. Several recently published results give some hope that such early diagnostic tests might be available in a near future. Agricultura 1: 1-7 (2002)

Key words: bovine spongiform encephalopathy; epidemiology; diagnosis; test

INTRODUCTION

Transmissible Spongiform Encephalopathies (TSE) are neurodegenerative diseases with a long incubation period. They result in typical pathological signs in the central nervous system, in particular spongiosis (presence of large neuronal vacuoles), neuronal loss, and accumulation of amyloid deposits of an aggregated form of the host protein PrP (abbreviate for "Prion protein). This abnormally folded form of a normal host protein, anchored to the neuronal membrane, namely PrP, is invariably present in all TSE. It is named PrPsc (for "scrapie") and after treatment with proteinase K, the resistant fraction of PrPsc, partially digested on N-terminus, becomes PrPres (for reviews, see Prusiner 1998, Liemann and Glockshuber 1998, Collinge 1999). The Prusiner's model proposes that PrPsc is by itself the infectious agent of TSE, with probably the help of a proteinaceous co-factor (Prusiner 1982, Huang et al. 1996). TSE infect animals, like sheep and goats (Scrapie), cattle (BSE), wild deers (Chronic Wasting Disease), and humans (Kuru, Creutzfeld-Jacob Disease, Fatal Familial Insomnia, etc).

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In march 1996, R.G. Will, a neuropathologist from Edinburgh, announced that 10 cases of a new variant of human Creutzfzeld Jacob Disease (vCJD) had emerged in Great Britain since 1995, and alerted his colleagues and human health authorities that the bovine spongiform encephalopathy (BSE) had probably been transmitted to humans through consumption of infected beef products. (Will et al. 1996). This announcement, which contradicted all previous reassuring claims of British officials that BSE was not a risk for humans, provoked the first major « mad cow crisis » with its well known consequences in agriculture, and justified fears for human health in GB and in continental western Europe. Unfortunately, the number of vCJD deaths steadily increased since then, and all available epidemiological data have confirmed that vCJD was nothing but the human version of BSE.

Since 1996, many experimental arguments have accumulated in favor of this hypothesis. One major argument has been the intracerebral transmission of BSE to macaque monkeys, which gave rise by about 3 years, to a disease much resembling human vCJD. In particular, neuropathology revealed the existence in the macaque brain of numerous « florid » plaques (amyloid deposits of PrPsc protein surrounded by spongiosis vacuoles), a hall mark that was as yet only found in the brain of vCJD patients (Lasmezas et al. 1996).

A second series of experimental data was obtained in mice (conventional or transgenic), showing that the bovine "strain" of BSE was the same as the human vCJD strain (Collinge et al. 1996, Hill et al. 1997, Bruce et al. 1997, Scott et al. 1999). The present view is therefore completely different from that of British experts and officials in charge in the 80ties, who claimed that BSE was not a risk for humans because it probably derived from ovine scrapie, a TSE which is considered safe for human health. However, the truth is that after the MBM (meat and bone meal) ban for ruminants in 88, the SBO (specified bovine offals) ban was implemented in 1989 in G.B., a measure that was taken in direct concern with public health.

Focusing on BSE, which is probably the origin of major health and economical problems, we review in this article the main epidemiological data of BSE in western Europe, then we give an overview of the quick diagnostic tests that are presently used by the European Union. Finally we give some indications on possible future pre-clinical tests that could be designed according to recent scientific results.

I - EPIDEMIOLOGY: BSE COULD BE MORE WIDELY SPREAD THAN EXPECTED

According to available epidemiological data in the UK, BSE is not a true epidemic, in that it is not horizontally transmitted, and little if any transmissible to offspring (Wilesmith et al. 1997, Wilesmith and Ryan 1997). It must rather be considered as an anademic disease, that is an infection of many individuals by a common exogenous source, most probably MBM. The UK has been by far, with around 180 000 cases, the main nation concerned with BSE. Started in 1986, the epizootic culminated in 1992-93, with over 35 000 new cases per year (Fig. 1). Due to an efficient epidemiological survey (Wilesmith et al. 1991), the probable origin of this spectacular "epizootic" was identified as soon as 1988, and the MBM ban resulted, 5 years later, in the expected drop of cases. However, it is clear that in 2001 (i.e. by 8-9 years after the ban), the infection is still there, at a slowly declining rate (1602 notified cases in 2000 in the UK), and the origin of this persistent infection is not known. One hypothesis put forward for the numerous BSE cases appeared in cattle born after the 1988 MBM ban (so-called "BAB" for born after the ban) is the possible cross-contamination of cattle meal (normally without MBM) with MBM fed to pigs and poultry. This contamination may take place either in the plants that produce the different meals, or in the distribution chain (vans, silos, etc), or at the farm (silos). It could also be the result of fraud, and many legal actions are in progress in Europe on this matter.

The UK exports of MBM, and of live cattle throughout the world, have allowed the spread of BSE in different European countries, although with low incidences as compared to the UK, and with timings that are not well understood (Fig. 2). For theses countries, the evolution of case numbers per year of notification (until 2000, where only Switzerland and France had started post-mortem tests) might suggest, rather than a record of true BSE incidence, the influence on notification of such parameters as the control policy and the indemnities given to farmers, and their modifications. Several arguments are in favour of an underestimation of BSE prevalence, with probably great variations between countries (vide infra).

In July 2000, the European Scientific Steering Committee (Directorate XXIV), on the basis of data provided by evaluated countries on a voluntary basis, gave an estimate of the so-called "Geographic BSE Risk" (GBR) for each country, either from EU, or from outside EU or

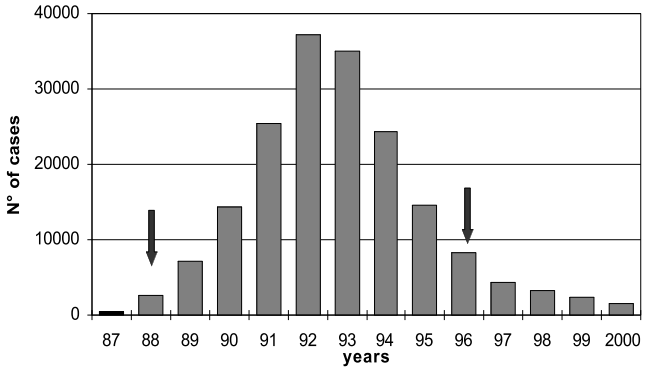

Fig. 1. BSE cases per year recorded in the United Kingdom since 1987. The arrows point to:
-The meat and bone meal (MBM) ban for ruminants (1988)
-The MBM ban for all farm species (1996).

Europe. This GBR took into account, in addition to BSE notification: 1- the risk of introduction of the infectious agent through cattle an/or MBM from the UK (or from other infected countries), and 2 - the risk of recycling the infectious agent inside the country, in particular through rendering practices. The SSC defined 4 levels of GBR (Table 1), in which the different European and non European countries were ranked. The border line between GBR level III and IV, in a way arbitrary, has been chosen as 100 confirmed cases per million cattle over 24 months of age (in the past 12 months). Hence the GBR level IV included only the UK and Portugal. Most remarkable, 3 countries belonging to EU, namely Germany, Italy and Spain, yet with no notified BSE, were classified at level III, like other countries with confirmed BSE cases (i.e. Ireland, France, Switzerland). It is worth noting that since January 2001, that is about 6 months after the SSC opinion, due to UE directives towards systematic tests of carcasses entering the human food, Germany, Spain and Italy all discovered

Table 1. Definition of GBR (geograpical risk of BSE) and its level.

GBR level	Presence of one or more cattle clinically or pre-clinically infected with the BSE agent in a geographical region/country
I	Highly unlikely
II	Unlikely, but not excluded
III	Likely, but not confirmed, or at a lower level
IV	Confirmed, at a higher level

source: European Union, Scientific Steering Committee

(http://europa.eu.int/comm/food/fs/sc/ssc/outcome_en.html#opinions)

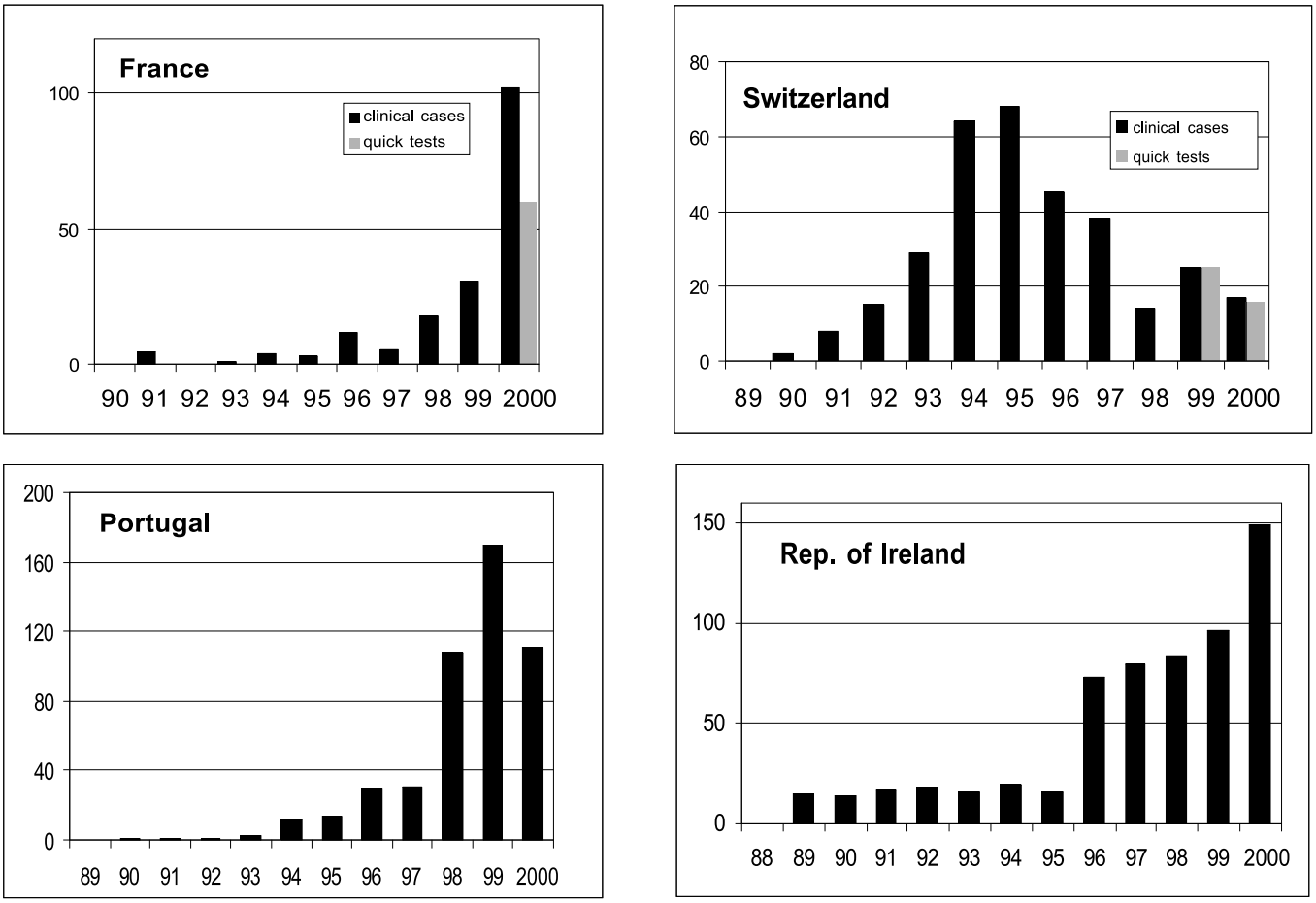

Fig. 2. BSE cases notified per year in four most affected countries outside the UK.: Portugal, France, Switzerland and Republic of Ireland. Black bars: clinical cases. Grey bars: positive by quick tests.

that they were infected with BSE. This is of great concern in particular in Germany, where the absence of clinic cases had exempted the authorities, until end of 2000, to take precaution measures like banning SBO for human food.

From February to May 2001, the SSC has published his evaluation of GBR for 23 other countries, providing now a total of 46 countries from all continents (Table 2). What emerges principally from this evaluation is that all countries where BSE is "likely" or confirmed (levels III and IV) are in Europe, and among those 21 countries, 11 belong to the EU (data for Greece are missing). Another point to note is that among the 9 recently evaluated countries from central Europe and Baltic (Romania, Albania, Czech Republic, Hungary, Slovenia, Slovak Republic, Poland, Estonia, Lithuania) all but Slovenia – at level II - are at level III. This means that there is a significant probability that BSE cases will appear in these countries. As a matter of fact, about ten weeks after the opinion was published, the first BSE case was confirmed in Czech Republic.

The remittance of BSE in the UK and its evolution in continental Europe, its possible future emergence in new countries and continents, pauses difficult questions from an epidemiological point of view: 1 – Is an unrecognised source of infection still present in Great Britain, that could explain the long lasting occurrence of BSE cases? 2 - Is the origin of BSE in countries outside UK, although at much lower incidence, the same as in the UK ? 3 - Is the general European MBM ban for all farm animals likely to eradicate the BSE epizootic ? Concerning the last question, one can have some doubts, since already in the UK, at least two cows born after august 1996 (the official ban on feeding any specie with MBM) have caught BSE. As it is likely that maternal transmission is not a major epidemic factor, if any, at least two possibilities remain: the first has to do with a possible contamination of fat added in the reconstituted milk fed to calves, as the origin and processing of fat supplement did not receive enough attention up to now. The second one could have to do with an infection of the "environment" in a wide sense, including soil and animal species that could be carriers of the infectious agent. In this regard, the study on "mites" as possible vectors of scrapie in Iceland could make some sense (Wisniewski et al. 1996, Carp et al. 2000), even though there is as yet no direct argument in favour of an environmental origin of BSE.

II – AVAILABLE POST MORTEM DIAGNOSTIC TESTS

The BSE pathogenesis differs from that of scrapie, where infectivity can be detected in many lymphoid organs and in the blood, even before the onset of clinical signs. In

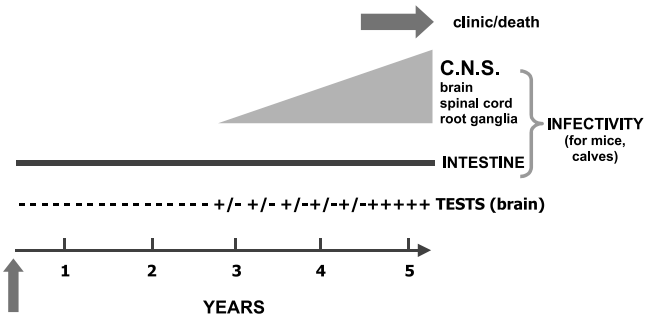

Fig. 3. BSE in cattle: schematic view of the progression of infectivity and PrPres-positivity as a function of time after infection. (After AFSSA, Paris, France)

cattle with BSE, only the CNS is infectious, and so only in the late phase of the disease, when clinical signs show (Fig. 3). The only other organ found slightly infectious in experimentally infected cattle is the distal ileum. No infectivity, nor abnormal PrPres protein was found so far in lymphoid organs nor in the blood, milk, or faeces from infected cattle (urine might be reconsidered, see chapter III). A safe diagnostic based on the presence of PrPres is therefore only possible in the brain or spinal cord, that is after slaughter.

In 1999, the European Commission validated 3 quick tests among 4 candidates in the frame of a world-wide call proposal. Table 3 gives the main characteristics of the 3 tests, one based on western blot (Prionics, CH) and two based on ELISA (Biorad/CEA, FR; Enfer-Sci, IRE). At least two of the tests - and probably the Enfer test too, but information is not available - have in common to specifically recognise PrPres by means of specific anti-PrP antibodies, after digestion of brain samples by the proteinase K, which renders the brain suspension free of protease-sensitive PrPc.

The measured performances of the tests, carried out in very strictly controlled conditions, consisted in their sensitivity (% of BSE samples found positive), their specificity (% of control samples found negative), and their detection limit (maximal dilution of BSE samples found positive in the test). The 3 tests have been validated mainly on the basis of their 100% scores in specificity and sensitivity. Concerning the detection limit, the BIORAD test performed the best, most probably because the ELISA step is preceded* with a quick PrPres concentration/purification step.

Table 3. Characteristics of the quick tests for BSE validated by the European Commission

TEST	BIORAD	ENFER TECH.	PRIONICS
inventor	C.E.A.	Proteus	Prionics
protocol			
tissue sample	brain stem	spinal cord	brain stem
type of test	ELISA (sandwich)	ELISA	western blot
specificity/PrPres	purif. + prot. K	?	prot. K
duration	4 -5 h	3 h	6 - 7 h
achievements			
sensitivity	100	100	100
specificity	100	100	100
detection limit*	10-2.5 (18/20)	10-1.5 (20/20)	10-1 (15/20)

* The maximal dilution at which at least 50% of 20 independent serial dilutions of a BSE sample are found positive in the test

By use of these validated tests, most countries of the EU have started the systematic testing of cattle over 30 months at slaughter. France for instance developed an important network of 66 approved laboratories, each one allowed to choose between Biorad or Prionics test. About 70% of these laboratories chose Prionics, 30% Biorad. As June 2001, about 797.000 tests have been performed, and 27 positive samples have been detected (prevalence: 0.03 p. 1000). A comparative research program aimed to compare the two tests is underway. It is important to remind here that research programs conducted initially in Switzerland, then in the west of France with the help of the Prionics test, have shown that categories of "risk animals" (dead-on-farm, euthanasied, emergency slaughtered) have indeed a much higher BSE prevalence: about 1.6 p.1000 in western France, that is a prevalence about 50-fold that of normally slaughtered cows. It is so far difficult to approach the real prevalence of BSE in each country in Europe, just because, at a given time, not all categories of cattle are tested.

Finally, five new post-mortem diagnostic tests are presently evaluated by the EU, among which one or several are expected to be more powerful, and therefore detect positive brains earlier than do presently available tests. However this generation of post-mortem tests will be certainly replaced in a near future by pre-clinical tests, and here follows some good news from this point of view.

III – WHAT EXPECTATIONS FOR A PRE-CLINICAL DIAGNOSTIC ?

There is a detection limit that cannot be overcome by any post-mortem test, which is the appearance of the first abnormal PrPres molecules in the CNS. Experimental infections performed in England on 40 calves, in order to analyse the time course of BSE infectivity in various organs, by use of i.c. injection to sensitive breeds of mice, suggest that it is not before 32 months that PrPres can be detected in the CNS (Wells et al. 1998). However, on a very large population concerned by the natural disease, like in the UK, several cattle have been found, by clinic and histo-pathology, positive for BSE before 30 mo of age. In France, 2 cows under 30 mo have been found BSE positive by quick tests. But these cases remain exceptional.

It is therefore very important to enable early detection of the disease on live animals in order to improve the efficiency of its eradication. This is still more important in terms of human health, because when available, any therapy will be probably most efficient if applied in the early stages of the infection, and also because such a test will secure the selection of blood and organ donors.

Several recent published data enable a reasonable optimism as to the emergence, in a near future, of one or several preclinical diagnostic tests for TSE, including, for some of them, BSE.

1- Ovine Scrapie: detection of PrPres in the blood

An American team (USDA) has succeeded to detect abnormal PrP in the blood of sheep infected with scrapie

Table 2. Opinion of the European Scientific Steering Committee (SSC) on geographical BSE risk.

GBR level	EUROPE (E. U.)	AFRICA	N-AMERICA	S-AMERICA	ASIA	PACIFIC (and mid East)	
I	Norway		Botswana Namibia Swaziland	Costa-Rica Brazil Chile Nicaragua Paraguay Uruguay	Argentina	Singapore	Australia New Zealand
II	Austria Finland Slovenia Sweden		Kenya Mauritius	Canada USA	Colombia	India Pakistan	
III	Albania Belgium Cyprus Czech Rep. Denmark Estonia France Germany Hungary Ireland Italy Lithuania Luxembourg Netherlands Poland Romania Slovak Rep. Spain Switzerland						
IV	Portugal United Kingdom						

(Schmerr et al. 1999). The test relies upon the capacity of a proteinase K-treated extract from blood leukocytes (possibly containing PrPres) to compete with a 15 amino-acid long, FITC-conjugated peptide, for binding to an anti PrP antibody. By capillary electrophoresis, in which molecules are separated according to their charge/mass ratio, the authors can readily distinguish each peak corresponding to free peptide, and to Ab-coupled peptide, respectively. For each sample, the relative ratio of peak areas is depending on the concentration of PrPres. This test was applied to genetically susceptible sheep, and among 5 that were found positive, only one was clinically infected. The authors conclude that the test must be of pre-clinical value, at least in the case of ovine scrapie. It is very likely that this test will not be applied to BSE, unless the detection limit of the USDA test would happen to be higher than the infectivity assay. However, the work of Schmerr et al. (1999) might be of interest in view of a future pre-clinical diagnostic of human vCJD, for which ovine scrapie seems to be a better model than cattle BSE.

2 – Selective binding of PrPsc to plasminogen

Since no workable PrPsc-specific antibody is available yet, all present diagnostic tests find their specificity for the abnormal PrPsc isoform in the proteinase K digestion step. This prot.K step is however a problem, because PrPres is only partially resistant, therefore the accuracy of any test is decreased upon digestion. In addition the efficiency of prot. K digestion greatly depends on the precise state and content of samples, and therefore this step may introduce a significant variability. It is therefore important to obviate this step and develop reagents that could specifically bind the prPsc molecules/aggregates. In that aim, the group of A. Aguzzi in Zurich has clearly demonstrated that the plasma protease plasminogen forms a selective complex with disease-associated prion protein derived from the brain of scrapie-infected mice (Fischer et al. 2000). When coupled to magnetic beads, plasminogen can efficiently bind and concentrate PrPsc from the brain, but not PrPc. One can imagine how useful such a step, very easy to perform, would be as a preliminary step in any kind of diagnostic. The initial data with mouse PrPsc have been recently extended to cattle, sheep and humans; in each case, plasminogen bound only PrPsc from affected individuals (Maissen et al. 2001).

Such a test will be valuable when it is shown that plasminogen can bind and concentrate PrPsc from blood or from organs amenable to biopsy, allowing early diagnosis of prion diseases.

3 – PrPres in urine early in infection ?

The plasminogen specific binding just described might be more easy to perform on urine, provided recent data from the group of R. Gabizon in Israël are confirmed (Shaked et al. 2001): this group shows that hamsters infected with the 263 K strain of hamster-adapted scrapie excrete in the urine a PrPres isoform, detectable only after dialysis of urine against saline. In addition, a prot. K-sensitive isoform (PrPc ?) is also found in the urine of all individuals, and this is also new. The urinary PrPres specie (UPrPres) has a higher MW than the usual “27-30” specie, and only one band is visible, proposed by the authors as being the bi-glycosylated form. UPrPres is detectable in hamsters as early as the mid-incu-

bation period of the disease. The authors have finally infected hamsters with UPrPres, and found that hamsters were still alive 270 days p.i. (unlike those infected with the same amount of brain-derived PrPres). More surprising, all urines from 12 UprPres-infected hamsters were found positive for UprPres from 60 days p.i. on. Importantly, Shaked et al. (2001) also show in the same paper that urinary PrPres is present in BSE clinically infected cows and human patients clinically affected with genetic CJD. Surprisingly, nobody before this group could find this PrPres in urine in any specie. No doubt that if confirmed, such data are fundamental in view of future ante mortem and moreover early diagnostic of most prion diseases, including BSE.

4 – A simple way to amplify abnormal PrP

In terms of diagnostic improvement, one of the most exciting results recently published by a group from SERONO in Geneva describes a very efficient way of *ex vivo* propagation of PrPres (Saborio et al. 2001). The authors demonstrate that simply mixing two brain extracts, one from a scrapie infected hamster, and one from a healthy control, results, after a 5-hrs incubation at 37°C, in a strong signal of PrPres in Western blot, in conditions where the PrPres signal of the "inoculum" (infected brain) was reduced, by appropriate dilution, to the minimal detectable signal. In addition, when the incubation (between 5 hrs to 40 hrs) was interrupted by repeated sonications every hour, the authors obtained a significant amplification of the PrPres signal over the control without sonication. This very simple and elegant demonstration of "ex vivo" short term "propagation" of the PrPres isoform by repeated incubation/disruption (of PrPres aggregates) might represent a major breakthrough for the comprehension of prion propagation (a strong support to the Prusiner's theory ?), and for numerous practical applications, obviously in early diagnostic: the possible amplification of low amounts of PrPres, in theory, opens the way for prion diagnosis in fluids and organs where PrPres is otherwise undetectable. One has however to be cautious, precisely because the data from the Serono team deal with crude brain extracts. In this organ, one or several necessary "cofactor(s)" for amplification are probably present (all previous in vitro conversion experiments performed with purified SAF did not amplify newly formed PrPsc) , but the said cofactor(s) could be absent or at much lower concentration in other organs and in the blood.

CONCLUSION

We have briefly reviewed some aspects of BSE epidemiology (so far the disease is only notified in Europe), we have described the present approved test for post-mortem diagnosis, and finally reviewed some encouraging results for future pre-clinical diagnostics. These data and the comments they suggest, make sense if one keeps in mind that the heavier consequence of BSE, not only in the UK, is the possible outbreak of human vCJD in all countries were consumers have been exposed to contaminated bovine offals. Most countries are now alerted about the risk that latent BSE (whether endogenous or "imported") might represent for human health. However nobody knows the real prevalence of BSEin. It is probably much underestimated, even in countries who have experienced this disease for 10 years and more. Before the quick post-mortem test were used (since 1998 in Switzerland, 2000 in France, 2001 in other EU countries), only clinical diagnosis was made, which is a very difficult one (Braun et al. 1998). It is therefore highly likely that many BSE cases have escaped surveillance even in countries where BSE was notified. For example, one very first, non specific sign of BSE infection on dairy cows is a drop in milk production. This quite common symptom may have been the cause of numerous entries of clinical cows in the food chain.

Now the post-mortem tests have been introduced, at least those cattle heavily infected, and therefore at the end of their incubation, can be detected and removed from the food chain. The fact that countries like Germany, Italy, Spain, discovered their first BSE-affected animals in 2001, that is as soon as quick tests were applied at slaughter, with very few clinical cases found in the same period, suggests that many clinical cases were already present in theses countries.

Systematic post-mortem tests provide at least a minimal mean of protection of consumers. But it must be clear that they cannot constitute adequate tools to measure the real BSE prevalence in a given country, nor to elaborate efficient policies for BSE eradication. BSE eradication in the UK is not yet effective, despite a significant advance over other countries concerning safety measures. Therefore, a total eradication of BSE in continental Europe in 2005-6, expected from the (provisional) ban of MBM for all farm species decided by EU in Nov. 2000, is not guaranteed.

The question must be addressed right now of whether other sources of BSE infection than contaminated MBM do exist. If there is a suspicion of contamination of fats present in the reconstituted milk, it is urgent to take all possible measures to secure this constituent part of the calf diet. Another possibility is that infectivity might persist or propagate in the environment (for example carrier wild species, mites being one possibility for scrapie infected areas). So far no data have been produced in this field concerning BSE, but it is true that the "paradigm" of contaminated MBM as the main and perhaps only source of cattle infection may have somewhat discouraged other tracks of investigation.

Whichever is the reason for persistent BSE in Europe, even in the UK, it is urgent to set up efficient pre-clinical tests that should not only improve the food safety for consumers, but greatly participate to a better understanding of this animal disease, its prevalence, its pathogenesis, in view of its eradication. The recently published results presented here are quite encouraging from this point of view. Indeed, not only they suggest that sensitive tests for prions could be soon available, but in addition they provide substantial contributions to the comprehension of the biology of PrP isoforms and of the pathogenesis of prion diseases.

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